DRAFT FINAL REPORT

on

MULTI-CHEMICAL EVALUATION OF THE SHORT-TERM REPRODUCTION ASSAY WITH THE FATHEAD MINNOW

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1.0 INTRODUCTION

The U.S. Environmental Protection Agency (EPA) has implemented an Endocrine Disruptor Screening Program (EDSP). In 1996, the Food Quality Protection Act and the Safe Drinking Water Act were enacted by Congress to authorize EPA to implement a screening program to evaluate whether pesticides and other chemicals found in food or water could affect endocrine systems in humans. In this program, comprehensive toxicological and ecotoxicological screens and assays are being developed to identify and characterize the endocrine effects of environmental contaminants, industrial chemicals, and pesticides. A two-tiered approach is being used: Tier 1 employs a combination of *in vivo* and *in vitro* screens, and Tier 2 involves *in vivo* testing using two-generation reproductive studies. Validation of the individual screens and assays is required, and the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) will provide advice and counsel on the validation assays.

The Fish Screening Assay was selected as a component of the Tier 1 screening by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to evaluate the potential toxicity of chemicals and mixtures on the endocrine system (EDSTAC 1998). The Tier 1 screening assays were selected to obtain minimum, yet sufficient estimates of potential endocrine disrupting activity. The committee has several stated goals for these assays. First, they should be relatively inexpensive, quick, and technically easy to perform. Second, they should be sensitive and specific, capture multiple endpoints, and be predictive across species, gender, and age. Third, they should be validated and standardized before they are used routinely by testing laboratories (EDSTAC 1998, Vol. 1, p 3-9). The purpose of using testing protocols within the EDSP is "to characterize the nature, likelihood of a dose-response relationship of endocrine disruption in humans and wildlife" (EDSTAC 1998; EPA 1997). Subsequently, EPA has requested the development of a screening protocol that identifies compounds having the potential to affect selected endocrine processes in fish.

The recommended protocol to be used as part of a Tier 1 screening (T1S) battery includes a fish screen assay, which complements the information from assays using mammals. Therefore, the inclusion of the fish-screening assay in Tier 1 is important, because estrogenic and androgenic controls in reproduction and development in fish differ enough from those in higher vertebrates that mammalian screening alone may not identify potential endocrine disrupting chemicals in this class of animals. It is expected that the fish-screening assay will complement the other screening assays such that through its completion, the following five criteria will be met.

1. The T1S battery should maximize sensitivity to minimize false negatives while permitting analysis of a yet undetermined, but acceptable, level of false positives. This criterion expresses the need to "cast the screening net widely" to not miss potential endocrine disruptors or estrogen-androgen-thyroid-active materials.
2. The T1S battery should include a range of organisms representing known or anticipated differences in metabolic activity. The battery should include assays from representative vertebrate classes to reduce the likelihood that important pathways for metabolic activation or detoxification of parent chemical substances or mixtures are not overlooked.

3. The T1S battery should be designed to detect all known modes of action for the endocrine endpoints of concern. All chemicals known to affect the action of estrogen, androgen, or thyroid hormones should be detected.
4. The T1S battery should include a sufficient range of taxonomic groups among the test organisms. Differences in endogenous ligands, receptors, and response elements among taxa can affect endocrine activity of chemical substances or mixtures.
5. The T1S battery should incorporate sufficient diversity among the endpoints and assays to reach conclusions based on "weight-of-evidence" considerations. Decisions based on the battery results will require weighing the data from several assays.

The Tier 1 screening must be relatively fast and efficient while meeting the criteria described above. The screening includes a fish reproductive assay, which fills important needs in the battery and complements the information from assays using mammals and other ecologically significant animal classes. Fish differ in steroid profiles from mammals. For example, 11-ketotestosterone, as opposed to testosterone, is the most important androgen in fish, and the estrogen receptor in fish appears to differ structurally and functionally from the corresponding mammalian receptor (Petit et al. 1995). In addition, steroid receptors in eggs and for hepatic vitellogenin (VTG) have no known analogous receptors in mammals, which would suggest sites of endocrine disruption unique to oviparous animals. Therefore, this assay is essential to address these known endocrine differences.

1.1 Purpose

EPA Mid-Continent Ecology Division (EPA MED 2002) described a short-term test with the fathead minnow that considers reproductive fitness as an integrated measure of toxicant effects, and also enables measurement of a suite of histological and biochemical endpoints that reflect effects associated with [anti-]estrogens and androgens. The test is initiated with mature male and female fish. During a 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics are observed, and fecundity monitored. Assessments of fertility and F1 development can be made, if desired. At the end of the test, measurements are made of a number of endpoints reflective of the status of the reproductive endocrine system, including the gonadal-somatic index (GSI), gonadal histology, and plasma concentrations of vitellogenin (VTG) and sex steroids (17β-estradiol, testosterone, 11-ketotestosterone).

