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The Effects of Aqueous Atorvastatin on Steroidogenesis of *Xenopus laevis* at Environmentally Relevant Concentrations

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THE EFFECTS OF AQUEOUS ATORVASTATIN ON STEROIDOGENESIS OF XENOPUS LAEVIS AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS.

by

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ABSTRACT

Statin drugs are a class of drug that work to reduce endogenous production of cholesterol by competitively inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase (*Hmgcr*) thus inhibiting production of mevalonic acid in the mevalonate pathway. Atorvastatin (Lipitor) is one of the most widely prescribed statin drugs and contamination of wastewater effluent is a growing environmental concern because of the potential to interfere with steroidogenesis in wildlife. Amphibians may be particularly susceptible to the effects of atorvastatin contamination because of their highly permeable integument. I used an amphibian model, *Xenopus laevis* to test the hypothesis that chronic exposure to low concentrations of atorvastatin in water has an adverse effect on steroid hormone levels, growth and development. This hypothesis was tested via a series of toxicity assays designed to evaluate potential endocrine disrupting effects of atorvastatin using traditional aquatic toxicology assays combined with modern molecular biology techniques to identify and report regulatory alteration of physiological pathways. Results indicated significant dose-dependent upregulation of steroidogenesis as confirmed by both qPCR and total RNA sequencing and supported by evidence of alteration of testosterone and estradiol concentrations. Such effects have the potential to significantly alter sex ratios in amphibian populations localized around wastewater effluent sites. This research provides insight into the potentially harmful effects of relatively low concentrations of aqueous atorvastatin.

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CHAPTER I – INTRODUCTION AND BACKGROUND

Statin drugs are a class of drug that work to reduce endogenous production of cholesterol by competitively inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase (*Hmgcr*) thus inhibiting production of mevalonic acid in the mevalonate pathway. This results in reduced cholesterol biosynthesis in humans and helps to reduce high cholesterol levels (hypercholesterolemia) and the risk of cardiovascular disease.

Atorvastatin (Lipitor) as one of the most widely prescribed statin drugs (Stagnitti, 2007). Since new statin drugs are created constantly, prescription rates and use have likely declined, however atorvastatin remains one of the most highly prescribed medications in the western world. A recent study of patients in the US Department of Veteran Affairs found that 79% of all patients at their facilities were prescribed a statin of some type (McBride et al., 2018). In the US, it is estimated that 27.8% of the population 40 years and older take statin medications, and the medical community widely considers these rates to be considerably lower than target rates for a healthy population (Salami et al., 2017). This is primarily due to the high rate of hypercholesterolemia in developed countries for which atorvastatin is designed to treat by reducing endogenous production of cholesterol.

1.1 Wastewater Effluent.

It has been suggested for quite some time that pharmaceutical contamination of wastewater effluent is a growing environmental concern. Numerous studies report active pharmaceutical compounds detected in both wastewater effluent and surface waters (Batt, Kostich, & Lazorchak, 2008; Chen et al., 2006; Ort et al., 2010; Richards & Cole, 2006).

The sources of these compounds vary from hospital input to improper disposal of expired medications and residential sewage and agricultural runoff.

Modern engineering solutions to effectively remove pharmaceutical contaminants from wastewater effluent are either too costly or impractical, and as a result, pharmaceuticals continue to be discharged in wastewater effluent at increasing rates (Halling-Sorensen et al., 1998). While designs of wastewater treatment plants vary from activated sludge digestion to spray field irrigation, almost all wastewater treatment methodologies involve mechanical, photochemical, and biological filtration. Photo degradation accounts for some removal of pharmaceuticals, but it is not significant enough to reduce pharmaceutical levels below detectable levels. A recent study of 3 conventional wastewater treatment plants in Spain over a 2-year period showed removal rates of ~50%, with atorvastatin effluent concentrations in the 2 ng L^{-1} range (Jelic et al., 2011). Moreover, accumulation in lakes and streams in locations near effluent sites is not uncommon.

Concentrations of atorvastatin ranging from 1-4 μg L^{-1} have been detected in waters adjacent to effluent sites in Canada (Lee, Peart, Svoboda, & Backus, 2009). As these chronic low levels of atorvastatin are continuously discharged into the surrounding ecosystem, small aquatic organisms such as fish and amphibians are exposed, and potentially affected by atorvastatin. Little is known about environmental degradation of atorvastatin. A 2005 study of photodegradation of atorvastatin suggested an environmental half-life of 0.6 – 2.4 hours in surface water and noted that photodegradation is enhanced in the presence of dissolved organic matter(Lam & Mabury, 2005). While this short half-life is favorable to the environment, high influent

rates of atorvastatin to wastewater treatment plants allow for replacement of degraded atorvastatin resulting the localized concentrations near wastewater effluent sites.

1.2 Endogenous Production of Cholesterol.

In humans, LDL/HDL cholesterol ratios are inflated in large part, due to endogenous production of cholesterol (Kapourchali, Surendiran, Goulet, & Moghadasian, 2016). Hypercholesterolemia is caused by high levels of LDL cholesterol proportional to HDL cholesterol in the body (Kapourchali et al., 2016).

Endogenous cholesterol production occurs across 3 primary pathways. These pathways are fatty acid metabolism, the mevalonate, or isoprenoid, pathway, and the squalene synthesis pathway (Figure 1.1).

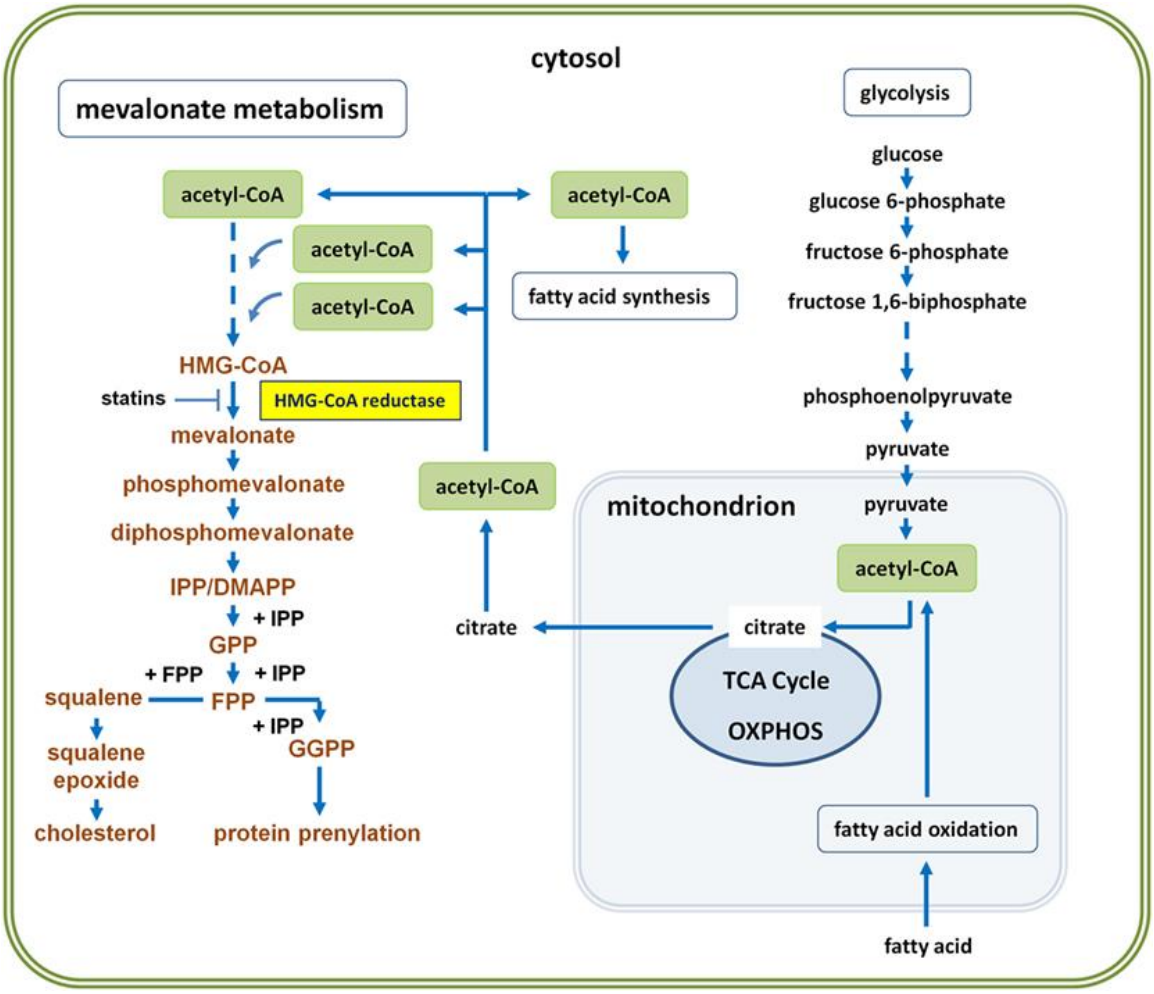

Figure 1.1 Cholesterol biosynthesis and regulation of the mevalonate pathway (Gruenbacher & Thurnher, 2017).

The fatty acid metabolism pathway converts carbohydrates via glycolysis, and lipids via β-oxidation into acetyl coenzyme A (Acetyl-CoA). Next, the mevalonate pathway converts Acetyl-CoA into isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP are then used in the squalene synthesis pathway for conversion into cholesterol.

In this general overview of endogenous cholesterol production, the mevalonate pathway can be considered the rate limiting step in conversion of fatty acids to

cholesterol. For animals with potentially low input of dietary cholesterol, this could reduce cholesterol levels significantly because endogenous production of cholesterol accounts for two thirds of all cholesterol in the body (Kapourchali et al., 2016). Atorvastatin blocks endogenous production of cholesterol by competitively inhibiting the active site of *Hmgcr*. This effectively blocks conversion of fatty acids to cholesterol and reduces the LDL/HDL ratio as treatment for hypercholesterolemia. An animal exposed to atorvastatin could experience hypocholesterolemia due to the reduced ability of the body to effectively regulate endogenous production of cholesterol to compensate for decreased dietary input such as in amphibians.

In amphibians, dietary cholesterol input has a far less significant contribution to overall cholesterol level than in the human model (Hadfield, Clayton, & Barnett, 2006). This is because amphibians, and frogs in particular, tend to rely on diets based heavily on plant matter and insects that contribute little to endogenous cholesterol levels(Hadfield et al., 2006). This natural high protein/low fat diet combined with naturally low body fat reserves suggests that shutting down endogenous cholesterol production via exposure to statin drugs could adversely affect frogs in ways not evident in humans.

1.3 Pharmacology and Pharmacodynamics of Atorvastatin.

Atorvastatin was developed to combat the hypercholesterolemia epidemic that currently plagues modern societies. Hypercholesterolemia, and by extension atherosclerosis and coronary heart disease are one of the leading causes of death in developed countries. Indeed, among the US population, heart disease is the leading cause of death, and has held this top spot for quite some time (CDC, 2019). This study shows that between 1999 and 2017 over 12 million deaths in the US were associated with

diseases primarily treatable by statin drugs. Atorvastatin, and statin drugs in general, work to reduce endogenous production of cholesterol and, in turn, reduce mean total cholesterol in the body. The prescribed dosage range in humans of atorvastatin is 10-80 mg/day (Lennernäs, 2003). Since the mass of an average human is approximately 80 kg, mathematically, this resolves to be a concentration of 0.125-1 mg kg^{-1} of body mass. At this dosage, smaller aquatic species undergoing chronic exposure have the potential to reach circulating atorvastatin concentrations in the range of efficacy for atorvastatin.

In humans, atorvastatin is administered orally as a tablet in the calcium salt form of the hydroxyl acid. Metabolism and uptake of atorvastatin occurs primarily in the gut. At the physiologically relevant pH of 6, the solubility of the acid form is high, and as a result, uptake into the intestinal lumen is high. The uptake mechanism of atorvastatin calcium is complex and not well understood. Atorvastatin is administered in its active form, and given its high lipophilicity ($\log K^{o/w} = 6.36$) (National Center for Biotechnology Information, n.d.), absorption across the gut wall is assumed to be rapid with the bulk of the uptake driven primarily by passive diffusion across the intestinal lumen and into the enterocyte. Following uptake, atorvastatin serves as a substrate for *Cyp3a4* in the intestinal lumen. The protein encoded by *cyp3a4* metabolizes a wide range of pharmaceuticals and is useful as a biomarker indicating uptake and metabolism of atorvastatin. When atorvastatin is administered, the acid undergoes glucuronidation and is converted to its lactone form. The lactone has a much higher affinity for the *Cyp3a4* protein, and conversion from the active acid form to the inactive lactone form in the liver is considered to be the primary route of elimination (Jacobsen et al., 2000). The *Cyp3a4* protein converts both the lactone and acid forms into their oxidized and μ-oxidized forms,

respectively. With respect to the acid forms, *Cyp3a4* converts the open acid to 2-hydroxy atorvastatin acid and 4-hydroxy atorvastatin acid. Of the two, 2-hydroxy atorvastatin acid is the dominant acid metabolite (Jacobsen et al., 2000). Figure 1.2 shows a hypothetical metabolic pathway for atorvastatin (Lennernäs, 2003).

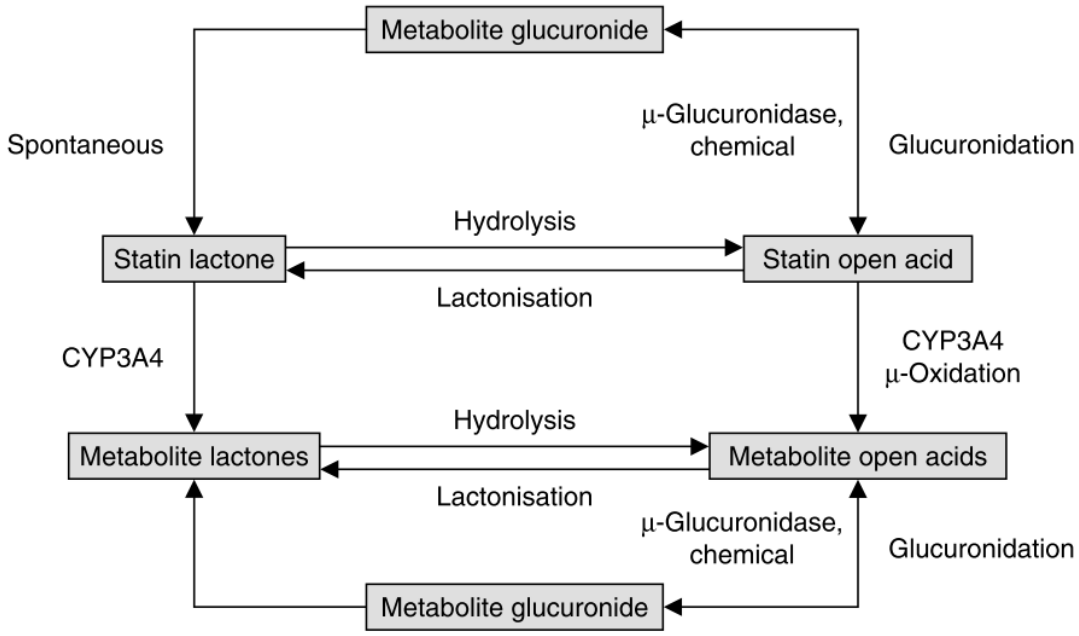

Figure 1.2 Hypothetical representation of the metabolic pathways associated with CYP3A4 metabolism of atorvastatin (Lennernäs, 2003).

Once inside the human body, it has a low bioavailability of 14% with serum concentrations reaching t_{max} within 1.5 hours of the initial dose (Gibson, Stern, Abel, & Whitfield, 1997). This high uptake and low bioavailability of atorvastatin allow comparatively high levels of the active compound to be excreted from the body via urine and feces. Repeated dose studies show that steady state is achieved within the first 72 hours, increases proportional to dose, and the remaining 70-80% of the dose is excreted as the parent compound within 11 to 14 days (Black et al., 1999; Cilla Jr., Whitfield, Gibson, Sedman, & Posvar, 1996).

Once metabolized and inside the body, atorvastatin works as a competitive inhibitor of *Hmgcr*. This competitive inhibition leads to decreased mevalonate levels, and consequently, reduced endogenous production of cholesterol. As mevalonate levels drop, upregulation of the genes encoding for *Hmcgr* are seen. This is one of several feedback loops regulating mevalonate production and makes the *hmgcr* gene a useful biomarker indicating exposure to atorvastatin. Multiple studies show evidence of dose dependent reductions in LDL-cholesterol as well as reductions in mean total cholesterol following administration of statin drugs in humans. (Cilla Jr. et al., 1996; Nawrocki et al., 1995).

In general, statin drugs show a wide variety of beneficial effects in humans. These beneficial effects include reduction in inflammation and reduced cholesterol plaque build-up in arterial walls (Lennernäs, 2003). These beneficial effects come with relatively few harmful side effects in humans, however, little work has been done to assess potential harmful side effects to atorvastatin exposure and associated cholesterol reduction in non-target species such as amphibians.

1.4 Steroidogenesis.

If exposure to environmental statins reduces circulating cholesterol levels in amphibians, it is possible that this reduction affects circulating hormone levels as well. In broad terms, cholesterol serves as a precursor molecule for steroidogenesis, following a well-described pathway (Figure 1.3). This pathway is responsible for conversion of cholesterol to the sex steroids estradiol and testosterone, in addition to production of the stress hormone cortisol. It is the close connection between cholesterol and sex steroid production that is the impetus for this study.

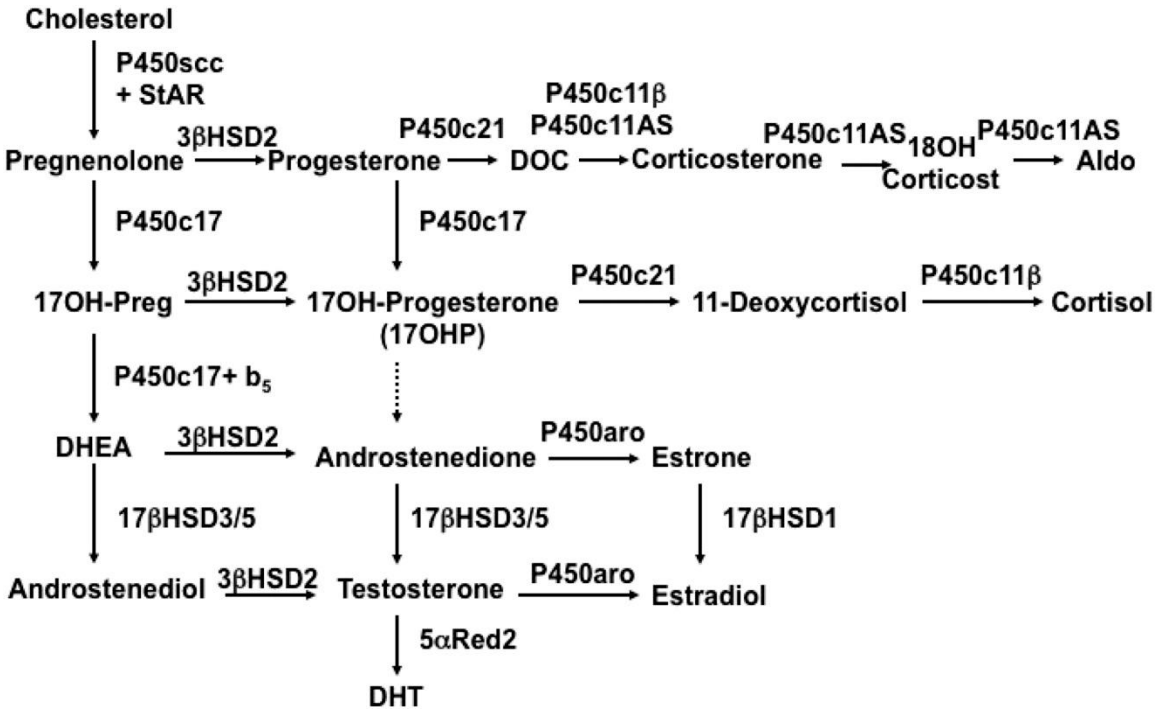

Figure 1.3 The steroidogenesis pathway (Miller, 2018).

