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DETERMINATION OF ENDOCRINE DISRUPTION RISK FOLLOWING  
EXPOSURE TO BETAMETHASONE IN SURFACE AND DRINKING WATER

by

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## ABSTRACT OF THE DISSERTATION

Determination of endocrine disruption risk following exposure to betamethasone in  
surface and drinking water

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To date, the presence of synthetic glucocorticosteroids in surface water and their potential endocrine disruption activity at environmental concentrations has not been fully investigated. Synthetic glucocorticosteroids (GC) may interfere with endogenous GC receptors within the hypothalamic-pituitary-gonadal (HPG) axis and disruptions of this pathway can result in decreased reproduction and/or adverse developmental effects in offspring. Much of the evidence for endocrine disruption in wildlife populations has been derived from aquatic animals such as fish, due to widespread contamination of surface water. The HPG axis is phylogenetically conserved across all vertebrate species and fish have the advantage over mammals as an experimental model of reaching maturity relatively quickly and have overall shorter life spans, which make them ideal for life cycle toxicology studies. Betamethasone, a synthetic glucocorticosteroid, has been on the market in the United States since the 1980's and is on the World Health Organization Model List of Essential Medicines. Betamethasone mimics the action of cortisol and may disrupt the HPG axis. Studying fish for the endocrine disruption potential of betamethasone is logical, as they could be exposed to pharmaceuticals in waste water

treatment plant effluent following normal patient use and excretion. In the present study, the Pharmaceutical Assessment and Transport Evaluation (PhATE) model estimated betamethasone concentrations to be <0.6 ng/L in 95% of all surface waters and <0.1 ng/L for 95% of the U.S. population. Environmentally relevant concentrations were then used in a two generation fish full life cycle (FFLC) study with Japanese medaka. Gross endpoints were evaluated, as well as secondary sexual characteristics and vitellogenin expression. The highest concentration at which no endocrine disruption outcomes are anticipated (NOEC) was determined to be 0.1 µg/L and a reference dose of  $7 \times 10^{-5}$  µg/kg-day for humans was derived from the NOEC. The average daily dose to humans was estimated from surface and drinking water concentrations and calculated margins of safety ranging from three to thirty indicate no adverse effects are anticipated from exposures to betamethasone at environmentally relevant concentrations.



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## LIST OF ABBREVIATIONS

AF .....	Bioavailability Factor
ADE .....	Acceptable Daily Exposure
ADD .....	Average Daily Dose
API .....	Active Pharmaceutical Ingredient
E2 .....	17 $\beta$ -estradiol
ED .....	Endocrine Disruption
EF .....	Exposure Factor
F0 .....	First generation of fish
F1 .....	Second generation of fish
FFLC .....	Fish Full Life Cycle
FSH .....	Follicle Stimulating Hormone
FSH $\beta$ .....	Follicle Stimulating Hormone $\beta$ -subunit
GC .....	Glucocorticosteroid
GnRH .....	Gonadotropin Releasing Hormone
HPG Axis .....	Hypothalamic-Pituitary-Gonadal Axis
IR .....	Intake Rate
LOEC .....	Lowest Observable Effect Concentration
LOAEL .....	Lowest Observed Adverse Effect Level
LH .....	Luteinizing Hormone
LH $\beta$ .....	Luteinizing Hormone $\beta$ -subunit
MF .....	Modifying Factor
MOS .....	Margin of Safety
NOEC .....	No Observable Effect Concentration
OECD .....	Organisation for the Economic Cooperation and Development
PEC .....	Predicted Environmental Concentration
PhATE .....	Pharmaceutical Assessment and Transport Evaluation
PNEC .....	Predicted No Effect Concentration
POTW .....	Publicly Owned Treatment Works
RfD .....	Reference Dose
UF .....	Uncertainty Factor
UF <sub>A</sub> .....	Uncertainty Factor to Account for Extrapolation from Animal to Human
UF <sub>H</sub> .....	Uncertainty Factor to Account for Inter-individual Variability
UF <sub>D</sub> .....	Uncertainty Factor to Account for an Incomplete Data Set
UF <sub>L</sub> .....	Uncertainty Factor to Account for an Extrapolation from a Lowest Observed Adverse Effect Level to a No Observed Adverse Effect Level
VTG .....	Vitellogenin
WWTP .....	Waste Water Treatment Plant

## BACKGROUND

### Pharmaceuticals in Surface Water

Since the early 1970's, pharmaceuticals have been detected in surface waters across the United States [1]. As technologies advance, these chemicals are able to be detected at very low concentrations, in some cases on the order of nanograms per liter [2]. The largest source of pharmaceuticals in the aquatic environment results from excretion following patient use [1, 2]. Often, active pharmaceutical ingredients (APIs) are excreted unchanged or only slightly transformed and then released into surface water via the effluent of wastewater treatment plants (WWTPs) [1]. Pharmaceuticals' attenuation following waste water treatment is mainly due to biodegradation and dilution, but other factors such as adsorption or their potential to bind to sludge and thus being removed from the water column also play a role in their removal [3]. They also may undergo photodegradation, or biotic or chemical transformations in water into metabolites [3]. The presence of minute quantities of pharmaceuticals has led to public concern for both aquatic and human health [1].

### Pharmaceuticals in Drinking Water

In general, drinking water processes are not designed to remove APIs so their elimination following treatment is largely dependent on their physical and chemical properties [4]. However, some common processes such as microfiltration, ultrafiltration, nanofiltration, reverse osmosis, electrodialysis, ozonation and chlorination are known to remove pharmaceuticals with some efficiency [3, 5]. For example, one study determined that removal following classic drinking water treatment steps of granular-activated-

carbon filtration, disinfection (chlorination) and clarification is 98% for acetaminophen, 88% for caffeine, and 85% for carbamazepine [4]. In general, for all of the compounds tested in the study, granular-activated-carbon filtration accounted for 53% API removal from the aqueous phase; disinfection accounted for 32%, and clarification accounted for 15% [4].

Granular and powdered activated carbon filtration systems are increasingly being adopted for drinking water treatment in order to remove pesticides and improve overall water taste and odor [1]. Both systems can achieve high removal of pharmaceuticals, especially hydrophobic compounds and removal efficacy is a function of contact time, organic loading, chemical structure, solubility and carbon type [5, 6]. However, unlike drinking water treatment for surface water sources, drinking water treatment for groundwater sources is mostly single-stage disinfection without multiple treatment barriers [1].

### Endocrine Disrupting Chemicals

Endocrine disruptors are chemicals that may interfere with an organism's endocrine system resulting in adverse developmental, reproductive, neurological, and immune effects. A wide range of substances, both natural and man-made, are thought to cause endocrine disruption, including pharmaceuticals. Endocrine disruptors may pose the greatest risk during prenatal and early postnatal development when organ and neural systems are forming [7]. Therefore, it is necessary to better understand the endocrine disrupting (ED) potential of compounds that humans may be exposed to every day over the course of their lives.

### Hypothalamic-Pituitary-Gonadal (HPG) Axis

The Hypothalamic-Pituitary-Gonadal Axis (HPG Axis) refers to the coordinated effects of the hypothalamus, pituitary gland, and gonads, which together help regulate reproduction [8]. The HPG axis is triggered when the pituitary gland is stimulated by hypothalamic neurons secreting gonadotropin-releasing hormone (GnRH) directly into the extracellular space of the hypophysis [9]. GnRH then stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. Release of LH stimulates final gamete maturation and induction of ovulation or sperm release and FSH induces oogenesis and spermatogenesis [8]. In males, sertoli cells in the testes respond to LH and FSH activity and foster the production and maturation of sperm [10]. Leydig cells are then stimulated to produce testosterone and 11-ketotestosterone, which induce masculine secondary sex characteristics and provide a negative feedback to the hypothalamus [9]. In females, the production of LH and FSH trigger the ovaries to produce progesterone and estrogen, which then provide the negative feedback to the hypothalamus [11] (Figure 1). GnRH, LH and FSH are collectively known as gonadotropins.

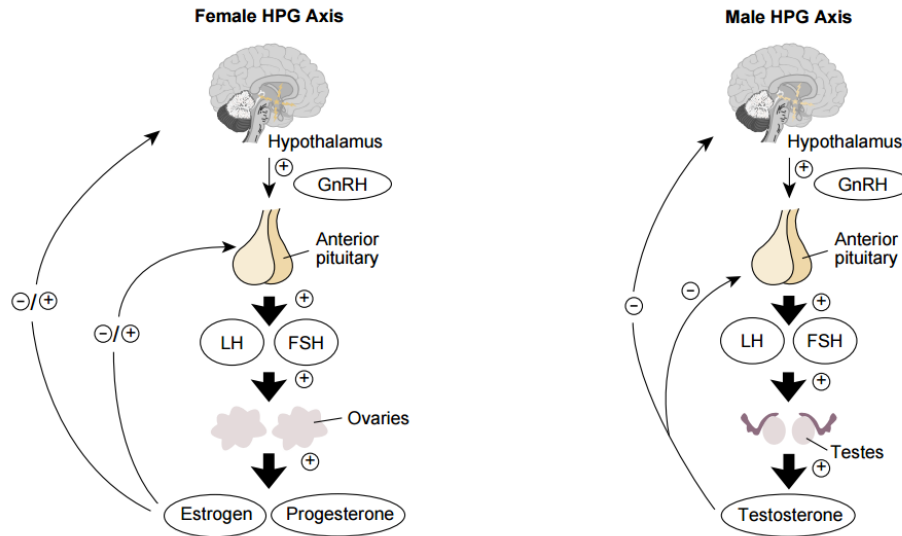


Figure 1. Schematic representation of the male and female HPG axis. Hypothalamic neurons secrete gonadotropin-releasing hormone (GnRH), which directly innervates the Pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH in turn stimulate sertoli cells, which foster production and maturation of sperm (males) and eggs (females). The ovaries and testes produce estrogen and testosterone, respectively, which provide negative feedback signals to the hypothalamus in order to signal termination of GnRH release. Figure taken from Hiller-Sturmhöfel & Bartke (1998) [11].

Currently, there are three known triggers of the HPG axis: hormone signals, neurotransmitter or neurohormone signals and paracrine signals. These signals initiate organizational or activational effects. Organizational effects, if disrupted, can result in malfunction of the differentiation of tissues and cells (such as the gonads) into organs with proper structure [9]. The organism may then have difficulty responding to internal or external cues. This process is often controlled by the hormones of the HPG axis [9]. Hormones of the HPG axis also can disrupt activational effects including the initiation of reproduction [9].

An important concept to note is that the HPG axis is phylogenetically conserved across all vertebrate species (Figure 2) [9]. Studies of the differences in gene sequences

of gonadotropins between teleost fish and mammals concluded that there is 47% homology for LH receptors [12], 53% for FSH receptors [13] and 45% for GnRH receptors [14]. The HPG axis of teleost fish also incorporates functions of the liver, which produces proteins such as vitellogenin (VTG), which is taken up into maturing oocytes during reproduction [9].

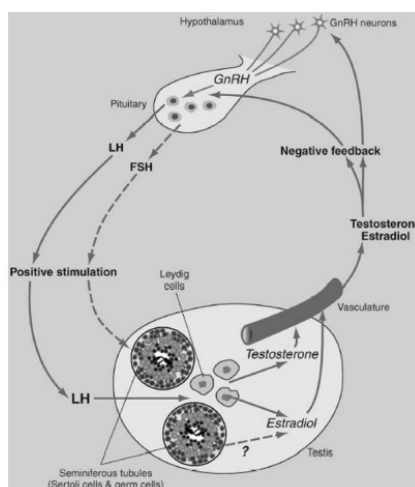


Figure 2. Schematic representation of the HPG axis in male teleost fish. This axis is phylogenetically conserved across all vertebrate species. Figure taken from Ankley & Johnson (2004) [9].

### Importance of Studying Synthetic Glucocorticoids

Corticosteroids are steroid hormones produced from cholesterol by the adrenal cortex. There are two major types: glucocorticosteroids and mineralcorticoids. Mineralcorticoids regulate cardiovascular function by influencing salt and water balances [15]. Glucocorticoids control numerous biological processes including carbohydrate, lipid and protein metabolism, anti-inflammation and regulation of the immune response [16]. Moreover, the presence of glucocorticoid receptors in reproductive tissues of vertebrates suggest that they may have positive and/or inhibitory effects on reproduction [16].

Often, naturally occurring hormones are more rapidly metabolized in humans or have a lower efficacy compared to synthetic hormones [17]. Many of the synthetic hormones were specifically designed to have greater pharmacologic potency, enhanced absorption properties, increased sensitivities and/or longer duration of action compared to the natural hormones [17]. Synthetic glucocorticoids are the most potent anti-inflammatory agents currently available for the treatment of chronic inflammatory diseases such as asthma and rheumatoid arthritis. Their clinical efficacy stems from their ability to mimic naturally occurring glucocorticosteroids in humans [18].

Synthetic glucocorticoids are known to affect the HPG axis at all levels: they decrease the synthesis and release of GnRH from the hypothalamus, they inhibit the synthesis and release of LH and FSH from the pituitary gland and they can modulate steroidogenesis or gametogenesis directly in the testes/ovaries [19] (Figure 3). Ultimately they can adversely affect ovarian steroidogenesis, which can disrupt reproduction in females or they can interfere with spermatogenesis by inducing spermatocyte apoptosis and decreasing sperm yield in males [16].

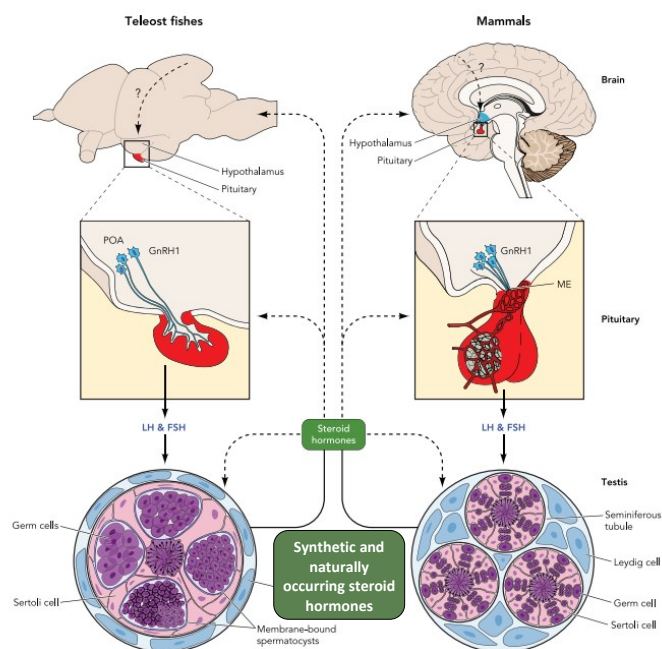


Figure 3. Diagram of the male Hypothalamic-Pituitary-Gonadal (HPG) axis in fish and in mammals. Steroid hormones (both natural and synthetic) affect the HPG axis at all levels. Figure taken from Maruska and Fernald (2011) [20].

It is known that LH and FSH are produced in the gonadotropic cells of the anterior pituitary [21]. These two hormones play vital roles in steroidogenesis, gametogenesis and ovulation. It has been shown that GnRH mediates the synthesis and secretion of LH and FSH and that glucocorticoids suppress GnRH expression [22]. In a study of sheep, it was determined that the majority of effects of glucocorticosteroids on the HPG axis occurred in the pituitary [10]. Furthermore, steroids are also known to control the transcription of LH $\beta$  and FSH $\beta$  subunits, which contribute to the biological specificity of both hormones and are the rate-limiting steps in their production [23]. Several previous studies have shown an increase in FSH $\beta$  gene expression in L $\beta$ T2 cells in response to glucocorticosteroids, and the level of induction is directly correlated with the amount of steroid dosed, indicating steroids are necessary for transcriptional activation of FSH $\beta$  [21, 24]. It was determined that glucocorticoids activate the FSH $\beta$



promoter and subsequently increase FSH $\beta$  gene expression in a saturable, dose-dependent manner [21]. In contrast to FSH $\beta$ , LH $\beta$  expression is actually suppressed in the presence of glucocorticosteroids, likely due to the decrease in circulating GnRH (lack of evidence of an upstream affected promoter region), indicating the two hormones are differentially regulated [21, 24].

A wealth of information on estrogens and their endocrine disruption activity has previously been collected [25, 26]. While these APIs cause adverse effects at very low levels, glucocorticosteroids are used in greater amounts and therefore have the potential to be found in greater concentrations [25]. While environmental levels are still expected to be much lower than therapeutic doses, little is known about the effects of continuous, low level lifetime exposures to humans and wildlife [25]. Moreover, there has been no systematic search for the presence of steroidal pharmaceuticals other than estrogens in the aquatic environment to date [25].

### Betamethasone

Betamethasone is a synthetic glucocorticosteroid that mimics the action of cortisol [19]. It has been on the market for sale in the United States since July of 1983 [27]. About 20,000 kg of betamethasone was sold worldwide in 2014, of which only about 240 kg was sold in the United States [28]. Additionally, it is listed on the World Health Organization Model List of Essential Medicines [29].

In clinical applications, clotrimazole/betamethasone is considered the most frequently prescribed topical cream in the United States [30]. Betamethasone may also be administered intranasally or via injection to treat conditions associated with decreased

adrenal gland function and to accelerate fetal lung maturation for women at risk of preterm birth [31-33].

In clinical trials, betamethasone displayed a high relative glucocorticoid activity compared to other commonly used glucocorticosteroids, and the duration of effect following administration was determined to be approximately 48 to 72 hours [34]. The bioavailability following inhalation or intranasal administration is unknown but is assumed to be approximately 35% based on data from other glucocorticosteroids [35]. A low clinical topical daily dose of betamethasone is estimated at 0.5 mg/day [36]. The recommended dose of betamethasone acetate and sodium phosphate suspension via intramuscular administration is 0.25 to 9 mg/day and the minimum effective daily dose via intranasal administration is 400 µg/day [36]. Adrenal suppression has been reported in children administered betamethasone intranasally for allergic rhinitis at doses as low as 66 µg/day for one year [33]. An acceptable daily exposure (ADE) value of 2 µg/day was previously derived using the daily dose of 66 µg/day via intranasal administration in children and adjusting to account for interindividual variability ( $UF_H=3$ ), extrapolation to a no-effect level ( $UF_L=3$ ) and the assumed 35% bioavailability by the intranasal route of administration [36]. The ADE value assumed 100% adsorption; however, additional studies of betamethasone in rats suggest that it has an oral bioavailability of 65% [37].

Studies of betamethasone in sheep suggest it has the potential to affect development during gestation; however, these studies only focused on impacts of exposure to therapeutic doses (0.5 mg/kg) [38, 39]. Other studies of 0.1 mg/kg administered to intramuscularly to pregnant rabbits resulted in decreased birth weight and fetal loss [40] as well as umbilical hernias, cephalocele and cleft palates at

0.05 mg/kg [36]. Pregnant rhesus monkeys treated with 2 mg/kg-day of betamethasone intramuscularly resulted in fetuses with smaller lungs, smaller brains and lower alveolar stability [41]. Therefore, at therapeutic doses, it is known that betamethasone can disrupt gestational development; however, the consequences from much lower, chronic exposures across the reproductive, postnatal and prenatal periods remain unknown.

#### Fish as a model for Endocrine Disruption Activity

As mentioned previously, the HPG axis is phylogenetically conserved across all vertebrate species [42]. It is no surprise that most of the evidence for endocrine disruption (ED) in wildlife populations has been derived from aquatic animals such as fish, due to widespread contamination of surface water [9, 43]. Much of this has involved strong-acting estrogen and androgen agonists, such as estradiol, ethinylestradiol and testosterone [26]. Little is known about the effects of other steroidal pharmaceuticals, which are used in greater amounts and are likely to enter the aquatic environment via a similar pathway [25].

Fish, like all other vertebrates, have the same HPG axis as humans and other mammals, but have the advantage over mammals as an experimental model of reaching maturity relatively quickly and have overall shorter life spans, which make them ideal for life cycle toxicology studies [9]. The toxicant delivery and exposure systems are generally more efficient for fish, and testing programs for ED chemicals have been previously developed, which use a tiered framework specifically to identify chemicals that may affect different mechanisms within the HPG axis [9, 44].

Another advantage of using fish as an experimental model for ED is that the females of nearly all egg-laying vertebrates express vitellogenin (VTG), which is an egg

yolk precursor protein [45]. VTG is synthesized in the liver and while is thought to be under multi-hormonal control, estrogens are known to play a dominant role [46]. Plasma VTG levels rise steadily during the sexual maturation of female fish and has been previously shown to increase with increasing circulating levels of  $17\beta$ -estradiol (E2) [47]. Adversely, very little (if any) VTG can be detected in male fish because naturally circulating concentrations of E2 are too low to trigger its expression [48]. VTG expression is regulated by the HPG axis, and in teleost fish, this system is referred to as the hypothalamus pituitary gonadal liver (HPGL) axis (Figure 4) [49]. The hypothalamus secretes GnRH, which then stimulates the release of pituitary gonadotropins (GtHs) from the pituitary [49]. Two GtHs, GTH I and GTH II, are structurally similar to human follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively [50]. GtH I (FSH) is involved in vitellogenesis and zonagenesis, while GtH II (LH) plays a role in final oocyte maturation and ovulation [50, 51]. GtH secretion is regulated through a feedback mechanism by E2 and testosterone [49]. E2 also stimulates the production of VTG in females [49]. Therefore, elevated levels of VTG expression in males is evidence of estrogenicity, and VTG can be used as a sensitive and specific biomarker of exposure to estrogen-active substances [48, 49].