A previous work assignment in this contract (WA 2-18; EPA 2003) has been completed to evaluate a short-term reproduction assay with fathead minnow and to compare the EPA MED (2002) method to two other related assays to inform the optimization of the assay for use as a screen in the EDSP. The purpose of work to be conducted under the current work assignment WA 2-29 is to perform a multiple chemical evaluation of the short-term reproduction assay with the fathead minnow as described in EPA MED (2002). Chemicals were selected based upon their known modes of action, with an emphases placed on weaker-acting compounds that can influence the hormonal control of the endocrine system. The chemicals selected were the following:

1 - Atrazine (alters neuroendocrine activity)
2 - Bisphenol A (weak estrogen receptor agonist)

3 - p,p'-DDE (weak androgen receptor antagonist)
4 - Perchlorate (iodine uptake inhibition; antithyroid)
5 - Cadmium chloride (altered steroidogenesis)
6 - Di-n-butylphthalate (weak antiandrogen).

2.0 MATERIALS AND METHODS

2.1 Test Material and Exposure Regime

The test chemical concentrations series used for this project were determined in consultation with representatives of EPA and are summarized in Table 2.1. Each chemical was chosen based on a suspected mode of action.

Table 2.1. Target Chemical Concentrations Used in the WA 2-29 Program

Test Chemical	Suspected Mode of Action	Low (µg/L)	High (µg/L)
Atrazine	Neuroendocrine activity	25	250
Bisphenol A	Weak estrogen receptor agonist	64	640
p,p'-DDE[(a)]	Weak androgen receptor antagonist	0.02	0.2
Perchlorate	Iodine uptake inhibition, antithyroid	5,000	50,000
Cadmium chloride	Altered steroidogenesis	1	10
Di-n-butylphthalate	Weak antiandrogen	5	50

a) DDE Dichlorodiphenyldichlorethylene.

Test-grade aliquots of each chemical were shipped to the EDSP chemical repository in Sequim, Washington. The chemicals were logged in for analysis following the procedures for sample receipt, handling, and storage: MSL-A-001, *Sample Log-In Procedure*, and MSL-A-002, *Sample Chain-of-Custody*. The test materials were stored under appropriate conditions; stock solutions were prepared and stored until transferred to the toxicology laboratory for use in the diluter exposure system. A copy of the chain-of-custody form accompanied all materials. Prior to conducting the chemical exposures, purity, and stability experiments were conducted. A summary of those results is located in Appendix A.

2.2 Preparation and Sampling of Chemical Exposure Water

Figure 2.1. Continuous flow proportional diluter system

A continuous-flow proportional diluter, similar in style to the commonly used Mount and Brungs diluter, was employed to deliver chemical concentrations to the test aquaria (Figure 2.1). The diluter was modified specifically for this study to deliver three chemical concentrations, including the control, with four replicates per concentration. This modified diluter was used for assays following the EPA methods. The chemical stock solution was metered into the mixing cell of the diluter using a fluid-metering pump. The diluter was set to add chemical-laden water to the test chamber every 12 min and was equal to six volume exchanges of water per day. The diluter casing was covered in black plastic to reduce biological activity, such as the development of algal growth, during the test.

Prior to introduction of the chemical, the diluter was calibrated using NaCl as an easily measured surrogate test chemical. After completion of the salt calibration and subsequent rinsing of the diluter, the chemical was added to the mixing chamber via a fluid metering pump, and concentrations in aquaria were verified prior to the introduction of organisms.

Bisphenol A was prepared by direct addition to water. Specifically, the bisphenol A stock was prepared by the chemical repository using 6.0979 g bisphenol A (CAS 80-05-7, CF 1825, Fisher Acros, Lot #A014744401,[1] expiration date 10/01/04), which was added to 15 L deionized (DI) water, brought to a volume of 19 L, and stirred. The stock solution was filtered and connected to the diluter system 6/18/03. This stock solution was used to calibrate the diluter. A second stock solution was prepared 7/9/02 by adding 2.2561 g of the same bisphenol A used for the first stock to 7 L DI water and stirring. This stock solution was used for the experiment. Triplicate 10 mL stock solution samples were collected 6/20/03 and 7/10/03 to confirm the integrity of the stock solution during the 21-day test exposure. In addition, 10-mL samples were collected weekly from the test aquaria to verify test concentrations (control, low, and high) in the exposure tanks 6/20/03 (pretest), 6/25/03 (Day 0), 6/30/03, 7/7/03, 7/10/03,[2] 7/14/03,[3] and 7/16/03 (termination). Data are provided in Appendix A.

Sodium perchlorate was prepared by direct addition to water. The Battelle Sequim Chemical Repository prepared the sodium perchlorate ($NaClO_4$) stock solution 7/30/03 using 1000.24 g $NaClO_4$ (7601-89-0, CF 1988, Sigma-Aldrich, Lot #17129CA, expiration date 3/08), which was

[1] Stated as Lot#A014744401 on the bottle, but as Lot#A0147444 on the certificate of analysis.
[2] Only one high concentration sampled.
[3] Only low and high concentrations sampled.

added to DI water and brought to a volume of 20 L. One stock solution sample was collected and analyzed 7/31/03. In addition, 10 mL samples were collected weekly from the test aquaria to verify test concentrations (control, low, and high) in each of four replicate exposure tanks (labeled C [control], L [low], and H [high]) 7/31/03 (pretest), 8/1/03 (pretest), 8/6/03 (Day 0), 8/11/03, 8/20/03, and 8/27/03 (termination).