The rate-limiting step in steroidogenesis is conversion of cholesterol to pregnenolone via enzymatic reaction with cholesterol side-chain cleavage enzyme (*P450scc)*. This reaction is mediated by Steroidogenic Acute Regulatory protein (*Star*), which functions to move cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where the *P450scc* reaction takes place. Since *Star* is believed to be constitutively expressed, and cholesterol availability is unlikely to be an issue, it is the rate at which *P450scc* can synthesize pregnenolone that determines substrate supply in subsequent reactions.

Pregnenolone then serves as the primary substrate for production of all steroids. As pregnenolone levels decrease, so do levels of testosterone and estradiol. Additionally, since pregnenolone also serves as a substrate for cortisol, stress has the risk to exacerbate the crisis of low pregnenolone and by association, cholesterol. These factors combine to

suggest that atorvastatin can have endocrine disrupting effects by altering the production of cholesterol critical for steroid synthesis.

1.5 Amphibian Toxicology.

Some amphibians, including *Xenopus laevis*, have an increased sensitivity to aquatic toxins due to cutaneous respiration (Feder & Burggren, 1985). Pre-metamorphosis tadpoles use gills and cutaneous respiration to supplement oxygen needs due to the high metabolic demands of metamorphosis. This provides an additional exposure route to aquatic toxins and allows for greater potential for uptake into the bloodstream. This is particularly problematic with regards to cutaneous respiration as the cutaneous membrane in these amphibians provides little barrier to environmental xenobiotics. As a result, amphibians have an enhanced sensitivity to chemicals in water (ASTM INTERNATIONAL, 2012).

If statins act in a similar way in amphibians as they do in humans, environmental exposure to atorvastatin could produce hypocholesterolemia, resulting in a net reduction of steroid hormone production, endocrine disruption, and at its most extreme, alteration of sexual differentiation and metamorphosis. Cholesterol is the primary substrate used in the endogenous production of testosterone, and estradiol (Miller & Auchus, 2011). In amphibians, endocrine disruption has been shown to alter sex ratios by affecting gonad differentiation (Miyata & Kubo, 2000; Ohtani, Miura, & Ichikawa, 2000; Yu, Hsu, Ku, Chang, & Liu, 1993). Many hormones produced via the cleavage of cholesterol also drive the physiological processes for embryonic development and metamorphosis. A recent study in zebrafish showed a 37% reduction in cholesterol, a 71% reduction in

cortisol, a ~60% reduction in testosterone, and a ~20% reduction in estradiol as a result of atorvastatin exposure (Al-Habsi, Massarsky, & Moon, 2015).

1.6 Amphibian Development.

Amphibians display a unique developmental apparatus that can be useful for toxicity studies: metamorphosis. In 1967 Nieuwkoop and Farber published "Normal table of *Xenopus laevis*". This book served as the springboard into amphibian genetics and teratogenesis assays. It laid out 66 developmental stages complete with criteria for morphological analysis that could be used to determine growth stage in a non-invasive manner. It defined times to each developmental stage at varying temperatures, making it possible for researchers to determine alteration of amphibian development on a large-scale basis and compare effects across laboratories.

Amphibians pass through four broad and generalized stages during development (Figure 1.4). The embryonic stage is immediately following fertilization, and during this time, embryos undergo cleavage, blastulation, gastrulation, neurulation, and tail-budding. Upon completion of the embryonic stage, the animals are free swimming and feeding tadpoles. In *Xenopus laevis*, this stage is complete in approximately 4 days.

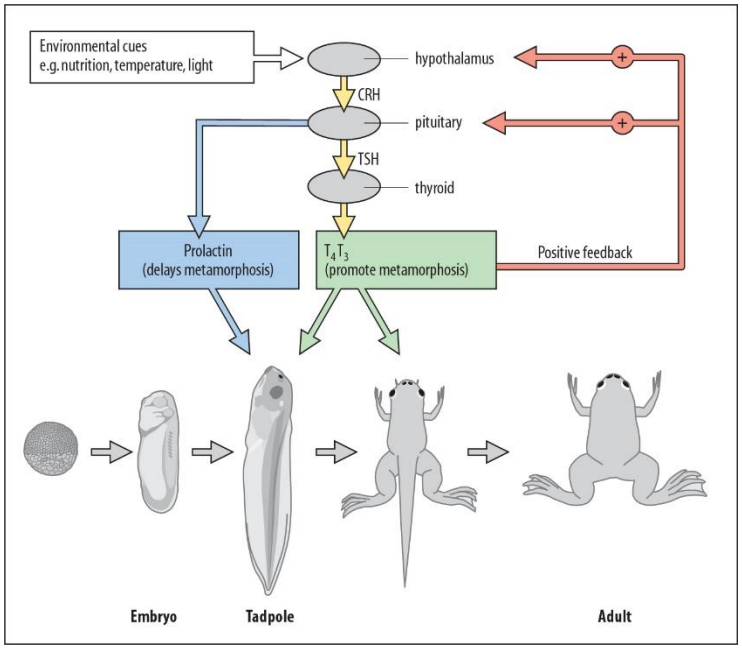

Figure 1.4 Developmental stages of amphibians with hormone mediation of metamorphosis.

Taken from: http://www.mun.ca/biology/desmid/brian/BIOL3530/DEVO_13/ch13f19.jpg

Following the completion of the embryonic stages, the embryos transition into the tadpole stage. The climax of this stage is defined primarily by metamorphosis. During metamorphosis, tadpoles undergo several progressive and regressive changes and extensive tissue remodeling. Hindlimb and forelimb development moves to completion, and the tail resorbs into the body. Gills are resorbed into the body, and the lungs become the primary respiratory organ. This stage comes with a variety of hormonal changes necessary for engaging the appropriate cellular response for tissue differentiation.

Regulatory control of metamorphosis is mediated by the release of thyroxine (T4) and triiodothyronine (T3) by the thyroid (Gilbert, 2007). Prolactin delays the onset of metamorphosis until levels of T3 and T4 exceed the levels of prolactin. This creates a positive feedback loop in that the high levels of T3 and T4 stimulate further release of T3

and T4 to push the animal through metamorphosis (Figure 1.4). Recent studies have shown an interaction between estrogen and testosterone levels with respect to thyroid function. The presence of high estrogen levels in thyroid tissues has been shown to cause cell proliferation, and negatively affect production of thyroid hormones(Santin & Furlanetto, 2011). Low levels of testosterone have also been shown to inhibit thyroid secretion of T3 and T4 (Meikle, 2004). It is the interaction between the thyroid and sex steroids that is a cause of concern with respect to frogs exposed to statins in the environment, specifically alteration of testosterone and estrogen levels in metamorphosing tadpoles. The interaction between sex steroids and the secretion of T3 and T4 suggests that alteration of hormone concentrations has the potential to alter metamorphic rates.

I hypothesized that chronic exposure to low concentrations of atorvastatin in water has an adverse effect on production of steroids regulating growth and development of *X. laevis*. This was tested via a series of toxicity assays. These assays first examined atorvastatin efficacy by evaluating biomarkers of exposure in a tightly controlled environment at doses well above environmental relevance. Once the efficacy of the drug was verified, a battery of assays were used to evaluate environmentally relevant doses across multiple life stages to examine the effects of atorvastatin exposure on amphibian steroidogenesis. Measures for the target and non-target effects of atorvastatin on *X. laevis* will be expression of several relevant genes, cholesterol measurement, and significant alteration of the steroidogenesis pathway.

1.7 Experimental Design.

The complete experimental design consisted of 3 distinct assays intended to test the effect of atorvastatin at different developmental endpoints and maximize data collection.

The first assay was used to determine the effects and verify the biomarkers used in follow-on assays. This assay followed the ASTM assay protocol for the Frog Embryo Teratogenesis Assay *Xenopus* (FETAX)(ASTM INTERNATIONAL, 2012) with minor modifications to assay termination to preserve samples for molecular analysis as described in the materials and methods chapter. This assay is performed at extremely high concentrations as defined by the FETAX protocol. The intent behind this assay was to elicit an effect and measure that effect. This assay served to hone tools for future data analysis and validate biomarkers for assays at lower concentrations.

The second assay was a chronic tadpole metamorphosis assay. This experiment was a 60-day chronic flow-through exposure under environmentally relevant concentrations of atorvastatin. Briefly, tadpoles (NF47) were exposed continuously for 60 days and time to and through metamorphosis was measured. At termination, tadpole liver tissue was taken for follow up molecular analysis. This experiment showed effects of chronic, low concentration exposures of atorvastatin in water to metamorphosing tadpoles.

The final assay was a chronic 60-day exposure of male and female adults to environmentally relevant concentrations of atorvastatin in water. This aqueous exposure showed the long term, chronic effects of exposure to atorvastatin. Endpoints in this assay

include transcriptional analysis of assigned biomarkers of exposure, and quantification of cholesterol, testosterone, and estradiol levels in plasma.

CHAPTER II – MATERIALS AND METHODS

2.1 Introduction.

The complete experimental design consisted of three distinct experimental assays. The FETAX assay was employed to evaluate the effects of high doses of atorvastatin on developing *Xenopus sp.* embryos. The metamorphosis assay integrated organismal responses determined from the FETAX to determine the effects of aqueous atorvastatin exposure at environmentally concentrations on metamorphosing tadpoles. Finally, the adult exposure evaluated the effects of chronic low concentrations of atorvastatin on adult *X. laevis*.

2.2 FETAX.

The Frog Embryo Teratogenic Assay (FETAX) is an acute 96-hour exposure to evaluate the effects of xenobiotic exposure during early life stages. The protocols and procedures used in this experiment were a modification of the "Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX)"(ASTM INTERNATIONAL, 2012). The FETAX follows the strictest of standards based on decades of research data, as such, these standards are ideal for testing the response of *Xenopus sp.* to xenobiotic exposure. The FETAX uses primarily morphometric data endpoints to determine growth and developmental effects of exposure. The standard protocol advises fixing the tadpoles in formalin for later analysis. This termination protocol would not preserve tissue in a manner useful for follow-on molecular analysis. In order to preserve the tissue in a manner more conducive to our needs, I have modified the termination procedure by preserving the samples either in RNALater for later extraction and analysis or by flash freezing for cholesterol analysis.

Briefly, embryos were collected and dejellied immediately following fertilization. Normal embryos between NF stage 8 and 11 were selected and randomly distributed into petri dishes. A selection of 5 increasing concentrations, determined using the ASTM selection criteria, plus a control were set-up with 4 replicates. The embryos were then allowed to grow out for 96 hours in a temperature-controlled incubator at 28°C under constant supervision. At termination, images of each tadpole were taken for morphometric analysis, and whole organisms were flash frozen for cholesterol quantification or stored in RNALater for qPCR analysis.

2.3 Metamorphosis Assay.

The metamorphosis experiment was a chronic 60-day exposure to evaluate effects during metamorphosis and growing into adulthood. The test was conducted with a control and 3 treatments (n=30) in duplicate. Frogs were exposed to aqueous concentrations of atorvastatin for the duration of the 60-day experiment. At termination, the froglets were staged to allow for morphometric analysis, and liver samples were taken for cholesterol quantification and qPCR analysis.

Tadpoles were commercially purchased from Xenopus Express and stage selected to be NF 47 at shipment. Tadpoles were shipped overnight and acclimated to room temperature upon arrival. Following acclimation, tadpoles were stage selected again to be NF 47 at test initiation. Tadpoles were then sorted into 100 mL beakers (n=40) and the beakers were covered with nytex mesh. The beakers were then placed into the flow through exposure facility at the University of Southern Mississippi's Gulf Coast Research Laboratory. This unique facility allows continuous flow through exposures at a wide

range of operating parameters. Table 2.1 describes the operational parameters of the exposure system for the duration of this experiment.

Table 2.1 Flow through chamber operating parameters for the metamorphosis assay.

Temperature	22° C
Salinity	0.15 ppt
Dissolved Oxygen	8.5 mg L^{-1}
pH	7.5
Flow Rate	1.5 L hr^{-1}
Residence Time	20 hr
Stock Renewal Frequency	48hr

Stock solutions were prepared by first diluting atorvastatin calcium (CAS#: 344423-98-9) into DMSO at a concentration of 250 g/L. This primary stock was then further diluted into a 10 g L^{-1} working stock for stock renewals. This working stock was then diluted into appropriate stock concentrations and volumes for exposure chamber protocols.

The exposure facility then continuously dilutes stock solutions and flows it through the exposure tanks as the prescribed flow rate. Water samples were taken at initiation, 30 days, and termination. These water samples were analyzed by Mississippi State Chemistry Laboratory using LC/MS-MS for validation of dosing concentrations.

Water quality was monitored continuously using the built-in sensor array and verified daily using a handheld YSI Quatro. As tadpoles grew, they were moved to a larger enclosure weekly. Tadpoles were fed Xenopus Express tadpole crumble food for the duration of the experiment. Initially, 1 pellet of crumble food per tadpole was left to soak for 1 hour in clean system water and once dissolved, the entirety of the liquid food

was fed to the tank. Animals were left at room temperature outside of flow-through conditions for 1 hour following feeding then returned to the flow-through chamber. As the tadpoles grew, an increasing amount of food was fed, and whole pellets were fed after tadpoles had transitioned from gills to lungs. Tanks were cleaned biweekly by siphoning out waste and detritus. Frogs were observed daily, and any injured or dead frogs were removed from the tank.

2.4 Adult Chronic Assay.

The chronic adult exposure was a 60-day flow-through exposure intended to investigate the response of adult frogs to chronic, low dose aqueous exposures at environmentally relevant concentrations. Frogs were ordered from Xenopus Express and shipped overnight. Upon arrival frogs were placed in 1cm of water and allowed to warm to room temperature for 30 minutes.

After the initial warming period, frogs were placed in individual 1 L tanks of water and visually inspected for any sign of injury or illness. Following visual inspection, frogs were transferred to 20 L holding tanks segregated by sex. The animals were physically isolated in the holding tanks, however both tanks shared the same water. The holding tanks were 30 L continuous flow holding tanks running at a flow rate of approximately 1.5 L/hr. Tank water was UV sterilized, mechanically filtered, and temperature adjusted artesian well water. Water testing performed within 1 year of the experiment verified that the water was within physical and chemical limits for experimentation. The holding tanks were maintained at 21 ±3°C and on a 12h day/12h night light cycle. Frogs were fed measured amounts of Zeigler Xenopus Diet 3 times weekly. Males received 3 pellets each per feeding, and females received 6 pellets each

per feeding. Approximately one hour after feeding, tanks were cleaned, and any uneaten food was siphoned from the tanks.

Table 2.2 Flow through chamber operating parameters for the adult assay.

Temperature	22° C
Salinity	0.15 ppt
Dissolved Oxygen	8.5 mg L^{-1}
pH	7.5
Flow Rate	1.5 L hr^{-1}
Residence Time	20 hr
Stock Renewal Frequency	48 hr

Similar to the metamorphosis experiment, this experiment was run in the flow-through exposure facility at the University of Southern Mississippi's Gulf Coast Research Laboratory. Exposure facility flow-through parameters can be found in Table 2.2. Water quality was continuously monitored using the facility's in-house sensor array and checked daily using a YSI Quatro. Water samples were collected from the effluent tube of the exposure tanks at set-up, 4 weeks, and termination. These samples were sent to Mississippi State Chemical Laboratory for analysis via LC-MS/MS. At termination, blood was drawn via cardiac puncture and plasma was separated and frozen, then the frogs were euthanized via double pith and cervical dislocation. Immediately following euthanasia, frogs were dissected, and liver, kidney, digestive tract, gonads, and spleen were removed for follow up analysis.

Preserved samples were then used for qPCR, cholesterol analysis, and sex steroid analysis. Samples used for qPCR underwent mRNA extraction using spin column extraction (Qiagen #74106) and reverse transcription to cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo #K1621). Extracted cDNA was then used for qPCR

to determine gene dysregulation as described in the molecular techniques. Samples tested for cholesterol and sex steroids used associated protocols described in the molecular techniques section of this document.

2.5 Molecular Techniques.

Spin column RNA isolation was performed following the RNeasy Mini Kit (Qiagen #74106). Following extraction and isolation of mRNA, samples were reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Thermo #K1621). Following cDNA synthesis, samples were used in follow up qPCR reactions. Primers were designed using Primer3 and purchased from Integrated DNA technologies. Primer efficiencies are listed in Table 2.3. Quantitative PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR system using Fast SYBR Green Mater Mix (Applied Biosystems #4385612) and associated protocol.

Table 2.3 Primer efficiencies for primers used in qPCR reactions.

Primer	Sequence	Efficiency
18s	CGT CGC TAC TAC CGA TTG GA CGC AGG TTC ACC TAC GGA AA	96.45 %
hmgcr	TGA TTG GCC GTA GGA AGA GC TGG TGA TTG TGA AGC TCG GG	95.68 %
cyp3a4	GAG CCC ATT ACT CAT CTT AGT GGT CAC GTC CCG AGT GAT CTC C	96.06%
cyp19a1	TGC ACG TCT CCT AAC TTT ACT GG GAG TGG GAA CAG CTT CTG TGA	94.54 %

Lipid extraction from tissues for follow-on cholesterol quantification was performed using a chloroform free Lipid Extraction Kit (abcam #ab211044). This protocol was only used in the FETAX assay for extraction of whole-body cholesterol from tadpoles. Cholesterol quantification was performed on plasma as well as tissue

extract using Amplex Red Cholesterol Assay Kit (Thermo #A12216) and associated protocol.

Testosterone and estradiol concentrations in plasma were measured using a Testosterone ELISA Kit (abcam #108666) and 17 beta Estradiol ELISA Kit (abcam #ab108667) respectively. These kits are designed for use in human plasma, therefore both ELISA kits were validated using *X. laevis* plasma to verify hormone binding to the antibody. Plasma used in the testosterone ELISA was diluted 100x and plasma for the estradiol ELISA was dilute 1000x to keep all sample RFU measurements within the standard curve.