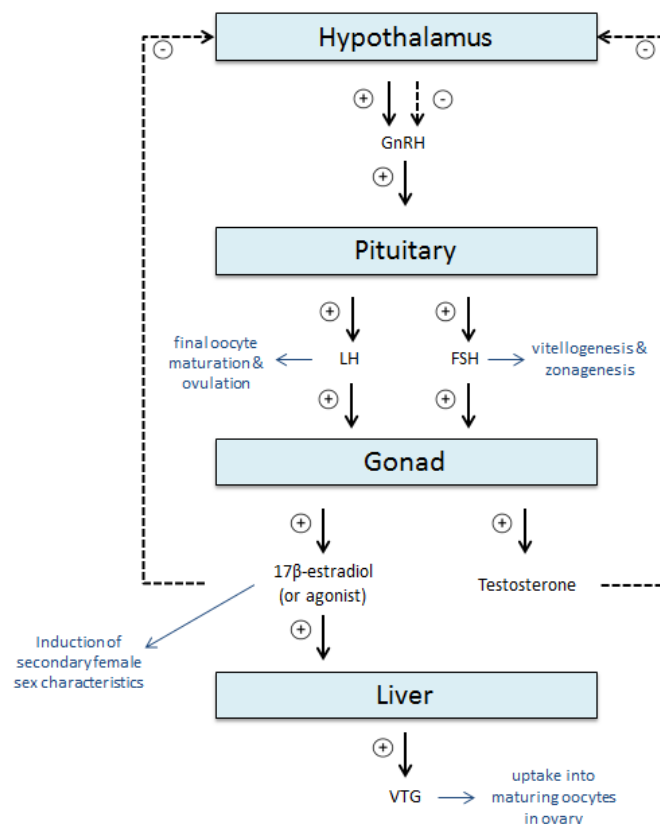


Figure 4. Schematic representation of the hypothalamus pituitary gonadal liver (HPGL) axis in female fish. The HPGL axis is regulated through a negative feedback mechanism triggered by 17β-estradiol (E2) and testosterone. Figure adapted from Arukwe and Goksøyr (2003) and Ankley et al (2004) [9, 49].

Historically, three species of small fish have been used as models for the screening and testing of ED potential of chemicals: the fathead minnow (*Pimephales promelas*), the zebrafish (*Danio rerio*) and the Japanese medaka (*Oryzias latipes*) [9]. All three fish species have advantages and disadvantages, as discussed below.

#### *Fathead minnow*

The fathead minnow (*Pimephales promelas*) is native to North America and adults are approximately 50 to 70 mm long, weigh 2 to 5 grams, and are large enough for collection of blood samples [52]. This species is sexually dimorphic: the adult male is

larger than the adult female and exhibits secondary sexual characteristics, such as dark banding, dorsal pads and nuptial tubercles [53]. These features are essential for visually identifying potential changes in normal female:male ratios following exposures to endocrine disrupting chemicals (EDCs). Male and female fathead minnows are phenotypically indistinguishable until they reach full maturity at approximately 4 months [54]. Therefore, tests to determine alterations in female:male ratios need to be prolonged until maturity is reached.

Adult breeding fathead minnow males are territorial and will actively defend their nest sites against other males or intruders [54]. Therefore, it is necessary to isolate breeding pairs during testing. Females generally have 30 to 50 eggs per spawn under optimal lab conditions every 3 to 4 days [54]. In a typical 21 day chronic study, 300 or more eggs can be produced. Some major disadvantages of the fathead minnow as a test species include the relatively little knowledge available on sex steroid dynamics and the specific processes that control sex determination [54].

### *Zebrafish*

The zebrafish (*Danio rerio*) is a member of the Cyprinidae family and is native to India and Burma [9]. They are approximately 40 to 50 mm long and weigh about 1.5 grams. The eggs are relatively transparent so developing embryos can be observed; however, it is difficult to collect blood from individual zebrafish, which poses a problem for the analyses of sex steroids [9].

This species reaches full maturity at approximately 2 to 3 months of age but does not exhibit sexual dimorphism, making this endpoint difficult to study in EDC tests [9]. Adult females have small spawns daily and relatively large spawns every 5 to 10 days,

which can sometimes contain in excess of 150 eggs. Zebrafish are known as “broadcast” spawners, which means they release eggs that settle to the bottom of the tank, making determination of the condition and number of prehatch zebrafish more intensive [9]. Additionally, the timing of sexual development and differentiation in this species is poorly understood.

### *Japanese medaka*

The Japanese medaka (*Oryzias latipes*) is a member of the Adrianichthyidae family and is distributed throughout southeastern Asia [9]. Adult medaka are approximately 25 to 50 mm long, and weight 0.7 to 0.8 grams [9]. This species reaches maturity in approximately 2 to 3 months, but unlike the zebrafish, does exhibit sexual dimorphism [9]. Therefore, secondary sexual characteristics are often used as an endpoint in EDC studies using this species. Unlike the fathead minnow, Japanese medaka do not display territorial behavior and can be bred in groups or pairs [9]. Females produce approximately 10 to 30 eggs per day and are not considered “broadcast” spawners, so the eggs are easier to analyze. Furthermore, the process of development and differentiation of the gonad in larval medaka is better characterized than both the fathead minnow and the zebrafish [9], and while blood collection in the Japanese medaka is more challenging than the fathead minnow, it is not as difficult as with the zebrafish.

One major advantage of the Japanese medaka species for ED testing is the ability to genetically determine the sex of individuals before full sexual maturity has been achieved. DMY, the DM-domain gene on the Y chromosome of non-mammalian vertebrates, has been proven to be required for normal development of male individuals [55]. Expression of DMY is sufficient for male development in medaka and multiple

studies suggest that the functional difference between the X and Y chromosomes in medaka is this single gene [55]. A reduction in DMY expression has also been shown to result in XY females [56]. Studies of XY fry exposed to 17 $\alpha$ -estradiol (a known EDC), resulted in 100% sex reversal [57]. Therefore, genetic sex can be compared to phenotypic and gonadal sex in order to determine feminization of males or masculinization of females.

Finally, male Japanese medaka normally have papillae that develop as a secondary sexual characteristic [58]. These bony structures grow during sub-adulthood and their total number can be positively or negatively affected by exposures to endocrine disrupting compounds [58, 59]. In males, it has been found that estrogen receptor agonists can lower the total number of bones or prevent them from growing at all [58, 59]. Androgen receptor agonists can induce growth of these papillae in females [58, 59].

### SimpleTreat

As discussed previously, betamethasone has the potential to enter the aquatic environment through effluent of wastewater treatment plants following excretion from normal patient use and subsequently be found in drinking water. Currently, very little is known about the fate of betamethasone in the aquatic environment. One study of the biodegradation of betamethasone determined it has a half-life of approximately 6.2 days in sludge [60].

Models may be used to estimate concentrations of betamethasone in surface and drinking water. SimpleTreat (version 4.0) was developed by a workgroup of the European Centre for Ecotoxicology and Toxicology of Chemicals, which simulates the fate of chemicals following waste water treatment and is recommended for use in the



environmental risk assessment of pharmaceuticals [61, 62]. This model is based on the physical-chemical properties of an individual compound, including (but not limited to) molecular weight, water solubility, vapor pressure, sludge degradation half-life, adsorption/desorption coefficient ( $\log K_{oc}$ ), and the *n*-octanol/water partition coefficient ( $\log K_{ow}$ ). Additionally, it assumes default waste water treatment sewage flow of 200 L/person-day.

#### Pharmaceutical Assessment and Transport Evaluation (PhATE) Model

The Pharmaceutical Assessment and Transport Evaluation (PhATE) model (version 4.0.1), was developed by the Pharmaceutical Research Manufacturers Association and estimates concentrations of APIs in U.S. surface waters resulting from normal human use of medicines. This model uses a mass balance approach to calculate predicted environmental concentrations (PECs) based on publicly owned treatment works (POTW) discharge loads from the population served, the API use per capita, the potential loss of API associated with human use and the portion of API mass removed in the POTW [63].

The PhATE model is used to calculate PECs for 12 watersheds that are considered to be representative of most hydrologic regions of the United States, and that in total, cover approximately 19% of the surface area of the contiguous 48 states [63, 64]. It is supported with data from the USGS nationwide reconnaissance of the occurrence of pharmaceuticals, hormones, and other organic wastewater contaminants in water resources [65]. This model is useful for estimating environmental concentrations when sampling data are unavailable and outputs are estimated for both surface and drinking water sources.

## Estimation of Risk

### *PEC/PNEC Ratio for Aquatic Life*

According to the Guidance for the Implementation of REACH (a regulation of the European Union, adopted to improve the protection of human health and the environment from the risks that can be posed by chemicals), a predicted no effect concentration (PNEC) for aquatic life can be determined based on the highest concentration at which no adverse effects are observed divided by appropriate assessment factors [66]. Since it is known that fish are more sensitive to estrogen-active compounds than other aquatic species [67], an assessment factor of 10 is appropriate. Therefore, risk to the aquatic environment is anticipated if the predicted environmental concentration (PEC) is greater than the PNEC [68].

### *Dose to Fish*

The total dose to fish (or uptake into fish plasma) is estimated based on the partitioning between the aqueous phase and arterial blood [69, 70]. Therefore, the highest concentration at which no observable adverse effects (NOEC) occur can be used to estimate an uptake dose in the fish at which no effects are anticipated.

### *Reference Dose (RfD)*

A reference dose (RfD), or the maximum acceptable oral dose for humans, can be derived using the total dose to fish and appropriate uncertainty factors (UFs) in order to extrapolate within and across species. A previous paper argued that a composite uncertainty factor of 1000 is appropriate between fish and humans, which accounts for

extrapolation of animals to humans ( $UF_A=10$ ), human interindividual variability ( $UF_H=10$ ), and non-mammalian to mammalian species (interspecies  $UF=10$ ) [71]. Given that the HPG axis is conserved, and fish to humans is already accounted for ( $UF_A$ ), a composite uncertainty factor of 100 seems more appropriate. However, species effect differences have been noted among fish exposed to endocrine disrupting compounds [72] and therefore, a modifying factor for intraspecies variability of 10 should be added to account for residue uncertainties regarding interspecies variability ( $MF=10$ ).

#### *Average Daily Dose (ADD)*

The average daily dose (ADD) to humans can also be estimated based on the concentration of betamethasone in surface and drinking water, the contaminant concentration, default intake rates, the oral bioavailability of betamethasone, the total time of exposure and body weight of adults and children [73, 74].

The contaminant concentration is the estimated betamethasone concentration in drinking and surface water. Surface water concentrations were used to represent groundwater drinking sources, conservatively assuming no attenuation. According to the U.S. EPA (2014) [74], the default drinking water ingestion rate (IR) for an average adult and child is 2.5 and 0.78 L/day, respectively, and the average body weight (BW) for adults and children is 80 and 15 kg, respectively. The oral bioavailability of betamethasone in humans is estimated to be 65% [37] and an exposure factor of 1 represents a daily exposure.

### *Margin of Safety (MOS)*

Using the derived RfD and the estimated ADD, a margin of safety (MOS) for humans can be calculated assuming daily ingestion of surface and drinking water (Equation 5). The MOS is the ratio of the threshold dose (RfD) and the actual dose (ADD) [75]. If the MOS is greater than 1, no adverse effects are anticipated [75].

### STUDY AIMS

1. Estimate concentrations of betamethasone in surface and drinking water following normal patient use in the United States using the SimpleTreat and PhATE models. Surface water is used as a surrogate for drinking water from ground water sources.
2. Develop a protocol and execute a fish full life cycle study of betamethasone using VTG as a biomarker of estrogenicity at environmentally relevant concentrations.
3. Compare fish results to mammalian studies to validate model.
4. Calculate the risk to aquatic life as well as the potential endocrine disruption risk to humans based on the results of the fish full life cycle study and the estimated concentrations of betamethasone in surface and drinking water.

## METHODS

### SimpleTreat

SimpleTreat (version 4.0) was used to predict the percentage of betamethasone that is expected to reach surface water following treatment in a POTW. The relevant physical-chemical properties of betamethasone used in the model are detailed in Table 1.

Table 1. Physical-chemical properties of betamethasone used in the SimpleTreat (version 4.0) and PhATE models (version 4.0.1).

Property	Input	Reference
Molecular Weight:	392.47 g/mole	[76]
Water solubility:	66.5 mg/L	[77]
Log Kow:	2.11 at pH 7	[78]
Vapor Pressure:	$7.75 \times 10^{-12}$ Pa	[79]
Biodegradation in sludge:	DT <sub>50</sub> = 6.2 days k <sub>e</sub> = 0.1122 /day	[60]
U.S. API Sales in 2014	240 kg	[28]

### PhATE

PhATE (version 4.0.1) was used to estimate concentrations of betamethasone in surface water and drinking water using the physical-chemical properties of betamethasone and loss to sludge following treatment (as predicted by SimpleTreat) using the input parameters detailed in Table 1.

### Model Species and Fish Full Life Cycle Study

The Japanese medaka was selected as an appropriate species to use in a fish full life cycle (FFLC) study of endocrine effects, specifically due their small size, the short time it takes for them to reach full sexual and reproductive maturity, and the ability to genotypically determine sex and compare to visible secondary sexual characteristics for discrepancies.

In a screening study, 0.1, 1.0, and 10 µg betamethasone/L were initially selected for the treatment groups. However, 100% mortality was observed within 7 days in the highest treatment group (10 µg/L) and it was therefore terminated. Exposure concentrations of 0.01, 0.1 and 1.0 µg/L were then selected as more appropriate for the definitive FFLC study. Additionally, a positive control (90 ng estradiol/L) was used, as well as a negative control (clean water). The concentrations were based on both the results of the PhATE model (in order to determine environmental relevance) and a previous fish early life stage study with the fathead minnow that resulted in an observed NOEC for betamethasone of 50 µg/L (in order to avoid general toxicity effects) [80].

The study was performed following Organisation for Economic Cooperation and Development (OECD) standard guidelines #210, 229 and 234 [81-83]. The OECD Guidelines are used most frequently to assess the potential effects of chemicals on human health and the environment. They are used in various settings such as industry and academia, and are internationally accepted as standard methods for safety testing by governmental organizations such as the European Medicines Agency and the United States Food and Drug Administration [84]. A brief description of the methodology is

outlined below. More detailed information on the assessment of endpoints and measurements can be found in Figure 5 and Appendix I.

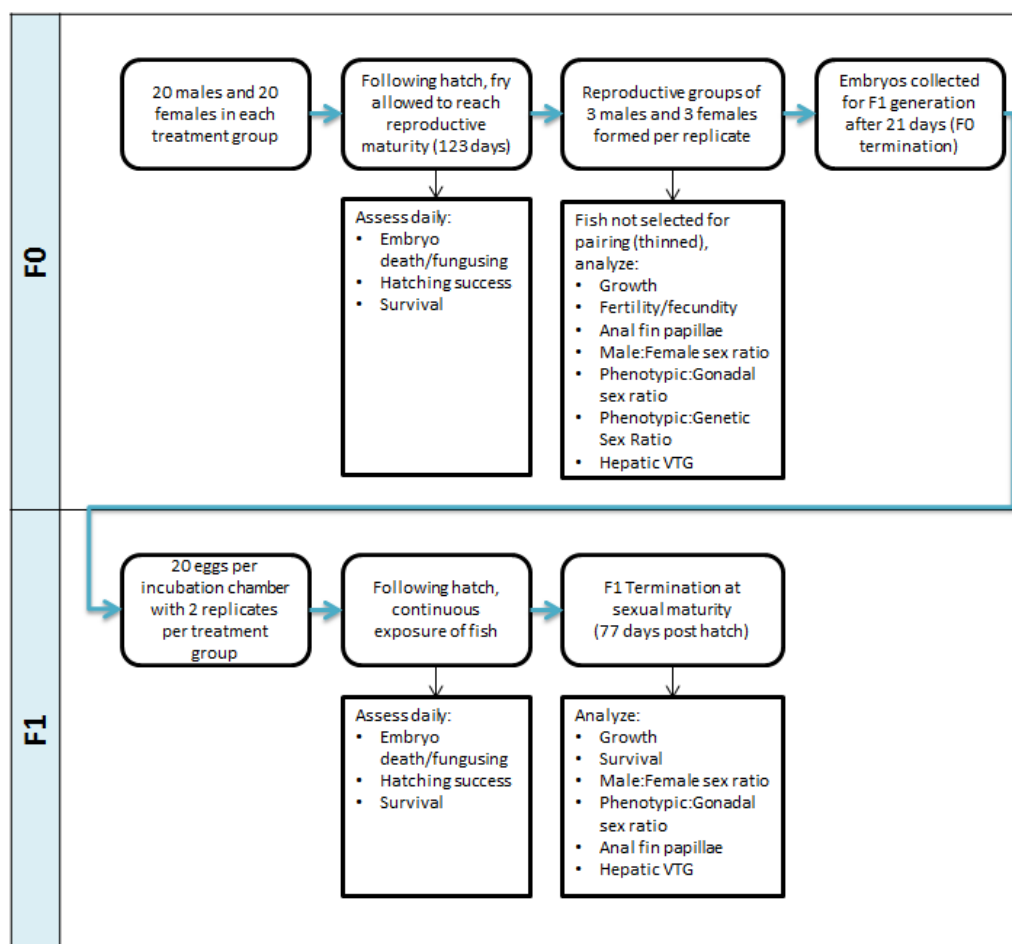


Figure 5. Schematic of sampling times and endpoints investigated in the fish full life cycle study.

### *Exposure System*

Betamethasone is not expected to degrade in water ( $DT_{50} > 1$  year) and thus nominal concentrations were used for the study [85]. Continuous flow-through diluter systems were used to provide each concentration of betamethasone, the negative (clean water) control, and the positive (estradiol) control at a flow rate of 45 mL/min. Test chambers were 6 L glass aquaria filled with 5 L of water. The temperature, hardness,

alkalinity, pH, specific conductance and total organic carbon (TOC) of the water were approximately:

Temperature:	24-26°C
Hardness:	140 mg/L as CaCO <sub>3</sub>
Alkalinity:	180 mg/L as CaCO <sub>3</sub>
pH:	8.2
Specific Conductance:	365 µS/cm
Total Organic Carbon:	<2 mg/L

Since it is known that environmental conditions can influence sex ratios [86], temperature was monitored daily, hardness, alkalinity, pH and specific conductance were measured weekly to ensure consistency of the well water and TOC was measured monthly. A separate diluter system was used for each generational exposure (F0 and F1) and embryos were held in incubation chambers (well plates) within each test chamber. For each system, a pump was used to deliver stock solutions to containers where betamethasone was mixed with dilution water in order to prepare the appropriate nominal concentrations prior to delivery to the test chambers. The flow of dilution water into each mixing container was controlled using rotameters. After mixing, betamethasone was delivered in approximately equal volumes to each replicate test chamber within an exposure group. The proportion of water delivered to each replicate was checked prior to initiation of each generational exposure and weekly (or as needed) to ensure that the flow rates varied by no more than  $\pm 10\%$  of the mean flow rate of the four replicates in each exposure group throughout the test.

### *Test Initiation*

Test systems and collection of eggs were performed according to the OECD Guideline for the Testing of Chemicals #229: Fish Short Term Reproduction Assay [81].



Male and female Japanese medaka of reproductive age were allowed to reproduce in clean water. Forty fertilized eggs produced by the coupling parentals were placed into two test chambers and continuously exposed to betamethasone in the flow-through system until they hatched and reached full reproductive maturity (F0 generation).

#### *F0 Embryo Exposure*

Eggs were checked daily for mortality and recorded. OECD guidelines consider >90% hatch rate of the controls to be valid [81]. Therefore, once >90% of viable embryos in the negative control replicates hatched, the newly hatched larvae in each incubation chamber were counted and released into the corresponding replicate test chamber in each experimental group. All unhatched embryos were kept in the incubators until they hatched, at which time they were distributed to the appropriate test chamber, or removed if death or fungusing of the embryo occurred. The rate of hatching was recorded.

#### *F0 Larval-Juvenile-Adult Reproductive Exposure Period*

Throughout the duration of exposure, fish were observed daily for mortality and transferred to clean tanks once per week. When full reproductive maturity was reached at 133 days (123 days post hatch), fin clips were collected for genetic sex determination using PCR methods as specified in OECD Guideline #234 [82]. The fish were randomly thinned to create one spawning group in each test chamber, which consisted of three males and three females. A total of four spawning groups per treatment and control were used for a total of 24 fish per group. When necessary (due to poor hatch), pairs were formed using other replicates of the same treatment group. Male and female fish were initially selected based on external phenotype including the presence (male) or absence

(female) of a notched dorsal fin and sail-like and transparent appearance of the anal fin. Phenotypic sex was then subsequently confirmed by analysis of genetic sex.

All fish not selected for use during the adult reproductive exposure period were euthanized with buffered MS-222 and measured for length and weight. Fin clips were taken to determine genetic sex via expression of DMY, gonads were examined for internal sex determination and presence of testis-ova, and anal fin papillae were counted in accordance with OECD Guideline #210 [83]. Additionally, livers were excised for determination of VTG concentration in 20 fish from each treatment group and were stored frozen (-80°C) until processing.

#### *F0 Adult Fertility & Fecundity*

Adult reproductive groups were allowed to mate for 21 days. All eggs either held by the female or deposited into the tank were removed and counted daily. On day 22 of the fertility and fecundity assessment, fish were euthanized with buffered MS-222 and the same endpoints were evaluated as the thinned fish.

#### *F1 Initiation (2<sup>nd</sup> Generation)*

On day 22 of the F0 fecundity and fertility assessment, eggs were collected from each replicate and pooled within each experimental group. Fertile, healthy embryos were selected and distributed to at least one incubation chamber (well plate) per experimental group, with a maximum of 20 embryos per incubator (one embryo per well).

### *F1 Embryo Exposure*

Eggs were checked daily for mortality and recorded. Once >90% of viable embryos in the negative control replicates hatched, the newly hatched larvae in each incubation chamber was counted and released into the corresponding replicate test chamber in each experimental group. All unhatched embryos were kept in the incubators until they hatched, at which time they were distributed to the appropriate test chamber, or removed if death or fungusing of the embryo occurred. The rate of hatching was recorded.

### *F1 Larval-Juvenile-Adult Reproductive Exposure Period*

The newly hatched larvae were evaluated until full sexual maturity was reached. Throughout the duration of exposure, fish were observed daily for mortality and transferred to clean tanks once per week. When sexual maturity was reached at 91 days (77 days post hatch), fin clips were collected for genetic sex determination via DMY expression using PCR methods as specified in OECD Guideline #234 [82].

Fish were euthanized with buffered MS-222 and measured for length and weight. Fin clips were taken for genetic sex, gonads were examined for internal sex determination and presence of testis-ova, and anal fin papillae were counted in accordance with OECD Guideline #210 [83]. Livers from five males and five females from each replicate (a total of 20 males and 20 females from each treatment and control group) were excised for determination of VTG concentration. Livers were stored frozen (-80°C) until processing.

### *Statistical Analysis*

SAS<sup>®</sup> 9.3 was used to calculate significant differences between male and female Japanese medaka in each treatment group compared to the control, which were considered significant at  $\alpha=0.05$ . Statistical tests that were used to analyze each endpoint are listed in Table 2.

Table 2. Statistical tests used to evaluate each endpoint studied in the FFLC test. All endpoints were evaluated separately for each sex.