RNA sequencing was performed on 6 samples at the University of Colorado's Core Microarray facility. The six samples were selected from the metamorphosis assay and consisted of three control samples and three samples from the 4.4 ug L-1 treatment. Briefly, RNA was isolated using the previously described protocol. Verification of RNA integrity was performed using a NanoDrop 2000 to evaluate purity, and an Agilent Bioanalyzer 2100 to evaluate RNA integrity. Once purity and integrity were determined to be acceptable for sequencing, samples were shipped overnight in dry ice to the University of Colorado. RNA sequencing and library preparation was performed on a NovaSEQ 6000 using 2x150 paired end reads.

CLC Genomics Workbench (CLC) was used for initial data preparation and mapping to the *X. laevis* reference genome (NCBI accession number: GCA_001663975.1). Data preparation included removal of failed and low-quality reads as well as adaptor trimming. Differential gene expression analysis was then performed on the control versus the treatment group.

Ingenuity Pathway Analysis (IPA) was used for determination of pathway dysregulation. One of the limitations of IPA is that reference genes must be mapped to their human ortholog as pathway data is not available for the amphibian genome. Since molecular pathways are widely considered to be highly conserved across taxa, this limitation was deemed to have a minimal impact on pathway analysis. The FDR cutoff was set to 0.05, and pathway analysis was determined using expression fold change determinations from CLC.

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CHAPTER III – FROG EMBRYO TERATOGENESIS ASSAY, XENOPUS

3.1 Introduction

The purpose for the FETAX assay in this project was to evaluate the uptake, activity, and efficacy of atorvastatin in the amphibian model. Uptake was defined as integration into the bloodstream. This is accomplished primarily by either osmosis via cutaneous respiration or *Cyp3a4* mediated transport across the intestinal lumen. Atorvastatin activity was defined by upregulation of *hmgcr* and was a measure of substrate binding activity allowing the drug to produce both target and non-target effects. Substrate efficacy was defined as target effects produced downstream of the site of activity and was be measured via cholesterol concentrations in tissue.

As previously discussed, the FETAX is an acute 96-hour assay. It is a highly sensitive, highly studied experimental protocol useful in determining growth and developmental abnormalities. Our purpose for choosing the FETAX protocol is its tight experimental parameters and its ability to rapidly evaluate the effects of atorvastatin exposure. Embryos were collected and dejellied immediately following fertilization. Normal embryos between NF stage 8 and 11 were selected and randomly distributed into petri dishes. A selection of 5 increasing concentrations determined from the FETAX experimental protocol, plus a control and carrier control were set-up with replicates (Figure 3.1). The embryos were grown out for 96 hours in a temperature-controlled incubator at 28C under constant supervision with water changes every 24 hours. At termination, images of each tadpole were taken for morphometric analysis, and the whole organism was preserved for cholesterol quantification and qPCR analysis by flash freezing in liquid nitrogen or storage in RNALater respectively.

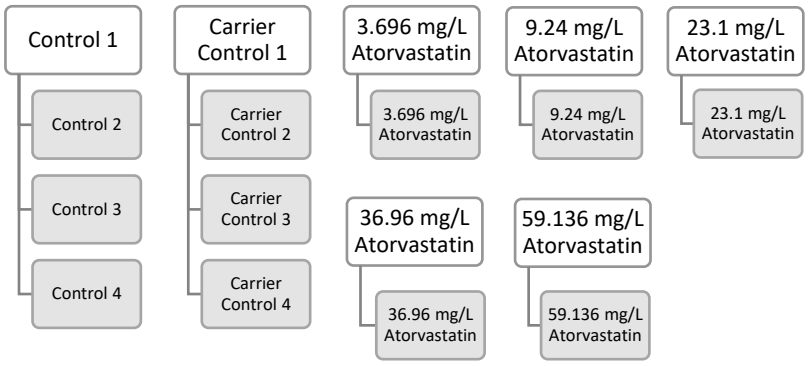

Figure 3.1 FETAX experimental design.

Frogs were bred as a single mated pair, and only the embryos from one single pair were used in the experiment. Frogs were injected with 350 and 700 IU human chorionic gonadotropin (HCG) for males and females respectively. Injections were given into the dorsal lymph sac at a concentration of 1000IU/mL in sterile 0.9 % NaCl using a 26-gauge needle. Twelve hours following injection, eggs were collected and dejellied immediately.

De-jellying embryos was performed as soon as egg collection was complete. Eggs were transferred to a clean 1L beaker and swirled for ~2 minutes using de-jellying solution described in the protocol(ASTM INTERNATIONAL, 2012). Once the jelly coat was sufficiently removed, embryos were rinsed 3 times in FETAX solution(ASTM INTERNATIONAL, 2012) and transferred to petri dishes for staging.

During staging and selection, embryos were double staged prior to selection for experimentation. First embryos were selected for normal cleavage consistent with “Atlas of Abnormalities”(Nieuwkoop P., 1994). Next embryos were selected that were between

NF 8 and NF 11. If selected, embryos were moved to petri dishes of 25 embryos per dish containing 10 mL FETAX solution.

Petri dishes were then randomly assigned to replicate groups. There were four control replicates, four carrier control replicates, and 2 dishes for each concentration of 0.003696, 0.00924, 0.0231, 0.03696, and 0.059136 mg mL^{-1} respectively. Dishes were then spiked with atorvastatin dissolved in DMSO, or DMSO only for carrier control. Spike solution did not exceed 1% of FETAX solution volume, and was temperature adjusted to 28C prior to introduction for petri dishes.

Dishes were then stored overnight in an incubator at 28C until renewal. Test solution was completely replaced every 24 hours. Prior to renewal, the pH of the control and the highest dose dishes was measured to monitor for large pH differences in the presence of toxin. For renewal, all test solution was removed using a pasteur pipette with an orifice large enough to allow the embryos to pass through to prevent damage. Next, new test solution was then immediately poured into the dish. Dishes were then returned to the incubator. This process was repeated daily for the duration of the 96-hour experiment.

At test completion, dishes were photographed using a Nikon SMZ1500 dissecting microscope fitted with a Nikon DXM1200C digital camera and embryos were euthanized using a 2.5% v/v tricaine solution. Embryos were then transferred into 1.5 mL microcentrifuge tubes containing 1 mL of RNALater. Tubes were allowed to rest at room temperature for one hour to allow complete infiltration of preservative, then transferred into -80C until downstream analysis.

Validation of test concentrations was performed by spectrofluorimeter using a previously validated method (Sharaf El-Din, Salama, Nassar, Attia, & Kaddah, 2012). This method allowed simple, and rapid, same-day analysis of FETAX test solution to monitor and verify test concentrations.

3.2 Endpoints and Protocols

Preserved embryos were used for qPCR and cholesterol analysis. Embryos used for qPCR underwent mRNA extraction using spin column and reverse transcription to cDNA. Extracted cDNA was then used for qPCR to determine gene dysregulation as described in the molecular techniques section of this manuscript.

The FETAX assay in this project was intended to verify that the response of the amphibian model is similar to the response in humans. As such, the biomarkers selected for this assay were carefully designed to map directly to the human response to atorvastatin exposure. The protein encoded by *cyp3a4* is responsible for transporting atorvastatin from the digestive system into the blood stream. Studies have shown that in the presence of atorvastatin, *cyp3a4* will be transcriptionally upregulated in order to maintain sufficient enzyme in circulation (Willrich et al., 2013). In the FETAX assay I expected to see a similar response in *X. laevis* with upregulation of *cyp3a4* indicating uptake of atorvastatin. Atorvastatin is a competitive inhibitor *hmgcr*, and exposure causes transcriptional upregulation of the genes encoding for *Hmgcr* (Brown & Goldstein, 2009). This provides a useful biomarker indicating activity of atorvastatin.

Finally, the target effect of atorvastatin is a reduction in circulating cholesterol (Lennernäs, 2003). While this study is intended to investigate non-target effects of atorvastatin, evaluating cholesterol levels serves as a measure of efficacy by determining

if atorvastatin has reached circulatory concentrations high enough to elicit the targeted response, in this case, reduction of circulating cholesterol. Eliciting this response is the primary reason for the use of concentrations many orders of magnitude above environmental relevance in the FETAX. If atorvastatin functions in amphibians in the same manner as in humans the molecular signature should be similar to humans. This is upregulation of *cyp3a4* and *hmgcr*, and reduction of total cholesterol levels. Once verified, this signature was then applied to follow-on studies for validation of atorvastatin dosing in chronic aqueous assays.

3.3 Results and Discussion

3.3.1 Water Chemistry

Because I had previous data concerning aquatic toxicity of atorvastatin (Pfizer, 2018), I was able to skip the range finder test, and move directly to the definitive test in the FETAX. Test concentrations tested were 0.03, 0.05, 0.08, 0.10, and 0.12 mg L^{-1}. These concentrations were defined based on the strict FETAX experimental protocol and allow future comparisons to other FETAX data. I chose to take the lowest NOEC in the available data, and test concentrations ranging down from that concentration. This enabled us to test at the lowest possible concentrations and was particularly useful considering the extremely low concentrations to be tested during the subsequent assays.

The mean atorvastatin concentrations measured daily at each water change were 0.0142, 0.0112, 0.0489, 0.0564, and 0.1193 mg L^{-1} (Figure 3.2). The test solution fell into three statistically significant groups based on a One-Way ANOVA with Holm-Sidak pairwise comparisons ($p < 0.001$ $F = 20.98$, $df = 13$). The control, carrier control, 0.0142, and 0.0112 mg L^{-1} concentrations were statistically similar, however they were

different than all other groups. The 0.0489 and 0.0564 mg L^{-1} concentrations were statistically similar, but significantly different than all other groups. The highest concentration of 0.1193 mg L^{-1} was significantly different than all other groups. Based on these data, molecular endpoints were evaluated for samples produced from the 0.0489 and 0.1193 mg L^{-1} concentrations as these concentrations were significantly different from the controls.

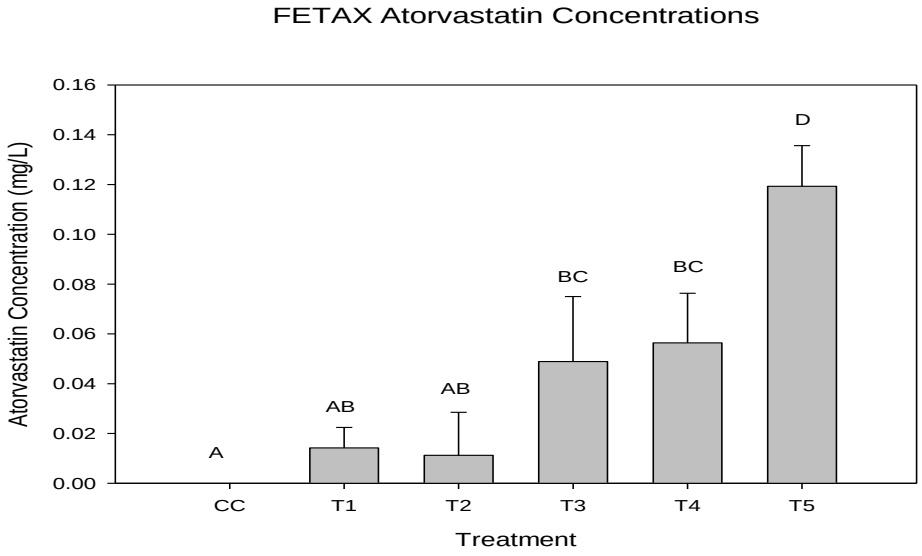

Figure 3.2 Mean atorvastatin concentrations and standard error of atorvastatin in exposure water immediately prior to water change.

Since I anticipated degradation of the atorvastatin over time for the duration of the experiment, I measured the concentration of atorvastatin in solution via fluorescence daily at water change (Figure 3.3). Overall, atorvastatin treated water saw a general degradation over time. This is expected since atorvastatin concentrations were measured prior to water change. The water had been in the exposure dishes for 24 hours, and some degradation of the stock is not uncommon. The highest concentrations saw the greatest decrease in statin levels over time. Collectively, all samples saw a degradation at each

water change. This is probably caused by stock degradation. The FETAX protocol requires a single stock solution created at the beginning of the experiment and used for all subsequent water changes if possible, meaning that stock degradation may occur over time.

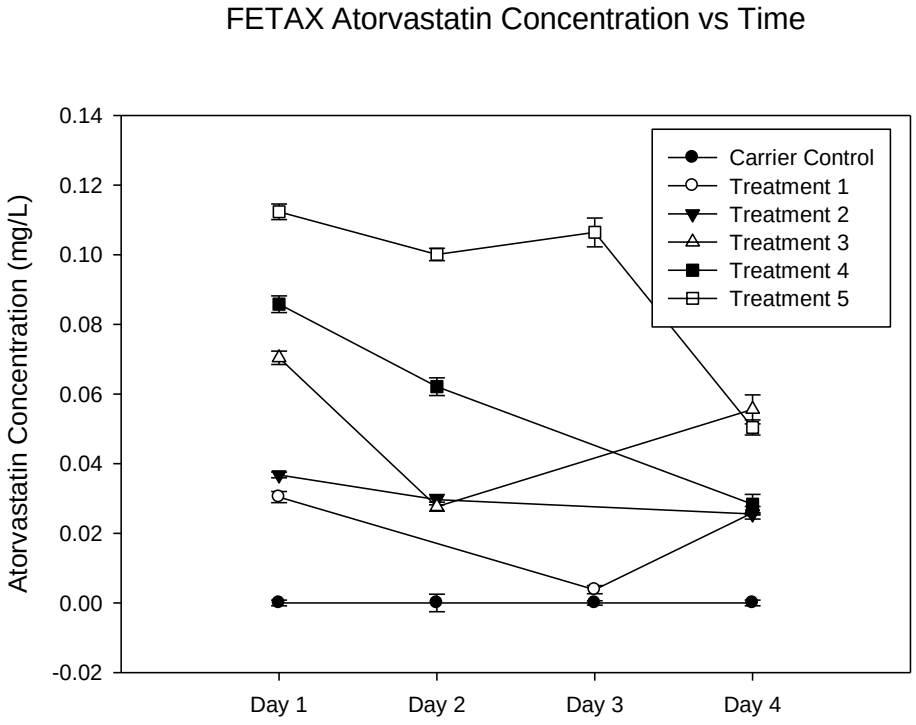

Figure 3.3 FETAX atorvastatin concentrations over time.

3.3.2 Molecular Endpoints

The metabolism and uptake of atorvastatin is mediated by the protein *Cyp3a4*. The qPCR data indicate statistically significant and dose dependent upregulation of *cyp3a4* (Figure 3.4). The control and carrier control treatments were not significantly different from each other but were statistically different from the atorvastatin treated doses base on One-way ANOVA with Holm-Sidak pairwise comparison ($p < 0.001$, $F = 25.56$, $df = 47$,). This indicates that the carrier did not have a significant effect on

transcriptional regulation of *cyp3a4*. The atorvastatin treatments of 0.0489 and 0.1193 mg L^{-1} were significantly upregulated relative to controls. The treated groups show increasing upregulation as dose increased. This significant upregulation indicates dose dependent metabolism of atorvastatin in the frog system as more *Cyp3a4* protein is produced to mediate uptake of atorvastatin as a greater concentration of substrate is available.

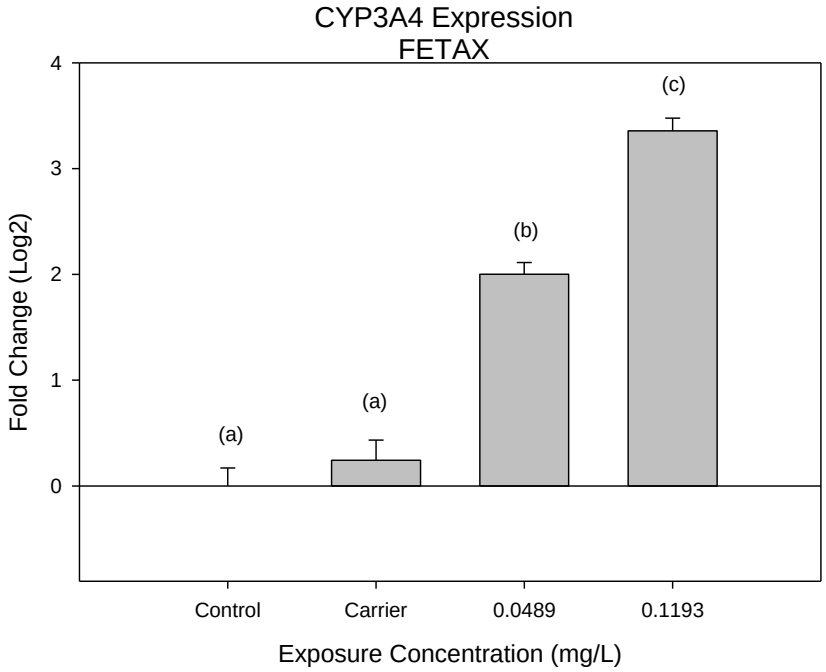

Figure 3.4 Gene expression of *cyp3a4* in the FETAX experiment.

The genes encoding for *Hmgcr* also presented significant upregulation of expression patterns (Figure 3.5). In this case, the control and carrier treatment were not significantly different from one another, and the lowest treatment of 0.0489 mg L^{-1}. The highest treatment of 0.1193 mg L^{-1} was significantly different from all other treatments. Upregulation of *hmgcr* expression was dose dependent, however upregulation of expression patterns was not as extreme as *cyp3a4* upregulation. The highest treatment

saw only 0.6833 upregulation relative to the carrier. This is consistent with a lag time associated with metabolism and uptake of atorvastatin prior to the distribution of atorvastatin throughout the circulatory system.

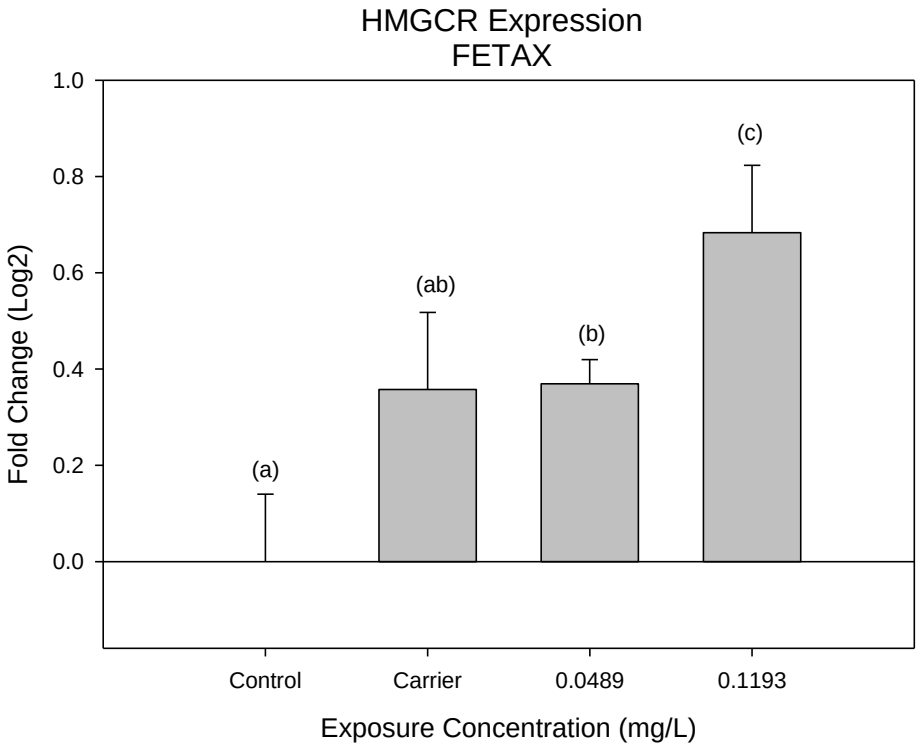

Figure 3.5 *hmgcr* expression in the FETAX experiment.

Overall, the molecular signature of atorvastatin dosing in humans was confirmed in the amphibian model. As expected, statistically significant upregulation of *cyp3a4* and *hmgcr* confirmed uptake and activity of atorvastatin. These data demonstrated *Cyp3a4* production increasing with increasing exposure concentrations resulting in statin uptake into the blood stream. Additionally, *hmgcr* follows a similar trend of upregulation, and this linkage confirms that *cyp3a4* upregulation is driving *hmgcr* upregulation as mevalonate is depleted and additional *Hmgcr* enzyme is created to attempt to convert additional mevalonate to fill this gap.

3.3.3 Cholesterol ELISA

Whole tissue cholesterol levels were analyzed via Amplex Red Cholesterol Assay. Samples were first extracted using a chloroform free lipid extraction kit (abcam #ab211044). Briefly, five tadpoles from each replicate were pooled (n=5) and homogenized prior to lipid extraction, and pooled samples were then analyzed for total cholesterol content. Replicates were then combined by treatment and mean cholesterol concentrations and standard deviation were evaluated against the standard curve associated with the previously discussed protocol.

Total cholesterol levels in the FETAX experiment showed a reduction in total cholesterol levels (Figure 3.6). One-way ANOVA with Holm-Sidak pairwise comparison of measured cholesterol ($p < 0.001$ $F = 56.54$, $df = 64$,) levels in the control and carrier control were not significantly different indicating the DMSO carrier had no effect on total cholesterol levels in the tadpoles. The lowest atorvastatin treatment of 0.0142 mg L^{-1} was not significantly different from the controls, however all higher concentration treatments were significantly different from the controls, and each other. This is indicative of the target action of atorvastatin dosing. These data show that a 96-hour exposure to statins at concentrations well above environmental relevance can elicit the targeted response of significant reduction in cholesterol. Furthermore, this reduction is achieved far faster than the 4-6 week timeframe suggested in the human model (Lennernäs, 2003).

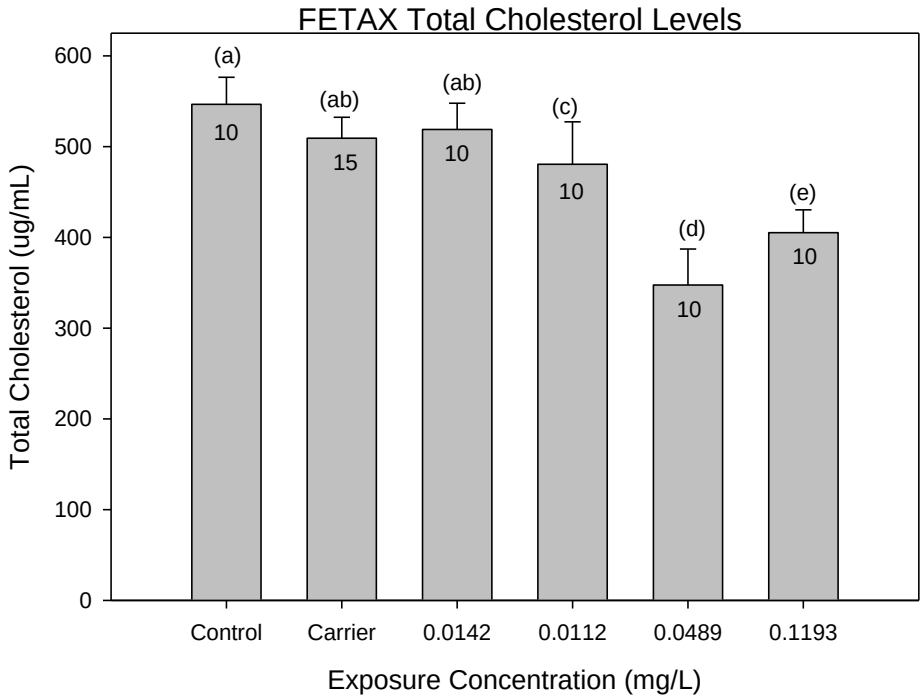

Figure 3.6 Total cholesterol levels of tadpoles in FETAX.

The number on each bar represents the number of samples represented in the data set.

3.4 Conclusions

The intent of the FETAX in this project was to confirm uptake, activity, and efficacy of atorvastatin on *X. laevis*. The data collected and analyzed show that *Cyp3a4* mediated uptake of atorvastatin and competitive inhibition of *Hmcgr* results in dose-dependent reduction of total cholesterol levels in tadpoles exposed to atorvastatin. These results are consistent with the previously discussed molecular and physiological signature associated with atorvastatin exposure in humans. These results lay the groundwork for the two follow-on exposures at environmentally relevant concentrations of atorvastatin and provide baseline reference data for predicting non-target effects of atorvastatin exposure in *X. laevis*.

CHAPTER IV – METAMORPHOSIS ASSAY

4.1 Introduction

The objective of the metamorphosis assay was to evaluate the effects of chronic, low concentrations of aqueous atorvastatin on developing tadpoles with additional emphasis on endocrine disruption. This assay used the molecular fingerprint confirmed in the FETAX to verify atorvastatin exposure and adds additional analysis of *cyp19a1* to evaluate the endocrine disruption potential of atorvastatin exposure. In addition, morphometric analysis of growth and developmental parameters was used to monitor for overt endocrine disruption and teratogenesis.

This experiment was a 60-day chronic flow-through exposure and concentrations for this experiment were 0.2, 0.7, and 0.5 $\mu g\ L^{-1}$ plus a control and there were two replicates of each concentration (n=40). Mortality was measured for the duration of the experiment only for confirmation of sub-lethal dosing. Due to the highly lipophilic nature of atorvastatin, DMSO was used as a solvent to assist the atorvastatin transfer into water. Atorvastatin was first diluted into DMSO to make the primary stock solution. The primary stock solution was then further diluted into water to created stock concentrations at the desired level. The same stock solution was used throughout the duration of the 60-day chronic exposure. Stock solution was renewed every 48 hours, and the primary stock was stored at -20 C to prevent degradation for the duration if the test.

4.2 Endpoints and Protocols

The endpoints for this experiment were morphometric analysis of growth and developmental parameters, and molecular analysis via qPCR of the previously described

biomarkers. Due to the small size of post-metamorphosis frogs, it was not possible to draw blood for cholesterol or steroid analysis.

Molecular biomarkers for this experiment were *cpy3a4*, *hmgcr* and *cyp19*. Statistical analysis of all qPCR data was performed using a One-way ANOVA with Holm-Sidak pairwise comparisons. The previously discussed biomarkers of *cyp3a4* and *hmgcr* were used to evaluate uptake and activity of atorvastatin in this exposure. Since the post-metamorphosis frogs were too small to collect sufficient quantities of blood for cholesterol analysis, yet far too large for whole tissue analysis of cholesterol content, these two biomarkers will serve to confirm the efficacy of atorvastatin in this exposure by confirming the same directionality of transcriptional dysregulation.

The addition of *cyp19a1* as a biomarker to evaluate endocrine disruption allows additional molecular data to support the morphometric analysis. While the morphometric analysis can detect overt, severe effects of endocrine disruption, *cyp19a1* is far more sensitive in detection of changing sex steroid levels, an early predictor of endocrine disruption. As previously discussed, the *Cyp19a1* protein is responsible for conversion of testosterone to estradiol. This conversion happens as a negative feedback loop when faced with declining estradiol levels. As less estrogen is available to bind the estrogen receptor, estrogen receptor related proteins dissociate from the estrogen receptor complex and bind to the promotor region of *cyp19a1*. This promotes transcription of the *Cyp19a1* enzyme to generate more enzyme for conversion of testosterone to estradiol. It is this negative feedback loop that makes *cyp19a1* an attractive biomarker for evaluating changing sex steroid levels. Upregulation of *cyp19a1* will be indicative of activation of this feedback loop due to declining estradiol levels relative to control.

Morphometric analysis included measurement of snout-vent length, mass, and NF stage. Snout-vent length and mass were recorded at experiment termination. Snout-vent length was measured using stainless steel analog calipers on live frogs. Mass was recorded by placing the live frog in a tared beaker of water and recording mass. Growth stage (NF stage) was measured visually by only one observer. As these were post metamorphosis frogs, microscopy and blind observers were not deemed necessary due the relative ease of determining growth stage on frogs post-metamorphosis.

4.3 Results and Discussion

4.3.1 Water Chemistry

Water samples were taken at set-up, the midpoint, and termination of the experiment. These samples were collected directly from the effluent lines for each replicate tank. The samples were then frozen and shipped to the Mississippi State Chemical Laboratory for analysis. The results of this independent analysis of atorvastatin concentrations in test water served to validate test parameters. Figure 4.1 contains average concentrations and standard deviation (represented by error bars) of atorvastatin in exposure water. The low treatment was not significantly different, based on One-way ANOVA ($p < 0.001$ $F = 612.699$, $df = 23$,), from the control, and thus was not used in follow-on data analysis.

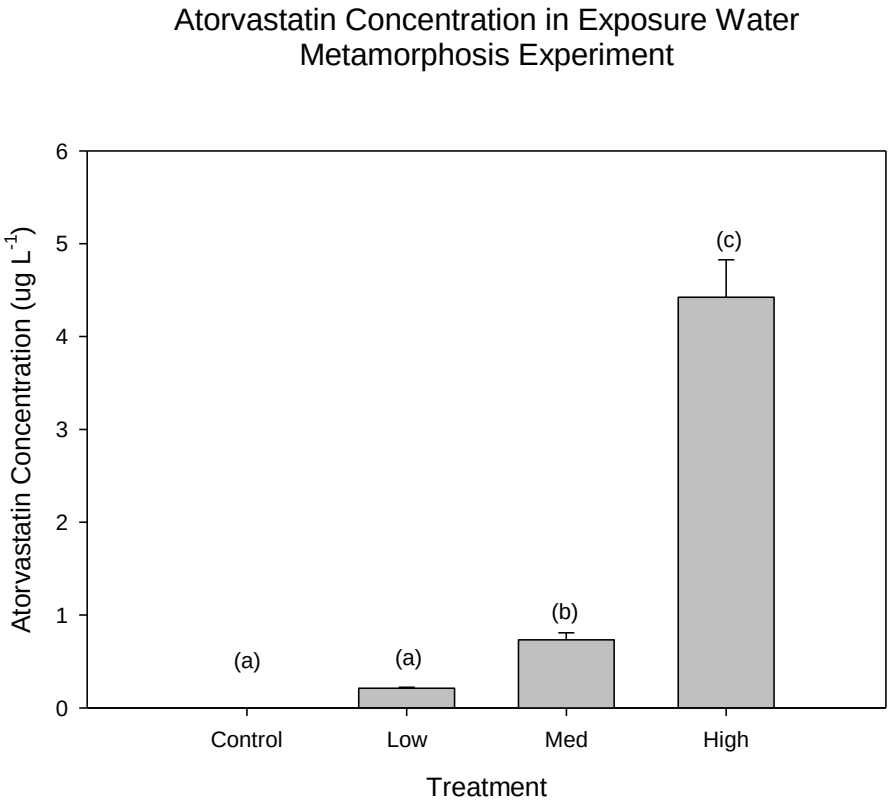

Figure 4.1 Atorvastatin concentrations in exposure water for the metamorphosis experiment.

The average water quality measurements and standard deviations are reported in figure 4.2. Water quality parameters were monitored continuously by the toxicology assay chamber, and randomly verified using hand held equipment daily. Ammonia levels were 0 ppt for the duration of the experiment and are not represented in graphs. All treatments were not significantly different from one another with regards to any water quality parameters.

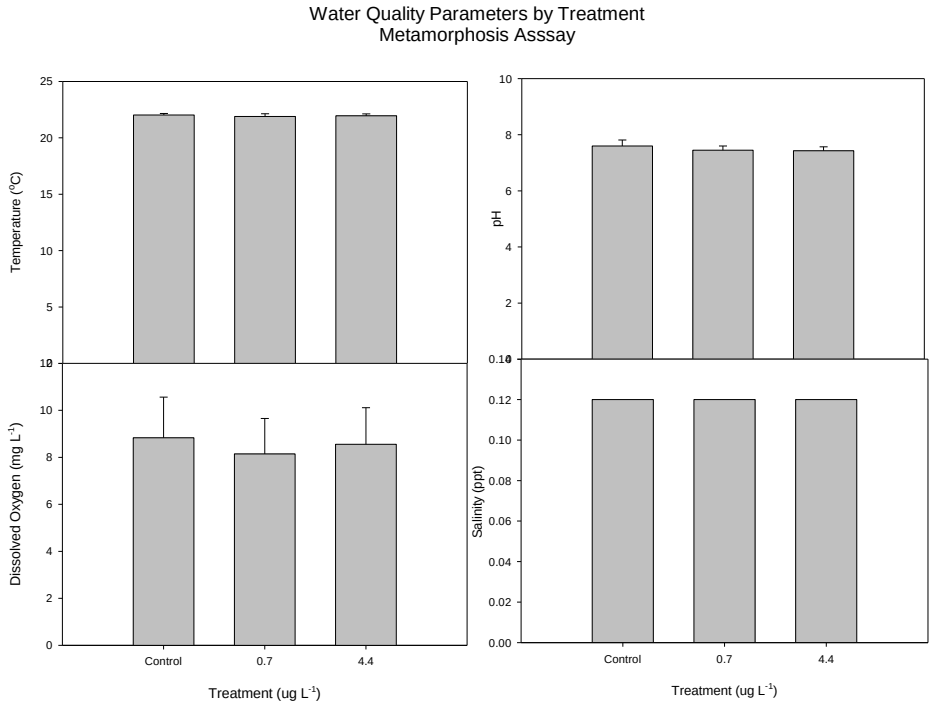

Figure 4.2 Exposure tank water quality parameters by treatment.

4.3.2 Molecular Analysis

At termination, liver samples were collected, and flash frozen for follow-on molecular analysis. The liver tissue was extracted and reverse transcribed following the previously described protocols.

Expression of *cyp3a4* is a biomarker of exposure to atorvastatin. As previously discussed, *cyp3a4* is used an indicator of atorvastatin uptake. Figure 4.3 shows differential gene expression of *cyp3a4* in tadpoles exposed to atorvastatin the metamorphosis experiment ($p < 0.05$, $F = 12.59$, $df = 8,$). These data indicate slight upregulation of *cyp3a4* relative to control in both the 0.7 and 4.4 ug L^{-1} concentrations, however this effect is not statistically significant. The reason for such insignificant upregulation may be differing exposure routes. As previously discussed, the primary site

of *cyp3a4* mediated uptake occurs in the lining of the digestive tract. This is somewhat problematic in that the gene expression analyzed here is from liver tissue. However, studies have shown that *cyp3a1* upregulation is also seen in the liver when an organism is exposed to atorvastatin, but not to the same extent as in digestive tissue. Additionally, this is an aqueous exposure that is bypassing the digestive tract and entering systemic circulation via cutaneous respiration as well as uptake at the gills pre-metamorphosis. In light of this, it is sensible to evaluate *cyp3a4* expression levels in the liver, and unsurprising that very little upregulation of *cyp3a4* expression is evident in this exposure. The 4.4 ug L^{-1} concentration showed less extreme upregulation than the 0.7 ug L^{-1} concentration.

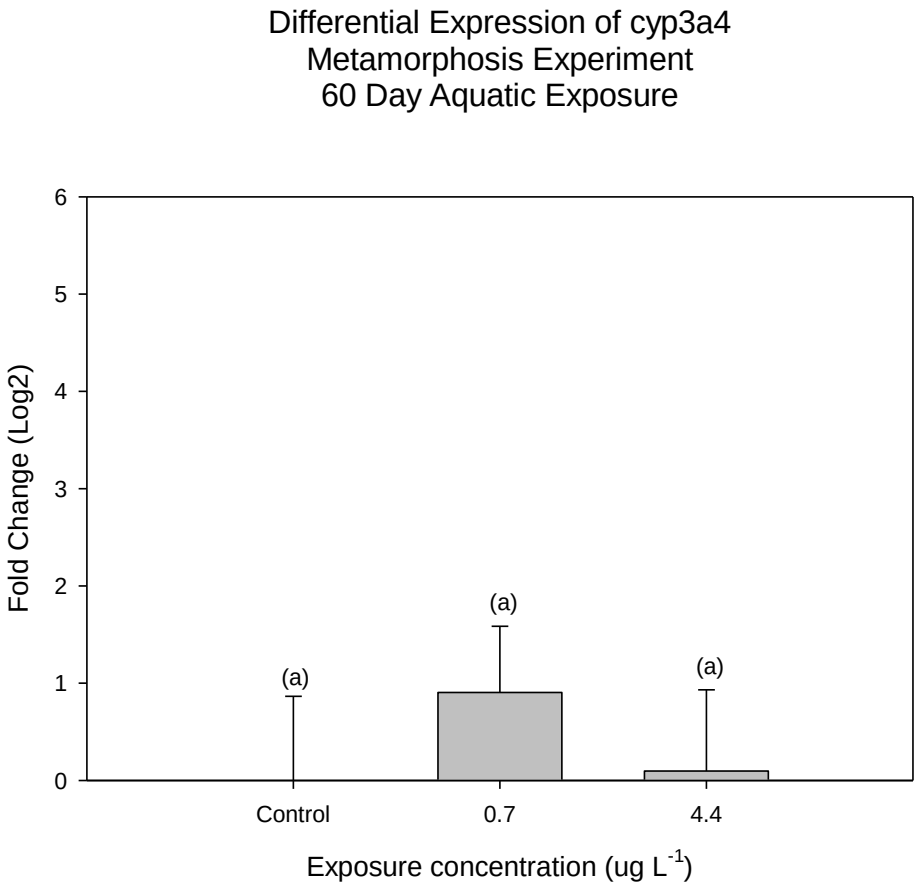

Figure 4.3 Differential expression of cyp3a4 in metamorphosing tadpoles exposed to the indicated concentrations of atorvastatin.

Expression of *hmgcr* served as a biomarker indicating activity of atorvastatin. Figure 4.4 shows differential gene expression of *hmgcr* in tadpoles exposed to atorvastatin in the metamorphosis experiment ($p < 0.05$ $F = 20.34$, $df = 8,$). These data indicate upregulation of *hmgcr* relative to controls for both the 0.7 and 4.4 ug L^{-1} concentrations. The expression levels were not statistically different from one another indicating similar activity of atorvastatin despite differences in *cyp3a4* expression levels previously discussed. This upregulation in *hmgcr* expression levels relative to controls indicates atorvastatin competitively inhibiting *Hmgcr*. When viewed in conjunction with the *cyp3a4* data, it becomes apparent that despite atorvastatin bypassing the digestive

exposure route, cutaneous respiration still allows significant enough levels of atorvastatin in the blood to maintain efficacy.