Endpoint <sup>1</sup>	Statistical Test
Hatching Success Percent Survival Fertilization Success Phenotypic:Gonadal Sex Ratio Phenotypic:Genetic Sex Ratio	Fisher's Exact Test with Bonferroni Adjustment
Fecundity Length Wet Weight VTG Concentration FSH Gene Expression LH Gene Expression	Dunnett's Multiple Comparison Test

<sup>1</sup>Endpoints evaluated separately by sex

The highest concentration at which no adverse effects were observed for reproductive success or development of secondary sexual characteristics, as well as at which the expression of VTG was not significantly altered was used as the NOEC for endocrine disruption.

## Estimation of Risk

### *Risk to Aquatic Life*

The risk to aquatic life is typically calculated based on the highest concentration in water at which no adverse effects are observed (NOEC). If a NOEC is not identified, then the lowest concentration at which affects are observed (LOEC) will be used in place of a NOEC as a screening value. The NOEC or LOEC is then divided by an assessment factor of 10 to derive a PNEC. The surface water concentration estimated by the PhATE model was used as the PEC.

### *Reference Dose (RfD)*

In order to determine the dose at which no adverse effects were observed in the FFLC, the plasma betamethasone concentration in fish was estimated using Equation 1 and Equation 2 [69, 70, 87]. The fish plasma betamethasone concentration in µg/L was then converted to µg/kg and the subsequent RfD was calculated using Equation 3.

Equation 1:  $\text{Log } P_{\text{blood:water}} = 0.73 * \text{Log } K_{ow} - 0.88$   
 where:  $\text{Log } K_{ow} = 2.11$

Equation 2:  $\text{Fish}_{\text{steady state}} \text{ Plasma Concentration} = \text{Log } P_{\text{blood:water}} * \text{PEC} * 1 \text{ L/kg}$

Equation 3:  $\text{RfD} = \frac{\text{Dose } (\mu\text{g/kg-day})}{\text{UF}_A \times \text{UF}_H \times \text{UF}_D}$

where:

Dose: from Equation 2

UF<sub>A</sub>: 10

UF<sub>H</sub>: 10

MF: 10

### *Average Daily Dose*

The ADD for children and adults was derived using default exposure factors, the bioavailability and betamethasone concentrations in surface and drinking water as estimated the SimpleTreat and PhATE models. As per the Agency for Toxic Substances and Disease Registry [73], the ADD is calculated using Equation 4.

$$\text{Equation 4: ADD} = \frac{C \cdot IR \cdot AF \cdot EF}{BW}$$

where:

- C: contaminant concentration
- IR: intake rate
- AF: bioavailability factor
- EF: exposure factor
- BW: body weight

### *Margin of Safety (MOS)*

The MOS for adults and children was calculated from the RfD and ADDs for both adults and children (Equation 5).

$$\text{Equation 5: MOS} = \frac{\text{RfD } (\mu\text{g/kg-day})}{\text{ADD } (\mu\text{g/kg-day})}$$

where:

- RfD: derived using Equation 3
- ADD: derived using Equation 4

## RESULTS

### Wastewater Modeling

According to SimpleTreat, approximately 4.4% of betamethasone is expected to bind to sewage sludge following waste water treatment, and the remainder is expected enter surface water (Figure 6). The PhATE model predicts that during average flow

conditions, betamethasone concentrations in 95% of all surface water segments will be less than 0.6 ng/L (Figure 7), and concentrations in drinking water for 95% of the US population will be less than 0.1 ng/L (Figure 8).

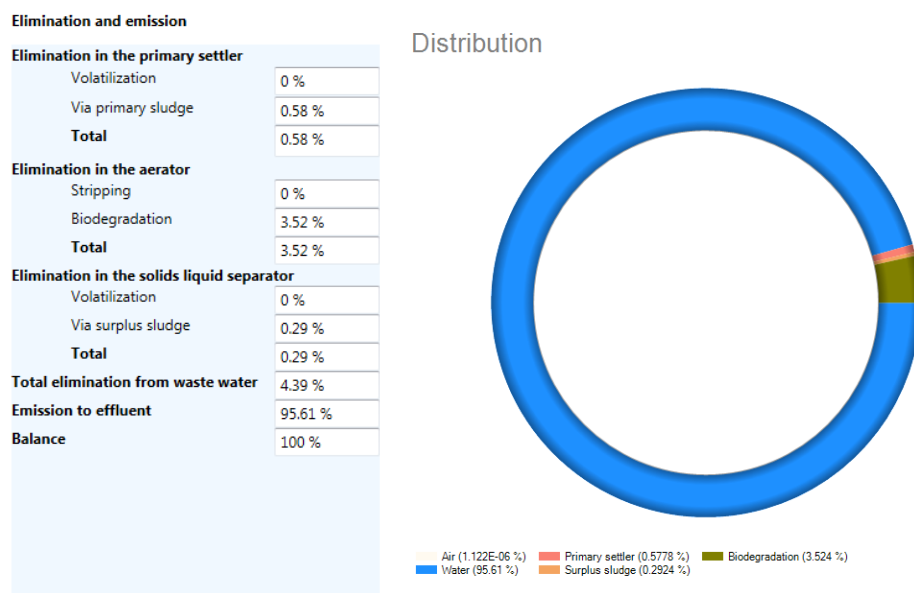


Figure 6. Outputs from SimpleTreat (version 4.0). Approximately 95.6% of all betamethasone entering a WWTP is expected be discharged to surface water via effluent, 0.9% is expected to settle or bind to sludge and 3.5% is expected to degrade.

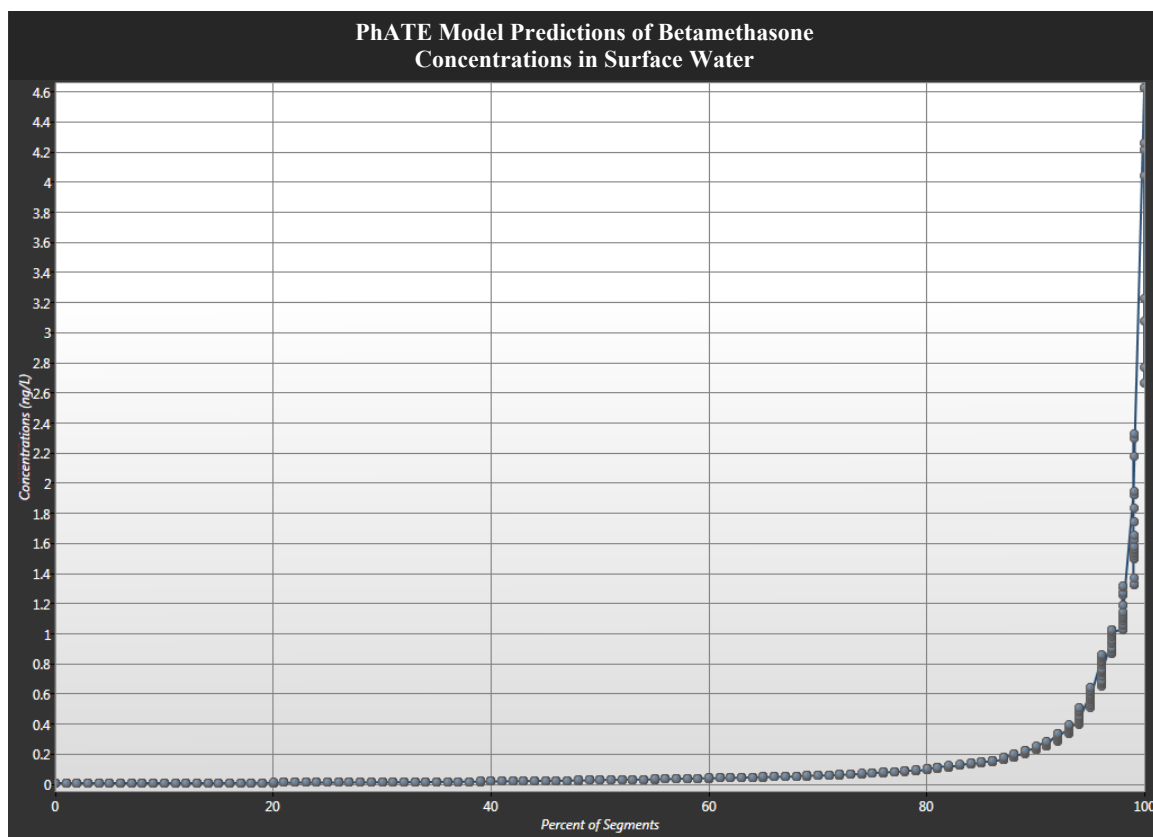


Figure 7. PhATE model predictions of average betamethasone concentrations in surface water segments across 12 watersheds in the United States based on sales in 2014.



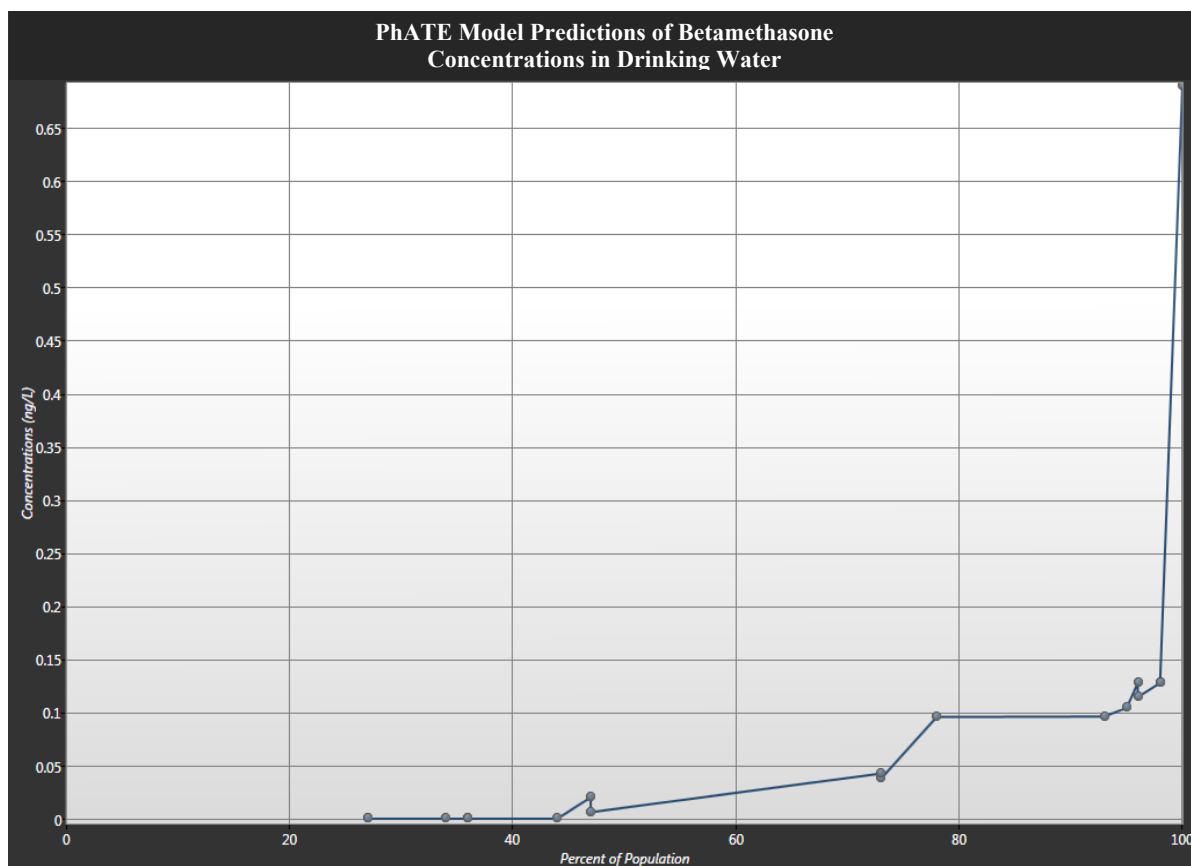


Figure 8. PhATE model predictions of average betamethasone concentrations in drinking water for the population of the United States based on sales in 2014.

### F0 Generation

#### *Definitive Study*

All of the fish in the 90 ng estradiol/L positive control were observed to be female at F0 termination, indicating the system was functioning properly. No reproductive pairs could be made to carry forward into F1 and no further analyses were performed for this group.

Approximately 57% of the eggs in the 0.01 µg/L treatment group succumbed to fungus but this was determined to not be treatment-related. Of the remaining eggs, there were no significant differences observed in percentage embryo survival and hatch (Figure

9,  $p>0.05$ ). No significant differences were also observed in overall F0 adult survival by termination at day 133 (91 days post hatch) (Figure 10,  $p>0.05$ ), ratio of females to males (Figure 11,  $p>0.05$ ), length (Figure 12 & Figure 13,  $p>0.05$ ) and male weight (Figure 14,  $p>0.05$ ) for any of the betamethasone treatment groups. However, a significant reduction in female wet weight was observed in the 1.0  $\mu\text{g/L}$  treatment group (Figure 15;  $p=0.0077$ ). Due to the low sample size of the 0.01  $\mu\text{g/L}$  treatment group, 12 of the 14 female fish were carried forward into reproductive groups. Therefore, sex ratio and survival statistics included both thinned fish and fish carried forward into reproductive groups. Length and weight analyses were only performed on reproductive groups because the sample size of females in the 0.01  $\mu\text{g/L}$  was too low for statistical power, and growth of the reproductive groups was measured approximately 3 weeks after growth of the thinned fish were recorded.

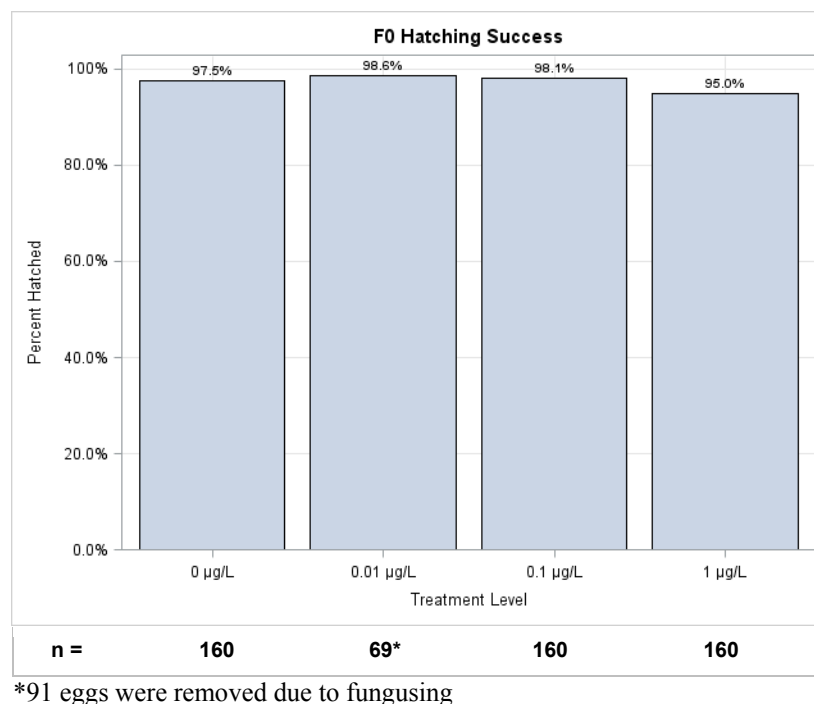
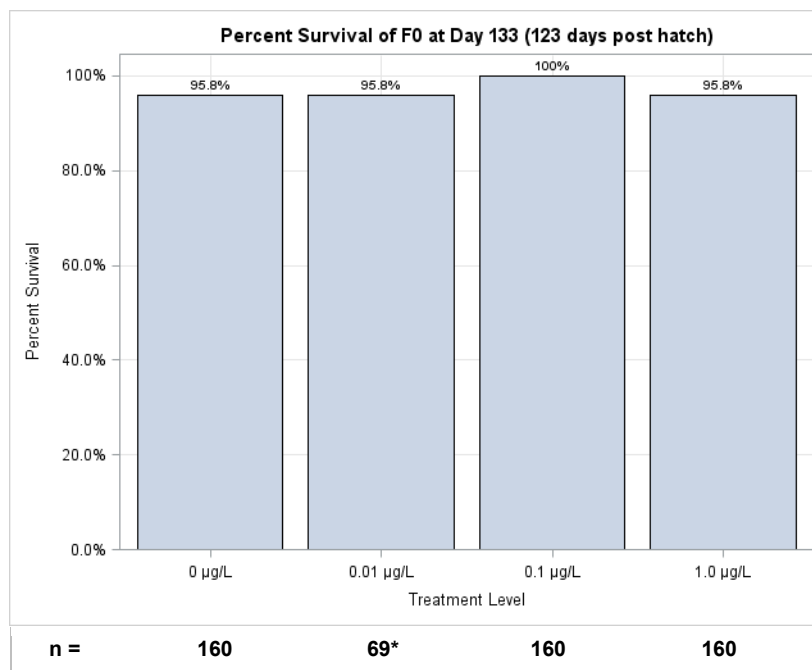


Figure 9. No significant differences were observed for hatching success of F0 fertilized eggs at  $\alpha=0.05$  (Fisher's Exact Test with Bonferroni Adjustment;  $p=0.2968$ ).



\*91 eggs were removed due to fungusing

Figure 10. No significant differences in percent survival of adult reproductive groups at day 133 (123 days post hatch) for the F0 generation were observed at  $\alpha=0.05$  (Fisher's Exact Test with Bonferroni Adjustment;  $p=0.7934$ ).

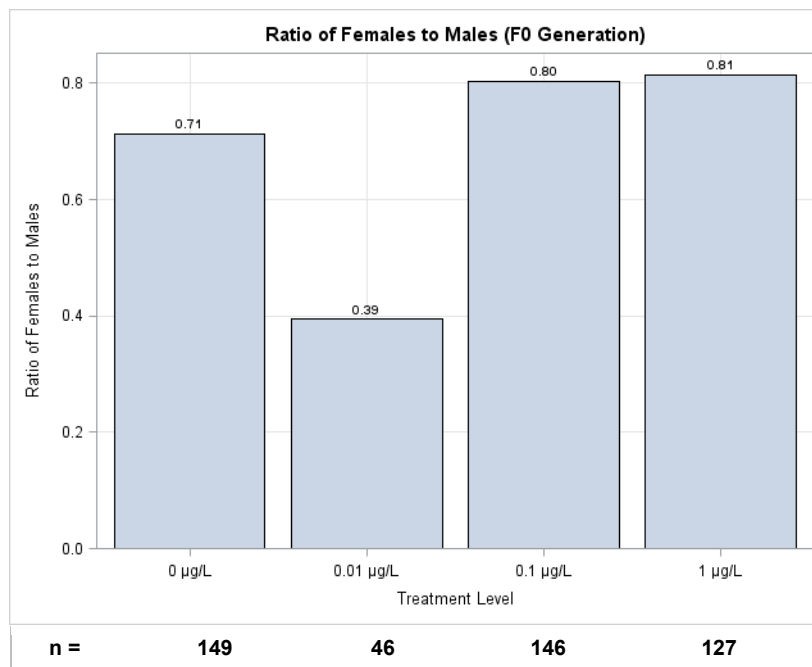
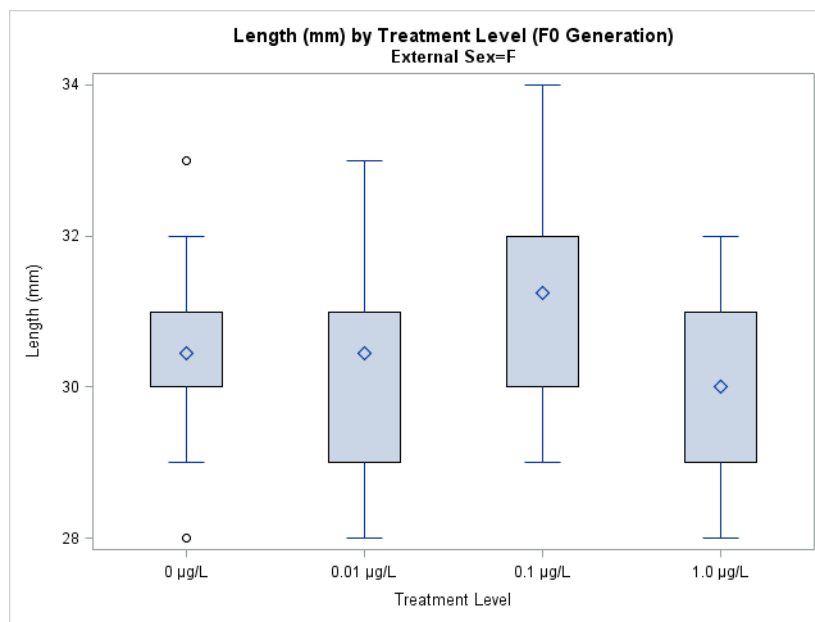
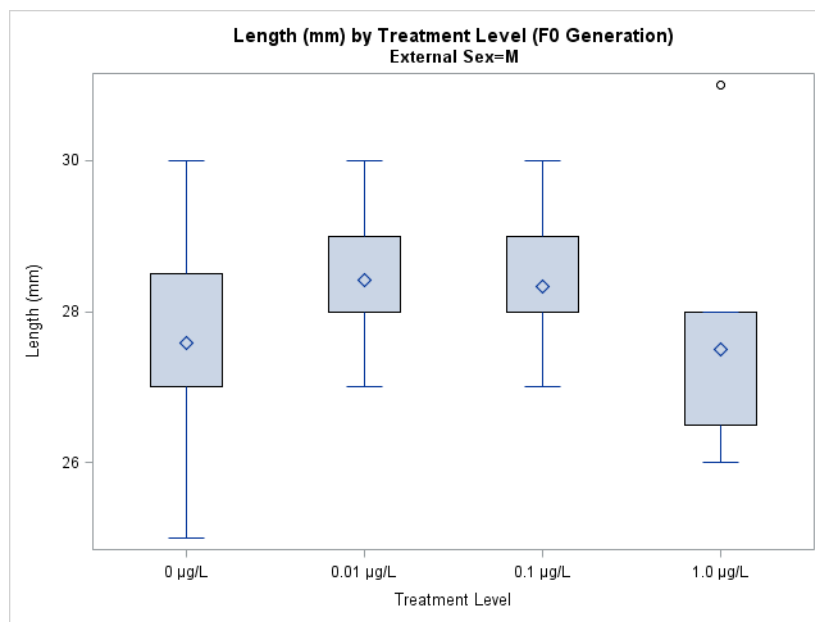


Figure 11. No significant differences were observed between the ratios of females to males in any treatment level of the F0 generation at  $\alpha=0.05$  (Fisher's Exact Test with Bonferroni Adjustment;  $p = 0.2230$ ).