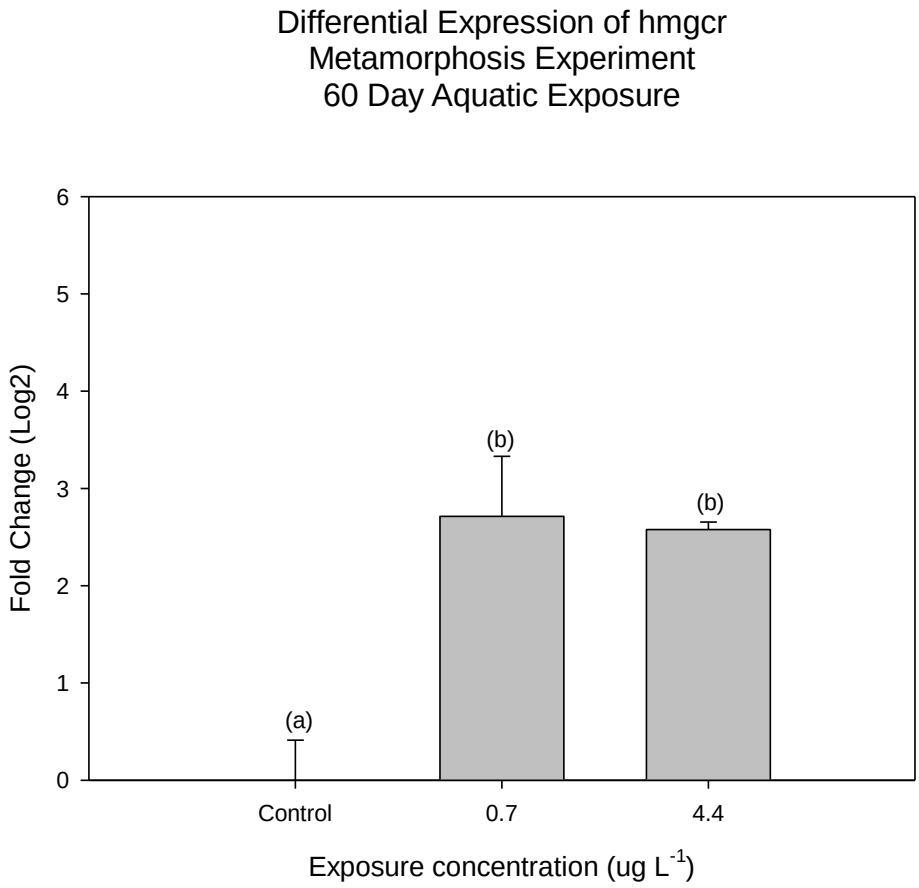

Figure 4.4 Differential expression of hmgcr in metamorphosing tadpoles exposed to the indicated concentrations of atorvastatin.

The protein encoded by the *cyp19a1* gene is responsible for conversion of testosterone to estradiol. This gene serves a biomarker indicating dysregulation of the steroidogenesis pathway. Figure 4.5 shows differential gene expression of *cyp19a1* in liver tissue of post-metamorphosis froglets exposed to atorvastatin through metamorphosis. These data show statistically significant, dose dependent, upregulation *cyp19a1*. As previously stated, this upregulation is indicative of declining estradiol levels

in the frogs. The dose dependent trend of *cyp19a1* indicates that as atorvastatin concentrations in water increase, the extent to which *cyp19a1* transcription is upregulated also increased. This is occurring despite similar activity levels in *hmgcr* transcription rates indicating that while the cholesterol biosynthesis pathway displays a degree of resistance to dysregulation, the steroidogenesis pathway is still altered dramatically. The cholesterol biosynthesis pathway is highly conserved and tightly regulated and is surprisingly resistant to xenobiotic alteration of transcriptional profiles, however, as seen in these data, only slight alteration of cholesterol biosynthesis is enough to significantly alter steroidogenesis.

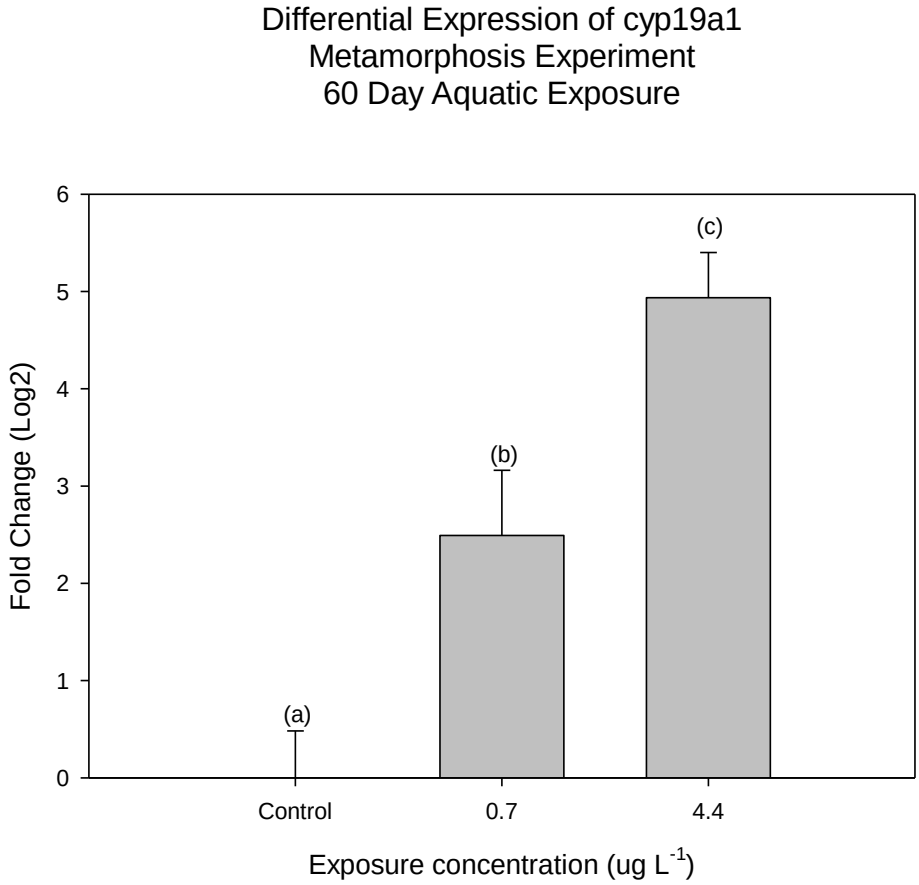

Figure 4.5 Differential expression of cyp19a1 in metamorphosing tadpoles exposed to the indicated concentrations of atorvastatin.

4.3.3 Morphometric Analysis

In addition to the previously discussed molecular endpoints, this experiment included morphometric endpoints. These endpoints were length, mass, and NF stage. NF stage was measured two times during the experiment, at set-up and take-down. Figures 4.6 and 4.7 show mean frog length and mass respectively with standard deviation represented by error bars. These data showed no significant difference in either endpoints based on One-way ANOVA ($p = 0.136$ $F = 2.01$, $df = 173$) indicating aqueous exposures at these concentrations have no significant effect on tadpole growth. Figure 4.8 shows mean NF developmental stage of post-metamorphosis tadpoles in this exposure (n=40/treatment). While a slight downward trend is evident, the difference is not enough to be statistically significant based on One-way ANOVA ($p = 0.981$, $F = 0.019$, $df = 119$). The lack of error bar on the control is due to the deviation being 0 as all animals in the control tanks completed metamorphosis. The downward trends in the treated tanks are driven by a small number of tadpoles that did not complete metamorphosis. As previously mentioned, this trend was not significant enough to indicate developmental dysfunction, however, this does warrant further observation in the future.

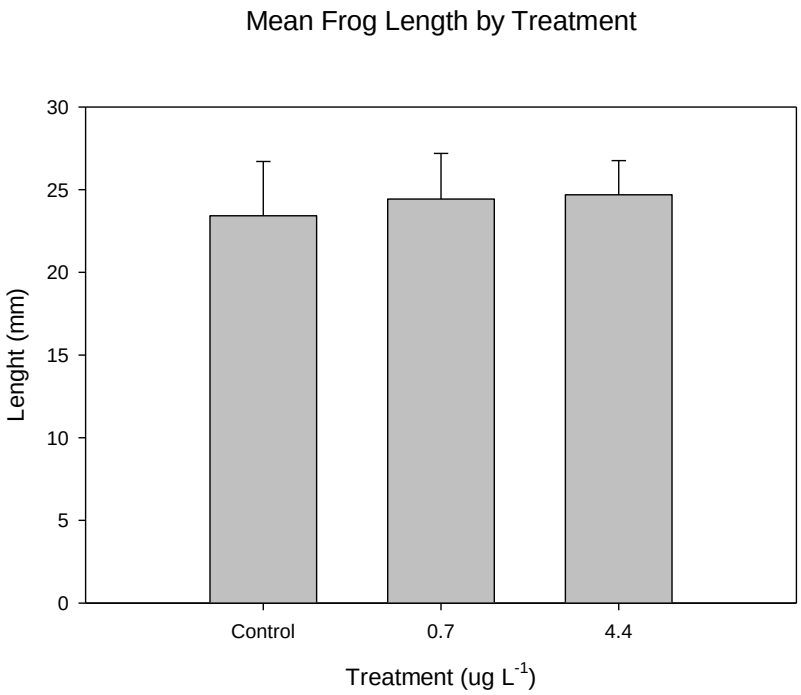

Figure 4.6 Mean length and standard deviation of tadpoles treaded with aqueous atorvastatin (n=40 per treatment).

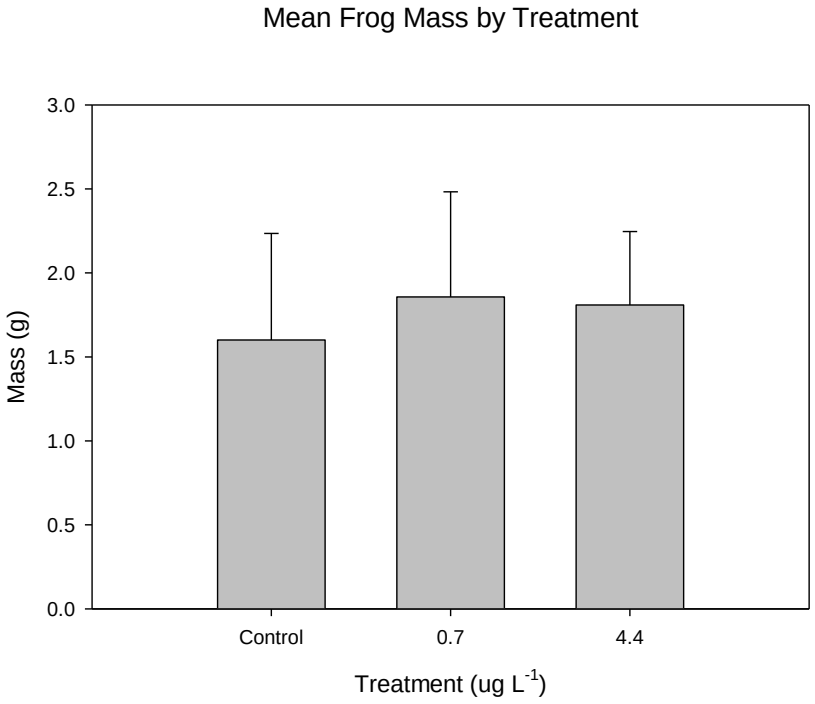

Figure 4.7 Mean wet mass and standard deviation of tadpoles treaded with aqueous atorvastatin (n=40 per treatment)

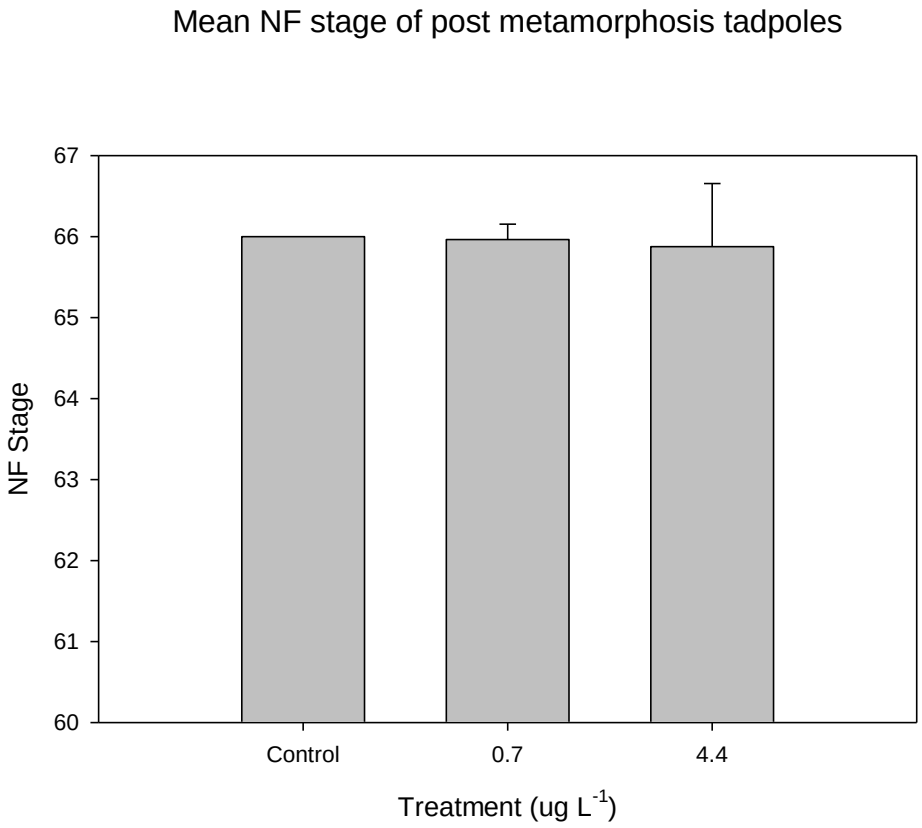

Figure 4.8 Mean NF developmental stage of post-metamorphosis tadpoles exposed to aqueous atorvastatin.

4.3.4 RNA Sequencing and Bioinformatic Analysis

In addition to the traditional qPCR analysis of gene expression, sequencing of total RNA transcripts and subsequent bioinformatic analysis was employed for the metamorphosis assay to evaluate dysregulation of molecular pathways associated with exposure to aqueous atorvastatin. Figures 4.9 and 4.10 show the top 30 most dysregulated canonical pathways as determined by IPA analysis of differentially expressed genes in the data set. The darker orange and red colors indicate activation of the pathway, and the corresponding shades of blue indicate suppression of the pathway. The grey bars indicate dysregulation; however, the software was unable to determine directionality in terms of activation or suppression. This is most often due to individual

genes in the pathway not behaving as predicted. For instance, if the software sees upregulation in a specific gene, and predicts corresponding upregulation of a subsequent gene in response, but instead the data indicate downregulation of that downstream gene, then the software is unable to determine directionality. This type of result should not be viewed as a negative result or lack of data, but rather it indicates that the pathway has been altered, and now it is not behaving as literature predicts is should behave.

Figure 4.9 Top 30 dysregulated pathways in X. laevis exposed to aqueous atorvastatin during metamorphosis.

Figure 4.10 Top 30 dysregulated pathways (continued) in X. laevis exposed to aqueous atorvastatin during metamorphosis.

These data indicate extensive DNA damage and associated repair mechanisms for responding to damage. Thirteen of the top 30 significantly dysregulated pathways are associated with this DNA damage response, indicating extensive DNA damage repair in metamorphosing tadpoles exposed to aqueous atorvastatin. Additionally, two of the top 30 pathways indicate oxidative stress response. This points to generation of reactive

oxygen species (ROS) causing DNA damage in exposed tadpoles. The link between ROS generations and DNA damage is well supported in literature (Friedberg, McDaniel, & Schultz, 2004), as is the link between atorvastatin and ROS generation (Bouitbir et al., 2011, 2016). Interestingly, human biomedical literature suggests the opposite effect of reduction of oxidative stress primarily driven by a reduction in endogenous production of cholesterol resulting in decreased metabolism of fatty acids and subsequently decreased generation of ROS (Briones et al., 2009; Ceriello et al., 2005; Sugiyama et al., 2005). These opposing findings suggest differing responses to atorvastatin in humans versus rats in the cited studies, and amphibians in this study. This gap in understanding warrants future investigation into causes of these differing responses.

Additionally, estrogen biosynthesis and aldosterone signaling are upregulated in the data set supporting the hypothesis of upregulation of the steroidogenesis pathway in atorvastatin exposure. Furthermore, Figure 4.11 shows the effect of atorvastatin exposure on master regulators of steroid metabolism. All master regulators in this data set were significantly upregulated relative to control, further supporting the hypothesis of upregulation of steroidogenesis concurrent with exposure to environmentally relevant concentrations of aqueous atorvastatin

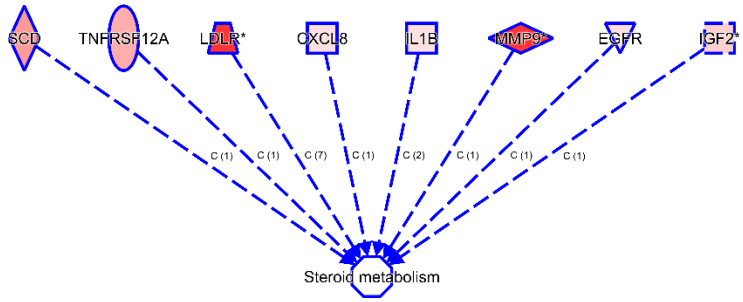

Figure 4.11 Effect of atorvastatin exposure of master regulators of steroid metabolism with upregulated genes indicated in red.

4.4 Conclusions

The purpose of this assay was to evaluate potential endocrine disrupting effects of environmentally relevant concentrations of aqueous atorvastatin on tadpoles undergoing metamorphosis. This experiment used molecular biomarkers previously validated by FETAX as measures of atorvastatin uptake and activity as well as introducing *cyp19a1* as a biomarker for dysregulation of the steroidogenesis pathway. Additionally, morphometric analysis of length, mass, and NF developmental stage were used as gross measurement of endocrine disruption.

Analysis of molecular biomarker expression levels indicated minimal *cyp3a4* mediated atorvastatin uptake. This is unsurprising considering the non-traditional exposure routes in this assay design. Cutaneous respiration is allowing for atorvastatin dosing despite lack of digestion. This is allowing sufficient quantities of atorvastatin to enter the blood stream for drug activity as indicated by the significant upregulation of *hmgcr*. Additionally, strong dose-dependent upregulation of *cyp19a1* suggests upregulation of the steroidogenesis pathway and declining estrogen levels corresponding to atorvastatin dose as previously discussed. This is further supported by RNA sequencing and bioinformatic analysis. These data showed upregulation of the aldosterone signaling and estrogen biosynthesis pathway as well as blanket upregulation of the master regulatory network associated with steroid metabolism.