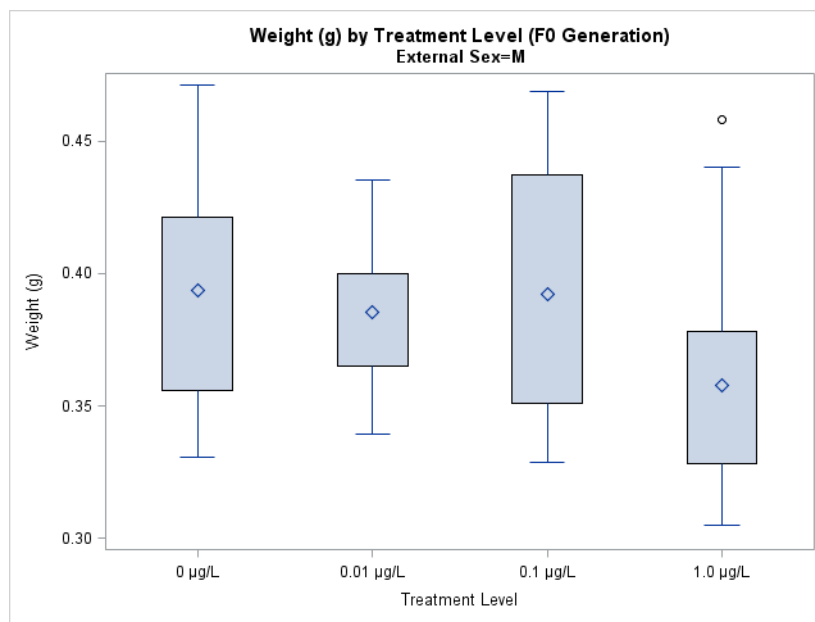
Treatment Level	n	Average Length (mm)	p-value
0 µg/L	11	30.45	1 0.3585 0.7651
0.01 µg/L	11	30.45	
0.1 µg/L	12	31.25	
1.0 µg/L	11	30.00	

Figure 12. No significant differences in length (mm) were observed for females in any of the treatment levels of the F0 generation at  $\alpha=0.05$  (Dunnett's Multiple Comparison Test). Diamonds indicate mean values.



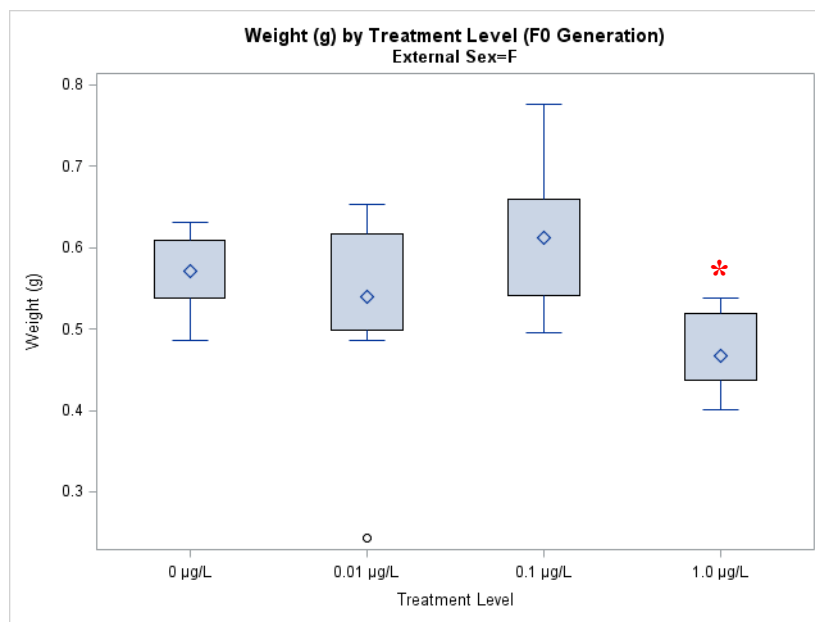
Treatment Level	n	Average Length (mm)	p-value
0 µg/L	12	27.58	
0.01 µg/L	12	28.42	0.2264
0.1 µg/L	12	28.33	0.3026
1.0 µg/L	12	27.50	0.9965

Figure 13. No significant differences in length (mm) were observed for males in any of the treatment levels of the F0 generation at  $\alpha=0.05$  (Dunnett's Multiple Comparison Test). Diamonds indicate mean values.



Treatment Comparison	n	Average Weight (g)	p-value
0 µg/L	12	0.39	
0.01 µg/L	12	0.39	0.9398
0.1 µg/L	12	0.39	0.9398
1.0 µg/L	12	0.36	0.1101

Figure 14. No significant differences in wet weight (g) were observed for males in any of the treatment levels of the F0 generation at  $\alpha=0.05$  (Dunnett's Multiple Comparison Test). Diamonds indicate mean values.



Treatment Comparison	n	Average Weight (g)	p-value
0 µg/L	11	0.57	
0.01 µg/L	11	0.54	0.6619
0.1 µg/L	12	0.61	0.4388
1.0 µg/L	11	0.47	0.0077*

\*Results indicate significant reduction in wet weight at  $\alpha=0.05$ .

Figure 15. A significant decrease in wet weight (g) was observed in females of the F0 generation in the 1.0 µg/L of the F0 generation at  $\alpha=0.05$  (Dunnett's Multiple Comparison Test;  $p=0.0077$ ). Diamonds indicate mean values.

One female fish in the highest treatment group developed a small number of anal fin papillae; however, the total count was not significant compared to the control. No other significant differences in the average number of anal fin papillae were observed for males or females in any treatment group (Figure 16 & Figure 17,  $p>0.05$ ).

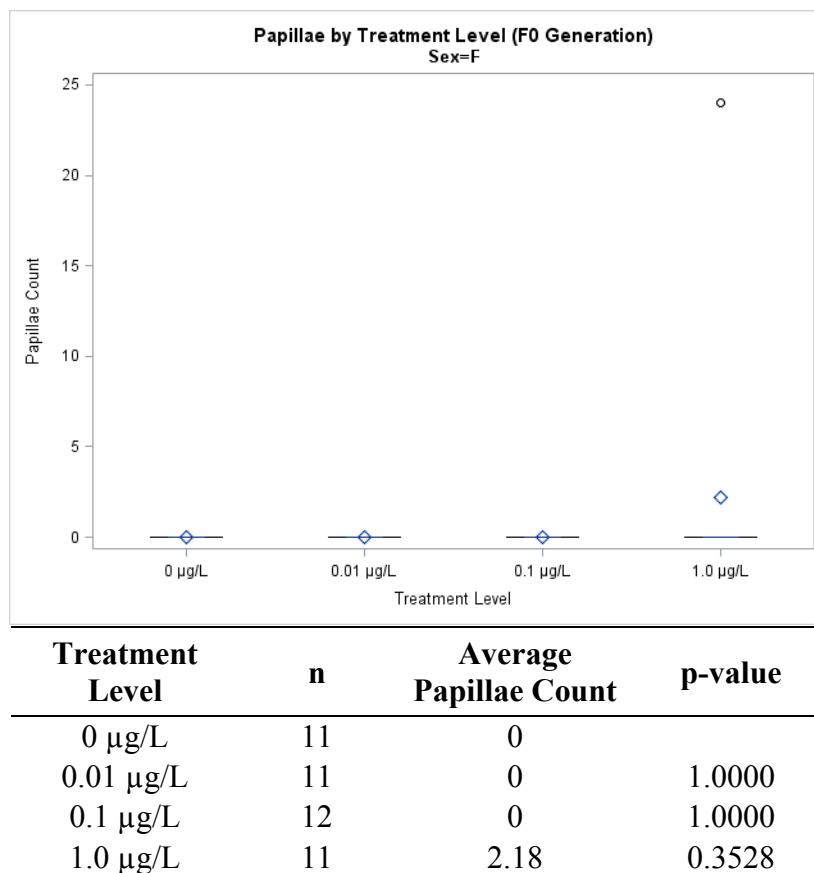


Figure 16. No significant differences in the average number of anal fin papillae were observed for females in any of the treatment levels of the F0 generation (Dunnett's Multiple Comparison Test,  $p>0.05$ ). Diamonds indicate mean values.



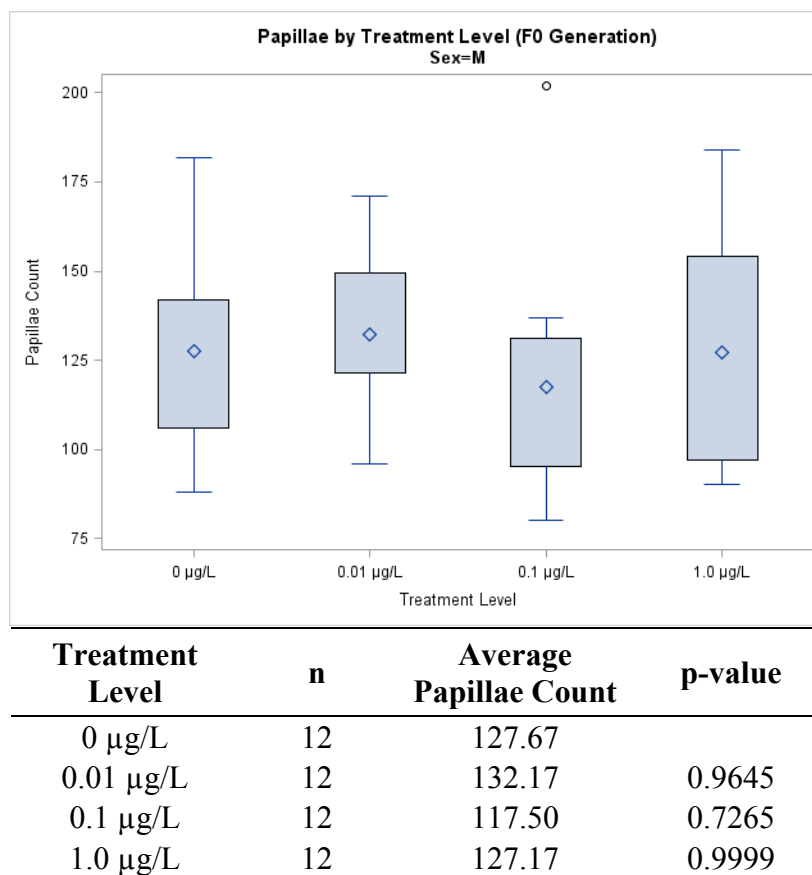
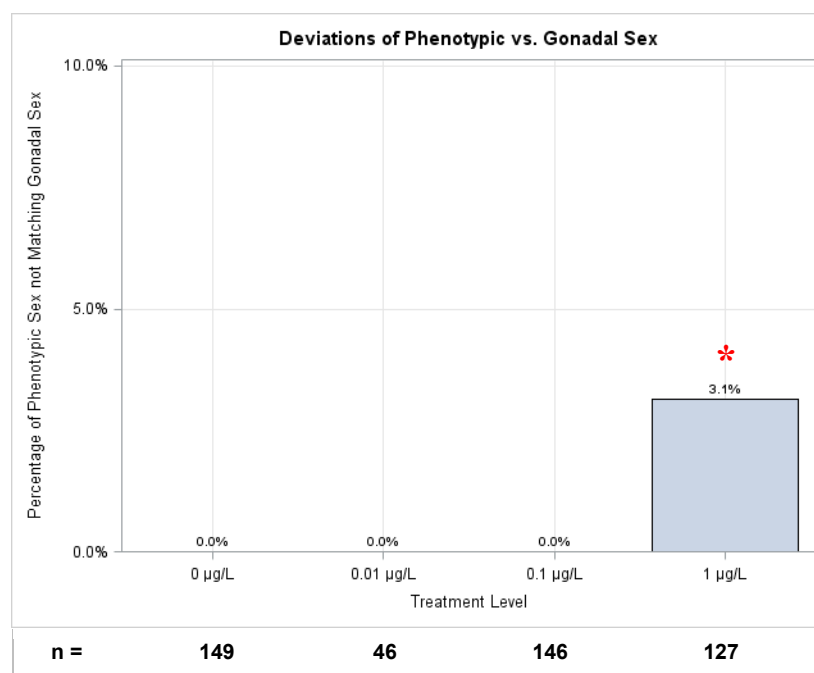


Figure 17. No significant differences in the average number of anal fin papillae were observed for males in any of the treatment levels of the F0 generation (Dunnett's Multiple Comparison Test,  $p > 0.05$ ). Diamonds indicate mean values.

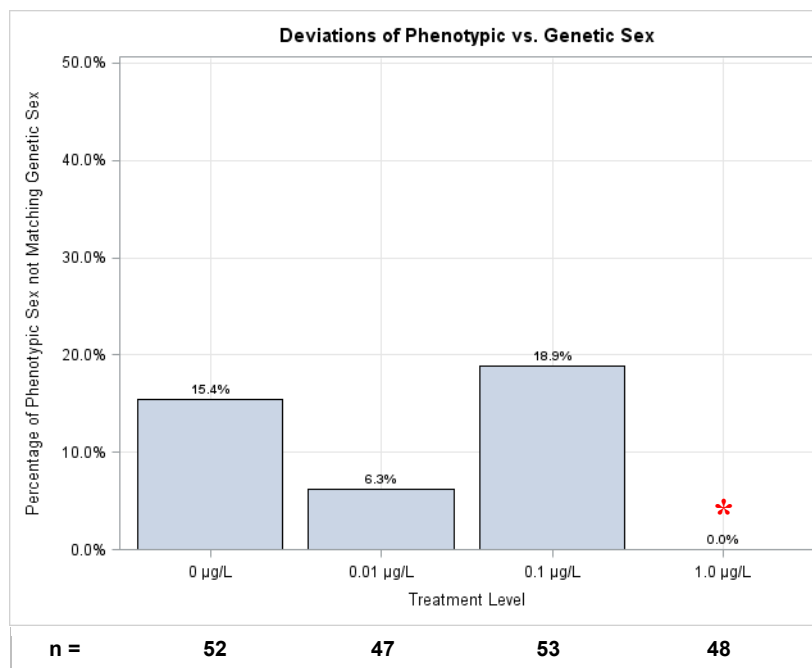
There was a significant increase in the percentage of cases where the observed phenotypic sex did not match gonadal sex in the highest treatment group (Figure 18, Fisher's Exact Test with Bonferroni Adjustment;  $p = 0.0127$ ). Four of the 127 total thinned fish in the 1.0 µg/L treatment group showed signs of feminization (phenotypically female but were noted to have testes upon internal examination). Additionally, fin clips taken from reproductive pairs of the F0 generation were analyzed using PCR to determine consistency of genetic sex with phenotypically determined sex by evaluating DMY expression. However, unlike phenotypic versus gonadal sex ratios in thinned fish, there

was a statistically significant decrease in the percentage of mismatches between phenotypic and genetic sex (Figure 19; Fisher's Exact Test with Bonferroni Adjustment;  $p=0.0135$ ). In all mismatch cases between phenotypic and genetic sex, fish were phenotypically and internally male, but genetically female ( $n=21$ ).



\*Results indicate significant increase in sex mismatch at  $\alpha=0.05$ . All mismatches were phenotypic female but were gonadal males.

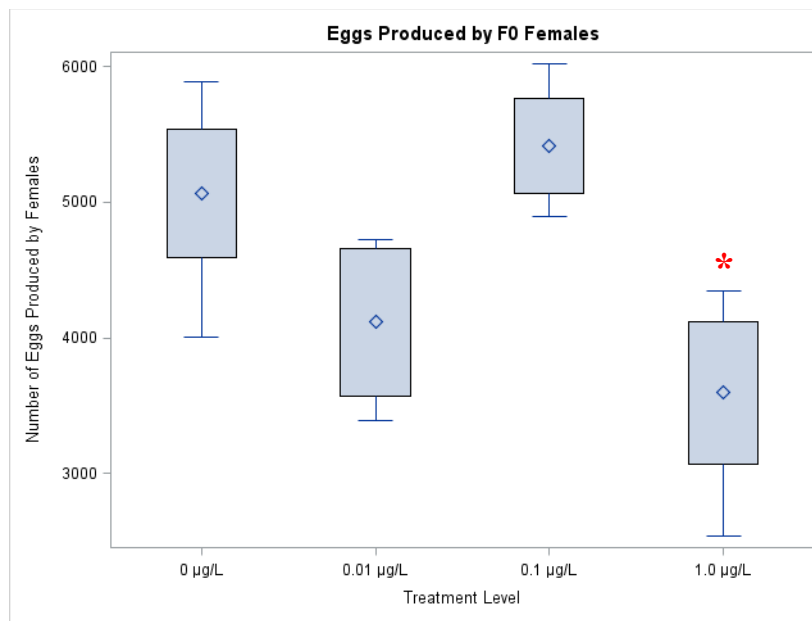
Figure 18. There was a significant increase in the percentage of cases where the observed phenotypic sex did not match gonadal sex in the highest treatment group (Fisher's Exact Test with Bonferroni Adjustment;  $p=0.0127$ ). There were 4 instances where fish were phenotypically female but were noted to have testes upon internal examination.



\*Results indicate significant decrease in sex mismatch at  $\alpha=0.05$ . All mismatches were phenotypic and internally male but were genetic females.

Figure 19. A statistically significant reduction in the percentage of cases where the observed phenotypic sex did not match genetic sex was observed in the highest treatment group (Fisher's Exact Test with Bonferroni Adjustment;  $p=0.0083$ ).

A significant reduction in the number of eggs produced by F0 females was observed in the highest treatment level of 1.0  $\mu\text{g/L}$  (Figure 20; Dunnett's Multiple Comparison Test;  $p=0.0251$ ). A decrease in fertilization success of those eggs was also observed in the highest treatment group; however, this decrease was not significant (Figure 21,  $p>0.05$ ).



Treatment Level	n	Average Egg Count	p-value
0 $\mu\text{g/L}$	11	5065	0.0251*
0.01 $\mu\text{g/L}$	11	4115	
0.1 $\mu\text{g/L}$	12	5411	
1.0 $\mu\text{g/L}$	11	3594	

\*Results indicate a significant decrease in number eggs produced at  $\alpha=0.05$

Figure 20. A significant decrease in the average number eggs produced by F0 females was observed in the 1.0  $\mu\text{g/L}$  treatment group compared to controls at  $\alpha=0.05$  (Dunnett's Multiple Comparison Test;  $p=0.0251$ ). Diamonds indicate mean values.

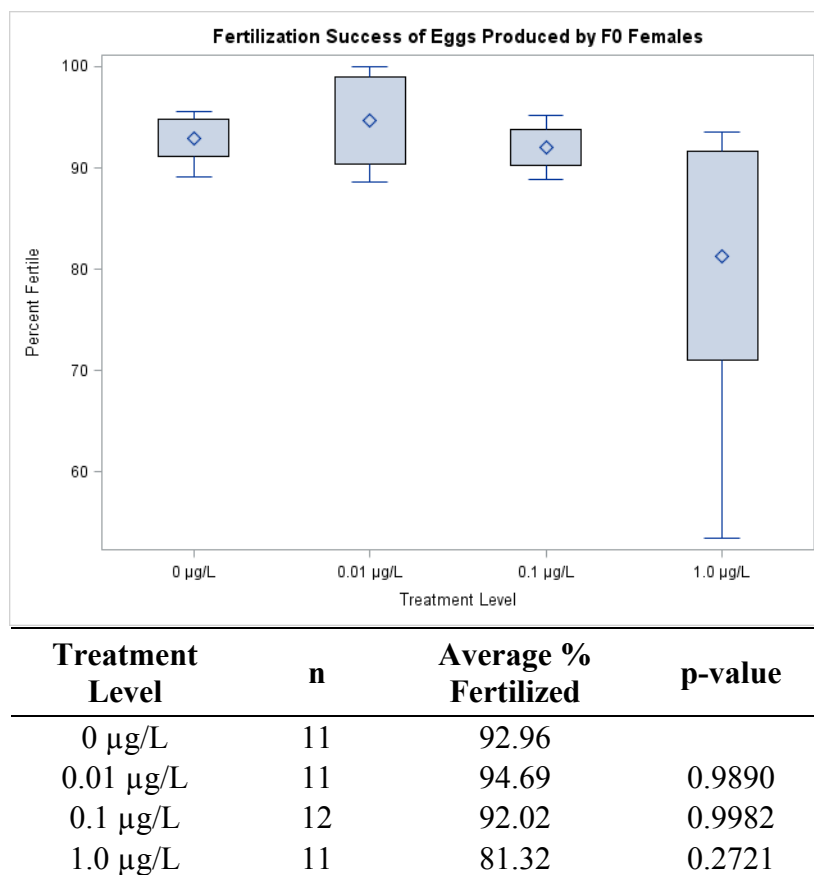


Figure 21. No significant differences in the percent of successfully fertilized eggs were observed in any of the treatment levels of the F0 generation (Dunnett's Multiple Comparison Test,  $p > 0.05$ ). Diamonds indicate mean values.

Due to low sample size of the 0.01 µg/L treatment group, only 2 females and 14 males were able to be evaluated for VTG expression. There were no significant differences observed in any treatment level for females (Figure 22, Dunnett's Multiple Comparison Test,  $p > 0.05$ ). Additionally, there was one sample where the total RNA could not be quantitated in the 0.1 and 0.01 µg/L treatment groups, resulting in 19 and 13 males available for analysis of VTG expression, respectively. There was a slight increase in the average number of ng/copies of RNA hepatic VTG levels in male fish, but this increase was not significant (Figure 23, Dunnett's Multiple Comparison Test,  $p > 0.05$ ).

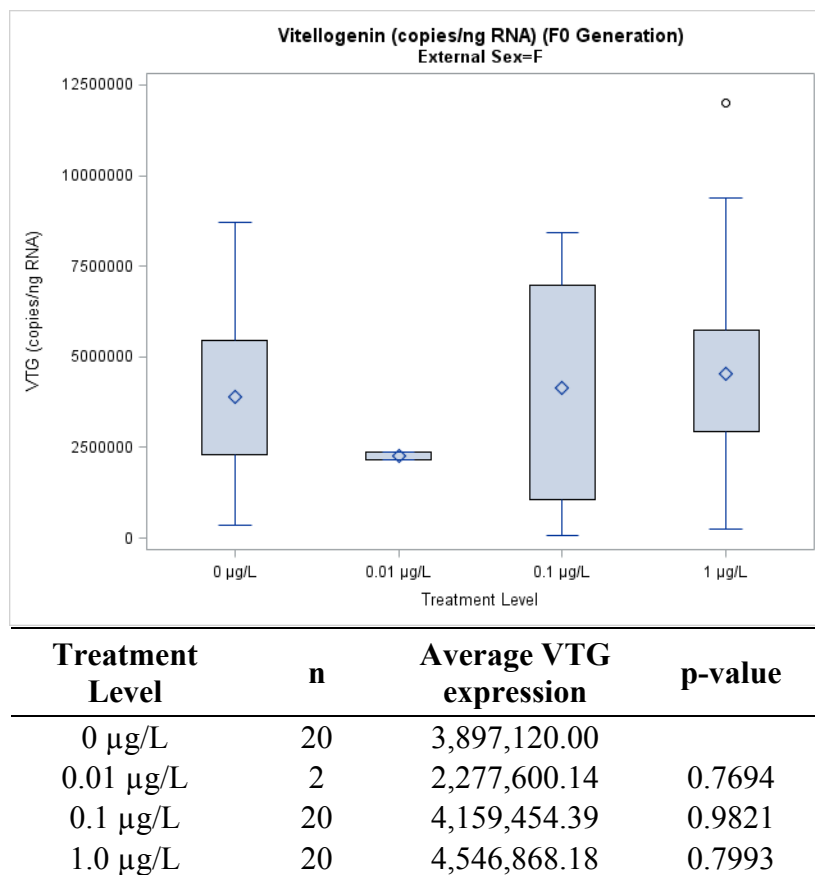


Figure 22. No significant differences were found in the average number of copies/ng of RNA hepatic VTG for F0 females in any treatment group (Dunnett's Multiple Comparison Test). Diamonds indicate mean values.

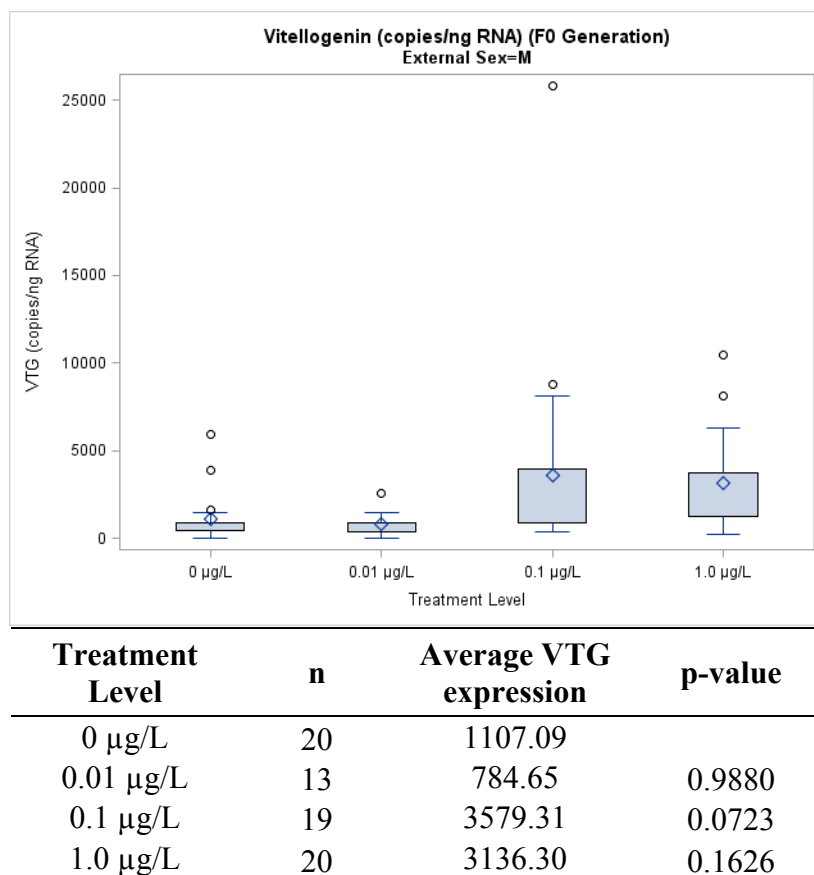
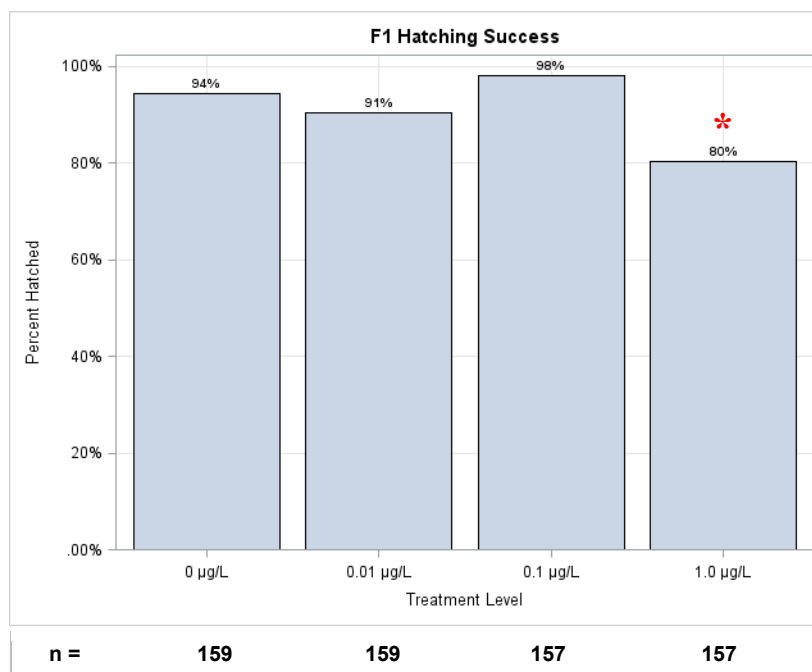


Figure 23. No significant differences were found in the average number of copies/ng of RNA hepatic VTG for F0 males in any treatment group (Dunnett's Multiple Comparison Test). Diamonds indicate mean values.

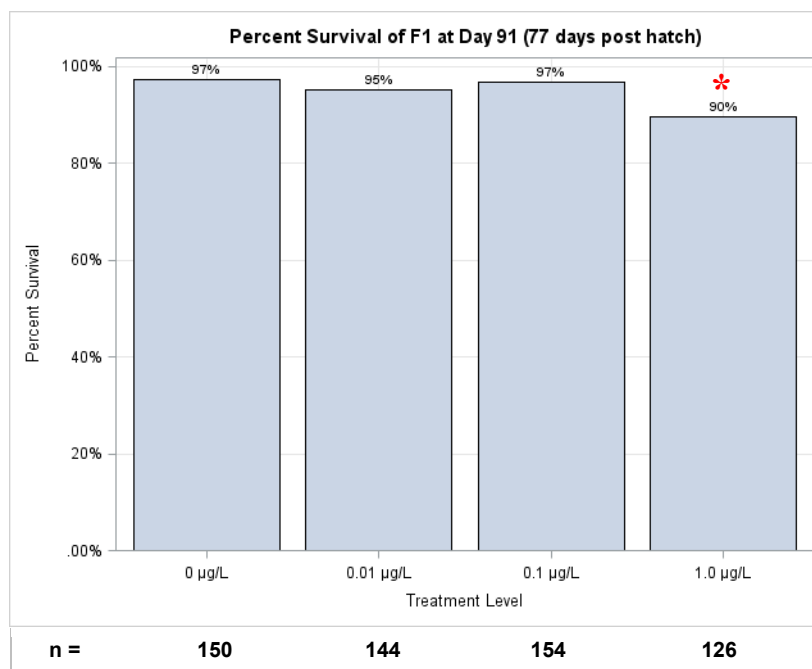
### F1 Generation

There was a significant reduction in hatching success of the F1 generation in the highest treatment group (Figure 24,  $p < 0.0001$ ). Additionally, at termination of the study on day 91 (77 days post hatch), a significant reduction in overall survival was also observed for the F1 generation in the highest treatment group (Figure 25,  $p = 0.0176$ ).



\*Results indicate a significant decrease in hatching success at  $\alpha=0.05$

Figure 24. A significant decrease in hatching success was observed in highest treatment group of the F1 generation at  $\alpha=0.05$  (Fisher's Exact Test with Bonferroni Adjustment;  $p<0.0001$ ).



\*Results indicate a significant decreased in percent survival at  $\alpha=0.05$

Figure 25. A significant decrease in percent survival of the highest treatment group was observed at study termination (77 days post hatch) for the F1 generation at  $\alpha=0.05$  (Fisher's Exact Test with Bonferroni Adjustment;  $p=0.0176$ ).



Large variability was observed in the ratio of females to males; however, the variability is not dose-related and none of the ratios were significantly different from the control (Figure 26,  $p>0.05$ ). No significant differences in length were observed for females in any treatment group (Figure 27,  $p>0.05$ ); however, a small, but significant decrease in male length was noted in the 0.1  $\mu\text{g/L}$  treatment group (Figure 28,  $p=0.0333$ ). It should be noted that the average length in the 0.1  $\mu\text{g/L}$  was only 0.03 mm smaller than the average length of the next highest treatment group, and may be due to biological variability. Fish in all treatment groups were had statistically significantly lower wet weight than the controls (Figure 29 & Figure 30,  $p<0.0001$ ).

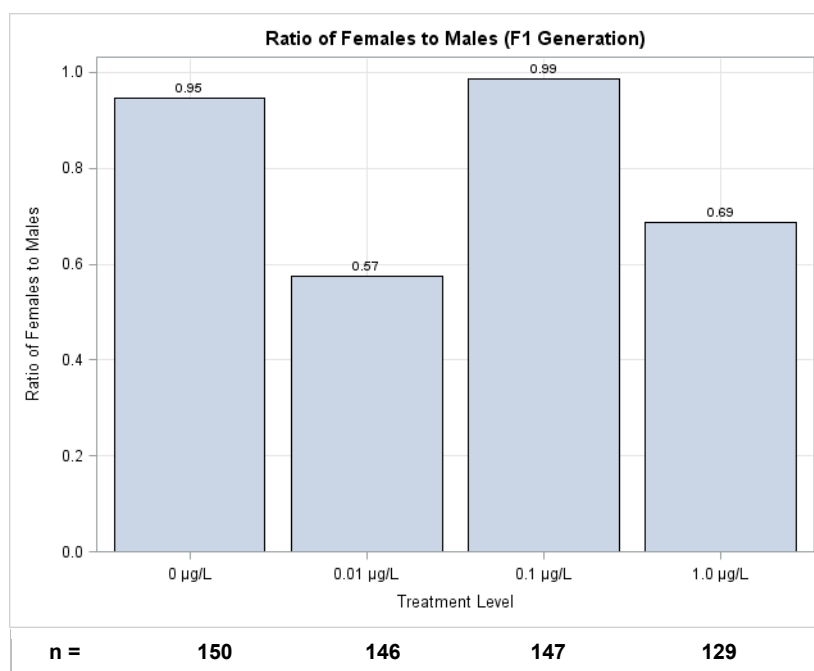
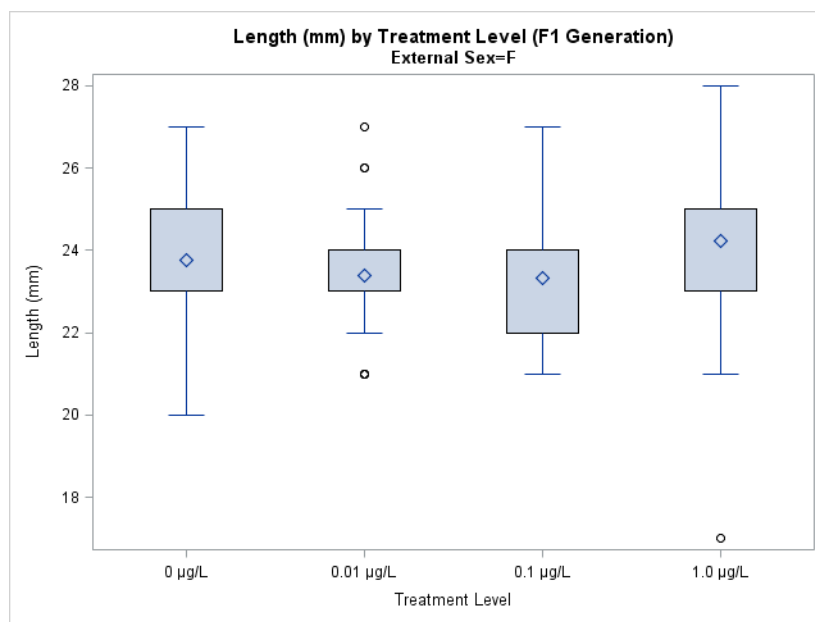
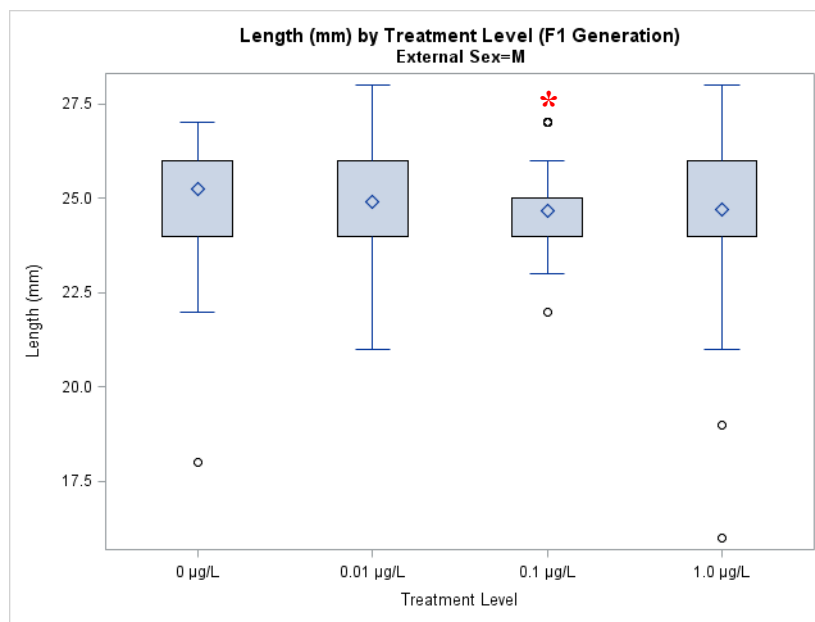


Figure 26. No significant differences were observed between the ratios of females to males in any treatment level of the F1 generation at  $\alpha=0.05$  (Fisher's Exact Test with Bonferroni Adjustment;  $p = 0.0779$ ;  $n=545$ ).



Treatment Level	n	Average Length (mm)	p-value
0 µg/L	71	23.76	
0.01 µg/L	50	23.38	0.3552
0.1 µg/L	74	23.32	0.1746
1.0 µg/L	46	24.22	0.2323

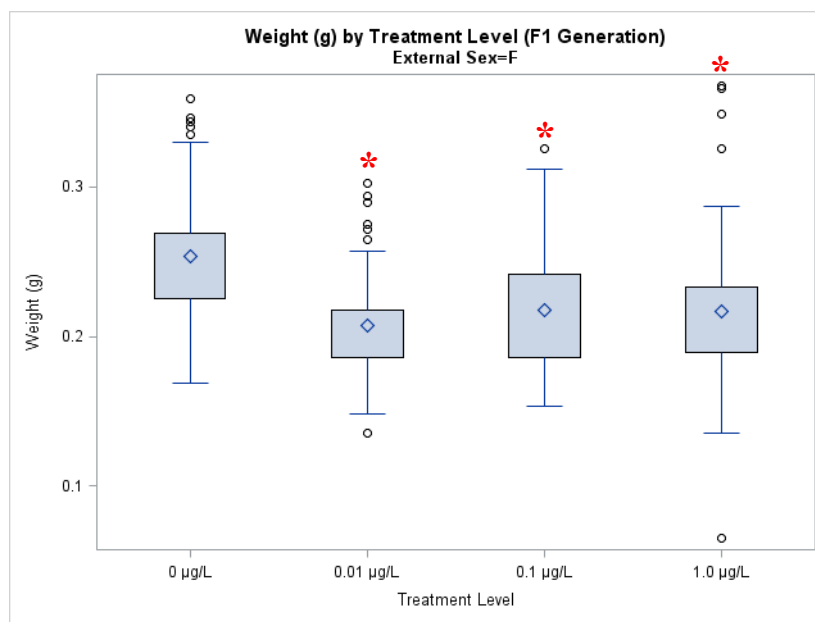
Figure 27. No significant differences in length (mm) were observed in females in any treatment group (Dunnett's Multiple Comparison Test). Diamonds indicate mean values.



Treatment Level	n	Average Length (mm)	p-value
0 µg/L	75	25.25	
0.01 µg/L	87	24.92	0.3155
0.1 µg/L	75	24.67	0.0333*
1.0 µg/L	67	24.70	0.0582

\*Results indicate a significant decrease in length at  $\alpha=0.05$

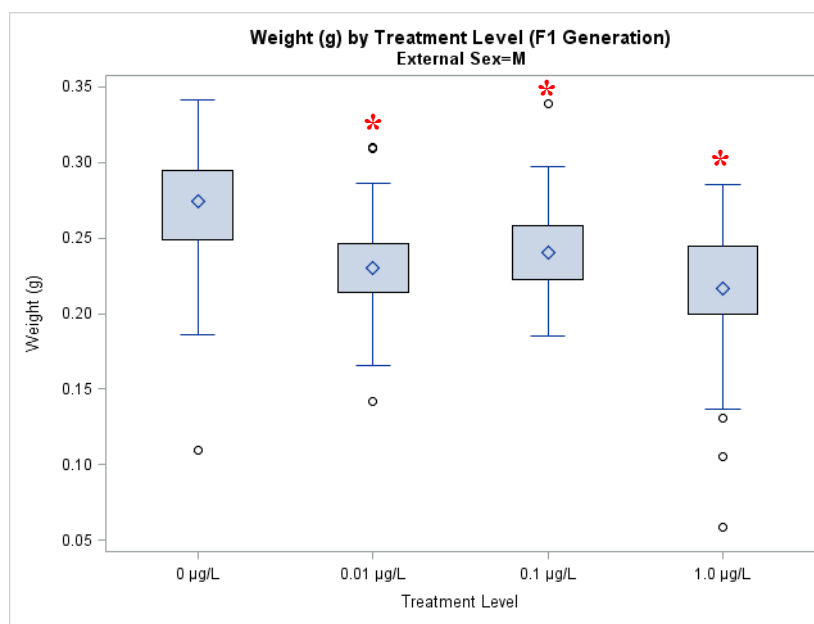
Figure 28. A significant decrease in length (mm) was observed in males in the 0.1 µg/L treatment group (Dunnett's Multiple Comparison Test;  $p=0.0333$ ). Diamonds indicate mean values.



Treatment Level	n	Average Weight (g)	p-value
0 µg/L	71	0.2534	
0.01 µg/L	50	0.2077	<0.0001*
0.1 µg/L	74	0.2179	<0.0001*
1.0 µg/L	46	0.2165	<0.0001*

\*Results indicate a significant decrease in weight at  $\alpha=0.05$

Figure 29. A significant decrease in wet weight (g) was observed in females in all treatment levels compared to the control (Dunnett's Multiple Comparison Test;  $p<0.0001$ ). Diamonds indicate mean values.



Treatment Level	n	Average Weight (g)	p-value
0 µg/L	75	0.2739	
0.01 µg/L	87	0.2296	<0.0001*
0.1 µg/L	75	0.2403	<0.0001*
1.0 µg/L	67	0.2166	<0.0001*

\*Results indicate a significant decrease in weight at  $\alpha=0.05$

Figure 30. A significant decrease in wet weight (g) was observed in males in all treatment levels compared to the control (Dunnett's Multiple Comparison Test;  $p<0.0001$ ). Diamonds indicate mean values.

No anal fin papillae were observed on any female in any treatment group of the F1 generation (Figure 31,  $p>0.05$ ). There was a significant increase in anal fin papillae count in males of the 0.1  $\mu\text{g/L}$  but not in 1.0  $\mu\text{g/L}$  (Figure 32, Dunnett's Multiple Comparison Test,  $p=0.0150$ ).

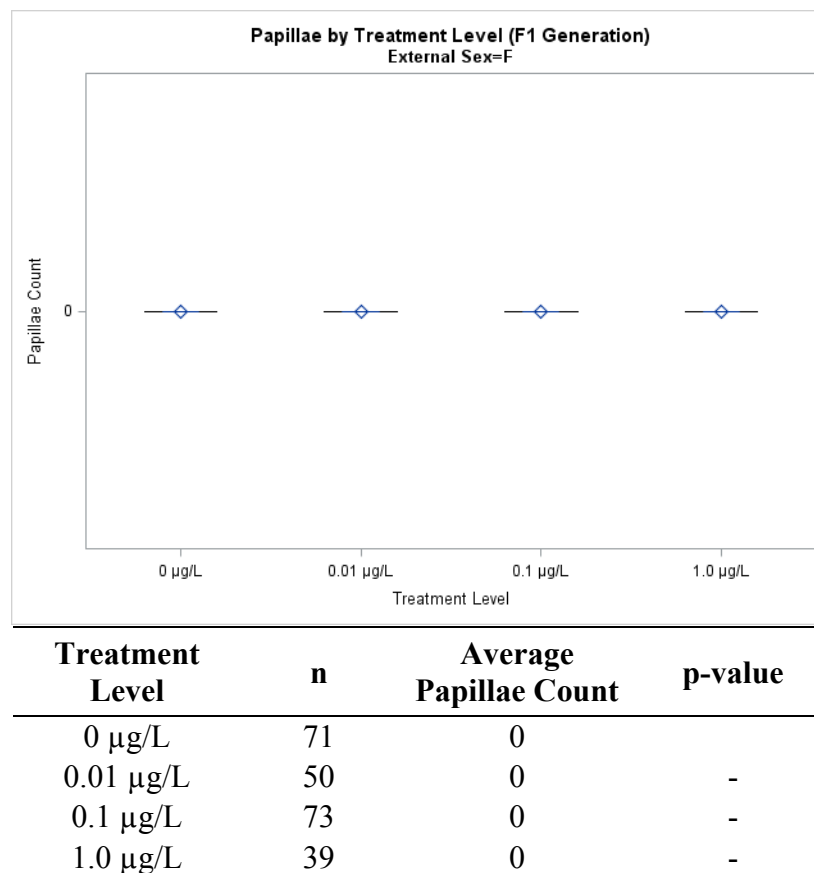
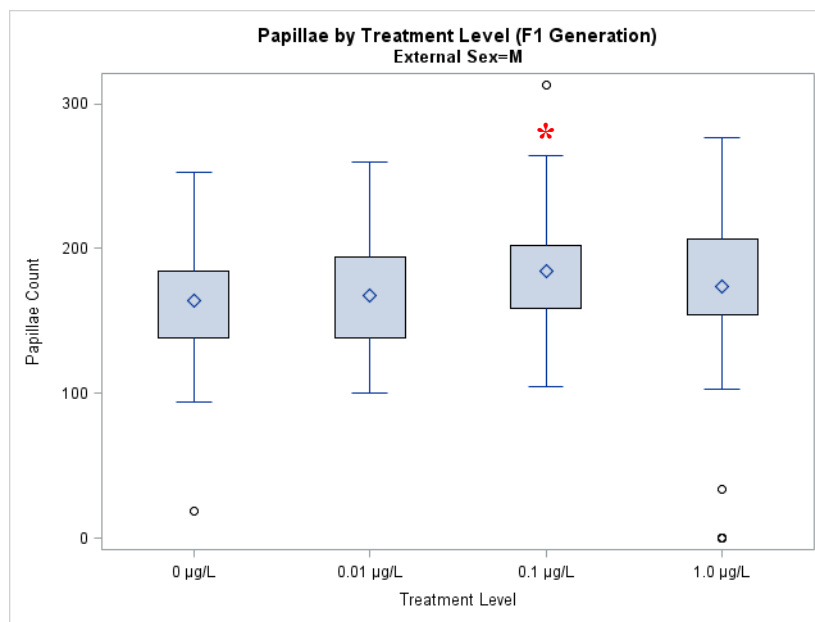


Figure 31. No significant differences in the average number of anal fin papillae were observed for females in any of the treatment levels of the F1 generation. Averages and p-values could not be calculated because no anal fin papillae were observed (Dunnett's Multiple Comparison Test). Diamonds indicate mean values.