Additionally, the RNAseq data also suggests significant generation of ROS and corresponding DNA damage repair. This effect is confirmed in rats, but the opposite response is seen in human biomedical literature. This differing response warrants further investigation and suggests a potential non-target effect not evident in humans.

The morphometric analysis of growth and developmental parameters did not show overt alteration of development suggesting that low concentration transient exposures to aqueous atorvastatin do not affect amphibian metamorphosis rates. However, the slight downward trend in NF developmental stage suggests that increased exposure concentrations or duration could magnify this effect and alter amphibian metamorphosis. This is particularly likely if tadpoles are exposed to atorvastatin from fertilization to NF 47, which is where this test initiated. Based on these data, environmentally relevant

concentrations of aqueous atorvastatin have an adverse effect of steroidogenesis by significantly dysregulating steroidogenesis.

CHAPTER V – ADULT ASSAY

5.1 Introduction

The purpose of the adult chronic assay was to evaluate potential endocrine disrupting effects of environmentally relevant concentrations of aqueous atorvastatin in *X. laevis*. By sampling at the 4-week and 8-week time points, we add an additional temporal analysis for better resolution of effects timelines. This assay was performed at the same environmentally relevant concentrations as the previous metamorphosis exposure and under the same flow-through conditions. At termination, liver and digestive tissue, as well as blood, were collected and preserved for future analysis. The liver and digestive samples were tested for the molecular biomarkers *cyp3a4* (digestive), *hmgcr* (liver), and *cyp19a1* (liver). These biomarkers were used in the previous assays to evaluate atorvastatin uptake and activity, and dysregulation of steroidogenesis, respectively. Those biomarkers were employed again in the adult assay for the same purposes. In addition to the molecular biomarkers, the larger size of adults allows blood draw and isolation of large enough volumes of plasma to perform additional analysis of testosterone and estradiol levels as well as total plasma cholesterol concentrations. These allow for additional investigation into potential endocrine disrupting effects of atorvastatin exposure on *X. laevis*.

5.2 Endpoints and Protocols

The endpoints for this assay were qPCR analysis of selected genes, hormone analysis of testosterone and estradiol, and plasma cholesterol quantitation. As previously discussed, the molecular biomarkers employed for this assay have particular molecular signatures that are useful in evaluating and confirming atorvastatin dosing. Statistical

analysis of all qPCR data was performed using a One-way ANOVA with Holm-Sidak pairwise comparisons. When dosed with atorvastatin, the expected response is transcriptional upregulation of *cyp3a4* and *hmgcr* in digestive and liver tissue, respectively. Previously, atorvastatin exposure caused transcriptional upregulation of *cyp19a1* and I anticipated a similar response in adults.

With the additional ability to collect plasma from blood samples, additional assays were performed to measure testosterone, estradiol, and total cholesterol concentrations in plasma. The testosterone and estradiol ELISA kits were previously validated for use in *X. laevis* using total cholesterol collected from whole body tissue of tadpoles from the FETAX experiment. The Amplex Red Cholesterol Assay was validated from the manufacturer for use in *X. laevis*, and additional validation was deemed unnecessary. Due to the extremely low concentrations of estradiol in males, and testosterone in females, it was not possible to test for opposing sex steroids. Thus, males were evaluated for testosterone concentrations, and females were evaluated for estradiol concentrations.

I anticipated a decline in testosterone and estradiol levels in both males and females, respectively, and large individual variability with respect to female estradiol concentrations. Frogs were shipped on ice overnight and thawed upon receipt then acclimated to the exposure chamber for 7 days under clean flow-through conditions. While this is not a perfect solution, I hope that the cold snap served to "reset" the ovulatory cycle in females to allow for similar ovulatory cycles during the 60-day exposure.

It was expected that total plasma cholesterol concentrations would decline when exposed to atorvastatin. The aqueous atorvastatin concentrations tested were several orders of magnitude below the equivalent recommended therapeutic dose in humans. Additionally, the exposure route bypassed the digestive system as with the metamorphosis exposure. Previously the transcriptional upregulation of *cyp19a1* had a very significant response to a relatively minor dysregulation of cholesterol biosynthesis. Based on this observation, even the slightest alteration of total cholesterol concentrations would significantly alter steroidogenesis.

5.3 Results

5.3.1 Water Chemistry

The adult chronic assay and metamorphosis assay were run in conjunction with one another, and as such, water quality was monitored continuously across both experiments to keep water chemistry parameters as well as atorvastatin concentrations consistent across both experiments. Figure 5.1 shows recorded water quality parameters and standard deviation. No water quality parameters were significantly different across any treatments or replicates.

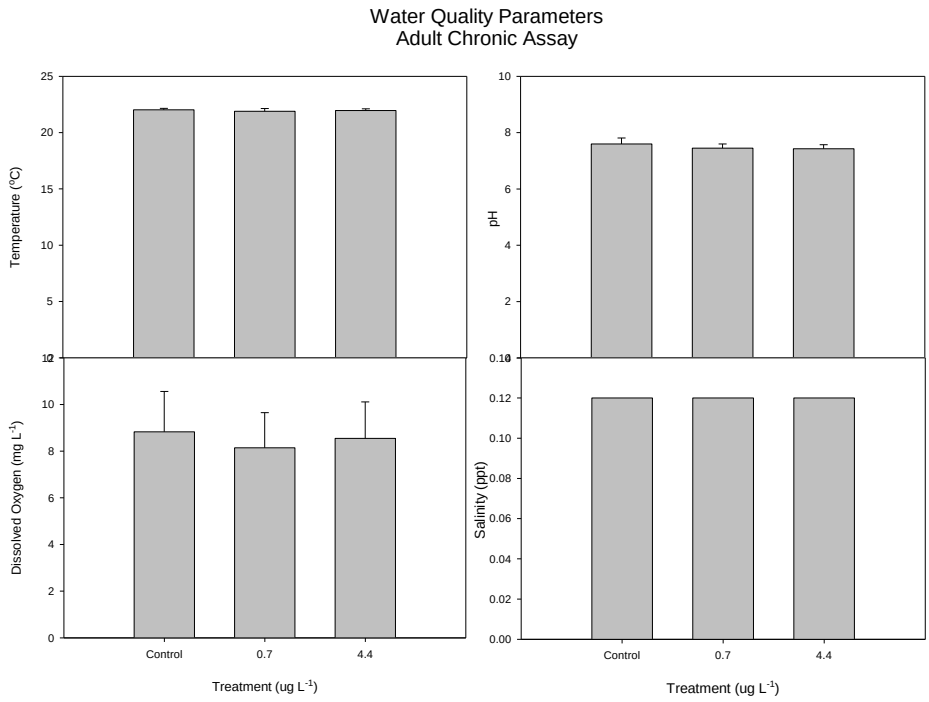

Figure 5.1 Exposure tank water quality parameters by treatment.

Similar to the metamorphosis experiment, water samples were collected at set-up, week-4, and termination (week-8). These samples were shipped to Mississippi State Chemical Laboratory for validation of exposure concentrations using the previously described protocol. Figure 5.2 shows atorvastatin concentrations in exposure water by treatment. All concentrations were significantly different from the other treatments.

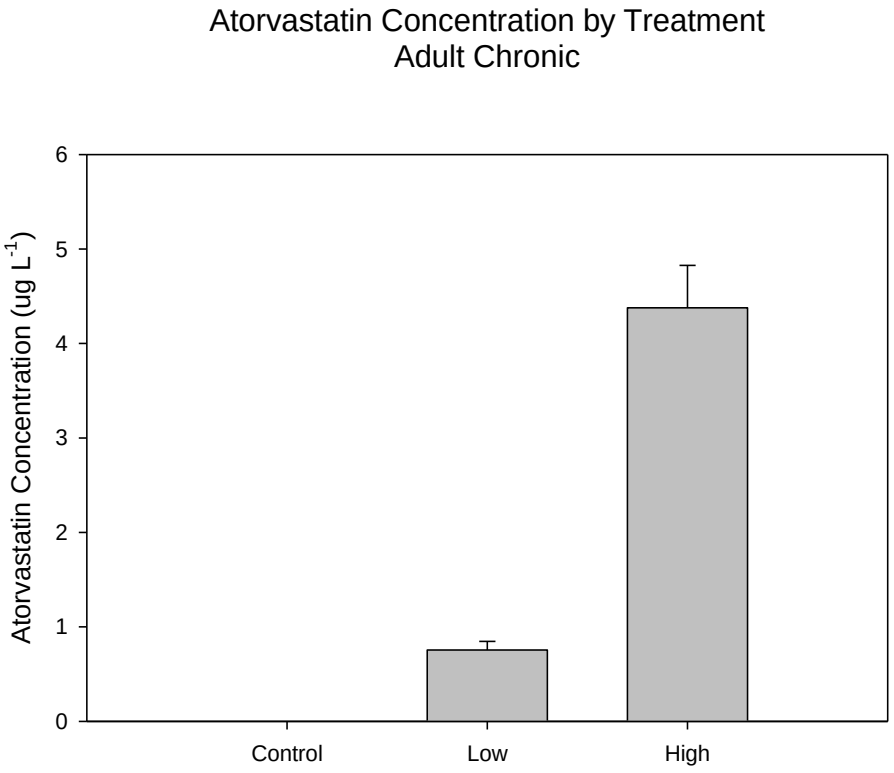

Figure 5.2 Atorvastatin concentration in exposure water by treatment.

5.3.2 Molecular Analysis

At termination, samples of liver and digestive tissue were collected, and flash frozen for later use in qPCR. These samples were then extracted and analyzed following the previously described protocols. The digestive tissue was analyzed for expression levels of *cyp3a4*, and the liver tissue was analyzed for *hmgcr* and *cyp19a1*. Additionally, these data are further subdivided into 4-week and 8-week sampling points. This sampling design allows for temporal analysis of exposure effects.

Expression levels of *cyp3a4* in digestive tissue is a biomarker of atorvastatin uptake as previously demonstrated. These data show statistically significant upregulation relative to control of *cyp3a4* at both the 4-week and the 8-week sampling point (Figure 5.3) ($p < 0.001$, $F = 110.96.5$, $df = 8$).

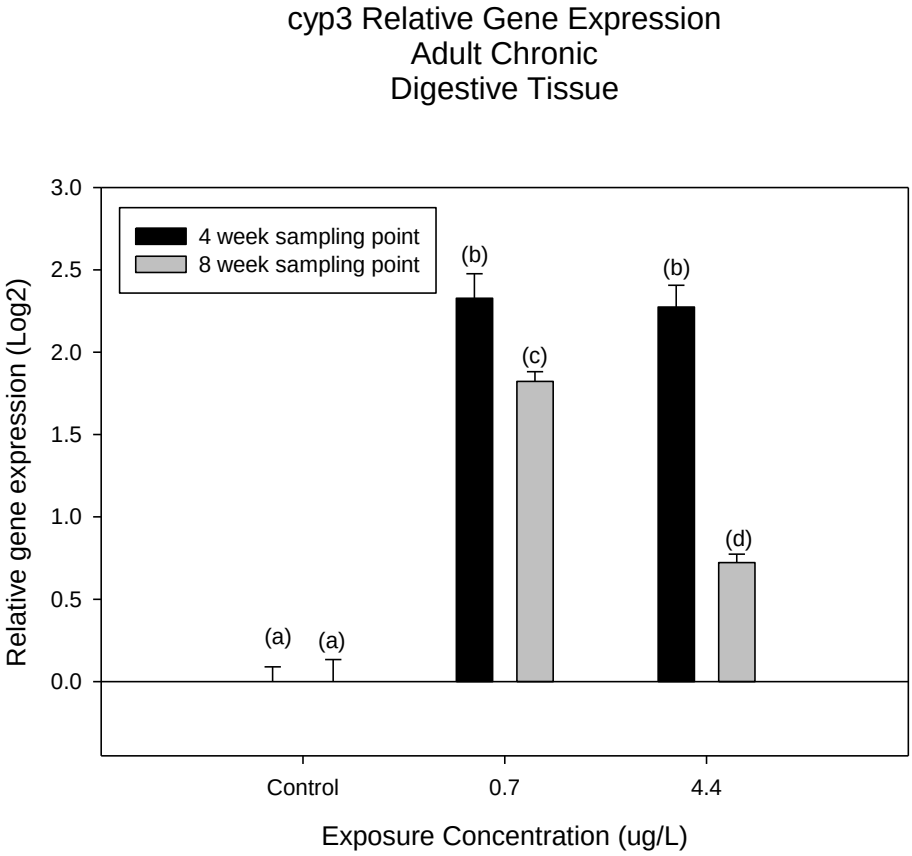

Figure 5.3 Relative gene expression of cyp3a4 in digestive tissue of X. laevis exposed to atorvastatin.

The 8-week sampling point shows a reduced upregulation significantly different from the 4-week sampling point indicating temporal differences in expression levels relative to control. These data are consistent with previous observations from the metamorphosis assay as well as the FETAX. It is of note that this upregulation is much higher than previously seen in the metamorphosis exposure despite similar exposure routes of primarily cutaneous respiration supplemented with digestion. One possible reason for differences in *cyp3a4* expression magnitude is lipid differences in food. Tadpoles and metamorphosing frogs are primarily filter feeders. At this age stage, their diet consists primarily of powdered algae dissolved in water. Adult frogs, however, were fed Xenopus Diet (Zeigler). This scientifically formulated diet has more fat content, and given the

high lipophilicity of atorvastatin, it is unsurprising that we would see increased binding to food relative to algae contributing to increased upregulation of *cyp3a4*. This statistically significant upregulation of *cyp3a4* indicates that atorvastatin uptake is occurring in the adult frogs.

Expression levels of *hmgcr* in liver tissue is a biomarker of atorvastatin activity. These data indicate statistically significant ($p < 0.001$ $F = 76.074$, $df = 8$), dose dependent upregulation of *hmgcr* at the 4-week time point, however, this effect is abolished over time (Figure 5.4). This indicates strong and rapid return to baseline expression levels as the enzyme reaches saturation. This threshold effect does not indicate reactivation of endogenous cholesterol production, rather it indicates the beginning of acclimation to continued inhibition of cholesterol biosynthesis.

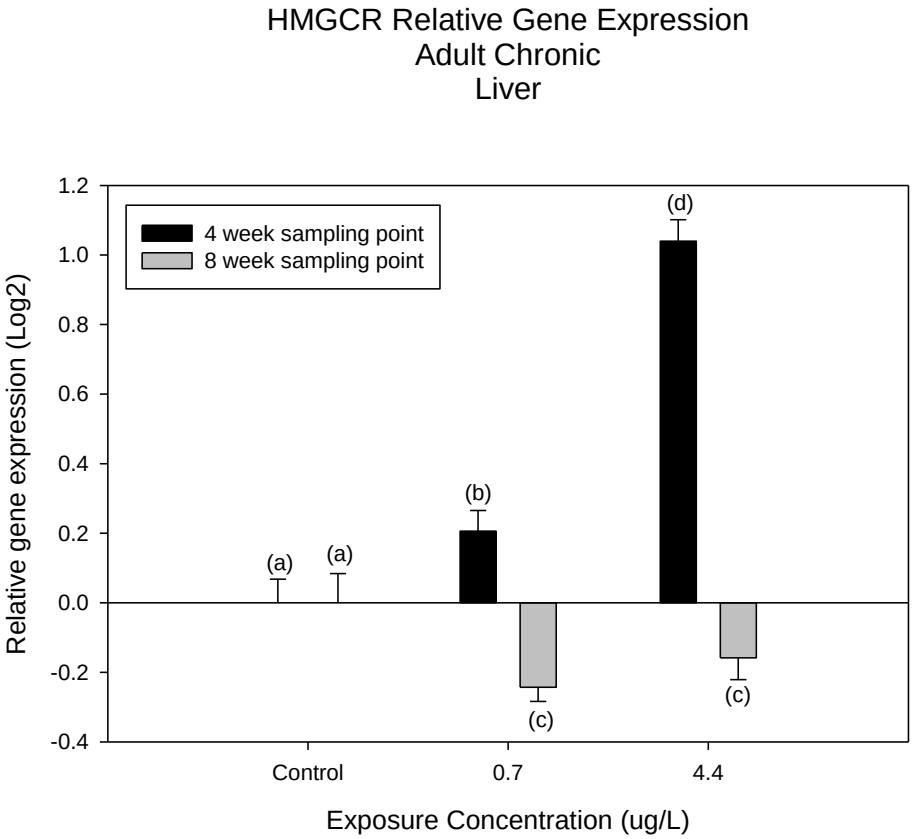

Figure 5.4 Relative gene expression of *hmgcr* in liver tissue of *X. laevis* exposed to atorvastatin.

Expression levels of *cyp19a1* in liver tissue is a biomarker of steroidogenesis dysregulation. Figure 5.5 shows *cyp19a1* expression levels in adult *X. laevis* exposed to aqueous atorvastatin. These data show statistically significant, dose dependent upregulation at the 4-week sampling point, with an even more pronounced effect at the 8-week sampling point ($p = 0.002$ $F = 22.84$, $df = 8$). This is indicative of rapid, significant dysregulation of steroidogenesis with the effect increasing over time and with dose. As previously discussed, this response is associated with increase conversion of testosterone to estradiol consistent with declining estradiol levels.

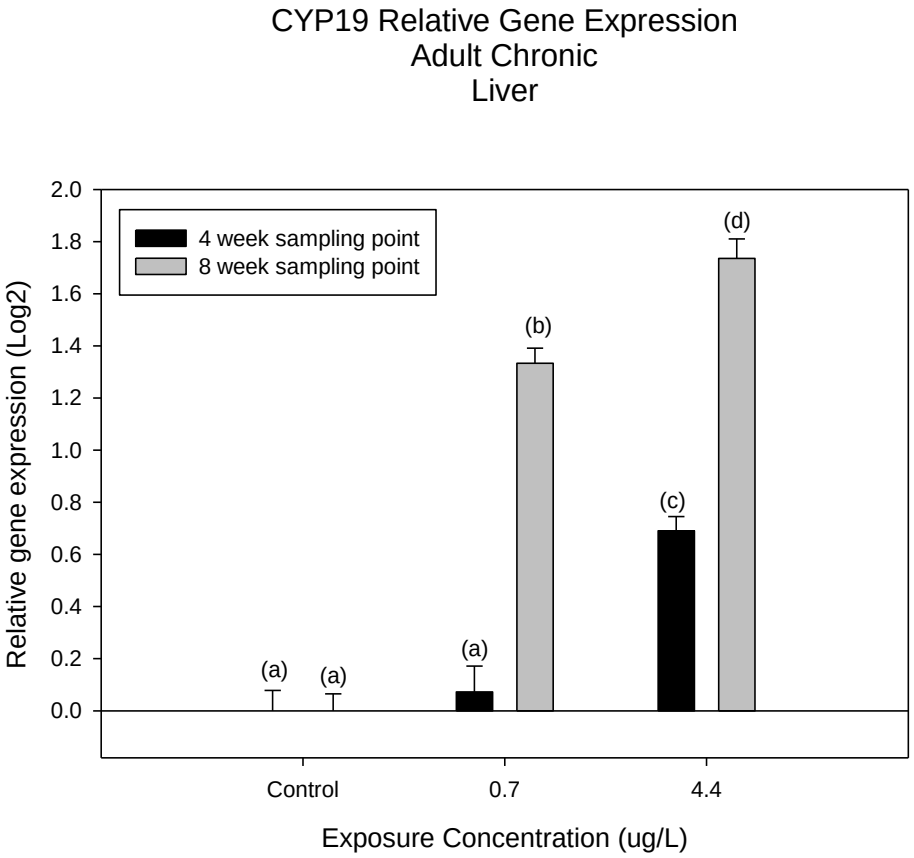

Figure 5.5 Relative gene expression of cyp19a1 in liver tissue of X. laevis exposed to atorvastatin.