Treatment Level	n	Average Papillae Count	p-value
0 µg/L	75	164.13	
0.01 µg/L	87	167.69	0.9179
0.1 µg/L	75	184.12	0.0150*
1.0 µg/L	67	173.55	0.4325

\*Results indicate a significant increase in anal fin papillae at  $\alpha=0.05$

Figure 32. There was a statistically significant increase in the average number of anal fin papillae for males in the 0.01 µg/L treatment level of the F1 generation (Dunnett's Multiple Comparison Test;  $p=0.0150$ ). Diamonds indicate mean values.

No significant differences were found in the number of phenotypic vs. gonadal female or male fish in any treatment group (Figure 33,  $p>0.05$ ).

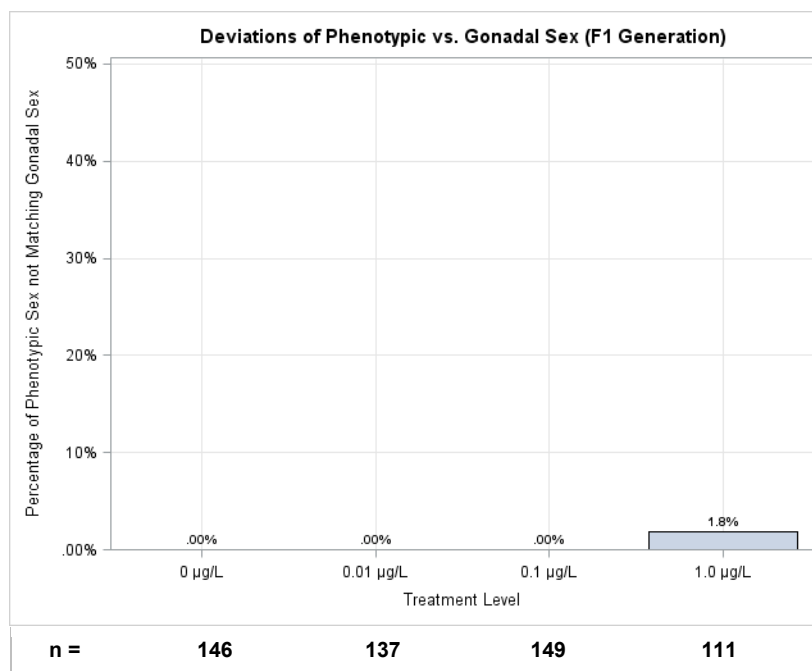


Figure 33. No significant differences were found in the percentage of cases where the observed phenotypic sex did not match gonadal sex in any treatment group at  $\alpha=0.05$  (Fisher's Exact Test with Bonferroni Adjustment).



There was an increase in female hepatic VTG expression as betamethasone concentrations increased; however, none of the treatment levels were statistically different from the control (Figure 34,  $p>0.05$ ). However, there was a significant increase in male hepatic VTG expression at 1.0  $\mu\text{g/L}$  (Figure 35, Dunnett's Multiple Comparison Test,  $p=0.0004$ ).

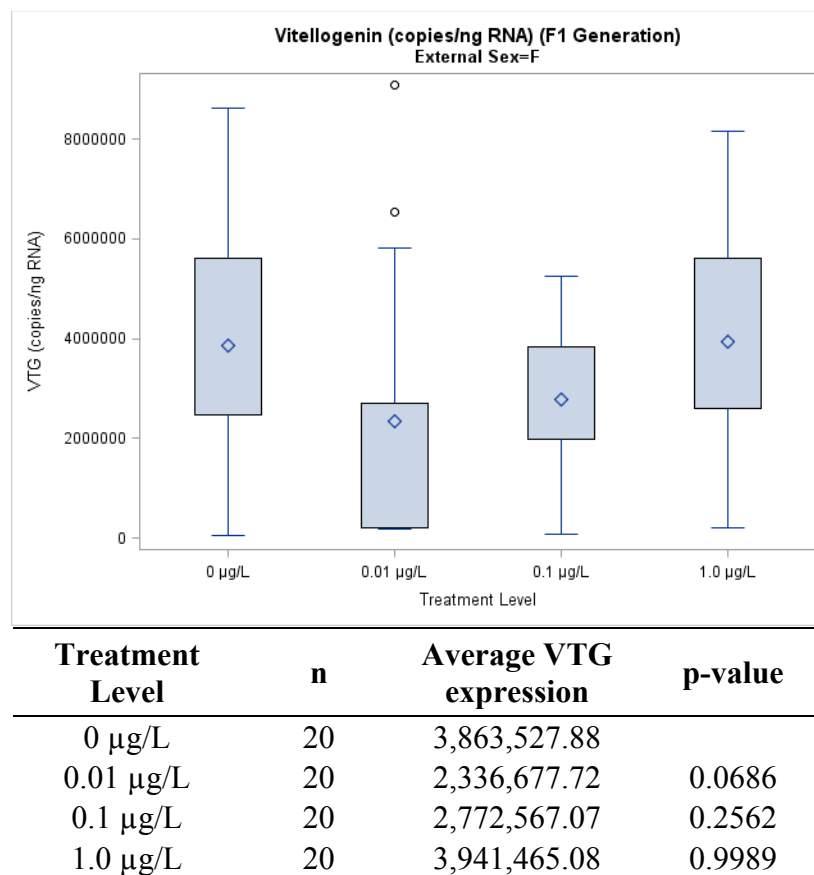
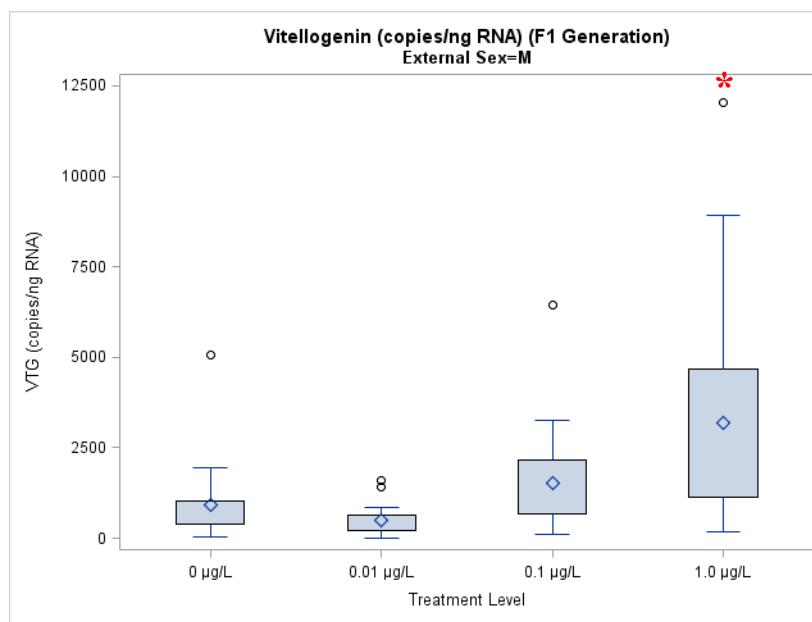


Figure 34. No significant differences were found in the average number of copies/ng of RNA hepatic VTG for F1 females in any treatment group (Dunnett's Multiple Comparison Test). Diamonds indicate mean values.



Treatment Level	n	Average VTG expression	p-value
0 µg/L	20	911.40	
0.01 µg/L	20	505.97	0.8145
0.1 µg/L	20	1524.96	0.5670
1.0 µg/L	20	3174.40	0.0004*

\*Results indicate a significant increase in VTG expression at  $\alpha=0.05$

Figure 35. A significant increase in hepatic VTG expression was observed for males in the 1.0 µg/L treatment group (Dunnett's Multiple Comparison Test;  $p=0.0004$ ). Diamonds indicate mean values.

In summary, NOECs were able to be identified in five of the 26 endpoints. Only LOECs could be identified in two endpoints and for another two endpoints, effects were noted at the middle concentration but not at the highest (Figure 36).

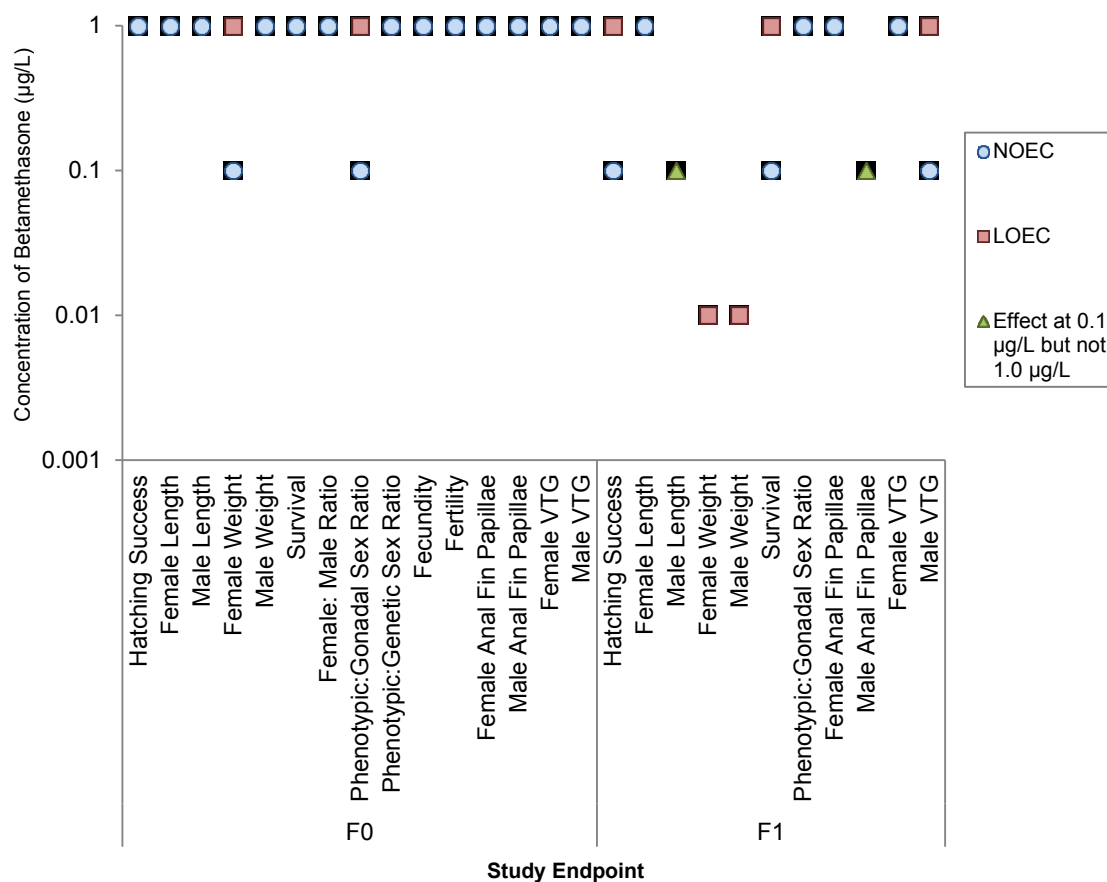


Figure 36. Summary of the no observable effect concentrations (NOECs) and lowest observable effect concentrations (LOEC) for each endpoint in the study. NOECs were identified in five of the 26 endpoints. Only LOECs could be identified in two endpoints and for another two endpoints, effects were noted at the middle concentration but not at the highest.

## Estimation of Risk

### *Concentration in Water*

Results from the SimpleTreat and PhATE models estimate concentrations of betamethasone in surface and drinking water to be 0.6 and 0.1 ng/L, respectively.

### *Predicted No Effect Concentration for Aquatic Life*

For all endpoints with the exception of weight of F1 males and females, the NOEC was determined to be  $\geq 0.01$   $\mu\text{g/L}$ . Since differences in length were not significant and all other effects were related to endocrine disruption, this effect in weight is considered to be mild but potentially biological significant to fish. Therefore, 0.01  $\mu\text{g/L}$  was used as a screening value to determine the magnitude of potential risk to fish from surface water exposures. Thus, using an assessment factor of 10, the PNEC is calculated to be 0.001  $\mu\text{g/L}$  (1 ng/L). Using the estimated surface water concentration 0.6 ng/L and a PNEC of 1 ng/L, the PEC/PNEC ratio is calculated to be  $>0.6$ .

### *Dose to Fish*

The NOEC from the FFLC study is determined to be 0.1  $\mu\text{g/L}$ , based on weight of F0 females and F0 phenotypic: gonadal sex ratio, as well as F1 hatching success, survival and hepatic VTG expression in males. Using Equations 1 and 2, the lowest dose that does not adversely affect fish is calculated to be 0.07  $\mu\text{g/kg-day}$ .

### *RfD*

Using Equation 3, the RfD with the applied composite uncertainty factor of 1000 is calculated to be  $7 \times 10^{-5}$   $\mu\text{g/kg-day}$  (0.07 ng/kg-day) for humans.

### *ADD*

Using Equation 4, the average daily dose for adults and children based on the concentration of betamethasone in surface water is calculated to be  $1 \times 10^{-5}$  and  $2 \times 10^{-5}$   $\mu\text{g/kg-day}$  for adults and children, respectively. The average daily dose for adults and children based on the concentration of betamethasone in drinking water is calculated to be  $2 \times 10^{-6}$  and  $3 \times 10^{-6}$   $\mu\text{g/kg-day}$  for adults and children, respectively.

### *Margin of Safety*

Using Equation 5, the calculated MOS for adults and children from surface water are 5 and 3, respectively. The calculated MOS for adults and children from drinking water are 30 and 20, respectively. Table 3 contains a summary of all risk calculations.

Table 3. Reference doses (RfD), average daily doses (ADD) and margins of safety (MOS) for adults and children following ingestion of betamethasone from surface and drinking water sources in the United States.

Water Source	Adults			Children		
	RfD ( $\mu\text{g/kg-day}$ )	ADD ( $\mu\text{g/kg-day}$ )	MOS	RfD ( $\mu\text{g/kg-day}$ )	ADD ( $\mu\text{g/kg-day}$ )	MOS
Surface	$7 \times 10^{-5}$	$1 \times 10^{-5}$	5	$7 \times 10^{-5}$	$2 \times 10^{-5}$	3
Drinking	$7 \times 10^{-5}$	$2 \times 10^{-6}$	30	$7 \times 10^{-5}$	$3 \times 10^{-6}$	20

## DISCUSSION

### Glucocorticosteroids

Synthetic glucocorticosteroids are pharmaceuticals routinely prescribed for the treatment of chronic inflammatory diseases such as asthma and rheumatoid arthritis [18]. Betamethasone has been on the market for sale in the U.S. since the early 1980's and is

listed on the WHO Model List of Essential Medicines [29, 43]. Despite this, there has been no systematic search for the presence of betamethasone in the environment.

Environmental fate modeling suggests that concentrations of betamethasone in the surface water are less than 0.6 ng/L for 95% of all watersheds analyzed in the PhATE model. Additionally, due to attenuation, drinking water concentrations are estimated to be below 0.1 ng/L for 95% of the U.S. population. While these concentrations are low, it is known that sub-lethal, endocrine disrupting effects can occur at minimal levels over the lifetime of an organism or can result in population-wide effects, even if general toxicity is not seen at higher doses [88]. Betamethasone is not expected to bioaccumulate based its *n*-octanol/water partition coefficient [78] but future efforts should focus on sampling of surface waters for its presence given that it is not expected to degrade.

#### Japanese medaka as an Experimental Model for Glucocorticosteroid Effects

A previous early life stage study using the fathead minnow resulted in a NOEC of 50 µg betamethasone/L based on weight [80], and a second study using fathead minnows and dexamethasone (another synthetic glucocorticoid) resulted in sub-lethal, endocrine effects at 500 µg dexamethasone/L [89]. Contrarily, the present study elicited nearly 100% mortality at only 10 µg betamethasone/L in Japanese medaka. This variability in species sensitivity has been seen in other studies with different ED toxicants. For example, in a study by Örn et al (2006), zebrafish and Japanese medaka were exposed to 10 and 100 ng ethinylestradiol/L [72]. At 10 ng/L, VTG concentrations were significantly elevated in zebrafish, whereas no increase was observed in medaka. Furthermore, zebrafish exhibited 100% mortality at exposures to 100 ng/L, while medaka only began to show increased VTG at this concentration. In humans, both betamethasone and

dexamethasone are considered to have high, but equivalent glucocorticoid activity [90]. Therefore, it is possible that Japanese medaka may be a more sensitive experimental model for glucocorticoid effects than the fathead minnow.

#### NOEC Selection for Aquatic Toxicity

For both generations of the FFLC, a NOEC  $\geq 0.01 \mu\text{g/L}$  was determined for all endpoints with the exception of wet weight of F1 males and females. Since differences in length were not significant, and all other effects were related to endocrine disruption, this effect in weight is considered to be mild and therefore,  $0.01 \mu\text{g/L}$  was used as a screening value to determine the magnitude of potential risk to fish from surface water exposures. The calculated PEC/PNEC ratio of 0.6, indicates no risk to aquatic life from current environmental concentrations of betamethasone. It is important to note that surface water concentration estimates are conservative because they assume that 100% of all purchased betamethasone was consumed. Patient compliance and adherence to prescription medication is estimated at approximately only 50% [91], and therefore, environmental concentrations are likely to be even lower. However, future evaluation of the effects, if any, from reduced weight across multiple generations is needed.

#### NOEC Selection for Endocrine Disruption Effects

Using a weight of evidence approach, the NOEC for endocrine effects was determined to be  $0.1 \mu\text{g/L}$ . This NOEC is based on female weight and deviations in phenotypic to gonadal sex ratios in F0, as well as hatching success, survival, and male hepatic VTG elevation in F1.

Wet weight was significantly reduced for both sexes of the F1 generation in all treatment levels (Figure 29 & Figure 30). This represents a mild toxic effect; however, it is unknown if this is specifically due to disruption of the HPG axis or if it is due to interference of transmembrane receptors that modify regulatory proteins or inhibition of hemopoietic growth factors that promote tissue proliferation, which are known to stunt growth [92]. Since VTG was not significantly altered in the 0.01 and 0.1  $\mu\text{g/L}$  treatment levels, and no other endpoints were affected in the 0.01  $\mu\text{g/L}$  treatment level, it is likely that this reduction in wet weight is due to general toxicity and not specifically due to interference of the HPG axis.

Anal fin papillae counts were significantly increased in the 0.1  $\mu\text{g/L}$  treatment group, but not the 1.0  $\mu\text{g/L}$  level (Figure 32). This was an interesting phenomenon that did not follow a typical dose-response relationship. In the present study, we hypothesized that feminization of males would occur due to increases in circulating plasma  $17\beta$ -estradiol. Anal fin papillae are secondary sexual characteristics that only develop in male Japanese medaka [93]. Similar to other studies with estrogen agonists, it was expected that phenotypic sex-reversal would occur in males and that anal fin papillae counts would be reduced following exposures to betamethasone [94]. While the deviation in average count from the control was statistically increased ( $p=0.0150$ ), these same males showed no significant increases in hepatic VTG expression, indicating that the effect may be due to biological variability or human error in counting of papillar processes, rather than disruption of the HPG axis.

There was a significant increase of intersex males in the highest treatment group of the F0 generation (Figure 18). It should be noted that four of the 127 fish were



phenotypically female but genetically male. While this percentage is low (3.1%), no other mismatches in sex were observed in any other treatment group. Furthermore, previous studies have identified <1% mismatches in phenotypic vs. genetic sex of medaka and no cases of spontaneous intersex (phenotypic vs. gonadal) in the wild, indicating that this rate of mismatch is biologically significant [95-97]. VTG levels in males increased with increasing betamethasone concentrations, but these increases were not significant. Therefore, this may be an indication of the beginning of HPG axis interference and would be considered biologically important, but the concentrations may have been too low to elicit a statistically significant response in this generation.

Most of the evidence for HPG axis disruption occurred in the second generation. The number of eggs produced by first generation females (F0) and the percentage of those eggs fertilized were unaffected by exposure to betamethasone; however, the hatching success of those eggs was significantly reduced in the highest concentration group. It is interesting to note that exposures of the F0 generation began 24 hours post fertilization and, unlike F1, hatching success was not affected. While not significant, hepatic VTG expression in females increased with increasing exposures to betamethasone (Figure 22) and thus, since VTG is an egg yolk precursor, it is likely that the reduced hatching success of F1 may actually be the result of poor quality eggs produced by F0 females. This reduction in reproductive success is consistent with previous studies that found a decrease in gamete quality and offspring as stress induced cortisol levels increase [98].

The strongest evidence of the estrogenicity of betamethasone is the elevated hepatic VTG expression in F1 males. Elevated VTG in males has been highly correlated

with increased E2 production following exposures to endocrine active substances, since endogenous levels of E2 in males are too low to induce production of VTG in the liver [46, 48]. However, the present study identified up to a 3-fold dose-related increase in expression of VTG in males of the 1.0 µg/L treatment group compared to controls, indicating that E2 production was artificially induced. Disruptions to multiple portions of the pathway can result in increased VTG concentrations and alterations in the development of secondary sexual characteristics. Therefore, future research should focus on identifying the exact macro-molecular interactions and cellular responses resulting in endocrine disruption in order to identify the adverse outcome pathway of betamethasone [99].

#### Estimation of Risk to Humans

The calculation of a reference dose for humans from a fish model has not been previously done. A number of studies have highlighted the benefits of using fish for toxicity and carcinogenicity bioassays, cancer research and *in vivo* mutagenesis studies [100-103]. In general, most of the molecular pathways involved in the initiation of toxic responses are conserved across vertebrate species [9]. Fish, specifically, have been extensively used as models for studies of endocrine disrupting compounds and allow for the testing of molecular, phenotypic and functional adverse effects [104].

Currently, the dose below which no adverse effects are expected in susceptible individuals following exposure to betamethasone for a lifetime by any route (ADE) is 2 µg/day [36]. The original ADE value was derived from the lowest clinical dose based on intranasal administration in children (66 µg/day) and a composite assessment factor of 9, which accounted for extrapolation to a no effect level ( $UF_L=3$ ), interindividual variability

( $UF_H=3$ ) and adjusted further to address differences in bioavailability following inhalation of 35% ( $\alpha=3$ ). Since the present study focuses on exposures via drinking water, the ADE was recalculated to be 2.6  $\mu\text{g}/\text{day}$  based on its oral bioavailability of 65% ( $\alpha=1.5$ ) [37]. The RfD for an 80 kg adult is then calculated to be 0.03  $\mu\text{g}/\text{kg}\cdot\text{day}$ .

A previous development and reproductive toxicity study in rabbits identified a lowest observed adverse effect level (LOAEL) of 0.05  $\text{mg}/\text{kg}\cdot\text{day}$  [40]. The subsequent RfD is calculated to be 0.5  $\mu\text{g}/\text{kg}\cdot\text{day}$  after converting to a human equivalent dose ( $HED = 3.1$  [105]) and adjusting for extrapolation to a no observable effect level ( $UF_L=3$ ) and interindividual variability ( $UF_H=10$ ) [105]. Another study of development and reproductive effects in monkeys identified a LOAEL of 2  $\text{mg}/\text{kg}$  [41]. The subsequent RfD is calculated to be 20  $\mu\text{g}/\text{kg}\cdot\text{day}$  after converting to a human equivalent dose ( $HED = 3.1$  [105]) and adjusting for extrapolation to a no observable effect level ( $UF_L=3$ ) and interindividual variability ( $UF_H=10$ ) [105].

The RfD based on the present fish study is almost 1000x lower than the lowest other calculated RfD, which was derived from human data (Figure 37). This indicates that the composite uncertainty factor used in the calculation may have been too conservative and over-predicted the differences in extrapolation from fish to humans based on disruptions of the HPG axis. Future studies are needed in order to fully validate the model. For example, an uncertainty factor of 10 was applied to account for extrapolation from fish to humans; however, given that the HPG axis is conserved across vertebrates, a high uncertainty factor may not be necessary.

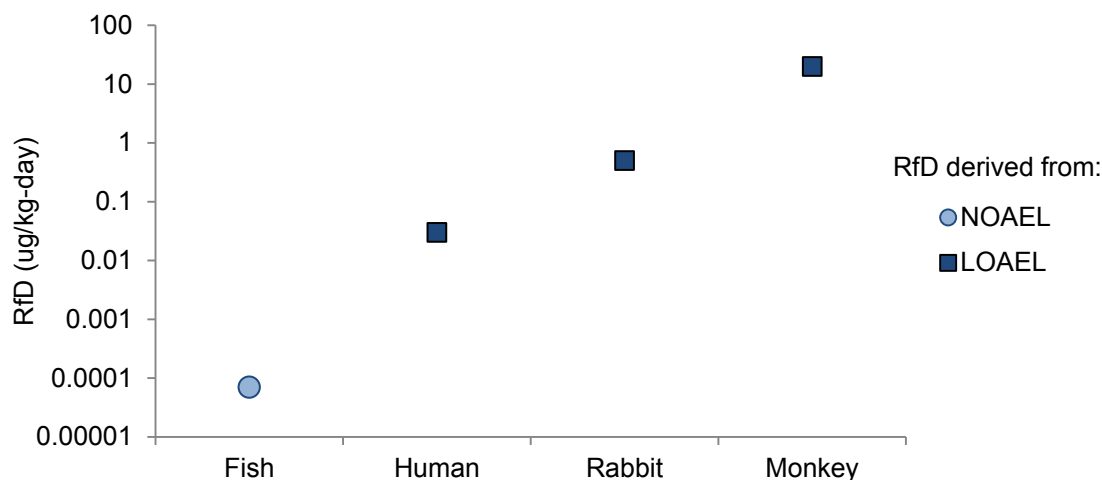


Figure 37. Comparison of the calculated RfD from the present fish study to the RfDs calculated from the human, rabbit and monkey studies.

Even with the low RfD calculated from fish, all of the derived MOS's are greater than 1, indicating no risk is anticipated from exposures to environmental concentrations of betamethasone. As noted previously, the estimations of the concentration of betamethasone in surface and drinking water conservatively assumes 100% patient compliance to prescriptions, and therefore, environmental concentrations are likely to be even lower. Furthermore, this study assumed that all ingestion exposures are from US surface water sources and did not account for bottled water use. However, one study suggested that not all pharmaceuticals detected in tap water are present in bottled water [106], and therefore this assessment may be overestimating human betamethasone exposures. Finally, surface water concentrations were used to represent water ingested from groundwater wells, which conservatively assumes no attenuation.

Other routes of exposure, such as dermal and inhalation, were not accounted for in the present study. Previous research has shown that the permeability of glucocorticosteroids across the skin is low, with only approximately 0.05 to 0.3% of applied steroid detectable in plasma after administration [107, 108]. In order to be

effective, various delivery methods are employed such as iontophoresis or chemical penetration enhancers, which increase drug diffusivity or solubility in the skin [109]. Therefore, direct dermal absorption of betamethasone from water is not expected to be a significant contributor to total body load. Similarly, the estimated vapor pressure of betamethasone is very low ( $7.8 \times 10^{-15}$  kPa) and is not likely to become an inhalable vapor [79, 110]. Therefore, inhalation is also not considered to be a major residential exposure pathway.

## CONCLUSION

Humans are exposed to potentially endocrine active substances every day. Little is known about the endocrine disruption potential from environmental concentrations of synthetic glucocorticosteroids, which are routinely prescribed as anti-inflammatory drugs and have the potential to enter surface water. Publicly owned treatment works and waste water treatment plants cannot always remove pharmaceuticals, as they are designed mainly to remove biodegradable organic matter. To date, no systematic search for glucocorticosteroids in the environment has been done, but because they are biologically active and little is known about their degradation potential, chronic, low level lifetime human exposures are a concern.

The present study was designed to assess potential impacts to the HPG axis in fish in order to assess potential effects in humans. The results indicate that adverse reproductive effects to vertebrates may occur at low level concentrations (1.0 µg/L) and could extend over multiple generations, but not at environmentally relevant concentrations.

In general, exposures of Japanese medaka to betamethasone during gestation through adulthood did not result in any significant adverse outcomes. Significant disruptions did begin to develop in the offspring of fish with lifetime exposures, indicating that betamethasone can interfere with reproductive success, resulting in poor outcomes across multiple generations and can potentially have population level effects that need to be explored further.

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APPENDIX I  
FISH FULL LIFE CYCLE PROTOCOL

## P R O T O C O L

BETAMETHASONE: A FLOW-THROUGH LIFE-CYCLE TOXICITY TEST  
WITH THE JAPANESE MEDAKA (*Oryzias latipes*)

OECD Detailed Review Paper on Fish Life-Cycle Tests (2008)

U.S. EPA OPPTS Number 850.1500 (1996)

Submitted to

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January 6, 2015



- 2 -

**BETAMETHASONE: A FLOW-THROUGH LIFE-CYCLE TOXICITY TEST  
WITH THE JAPANESE MEDAKA (*Oryzias latipes*)**

**SPONSOR:** Merck & Co., Inc.  
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**SPONSOR'S REPRESENTATIVE:** Jessica Vestel

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Proposed Dates:	
Experimental	Experimental
Start Date: _____	Termination Date: _____
Project No.: 105A-213	
Test Concentrations: 0.10, 1.0 and 10 µg betamethasone/L and 90 ng 17β-estradiol/L	
Test Substance No.: _____ Reference Substance No. (if applicable): _____	

**PROTOCOL APPROVAL**

Suzanne Z. Schneider  
STUDY DIRECTOR

7 January 2015  
DATE

Sean P. Gallagher  
LABORATORY MANAGEMENT

7 January 2015  
DATE

\_\_\_\_\_  
SPONSOR'S REPRESENTATIVE

\_\_\_\_\_  
DATE

PROTOCOL NO.: 105/010615/MED-LC/SUB105



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## INTRODUCTION

Wildlife International will conduct a flow-through life cycle toxicity test with the Japanese medaka (*Oryzias latipes*) for the Sponsor at the Wildlife International aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in the OECD Detailed Review Paper on Fish Life-Cycle Tests (1); and the U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1500: *Fish Life Cycle Toxicity* (2).

## OBJECTIVE

The objective of this study is to determine the effects of betamethasone on the Japanese medaka (*Oryzias latipes*) over a full life-cycle under flow-through test conditions. The parental (F0) generation will be monitored from the embryonic stage through sexual maturity and reproduction, and evaluated for embryo time-to-hatch, hatching success, survival, growth, fecundity, fertility, secondary sexual characteristics (anal fin papillae evaluation), hepatic vitellogenin concentration, internal phenotypic sex identification and sex ratio, genetic sex identification, and detailed gonad, liver and kidney histopathology. If requested by the Sponsor, analysis for gonadotropin and steroidogenic enzyme gene expression, and blood plasma concentrations of betamethasone may be conducted. The second (F1 or first filial) generation will be monitored from the embryonic stage through sexual maturation, and evaluated for embryo time-to-hatch, hatching success, survival, growth, internal phenotypic sex identification and sex ratio, and genetic sex identification. If requested by the Sponsor, analysis of hepatic vitellogenin concentrations, analysis for gonadotropin and steroidogenic enzyme gene expression, and blood plasma concentrations of betamethasone may be conducted.

## EXPERIMENTAL DESIGN

Japanese medaka will be exposed to three test concentrations (0.10, 1.0 and 10 µg betamethasone /L), a negative (dilution water) control and a positive control (90 ng 17β-estradiol/L) under flow-through test conditions. Nominal test concentrations were selected in consultation with the Sponsor, based upon information such as chronic toxicity data, physical/chemical properties of the test or reference substances and/or other relevant information. The concentrations need not exceed the solubility limit of the test substance in water. The nominal test substance concentrations will be 0.10, 1.0 and 10 µg betamethasone/L based on a fish, early life stage study conducted with fathead minnow, the expected introductory concentration (EIC) in the United States and concentrations used in a previous endocrine



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disrupting study of dexamethasone. Water samples will be collected at specified intervals for analysis of the test substance. An abiotic replicate test chamber in each treatment and control chamber will be maintained at least during the F0 generation exposure to aid in monitoring test concentrations.

The study will be conducted using two generations of medaka, the parental or F0 generation, and the second or first filial/F1 generation. The F0 generation exposure will be initiated with newly fertilized eggs. Each F0 generation treatment and control group will have four replicate test chambers. Each replicate will be initiated with 40 eggs (20 eggs per incubation chamber, with two incubation chambers in each replicate), for a total of 160 eggs per concentration. Following hatch, the fry will be allowed to grow to maturity. Once the fish reach sexual maturity (typically around 84 days post-fertilization (around 70 days post-hatch)), reproductive groups of three males and three females (confirmed by genetic sex) will be formed in each of four replicate test chambers in each treatment and control group (a total of 24 fish per concentration). The reproductive groups will be maintained for a minimum of three weeks. During the F0 generation exposure period, assessments of embryo time-to-hatch, hatching success, survival and gross morphology will be made daily, with measurement of growth at 28-days post-hatch. Evaluations of secondary sex characteristics (anal fin papillae evaluation), internal phenotypic sex identification, and measurements of hepatic vitellogenin concentration will be conducted on selected F0 generation fish not continuing exposure after the formation of reproductive groups, fin clips will be collected for possible genetic sex identification (PCR). If requested by the Sponsor, analysis for gonadotropin and steroidogenic enzyme gene expression, and blood plasma concentrations of betamethasone may also be conducted. Following formation of reproductive groups, a 21-day evaluation of fecundity and fertility will be conducted. At termination of the F0 generation reproductive groups, evaluations of growth, survival, secondary sex characteristics, internal phenotypic sex identification, and detailed gonad, liver and kidney histopathology will be conducted.

Following the 21-day reproduction evaluation in the F0 generation, the second or F1 generation exposure period will be initiated with embryos from the F0 generation reproductive groups. Each F1 generation treatment and control group will have four replicate test chambers. Each replicate will be initiated with 40 eggs (20 eggs per incubation chamber, with two incubation chambers in each replicate), for a total of 160 eggs per concentration. Post-hatch fry will be maintained through sexual maturity (typically approximately 60 – 70 days post-hatch). During the F1 generation exposure period, assessments



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of embryo time-to-hatch, hatching success, survival and gross morphology will be made daily, with measurement of growth at 28-days post-hatch. At termination of the F1 generation, evaluations of growth, survival, secondary sex characteristics (anal fin papillae evaluation), internal phenotypic sex identification, and measurements of hepatic vitellogenin concentration will be conducted, and fin clips will be collected for possible genetic sex identification (PCR). If requested by the Sponsor, analysis for gonadotropin and steroidogenic enzyme gene expression, and blood plasma concentrations of betamethasone may also be conducted.

Observations of the effects of betamethasone on the F0 and F1 generation exposures will be used to calculate the no-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC).

## MATERIALS AND METHODS

### Test Substance

The Sponsor is responsible for all information related to the test substance including the retention of a reserve sample of the lot or batch of the test substance used in this study. The Sponsor also agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

### Dilution Water

Water used for holding and testing will be obtained from a well approximately 40 meters deep located on the Wildlife International site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use in the test system, the water will be filtered to 0.45 µm in order to remove fine particles and may be UV-sterilized. The water is characterized as moderately hard. Typical values for hardness, alkalinity, pH, specific conductance and total organic carbon (TOC) are approximately:

Hardness, mg/L as CaCO <sub>3</sub>	140
Alkalinity, mg/L as CaCO <sub>3</sub>	180
pH	8.2
Specific Conductance, µS/cm	365
Total Organic Carbon, mg/L	<2



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Hardness, alkalinity, pH and specific conductance are measured weekly to monitor the consistency of the well water. TOC is measured monthly. Means and ranges of the measured parameters for the approximate four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the analyses will be summarized in the final report.

#### **Test Organism**

The Japanese medaka (*Oryzias latipes*) has been selected as the test species for this study. This species is representative of an important group of aquatic vertebrates and was selected for use in the test based upon past history of use in the laboratory and the recommendation of the test guideline (1). It is also successfully being used as a test species in long-term fish tests with endocrine endpoints. Embryos used to start the test will be obtained from cultures maintained at Wildlife International, Easton, Maryland. The identity of the species will be verified by the supplier of the original culture, or by Wildlife International personnel using appropriate taxonomic keys. The fish will not receive treatment for disease in the two-week period preceding the test or during the test.

On the day immediately prior to initiation of the test, all eggs will be removed from both the culture tanks and the females in the breeding groups to guarantee an accurate egg collection to start the F0 generation exposure on the following day. On Day 0 of the test, eggs will be collected from the culture tanks and females, and pooled. Embryos will be examined under a microscope to select healthy specimens in approximately the same stage of development. The embryonic stage of development at the initiation of exposure will be determined as closely as possible using a representative sample of the embryos. To avoid genetic bias, embryos from several breeding groups will be combined and used to initiate testing.

Loading, defined as the total wet weight of fish per liter of test solution, will not exceed 0.5 grams per liter of solution passing through the chamber in 24 hours, nor 5 g/L of test solution present in the test chamber at any given time.

#### **Feeding**

Feeding of fish in both the F0 and F1 generations will begin after the newly-hatched larvae are transferred from the incubation chambers to the test chambers. Newly hatched larvae will be fed Sera





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Micron at least twice per day during the first 14 days post-hatch, supplemented with newly-hatched brine shrimp nauplii (*Artemia* sp.) at least during the second week post-hatch. For the remainder of the exposure, the fish will be fed brine shrimp nauplii (*Artemia* sp.) at least twice per day. Feeding rates will be optimized for growth and reproduction. Following the first week post-hatch, feeding rates will be adjusted at least weekly, if necessary, to ensure that the feeding rate per fish remains constant. Feeding rates and frequency will be documented in the raw data. Food will be withheld from the fish for at least 24 hours prior to exposure termination to allow the fish to clear their digestive tracts before measurements of weight are made. Uneaten food and fecal material will be removed from the test vessels at least twice weekly by siphoning. Alternatively, tanks may be replaced periodically with clean tanks.

Specifications for acceptable levels of contaminants in fish diets have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents in the diet and results of the analyses will be summarized in the final report.

#### **Preparation of Test Concentrations**

The test substance will be administered to the test organism in water. This route of administration was selected because it represents the most likely route of exposure to aquatic organisms.

The test substance and positive control substance will be delivered using solvent-free methods, if practical. The materials typically will be mixed in dilution water or reverse-osmosis water to form one or more stock solutions. Additional stock solutions may be prepared by dilution from a primary stock. Aliquots of the stock solutions will subsequently be mixed with dilution water in the diluter system to prepare the test solutions.

The specific methodology used to prepare the test solutions will be documented in the raw data and summarized in the final report.



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#### **Test Substance Delivery System**

Continuous-flow diluter systems will be used to provide each concentration of the test substance, a negative (dilution water) control, and a positive (estradiol) control. A separate diluter system typically will be used for each generational exposure (F0 and F1). For each system, one or more pumps (e.g., syringe pump, peristaltic pump or metering pump) or a similar device will be used to deliver test substance stock solutions to mixing chambers where the test substance will be mixed with dilution water to prepare the test solutions at the appropriate nominal concentrations prior to delivery to the test chambers. The flow of dilution water into each mixing chamber will be controlled using rotameters. After mixing, test solutions will be delivered in approximately equal volumes to each replicate test chamber within an exposure group. The proportion of water delivered to each replicate will be checked prior to initiation of each generational exposure and approximately weekly or as needed during each exposure to ensure that these flow rates vary by no more than  $\pm 10\%$  of the mean flow rate of the four replicates in each exposure group throughout the test.

The diluters will be adjusted so that each test chamber receives at least 5 volume additions of test solution every 24 hours. Peristaltic or metering pumps, if used, and rotameters will be calibrated prior to initiation of each generational exposure, and recalibrated and/or verified approximately weekly or as needed during the exposure. Syringe pumps, if used, will be calibrated prior to initiation of each generational exposure and as needed during exposure. The delivery of test substance to test chambers will begin at least one day prior to exposure in order to establish equilibrium concentrations of the test substance. The duration of the equilibration period typically will be dependent on the physical/chemical properties of the test substance. The general operation of the diluter systems will be checked visually at least two times per day during each exposure period and at least once on the first and last days of exposure. The delivery systems and chambers will be cleaned periodically during the test, as necessary.

#### **Test Chambers**

Test chambers for each generational exposure will be positioned in a temperature-controlled environmental chamber, and will be labeled with the project number, test concentration and replicate designation. During the F0 and F1 generation embryo exposure periods, embryos will be held in incubation chambers (well plates) within each test chamber. Each well plate consists of a 24-well polystyrene tray covered with a mesh screen lid. Test chambers used in both the F0 and F1 generation



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exposure periods typically will be 6-L glass aquaria filled with approximately 5 liters of water. The embryo incubation chamber and test chamber type (e.g., glass), size and volume of test solution used in each exposure period will be documented in the raw data and presented in the final report.

#### **Environmental Conditions**

The test systems will be illuminated using fluorescent tubes that emit wavelengths similar to natural sunlight. The lights will be controlled by an automatic timer to provide a photoperiod of 16 hours of light and 8 hours of darkness. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured in five locations within each environmental chamber at approximately weekly intervals beginning at initiation of each generational exposure period, using a SPER Scientific Ltd. light meter or equivalent.

The test will be conducted at a target water temperature of  $26 \pm 1^\circ\text{C}$ . Water temperature will be monitored continuously during the F0 and F1 generation exposure periods using an automatic monitoring system. The system measurements will be verified with a liquid-in-glass or digital thermometer or equivalent prior to initiation of each generational exposure, and approximately weekly or as needed during exposure. The temperature in each test chamber will be measured at the beginning of the each generational exposure, approximately weekly during each exposure, and at the end of the exposure periods using a liquid-in-glass or digital thermometer or equivalent.

Dissolved oxygen will be measured in all test chambers at the beginning of the F0 and F1 generation exposures, and in one replicate test chamber of each treatment and control group approximately weekly during each exposure and at the end of the exposure periods using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent. Measurements typically will rotate among the replicates in each group at the weekly measurement intervals. In the event that dissolved oxygen levels approach or fall below 60% saturation, appropriate corrective actions (e.g., gentle aeration or increased flow rates) will be taken, if necessary, to maintain dissolved oxygen levels above 60% saturation. The Sponsor will be notified if corrective actions are taken.

Measurements of pH will be made in one replicate test chamber of each treatment and control group at the beginning of the F0 and F1 generation exposures, approximately weekly during each exposure, and



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at the end of the exposure periods using a Thermo Orion Model 525Aplus pH meter, or equivalent. Measurements typically will rotate among the replicates in each group at each measurement interval.

Hardness, alkalinity, and specific conductance will be measured in one replicate of the negative (dilution water) control and the highest concentration treatment group at the beginning of the F0 and F1 generation exposures, approximately weekly during each exposure, and at the end of the exposure periods. Measurements typically will rotate among the replicates in each group at each measurement interval. Hardness and alkalinity measurements will be made by titration based on methods in *Standard Methods for the Examination of Water and Wastewater* (3). Specific conductance will be measured using an Acorn Series Model CON6 Conductivity-Temperature meter, or equivalent.