In its entirety, this molecular fingerprint is consistent with previous data indicating uptake and activity of atorvastatin. Despite the uptake and activity biomarkers indicating acclimation to atorvastatin dosing occurring around the 4-week time point, the upregulation of steroidogenesis persists and continues to increase by week 8. This effect further highlights how a minor inhibition of cholesterol biosynthesis significantly contributes to dysregulation of steroidogenesis.

5.3.3 Hormone Analysis

Samples collected at both the four- and eight-week time points were analyzed via ELISA using the previously described protocol. Plasma samples were isolated from collected blood via centrifuge and were analyzed for testosterone concentration in males and estradiol concentration in females.

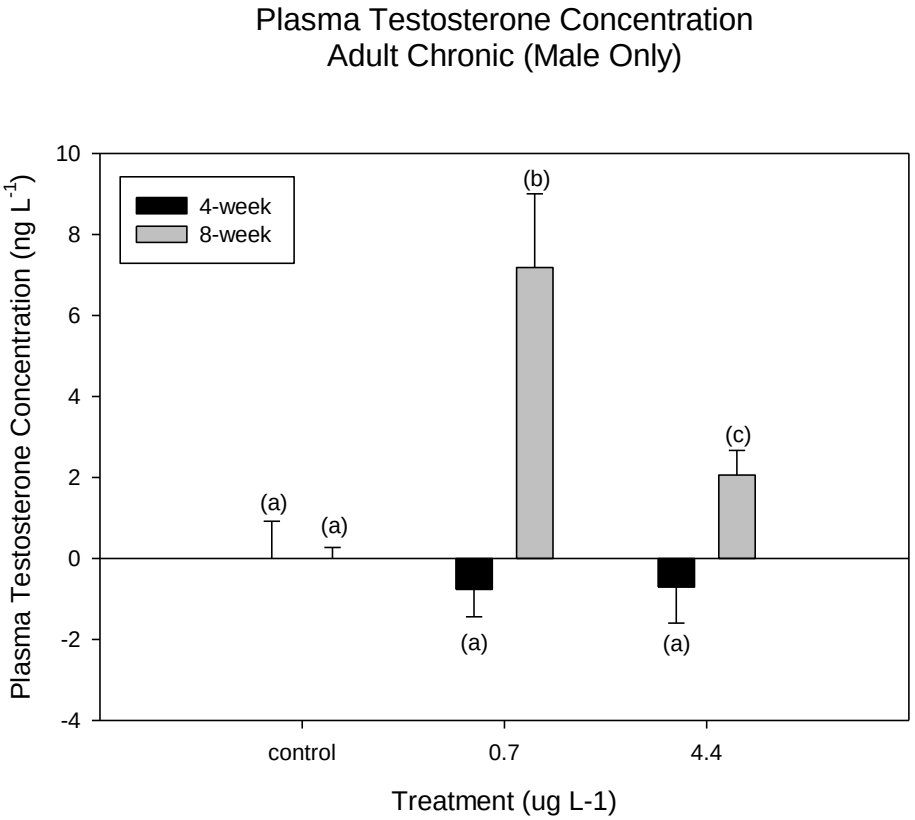

Figure 5.6 Plasma testosterone concentrations relative to control in adult male X. laevis exposed to aqueous atorvastatin sampled at multiple time points.

Figure 5.6 shows testosterone concentrations by treatment relative to control at both the four and eight-week sampling points. At the four-week time point, testosterone concentrations were not significantly different from control ($p = 0.01$, $F = 10.94$, $df = 8$). At the eight-week time point, testosterone concentrations differed significantly from

control. In the 0.7 ug L^{-1} treatment, testosterone concentrations were increased significantly relative to control and the high treatment. Control frogs had a mean plasma testosterone concentration of 1.09±0.27 ng L^{-1}(n=3) and the 0.7 ug L^{-1} atorvastatin treatment had a mean plasma testosterone concentration of 8.27±1.82 ng L^{-1}(n=3). This is a 658% increase in plasma testosterone concentrations at the lower atorvastatin treatment in the assay. The higher 4.4 ug L^{-1} treatment was also significantly increased relative to control although not to the extreme that the lower treatment was increased. The mean plasma testosterone concentration in the higher treatment was 3.15±0.60 ng L^{-1}, an increase of 189% relative to control. These data indicate that at 4 weeks, plasma testosterone concentrations were unaffected by atorvastatin, however, at the 8-week time point, elevated testosterone levels are evident with the greatest magnitude of change in the mid treatment. This lag in testosterone production is unsurprising as males will have significant enough quantities *Cyp19a1* to sustain testosterone conversion for a considerable amount of time before additional protein production is required. This is likely resulting in an overcorrection in treated groups due to the previously seen increase in *cyp19a1* expression over time. While these data are not sufficient to predict a decline in estradiol levels, they are significant enough to indicate an endocrine disrupting effect in male frogs exposed to environmentally relevant concentration of aqueous atorvastatin.

As previously discussed, the significant upregulation of *cyp19a1* points to a potential alteration in plasma estradiol levels increasing in severity as atorvastatin dose increases. Plasma estradiol concentrations of adult females exposed to atorvastatin are shown relative to control in Figure 5.7.

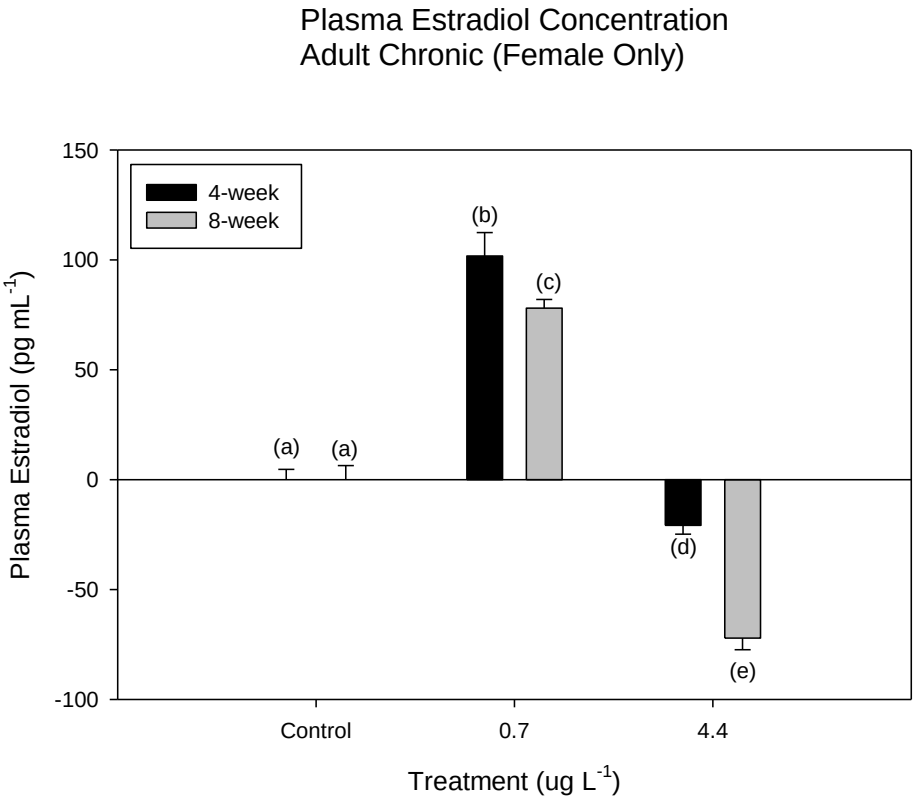

Figure 5.7 Plasma estradiol concentrations relative to control in adult female X. laevis exposed to aqueous atorvastatin sampled at multiple time points.

A similar dose response to the male testosterone levels is present in the lower 0.7 ug L^{-1} treatment. Mean plasma estradiol concentrations were increase substantially at the four-week time point. Unlike the male testosterone levels, however, there is a significant decrease in circulating estradiol levels in the higher treatment at the 4-week time point with the decline continuing into the eight-week time point. These data strongly correlate with the strong, dose dependent upregulation of *cyp19a1* in the highest treatment and confirms predictions of dysregulation of steroidogenesis accompanied by a significant decline in circulating estradiol levels at 4.4 ug L^{-1} concentrations. The 0.7 ug L^{-1} treatment shows significantly increased plasma estradiol concentrations with the effect

declining with time. While this is contradictory to expectations, it can be explained primarily by gender differences in the frogs.

As discussed in the materials and methods, adult female *X. laevis* are twice the size of their male counterparts. The males used in this assay were 50±1 g and the females used in this experiment were 100±1 g in mass. This drastic mass difference will drive a different dose response relationship in males versus females when exposed to the same dose. This affects two main dose response relationships. As cutaneous respiration accounts, at least in part, for atorvastatin uptake, body mass to surface area ratio is of significance. It is well known that as mass increases in an organism, the surface area to volume ratio decreases. This is beneficial for uptake via cutaneous respiration as the larger female frogs will experience reduced uptake when compared to their male counterparts.

Additionally, the increased mass of the female frogs means that they must receive a higher dose per organism to match the dose of their male counterparts. The goal of this experiment was environmental relevance, as such, the aqueous dose administered was based on environmentally relevant concentrations rather than equivalent dosing by mass. This means that the dose received by the female frogs was lower than the dose received in the male frogs. The threshold effect previously described in the *hmgcr* data indicate that at the lower concentrations, the larger females received a lower dose per body mass allowing compensation of endocrine disrupting effects. In contrast, the threshold was breached in the higher concentration resulting in the non-target effects previously described.

5.3.4 Cholesterol Analysis

In addition to sex steroid analysis, plasma cholesterol levels were also evaluated. The protocol for the Amplex Red cholesterol assay has been previously discussed. Simply put, this colorimetric assay is capable of determining free and bound cholesterol in plasma and was previously validated for use with *X. leavis.*

Figure 5.8 shows plasma cholesterol concentrations in males exposed to aqueous atorvastatin for 60 days. Plasma cholesterol concentrations were significantly reduced at both the four- and eight-week time-points ($p < 0.001$, $F = 42.26$, $df = 8$). In general, the higher treatment saw a lesser reduction, however across all treatments and time points plasma cholesterol levels were significantly lower than control suggesting targeted effects of atorvastatin correlating with increased uptake and activity relative to control. Based on these data, male frogs exposed to atorvastatin experience hypocholesterolemia relative to control when exposed to environmentally relevant concentrations of aqueous atorvastatin.

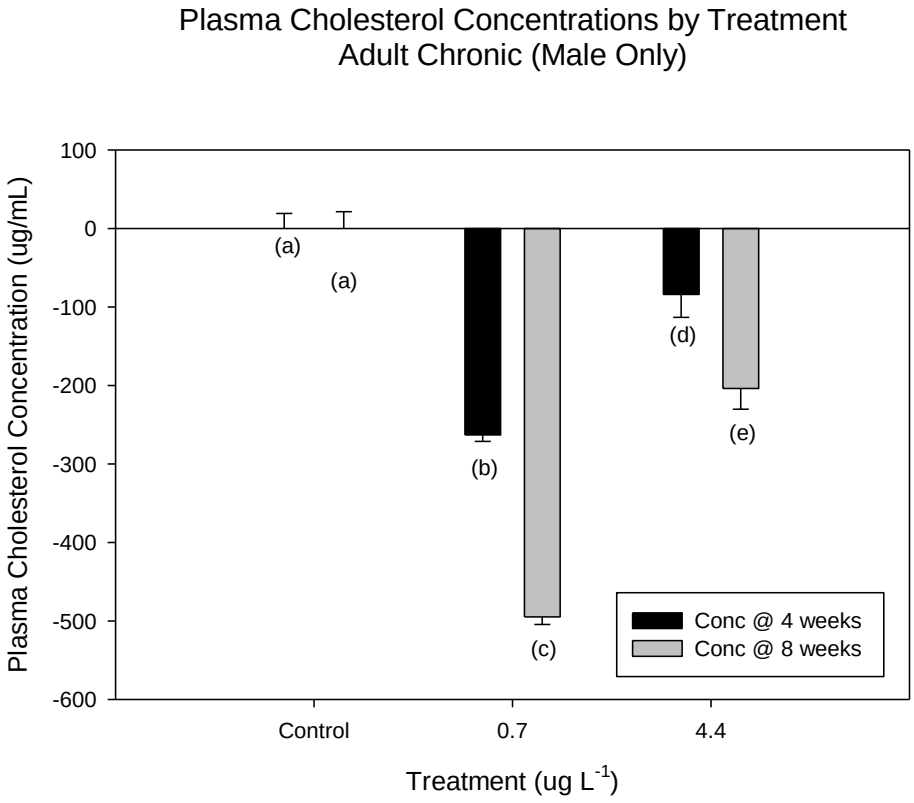

Figure 5.8 Plasma cholesterol concentrations relative to control in adult male X. laevis exposed to aqueous atorvastatin sampled at multiple time points.

Plasma cholesterol concentrations for the females showed a very different effect. Figure 5.9 shows plasma cholesterol concentrations relative to control in adult female *X. laevis* exposed to aqueous atorvastatin ($p < 0.001$, $F = 51.76$, $df = 8$). Only the lower 0.7 ug L^{-1} treatment showed a reduction in plasma cholesterol and only at the 4-week time point. Plasma cholesterol was reduced 24.5% from 1449.57±28.29 ug mL-1 to 1093.32±20.70 ug mL^{-1} relative to control. This reduction was completely abolished by the eight-week time point and showed an increase of 630% to 2876.17±99.00 ug mL^{-1}. The higher 4.4 ug L^{-1} treatment showed increases of 35% at four weeks to 1962.9±28.36 ug mL^{-1} and a 16% increase at eight weeks to 2047.58±84.50 ug mL^{-1} relative to controls. As previously discussed, it is possible that the larger mass of the female frogs contributed

to a lower overall dose received relative to body mass. This dose may have been sufficiently low enough to fail to elicit target effects in the female frogs. The cholesterol response from the females in this experiment may have been a byproduct of non-target effects which have been previously shown in this study to be quite dramatic despite relatively minor dysregulation of cholesterol biosynthesis.

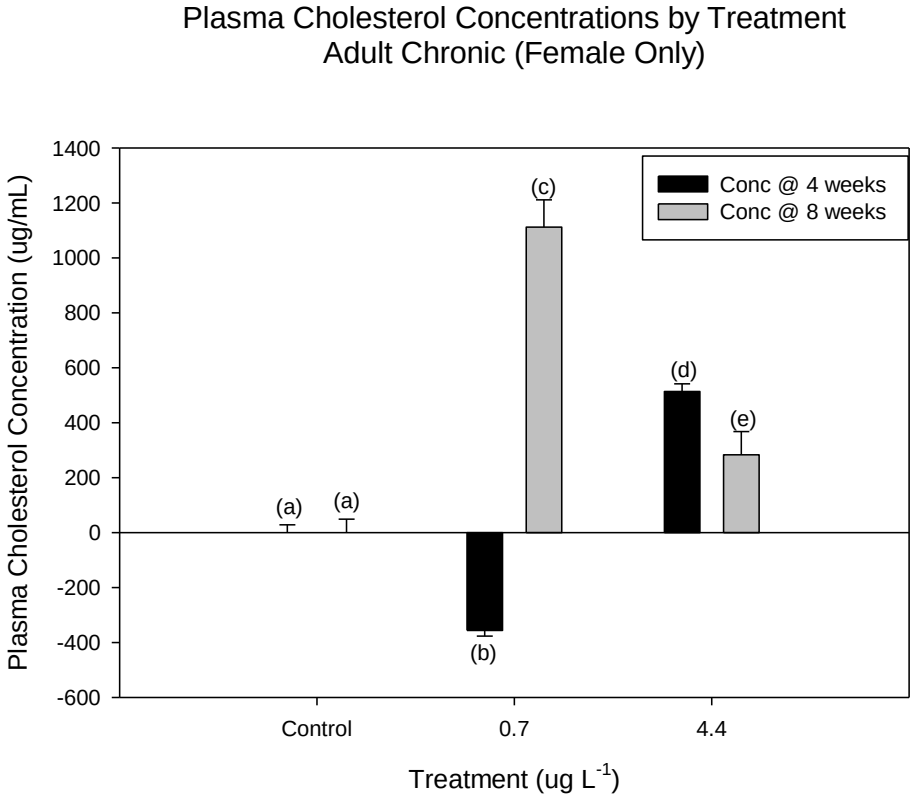

Figure 5.9 Plasma cholesterol concentrations relative to control in adult female X. laevis exposed to aqueous atorvastatin sampled at multiple time points.

5.4 Conclusions

The goal of the adult chronic assay was to evaluate potential endocrine disrupting effects of aqueous exposure to environmentally relevant concentrations of atorvastatin at multiple time points in adult males and female *X. laevis*. The endpoints measured for this

assay were transcriptional regulation of *cyp3a4*, *hmgcr*, and *cyp19a1* as well as measurement of plasma cholesterol, testosterone, and estradiol concentrations.

The molecular signature in this assay confirmed atorvastatin uptake and activity by *cyp3a4* and *hmgcr* transcriptional upregulation particularly at the 4-week time point. This effect diminished at the 8-week time point. Despite this, *cyp19a1* upregulation continued to increase through the 8-week time point indicating dysregulation of steroidogenesis and declining estradiol concentrations.

Plasma testosterone and estradiol concentrations were evaluated to confirm non-target effects of atorvastatin predicted by the upregulation of *cyp19a1*. Testosterone concentrations in males were unchanged relative to control at the 4-week time point with elevated testosterone levels by week 8. It is hypothesized that this increase is a byproduct of increased testosterone production consistent with general upregulation of steroidogenesis. This hypothesis is further supported by the plasma estradiol concentrations measured in this experiment. Plasma estradiol was elevated in the lower atorvastatin dose and significantly decreased in the higher treatment. The differences in this response are hypothesized to be due primarily to the larger mass of female *X. laevis* contributing to a reduced dose per body mass. It is believed that this reduced dose failed to achieve the threshold necessary to elicit non-target effects.

Plasma cholesterol concentrations confirmed target effects in males with significant reduction in circulation cholesterol increasing over time. However, the female cholesterol concentrations increased with atorvastatin dosing in all but the low treatment and only at the 4-week time point. This effect further confirms the hypothesis that the larger mass of females allows them to withstand higher concentrations of atorvastatin

before target effects are apparent. Taken into context with the previously discussed estradiol data, it appears that the threshold for eliciting non-target effects of reduced plasma estradiol is lower than the threshold to elicit target effects of reduction of circulating cholesterol.