If 100% mortality occurs in a treatment replicate, temperature, dissolved oxygen and pH measurements will be taken in that replicate at that time, and then discontinued. Additional measurements of environmental conditions may be taken as deemed necessary by study personnel in consultation with the Study Director. The reason for the additional measurements will be documented in the raw data and summarized in the final report.

## PROCEDURES

The study will be conducted using two generations of medaka (F0 or parental generation, and one generation of offspring, F1). An overview of the procedures for each generation is presented below, and greater detail regarding the endpoints and measurements to be made follows these procedures.

### Parental (F0) Generation Exposure

*Initiation of Test* - On the day of test initiation, embryos will be viewed using a dissecting microscope to confirm fertilization and general health. The embryonic stage of development at the initiation of exposure will be determined as closely as possible using a representative sample of the embryos. Any unfertilized eggs, embryos that appear abnormal, or embryos contaminated with fungus will be discarded. From the aggregate of healthy normal embryos, 20 will be impartially distributed to each of the embryo incubation chambers (well plates). Embryos will be added sequentially by placing no more than three embryos at a time in each incubation chamber until all chambers contain 20 embryos, with one embryo in each well. Once all incubation chambers contain their complement of embryos, two





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incubation chambers will be impartially assigned to and placed in each test chamber. The test will be considered initiated when the last incubation chamber is placed in its assigned test chamber.

*Embryo Exposure* - The eggs will be checked daily for mortalities, and all mortalities will be recorded and removed. Hatching typically begins on or about day 7 post-fertilization and will be monitored until all viable embryos have hatched. Once >90% of viable embryos in the negative control replicates have hatched, the newly hatched larvae in each incubation chamber will be counted and released into the corresponding replicate test chamber in each experimental group. Any unhatched embryos will be kept in the incubators until they have hatched, at which time they will be distributed to the appropriate test chamber, or until death of the embryo occurs.

*Larval-Juvenile Exposure* - Observations of the fish will be made at least once per day during the larval-juvenile period. During this period and throughout the parental generation exposure period, organisms periodically will be transferred to clean tanks (e.g., once a week) and the approximate number of live individuals will be counted at each transfer. At 28 days post-hatch (typically about 38-42 days post-fertilization), the number of surviving fish will be counted and standard lengths of the live fish in each test chamber will be measured using photographic methods.

*Adult Reproductive Exposure Period* - When the fish reach sexual maturity (typically about 84 days post-fertilization (about 70 days post-hatch)), fin clips will be collected from the fish for genetic sex identification by PCR methods, to enable selection of identified male and female medaka for reproduction groups. The fish will be thinned to create one spawning group in each test chamber (4 spawning groups per treatment and control group). Each spawning group will consist of three male fish and three female fish, for a total of 24 fish per concentration. The selection of male and female fish will initially be based upon external phenotype including the presence (male) or absence (female) of a notched dorsal fin and sail-like and transparent appearance of the anal fin, and confirmed by analysis of genetic sex. Fish with obvious abnormalities will be excluded when establishing spawning groups.

All fish not selected for use during the adult reproductive exposure period will be euthanized with buffered MS-222 and measured for growth (standard length and wet weight). Due to the number of fish to

be processed, the termination of the fish will be conducted over a 4-day period, with one replicate (e.g., Replicate A) from each treatment and control group terminated on the first day, a second replicate (e.g., Replicate B) terminated on the second day, and so forth. At termination, the internal phenotypic sex of each fish will be determined, when possible, based on a gross examination of the gonads. Fin clips will be collected from the fish and stored frozen (e.g., -80°C) for possible analysis by PCR methods for determination of genetic sex as requested by the Sponsor. The liver will be excised from each fish and frozen (-80°C) if necessary, and the livers of selected fish (initially 5 males and 5 females from each replicate; 20 males and 20 females from each treatment and control group, if available) will be processed for measurement of hepatic vitellogenin concentration. The remaining livers will be stored frozen for possible analysis if requested by the Sponsor. An evaluation for secondary sex characteristics (anal fin papillae) will be conducted on each fish, and the fish carcasses will be preserved for possible future analyses if requested by the sponsor. If requested by the Sponsor, the brain and gonads of selected fish may be collected for analysis of gonadotropin and steroidogenic enzyme gene expression. Blood plasma concentrations of betamethasone may be conducted if requested by the Sponsor.

The F0 generation reproductive groups will be formed over the 4-day period used to process those fish not carried forward in the reproductive exposure period. Beginning shortly after the formation of the final reproductive groups and continuing for at least 21 days, an assessment of fecundity and fertility in the F0 generation will be conducted. Any eggs present in the tanks or held by females will be removed prior to the start of the reproductive assessment. Each day during the assessment period, all eggs, either held by the female or deposited in the tank, will be removed and counted. During the entire F0 reproductive exposure period, assessments of survival and general observations of the fish will be made daily.

Exposure of the reproductive groups, and monitoring of fecundity and fertility, will continue until after successful hatch has been observed in the F1 generation exposure. At termination of the reproductive groups, the fish will be euthanized with buffered MS-222 and measured for growth (standard length and wet weight). The internal phenotypic sex of each fish will be determined, when possible, based on a gross examination of the gonads, and an evaluation for secondary sex characteristics (anal fin papillae) will be conducted on each fish. The fish will be processed for evaluation of detailed gonad, liver and kidney histopathology on selected fish requested by the Sponsor.

**Second (F1) Generation Exposure**

*Initiation* – On Day 21 of the 3-week assessment of fecundity and fertility, any eggs in the F0 generation tanks and on the females will be removed to ensure an accurate collection of new egg production on the following day to start the F1 generation exposure. On Day 22 of the fecundity assessment, eggs will be collected from each F0 generation replicate, if available, and pooled within each experimental group. Fertile, healthy embryos will be selected and systematically distributed to at least one incubation chamber (well plate) per experimental group, with a maximum of 20 embryos per incubator (one embryo per well). If there are a sufficient number of embryos available on that day, 20 embryos will be distributed to each of two incubators in each replicate. However, if there are only enough embryos for one incubator per replicate, the process will be repeated on Day 23 of the fecundity assessment, using the second incubation chamber in each test chamber. If necessary to ensure sufficient embryos, a third spawn may be collected on Day 24 of the fecundity assessment. The incubators will be placed in test chambers within their respective experimental group. In the event there is an insufficient number of embryos available to form replicates ( $n = 40$  per replicate), other replicates at the same test concentration may be used as a source of eggs to complete formation of the replicate. For example, if embryos are not being produced in replicate A of one treatment, then replicate B, C, or D of that treatment may be used to establish replicate A to move forward in the study.

*Embryo Exposure* - The embryos will be checked daily for mortalities, and any dead embryos will be recorded and removed. Hatching typically begins on or about day 7 post-fertilization and will be monitored until all viable embryos have hatched. Once >90% of viable embryos in the negative control replicates have hatched, the newly hatched larvae in each incubation chamber will be counted and released into the test chambers. Any unhatched embryos will be kept in the incubators until they have hatched, at which time they will be released into the appropriate test chamber, or until death of the embryo occurs.

*Larval-Juvenile-Adult Exposure* - The newly-hatched larvae will be evaluated until they achieve sexual maturity (typically approximately 60 to 70 days post-hatch) to monitor effects of the test substance on the second generation. Observations of the fish will be made at least once per day during the growth period. During this period, organisms periodically will be transferred to clean tanks (e.g., once a week) and the approximate number of live individuals will be counted at each transfer. At 28 days post-hatch, (typically



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about 38-42 days post-fertilization), the number of surviving fish will be counted and standard lengths of the live fish in each test chamber will be measured using photographic methods.

At termination, the fish in each replicate will be euthanized with buffered MS-222 and measured for growth (standard length and wet weight). Due to the number of fish to be processed, the termination of the fish will be conducted over a 4-day period, with one replicate (e.g., Replicate A) from each treatment and control group terminated on the first day, a second replicate (e.g., Replicate B) terminated on the second day, and so forth. At termination, the internal phenotypic sex of each fish will be determined, when possible, based on a gross examination of the gonads. Fin clips will be collected from the fish and stored frozen (e.g., -80°C) for possible analysis by PCR methods for determination of genetic sex as requested by the Sponsor. The liver will be excised from each fish and frozen (-80°C) if necessary, and the livers of selected fish (initially 5 males and 5 females from each replicate; 20 males and 20 females from each treatment and control group, if available) will be processed for measurement of hepatic vitellogenin concentration. The remaining livers will be stored frozen for possible analysis if requested by the Sponsor. An evaluation for secondary sex characteristics (anal fin papillae) will be conducted on each fish, and the fish carcasses will be preserved for possible future analyses if requested by the sponsor. If requested by the Sponsor, the brain and gonads of selected fish may be collected for analysis of gonadotropin and steroidogenic enzyme gene expression. Blood plasma concentrations of betamethasone may be conducted if requested by the Sponsor.

Endpoints and measurements to be made for the parental and second generations are described in greater detail below.

#### ***Survival and General Observations***

Assessments of survival and general observations will be made daily during both generational exposures in order to assess the health of the fish. Any mortalities or external abnormalities (such as hemorrhage, discoloration) will be noted. Abnormal behavior (relative to controls), such as hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and feeding abstinence, will be noted. Mortalities will be recorded and dead fish will be removed from test chambers as soon as possible and discarded. Dead fish will not be replaced in either the control or treatment vessels.





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#### ***Body Length and Weight***

At 28 days post-hatch, the standard lengths of the live F0 and F1 generation fish in each test chamber will be measured using photographic methods. Wet weights and standard lengths of each F0 and F1 generation fish will be recorded following euthanasia. Fish will be blotted dry and weighed to the nearest 0.1 mg. Standard lengths will be measured to the nearest millimeter.

#### ***Reproduction (Fecundity and Fertility)***

Fecundity and fertility of each reproductive group will be assessed over a period of at least three weeks in the F0 generation exposure. Egg clumps will be gently removed from netted females and/or from the aquarium bottom each morning. The number of eggs spawned and the number of fertile eggs will be determined for each breeding group daily during a 21-day reproductive assessment. Monitoring of fecundity and fertility will continue until termination of the F0 generation exposure. Any embryos not used to initiate the F1 generation exposure will be discarded after enumeration.

#### ***Hatchability***

During the F0 and F1 generation embryo exposure periods, each incubation chamber (well plate) will be evaluated daily for newly hatched embryos. The number of embryos hatched each day will be documented and the appropriate data will be collected to calculate the hatching success and time to hatch of each incubator. Any dead embryos or embryos infected with fungus will be removed each day and discarded. If a high rate of mortality is observed among embryos in a replicate due to fungal infection, the replicate may be reinitiated with eggs from the same F0 generation replicate, following evaluation by the Study Director. Any unhatched embryos will be kept in the incubators until they have hatched, at which time they will be released into the appropriate test chamber, or until death of the embryo occurs.

#### ***Determination of Genetic Sex***

Fin clips will be collected from F0 generation fish prior to formation of spawning groups, and from all fish from the F0 and F1 generation at termination for determination of genotypic sex in selected fish as requested by the Sponsor. Either the dorsal or ventral tip of the caudal fin of each fish will be clipped, and the sample will be placed in RNA stabilizing reagent (RNAlater) and stored at -80°C, unless the sample will be processed immediately for analysis. The genotypic sex of medaka is determined by an identified and sequenced gene (DMY) which is located on the Y chromosome. DNA from each fin clip

can be extracted and the presence or absence of DMY will be determined by PCR methods. The presence of DMY indicates an XY individual, regardless of phenotype, while the absence of DMY indicates an XX individual, regardless of phenotype. Fin clips from selected fish will be evaluated in the Wildlife International laboratory for determination of genetic sex by PCR methods. Fin clips from the remaining F0 and F1 generation fish will be stored for possible analysis if requested by the Sponsor.

#### ***Necropsy and Secondary Sexual Characteristics (Anal Fin Papillae)***

Following euthanasia, the tail with the anal fin of each F0 and F1 generation fish will be spread and fixed (e.g., in Davidson's solution) for subsequent assessment of the number of anal fin papillae. The body cavity of those F0 generation fish not selected for reproductive groups, and F1 generation fish will be opened and the liver will be dissected for measurement of vitellogenin concentration in selected fish. The body cavity will be perfused with fixative prior to submersing the entire body in fixative (e.g., Davidson's solution). F0 generation fish from the reproductive groups will be opened, taking care not to disturb the internal organs, and the body cavity will be perfused with fixative prior to submersing the entire body in fixative. After at least 24 hours of fixation, the samples will be rinsed with 70% ethanol and placed in 10% neutral-buffered formalin.

#### ***Vitellogenin (VTG)***

Collected livers will be stored frozen (e.g., -80°C) if necessary, prior to measurement for vitellogenin (VTG) concentration in selected fish. Male fish identified by external phenotype will be processed before female fish, when possible, to avoid the potential for contamination of male samples with VTG from female samples. The livers of selected fish will be individually homogenized and measured for hepatic vitellogenin concentration with Enzyme-Linked Immunosorbent Assays (ELISA) using commercially available test kits. Quality control of vitellogenin analysis will be accomplished through the use of standards, blanks and duplicate analyses. Each ELISA plate used for analysis will have at least six calibration standards covering the range of expected vitellogenin concentrations, and at least one blank. On each day that vitellogenin analyses are performed, a fortification sample will be analyzed. The procedures used to collect, prepare and analyze the samples will be based upon methodology provided by the ELISA system manufacturer and will be documented in the raw data and summarized in the final report. Alternately, the livers may be measured for vitellogenin concentration using PCR methodology. The methodology used will be documented in the raw data and summarized in the final report.

***Gonadotropin and Steroidogenic Enzyme Gene Expression Analysis***

If requested by the Sponsor, the brain and gonads will be excised from selected male and female fish and stored at -20°C in RNAlater for RT-qPCR analysis of selected gonadotropin (FSH $\beta$  and LH $\beta$  in brain) and steroidogenic enzyme gene expressions (for example, cyp19a1a or aromatase in gonads). RNA will be extracted from tissues using the Qiagen® RNeasy mini kit as per manufacturer recommended protocol. Upon extraction, RNA concentration and purity based on 260:280 (nucleic acid:protein) ratio will be quantified and RNA will be diluted to 40 ng/ $\mu$ L. SYBR Green detection will be used to quantify gene expression changes using the Qiagen® Rotor-Gene SYBR Green RT-PCR kit. RT-qPCR data will be normalized relative to an appropriate reference gene (such as  $\beta$ -actin or ribosomal protein) and quantified using the delta delta cycle threshold ( $\Delta\Delta$ Ct) method.

If requested by the Sponsor, concentrations of betamethasone in blood plasma may be evaluated in selected fish. The methods used for the analysis will be added to the protocol by amendment.

***Histopathology***

Histopathology samples from selected fish will be processed at the Wildlife International in-house histopathology laboratory, or at another facility if requested by the Sponsor. The fish carcasses will be processed, infiltrated with paraffin, and sectioned as appropriate to the species. Sections containing gonads, liver, and kidney will be collected. These sections will be stained with hematoxylin and eosin, and will be protected by a coverslip. The stained tissues will be examined and evaluated by an experienced pathologist, who will assign a grade to each histopathologic change observed using a four point scale.

***Sampling for Analytical Measurements***

Water samples will be collected from each treatment and control group prior to the start of each generational exposure to confirm the operation of the diluter systems. More than one pretest sampling interval may be conducted if needed. At a minimum, water samples will be collected from one replicate test chamber of each treatment and control group at the beginning of each generational exposure, at approximately weekly intervals during each exposure and at the end of each exposure to determine concentrations of the test substance. Sampling typically will rotate among the replicates in each group at each sampling interval. In the event that 100% mortality occurs in any treatment, final sampling of that



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treatment will be conducted on that day. Samples will be collected at mid-depth, and will be placed in an appropriate storage container (e.g., glass or polypropylene bottle). If not analyzed immediately, the samples will be stored under the appropriate conditions (e.g., refrigeration or ambient) until analyzed. Samples typically will be analyzed every other week during the study, but the analysis interval may change based on the stability of the samples. Stock solutions may be sampled for analysis as needed during the test to confirm the concentrations being delivered to the diluter system. Additional samples from one or more appropriate test chambers and/or stock solutions may be collected for analysis if an error in sampling or analysis is suspected, or if a malfunction in the test substance delivery system occurs. The reason for the additional samples will be documented in the raw data and summarized in the final report.

#### **Analytical Chemistry**

Chemical analysis of test solution and/or stock solution samples will be performed by Wildlife International. The analytical methods used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

#### **Data Analyses**

Statistical analyses will be performed to evaluate differences between treatment and control groups for each of the following biological endpoints, as appropriate:

- F0 and F1 generations embryo time-to-hatch
- F0 and F1 generations hatching success
- F0 and F1 generations survival
- F0 and F1 generations wet weight
- F0 and F1 generations body length
- F0 generation fecundity (time to first spawning, eggs per female reproductive day and cumulative eggs produced)
- F0 generation fertility (fertile eggs of the total eggs laid)
- F0 and F1 generations secondary sex characteristics (anal fin papillae)
- F0 and F1 generations hepatic vitellogenin concentration
- F0 and F1 generations genetic sex/sex ratio
- F0 generation histopathology

The total number of live embryos with fungus that are removed during the embryo incubation periods will be subtracted from the total number of embryos added to the test system at the beginning of





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the exposure periods to calculate hatching success, if applicable. Percentage data (e.g., survival, hatching success and sex ratio) will be subjected to arcsine square-root transformation prior to analysis. With the exception of histology endpoints discussed below, the unit of statistical analysis for all variables is the replicate test chamber. Males and females will be analyzed separately for each endpoint when appropriate. Vitellogenin data is inherently variable and boxplots of log transformed VTG values will be used to evaluate the data to identify potential outliers using Tukey's method (4).

The data for all variables except histopathology endpoints discussed below will be examined to determine whether concentration-response is fundamentally monotonic (trending in one direction, e.g., response not trending up and then down as concentration increases) or non-monotonic. Monotonicity of the dose-response for each endpoint will be determined by converting the variable responses to rank values, and then performing a regression of the response ranks versus concentration using a quadratic polynomial as the model. The concentration-response relationship will be considered to be monotonic if the quadratic term is not significant ( $p > 0.05$ ) or if both the linear and quadratic terms are significant ( $p \leq 0.05$ ). The dose-response will be considered to be non-monotonic if the quadratic term is significant and the linear term is not significant.

Those variables consistent with a monotonic concentration response will be analyzed using the Jonckheere-Terpstra trend test applied in a step-down procedure. The use of a trend-based test is desirable because such tests have superior power characteristics when the dose response is monotonic (5). The Jonckheere-Terpstra trend test also has the advantage that it is a non-parametric test, and data do not have to satisfy assumptions of normality and homogeneity of variance. Data that is determined to have a non-monotonic concentration response will be analyzed using Dunnett's test or the Wilcoxon rank-sum test.

The variables also will be assessed for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test ( $\alpha = 0.01$ ; applied to residuals of ANOVA). If the data meet assumptions of normality and homogeneity of variance, the variable will be analyzed using Dunnett's test. When data fail to meet these assumptions, normalizing or variance stabilizing transformations may be applied. Should these transformations fail to normalize the data or fail to result in homogeneous variances, a Mann-Whitney-Wilcoxon-U test will be performed to compare each treatment group to the control.



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Gonadal histology will be analyzed based on the scoring system. For each finding, the numbers for each sex of fish in each replicate with a grade greater than zero, greater than one, greater than two, and greater than three will be determined. A Jonckheere-Terpstra trend test applied by sex to the numbers above each grade in each treatment group will be used to evaluate the prevalence and severity of histological findings.

Statistical tests will be performed at  $\alpha = 0.05$  and will be performed using a personal computer with commercially available computer software programs such as SAS (6). Additional analyses of data may be conducted if deemed appropriate by the Study Director or requested by the Sponsor. The results of additional analyses will be documented in the raw data and summarized in the final report.

#### **RECORDS TO BE MAINTAINED**

Records to be maintained for data generated at Wildlife International will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. History of the test organism.
5. Stock solution calculation and preparation, if applicable.
6. Calculation and preparation of test concentrations.
7. Biological observations and measurements made during the test.
8. Test conditions and physical/chemical measurements.
9. The methods used to analyze test substance concentrations and the results of analytical measurements.
10. Statistical calculations.
11. A copy of the final report.

#### **FINAL REPORT**

A final report of the results of the study will be prepared by Wildlife International. The report will include, but not be limited to the following, when applicable:

1. Name and address of the facility performing the study.

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2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. Objectives and procedures as stated in the approved protocol, including all changes to the protocol.
4. The test, control and reference substances identified by name, chemical abstracts number or code number, strength, purity, and composition or other appropriate characteristics, if provided by the Sponsor.
5. Stability and solubility of the test, control and reference substances under the conditions of administration, if provided by the Sponsor.
6. A description of the methods used to conduct the test.
7. A description of the test organisms, including the source, scientific name, age or life stage, observed diseases and treatments.
8. A description of the preparation of the test solutions.
9. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, the duration of the test and environmental conditions during the test.
10. A description of circumstances that may have affected the quality or integrity of the data.
11. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
12. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.
13. Statistical methods used to evaluate the data.

If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment by the Study Director. The amendment shall clearly identify the part of the final report that is being added to or corrected and the reasons for the addition or correction. Amendments shall be signed and dated by the Study Director.

#### CHANGES TO PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the



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protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

#### **GOOD LABORATORY PRACTICES**

Data collection, recordkeeping, training and facility records will comply with Good Laboratory Practices; however, the report and raw data will not be audited. Raw data generated by Wildlife International will be stored in the archives at Wildlife International and will be available to the Sponsor. Wildlife International does not provide permanent storage for histopathological specimens. It is the responsibility of the Sponsor to arrange for transfer and archiving of histopathological specimens (slides, blocks and remnants of preserved tissue) at the end of the study.



## REFERENCES

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- 2 **U.S. Environmental Protection Agency.** 1996. Series 850-Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1500: *Fish Life Cycle Toxicity*.
- 3 **APHA, AWWA, WEF.** 2012. *Standard Methods for the Examination of Water and Wastewater*. 22<sup>nd</sup> Edition, American Public Health Association. American Water Works Association. Water Environment Federation, Washington, D.C.
- 4 **Tukey, J.W.** 1977. *Exploratory Data Analysis*. Reading, MA. Addison-Wesley.
- 5 **Organization for Economic Cooperation and Development.** 2006. *Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application*. OECD ENV/JM/MONO(2006)18.
- 6 **SAS System for Windows.** 2001. Version 8.2. SAS Institute, Inc. Cary, North Carolina.