The data from this assay indicate environmentally relevant concentrations of aqueous atorvastatin have an adverse effect of steroidogenesis by significantly upregulating conversion of testosterone to estradiol in the face of declining estradiol levels. This effect is more pronounced in males primarily due to their smaller size. Female *X. laevis* demonstrate a greater tolerance for aqueous atorvastatin with target effects not apparent in this assay, and non-target effects appearing only in the 4.4 ug L^{-1} treatment. This supports the hypothesis the aqueous atorvastatin has endocrine disrupting potential at environmentally relevant concentrations.

CHAPTER VI – CONCLUSION

This research investigated the non-target effects of exposure to environmentally relevant concentrations of aqueous atorvastatin. Previous research has shown that wastewater treatment plants do not sufficiently remove atorvastatin from wastewater during the treatment process(Halling-Sørensen et al., 1998; Jelic et al., 2011; Lee et al., 2009; Ort et al., 2010) resulting in discharge of atorvastatin in wastewater effluent at or below 2ug L^{-1}(Jelic et al., 2011; Lee et al., 2009). I hypothesize that these low concentrations of aqueous atorvastatin are sufficient to elicit the non-target effects of alteration of circulating testosterone and estradiol as a consequence of inhibition of endogenous production of cholesterol potentially resulting in alteration of growth, development, and sexual differentiation in amphibians localized near wastewater effluent sites.

This research sought to investigate these non-target effects using traditional aquatic toxicology assays combined with modern molecular biology techniques to identify and report regulatory alteration of physiological pathways in a model amphibian, *Xenopus laevis*. This was investigated via three primary assays. First, a modified FETAX assay was employed to evaluate the target and non-target effects of atorvastatin exposure at high concentrations on developing tadpoles. Second, the molecular fingerprint developed from the FETAX assay was used to verify exposure effects of chronic exposure to aqueous atorvastatin at environmentally relevant concentrations on metamorphosing tadpoles. Finally, a chronic adult exposure at environmentally relevant concentrations was used to determine the non-target effects of atorvastatin exposure on circulating testosterone and estradiol concentrations in amphibians.

The results of the FETAX validated biomarkers of uptake, activity and efficacy of atorvastatin in *X. laevis*. These biomarkers were upregulation of *cyp3a4*, and *hmgr* for uptake and activity respectively, and efficacy was validated by significant reduction in total cholesterol. The directionality of this dysregulation was consistent with the response of the same biomarkers in humans administered atorvastatin as treatment for hypercholesterolemia. These data confirm that atorvastatin has the potential to elicit the same targeted responses to atorvastatin exposure as humans.

Investigation into non-target effects of atorvastatin at environmentally relevant concentrations began with the chronic metamorphosis assay. The results of this assay indicated significant upregulation of biomarkers related to inhibition of cholesterol biosynthesis and showed dose-dependent upregulation of *cyp19a1* suggesting a molecular response to declining estrogen concentrations. This non-target effect was confirmed via total RNA sequencing, and showed significant upregulation of master regulators associated with steroid metabolism. Furthermore, RNA sequencing confirmed activation of the canonical pathways for estrogen biosynthesis and aldosterone signaling. Combined, these data indicate that exposure to aqueous atorvastatin alters steroidogenesis and results in declining sex steroid concentrations consistent with findings in zebrafish exposed to atorvastatin(Al-Habsi et al., 2015).

Morphometric analysis of length, mass and developmental stage did not indicate overt alteration of growth or development suggesting that transient exposure to environmentally relevant concentrations of atorvastatin was not sufficient to alter growth and development, however the data do suggest that longer term exposures or higher

concentrations may still alter rates of metamorphosis highlighting a need for further study into complete life cycle exposures.

Additionally, RNA sequencing confirmed generation of ROS and concurrent DNA damage repair consistent with findings from other researchers (Bouitbir et al., 2011, 2016). This response is contradictory to findings in the medical community suggesting that atorvastatin serves to ameliorate the effects of ROS generation and oxidative stress(Briones et al., 2009; Ceriello et al., 2005; Sugiyama et al., 2005). These contradictory findings suggest a potential difference in the response of humans versus amphibians exposed to atorvastatin and warrant future investigation into these differences.

Circulating cholesterol concentrations in males and females exposed to atorvastatin are indicative of targeted effects of atorvastatin exposure. Male *X. laevis* showed a dose-dependent and time dependent reduction in circulating cholesterol indicating the targeted effect of atorvastatin exposure is evident in in the smaller males even at the low environmentally relevant concentrations. Cholesterol concentrations in females show a general increase in cholesterol concentrations suggesting that the larger body mass of females provide some degree of protection from targeted effects of atorvastatin exposure at environmentally relevant concentrations. These sexual differences in body mass are also evident in testosterone and estradiol concentrations as well.

The adult chronic assay further confirms findings from the metamorphosis assay with relation to dysregulation of steroidogenesis. These data show strong, dose-dependent upregulation of steroidogenesis and declining estradiol concentrations in adult

female *X. laevis* at 4.4 ug L^{-1}. While the lower 0.7 ug L^{-1} did not show a decrease in circulating estradiol concentration, this is believed to be an artifact of mass differences in adult males and females as female *X. laevis* are significantly larger than their male counterpart. This may result in female *X. laevis* receiving a lower dose per body mass resulting in decreased non-target effects at lower concentrations. Testosterone concentrations in males were unaffected at 4-weeks, with elevated testosterone concentrations at 8-weeks. These findings suggest that testosterone concentrations are unaffected in the short term and are unsurprising given the increased capacity for testosterone production in males of any species. Relative to control, adult males and females show alteration of hormone level inconsistent with predictions suggesting that dysregulation of steroidogenesis is occurring in frogs exposed to atorvastatin in an unpredictable manner. The directionality of this dysregulation is inconsistent with predictions indicating the need for further research into the exact nature of this dysregulation.

This research provides further insight into the potentially harmful non-target effects of relatively low concentrations of aqueous atorvastatin discharged in bodies of water inhabited by amphibians. The data indicate significant dose-dependent upregulation of steroidogenesis as confirmed by both qPCR and total RNA sequencing. While these data do not indicate overt morphological alteration of metamorphosis in these short exposures, on a molecular level, the early indicators of endocrine disruption are evident, and this conclusion is further supported by alteration of testosterone and estradiol concentration in adults. While it was not possible to measure testosterone and estradiol concentrations in metamorphosing tadpoles, the increased sensitivity of earlier

life stages to aquatic toxins combined with the molecular signature indicating upregulation of steroidogenesis suggest that alteration of sex steroid levels is occurring and has the potential to significantly alter sex ratios in populations localized around wastewater effluent sites. Based on these data, I fail to reject the hypothesis that environmentally relevant concentrations of aqueous atorvastatin are sufficient to elicit the non-target effects of alteration of circulating testosterone and estradiol as a consequence of inhibition of endogenous production of cholesterol potentially resulting in alteration of growth, development, and sexual differentiation in amphibians.

REFERENCES

Al-Habsi, A. A., Massarsky, A., & Moon, T. W. (2015). Exposure to gemfibrozil and atorvastatin affects cholesterol metabolism and steroid production in zebrafish (Danio rerio). *Comparative Biochemistry and Physiology Part - B: Biochemistry and Molecular Biology*, *199*, 87–96. https://doi.org/10.1016/j.cbpb.2015.11.009

ASTM INTERNATIONAL. (2012). Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) (pp. 1–16). https://doi.org/10.1520/e1439-12

Batt, A. L., Kostich, M. S., & Lazorchak, J. M. (2008). Analysis of Ecologically Relevant Pharmaceuticals in Wastewater and Surface Water Using Selective Solid-Phase Extraction and UPLC-MS/MS. *Anal Chem*, *80*(13), 5021–5030.

Black, A. N. N. E., Hayes, R. N., Roth, B. D., Woo, P., Woolf, T. F., Pharmacokinetics, D., … Chemistry, B. D. R. (1999). Metabolism and Excretion of Atorvastatin in Rats and Dogs : *Pharmacology*, *27*(8), 916–923.

Bouitbir, J., Charles, A.-L., Rasseneur, L., Dufour, S., Piquard, F., Geny, B., & Zoll, J. (2011). Atorvastatin treatment reduces exercise capacities in rats: involvement of mitochondrial impairments and oxidative stress. *Journal of Applied Physiology*, *111*(5), 1477–1483. https://doi.org/10.1152/japplphysiol.00107.2011

Bouitbir, J., Singh, F., Charles, A.-L., Schlagowski, A.-I., Bonifacio, A., Echaniz-Laguna, A., … Zoll, J. (2016). Statins Trigger Mitochondrial Reactive Oxygen Species-Induced Apoptosis in Glycolytic Skeletal Muscle. *Antioxidants & Redox Signaling*, *24*(2), 84–98. https://doi.org/10.1089/ars.2014.6190

Briones, A. M., Rodríguez-Criado, N., Hernanz, R., García-Redondo, A. B., Rodrigues-Díez, R. R., Alonso, M. J., … Salaices, M. (2009). Atorvastatin Prevents Angiotensin II–Induced Vascular Remodeling and Oxidative Stress. *Hypertension*, *54*(1), 142–149. https://doi.org/10.1161/HYPERTENSIONAHA.109.133710

Brown, M. S., & Goldstein, J. L. (2009). Cholesterol feedback : from Schoenheimer ' s bottle to Scap ' s MELADL, 15–28. https://doi.org/10.1194/jlr.R800054-JLR200

CDC. (2019). Centers for Disease Control and Prevention, National Center for Health Statistics. Underlying Cause of Death 1999-2017 on CDC WONDER Online Database. Retrieved March 12, 2019, from http://wonder.cdc.gov/ucd-icd10.html

Ceriello, A., Assaloni, R., Da Ros, R., Maier, A., Piconi, L., Quagliaro, L., … Giugliano, D. (2005). Effect of Atorvastatin and Irbesartan, Alone and in Combination, on Postprandial Endothelial Dysfunction, Oxidative Stress, and Inflammation in Type 2 Diabetic Patients. *Circulation*, *111*(19), 2518–2524. https://doi.org/10.1161/01.CIR.0000165070.46111.9F

Chen, M., Ohman, K., Metcalfe, C., Ikonomou, M. G., Amatya, P. L., & Wilson, J. (2006). Pharmaceuticals and endocrine disruptors in wastewater treatment effluents and in the water supply system of Calgary, Alberta, Canada. *Water Quality Research Journal of Canada*, *41*(4), 351–364. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-38949136691&partnerID=tZOtx3y1

Cilla Jr., D. D., Whitfield, L. R., Gibson, D. M., Sedman, A. J., & Posvar, E. L. (1996). Multiple-dose pharmacokinetics, pharmacodynamics, and safety of atorvastatin, an inhibitor of HMG-CoA reductase, in healthy subjects. *Clinical Pharmacology & Therapeutics*, *60*(6), 687–695. https://doi.org/10.1016/S0009-9236(96)90218-0

Feder, M. E., & Burggren, W. W. (1985). Cutaneous gas exchange in vertebrates: design, patterns, control and implications. *Biological Reviews of the Cambridge Philosophical Society*, *60*, 1–45. https://doi.org/10.1111/j.1469-185X.1985.tb00416.x

Friedberg, E. C., McDaniel, L. D., & Schultz, R. A. (2004). The role of endogenous and exogenous DNA damage and mutagenesis. *Current Opinion in Genetics & Development*, *14*(1), 5–10. https://doi.org/10.1016/J.GDE.2003.11.001

Gibson, D. M., Stern, R. H., Abel, R. B., & Whitfield, L. R. (1997). Absolute bioavailability of atorvastatin in man. *Pharmaceutical Research*, *14*(New York), S253.

Gilbert, S. (2007). *Developmental Biology*. *Developmental Biology* (Vol. 311). https://doi.org/10.1016/j.ydbio.2007.08.033

Hadfield, C. A., Clayton, L. A., & Barnett, S. L. (2006). Nutritional Support of Amphibians. *Journal of Exotic Pet Medicine*, *15*(4), 255–263. https://doi.org/10.1053/j.jepm.2006.09.004

Halling-Sorensen, B., Nors Nielsen, S., Lanzky, P. F., Ingerslev, F., Holten Lutzhoft, H. C., & Jorgensen, S. E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment--a review. *Chemosphere*, *36*(2), 357–393.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P. F., Ingerslev, F., Holten Lützhøft, H. C., & Jørgensen, S. E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment- A review. *Chemosphere*, *36*(2), 357–393. https://doi.org/10.1016/S0045-6535(97)00354-8

Jacobsen, W., Kuhn, B., Soldner, A., Kirchner, G., Sewing, K. F., Kollman, P. A., … Christians, U. (2000). Lactonization is the critical first step in the disposition of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor atorvastatin. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *28*(11), 1369–1378.

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M., & Barcelo, D. (2011). Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. *Water Research*, *45*(3), 1165–1176. https://doi.org/10.1016/j.watres.2010.11.010

Kapourchali, F. R., Surendiran, G., Goulet, A., & Moghadasian, M. H. (2016). The Role of Dietary Cholesterol in Lipoprotein Metabolism and Related Metabolic Abnormalities: A Mini-review. *Critical Reviews in Food Science and Nutrition*, *56*(14), 2408–2415. https://doi.org/10.1080/10408398.2013.842887

Lam, M. W., & Mabury, S. A. (2005). Photodegradation of the pharmaceuticals atorvastatin, carbamazepine, levofloxacin, and sulfamethoxazole in natural waters. *Aquatic Sciences*, *67*(2), 177–188. https://doi.org/10.1007/s00027-004-0768-8

Lee, H.-B., Peart, T. E., Svoboda, M. L., & Backus, S. (2009). Occurrence and fate of rosuvastatin, rosuvastatin lactone, and atorvastatin in Canadian sewage and surface water samples. *Chemosphere*, *77*(10), 1285–1291. https://doi.org/10.1016/j.chemosphere.2009.09.068

Lennernäs, H. (2003). Clinical Pharmacokinetics of Atorvastatin. *Clinical Pharmacokinetics*, *42*(13), 1141–1160. https://doi.org/10.2165/00003088-200342130-00005

McBride, C. L., Akeroyd, J. M., Ramsey, D. J., Nambi, V., Nasir, K., Michos, E. D., … Virani, S. S. (2018). Statin prescription rates and their facility-level variation in patients with peripheral artery disease and ischemic cerebrovascular disease: Insights from the Department of Veterans Affairs. *Vascular Medicine*, *23*(3), 232–240. https://doi.org/10.1177/1358863X18758914

Meikle, A W. (2004). The interrelationships between thyroid dysfunction and hypogonadism in men and boys. *Thyroid : Official Journal of the American Thyroid Association*, *14 Suppl 1*, S17-25. https://doi.org/10.1089/105072504323024552

Miller, W. L., & Auchus, R. J. (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews*, *32*(1), 81–151. https://doi.org/10.1210/er.2010-0013

Miyata, S., & Kubo, T. (2000). In Vitro Effects of Estradiol and Aromatase Inhibitor Treatment on Sex Differentiation in Xenopus Laevis Gonads. *General and Comparative Endocrinology*, *119*(1), 105–110. https://doi.org/10.1006/gcen.2000.7497

National Center for Biotechnology Information. (n.d.). PubChem Database. Atorvastatin, CID=60823. Retrieved May 28, 2019, from https://pubchem.ncbi.nlm.nih.gov/compound/Atorvastatin

Nawrocki, J. W., Weiss, S. R., Davidson, M. H., Sprecher, D. L., Schwartz, S. L., Lupien, P. J., … Black, D. M. (1995). Reduction of LDL cholesterol by 25% to 60% in patients with primary hypercholesterolemia by atorvastatin, a new HMG-CoA reductase inhibitor. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *15*(5), 678–682.

Nieuwkoop P., F. J. (1994). *Normal Table of Xenopus Laevis (Daudin): A Systematical & Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis*. *Http://Www.Xenbase.Org/Anatomy/Alldev.Do*. Retrieved from http://www.xenbase.org/anatomy/alldev.do

Ohtani, H., Miura, I., & Ichikawa, Y. (2000). Effects of dibutyl phthalate as an environmental endocrine disruptor on gonadal sex differentiation of genetic males of the frog Rana rugosa. *Environmental Health Perspectives*, *108*(12), 1189–1193. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11133400

Ort, C., Lawrence, M. G., Reungoat, J., Eaglesham, G., Carter, S., & Keller, J. (2010). Determining the fraction of pharmaceutical residues in wastewater originating from a hospital. *Water Res*, *44*(2), 605–615. https://doi.org/10.1016/j.watres.2009.08.002

Pfizer. (2018). Safety Data Sheet. Lipitor® (Atorvastatin Calcium), 1–10.

Richards, S. M., & Cole, S. E. (2006). A toxicity and hazard assessment of fourteen pharmaceuticals to Xenopus laevis larvae. *Ecotoxicology*, *15*(8), 647–656. https://doi.org/10.1007/s10646-006-0102-4

Salami, J. A., Warraich, H., Valero-Elizondo, J., Spatz, E. S., Desai, N. R., Rana, J. S., … Nasir, K. (2017). National trends in statin use and expenditures in the US adult population from 2002 to 2013: Insights From the Medical Expenditure Panel Survey. *JAMA Cardiology*, *2*(1), 56–65. https://doi.org/10.1001/jamacardio.2016.4700

Santin, A. P., & Furlanetto, T. W. (2011). Role of estrogen in thyroid function and growth regulation. *Journal of Thyroid Research*, *2011*, 875125. https://doi.org/10.4061/2011/875125

Sharaf El-Din, M. M. K., Salama, F. M. M., Nassar, M. W. I., Attia, K. A. M., & Kaddah, M. M. Y. (2012). Validated spectrofluorimetric method for the determination of atorvastatin in pharmaceutical preparations. *Journal of Pharmaceutical Analysis*, *2*(3), 200–205. https://doi.org/10.1016/j.jpha.2012.01.005

Stagnitti, M. N. (2007). The Top Five Outpatient Prescription Drugs Ranked by Total Expense for Children, Adults, and the Elderly. *Agency for Healthcare Research and Quality*, *180*(July), Brief 180 1-4.

Sugiyama, M., Ohashi, M., Takase, H., Sato, K., Ueda, R., & Dohi, Y. (2005). Effects of atorvastatin on inflammation and oxidative stress. *Heart and Vessels*, *20*(4), 133–136. https://doi.org/10.1007/s00380-005-0833-9

Willrich, M. A. V, Rodrigues, A. C., Cerda, A., Genvigir, F. D. V, Arazi, S. S., Dorea, E. L., … Hirata, R. D. C. (2013). Effects of atorvastatin on CYP3A4 and CYP3A5 mRNA expression in mononuclear cells and CYP3A activity in hypercholeresterolemic patients. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *421*, 157–163. https://doi.org/10.1016/j.cca.2013.03.007

Yu, N.-W., Hsu, C.-Y., Ku, H.-H., Chang, L.-T., & Liu, H.-W. (1993). Gonadal differentiation and secretions of estradiol and testosterone of the ovaries ofRana catesbeiana tadpoles treated with 4-hydroxyandrostenedione. *Journal of Experimental Zoology*, *265*(3), 252–257. https://doi.org/10.1002/jez.1402650307