Cadmium chloride was prepared by direct addition to water. The Battelle Sequim Chemical Repository directed the preparation of the cadmium chloride hydrate (CdCl) stock solution (CF 1984, CAS 34330-64-8, Aldrich, Lot #23901HI, expiration date 3/07). Approximately 19 mL concentrated hydrochloric acid (HCl) and 41 mL DI water were added to 18 L DI water in a Pyrex glass carboy. Then, 0.1933 g CdCl was added, and the container was filled to 19 L and stirred overnight using a stir bar and magnetic stirrer. The prepared stock was connected to the diluter system, and 10 mL samples were collected 5/27/03 and 6/18/03. Stock solution samples were diluted 100 times prior to analysis so that the concentrations would fall within the calibration range. These samples are "D" flagged in the data. For the test concentration samples collected from the test aquaria, 20 mL samples were collected into acid-cleaned polyethylene 30 mL containers to verify the concentration of the test solutions. Pretest samples were collected 5/27/03 and 6/18/03. Test samples were collected 6/24/03 (Day 0), 6/30/03, 7/7/03, and 7/15/03 (termination). Samples were hand-carried to the laboratory and delivered on the same day collected. Samples were preserved with 0.2% nitric acid (HNO_3, Seastar, distributed by Fisher, Lot #1202040, acids are not assigned expiration dates) to pH < 2.0. Samples from replicate diluters provided a measure of precision.

Atrazine and p,p'-dichlorodiphenyldichlorethylene (p,p'-DDE) were prepared using a saturator column (Figure 2.2) similar to the method described in Kahl et al. (1999). A complete description of sample preparation and collection can be found in chemical stability plans included in Appendix A. The saturator-column method was chosen, because these compounds were found to have low water solubility, and concentrated stock solutions approaching the limit of aqueous solubility were found not to be chemically stable over time. This was documented by chemical stability studies performed by the EDSP chemical repository.

Specifically, the saturator column was used to coat the chemical onto a large surface area (i.e., glass wool) and to expose the water to the surfaces until equilibrium chemical concentration was reached. To prevent the need for daily preparation of any stock solutions, large volumes were prepared in a high-density polyethylene (HDPE) drum lined with a removable Teflon bag, which provided an inert absorption surface. The stock solution was prepared by pumping water through the column with a fluid metering stainless-steel pump at a flow rate of about 0.1 L/min. The stock solution in the drum was recirculated through the saturator-column system. The stock solution was sampled daily until a stable concentration was reached, then the drum was sampled to determine stability and duration of stability for each solution.

The Battelle Sequim Chemical Repository directed the preparation of the atrazine stock solution. On 8/1/03, a solution was made from half of the contents of a 1 g atrazine container (CF 1826, CAS 1912-24-9, Chem Service Lot #277-93B, expiration date 1/05) added to 50 mL acetone (Fisher Chemical, Optima, Lot #967647) in a 50 mL amber glass container, which was capped and hand-shaken to mix. A second solution was made from the second half of the atrazine in the bottle, which was added to 50 mL acetone, also capped and shaken to mix. This approach was followed to enhance solubility and mixing. The two solutions were used the following day to

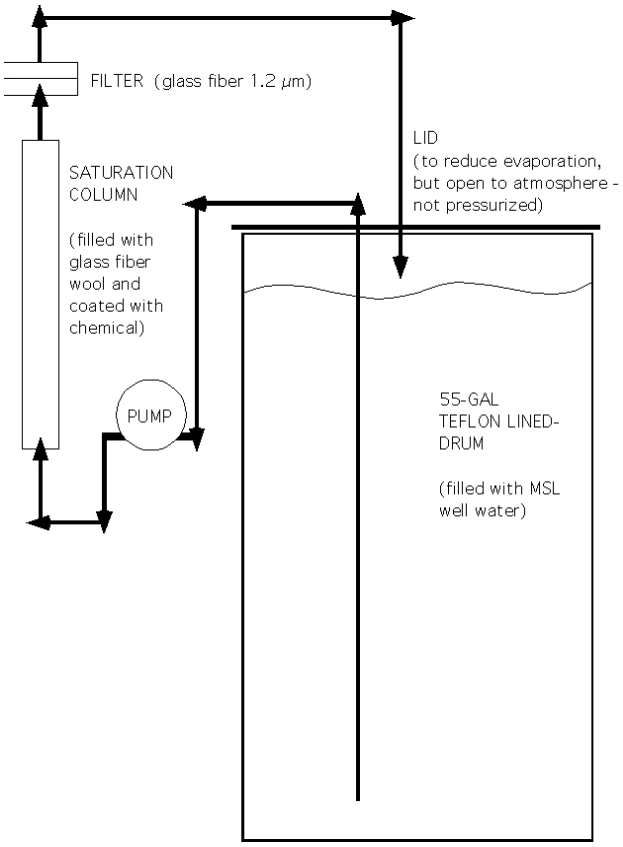

Figure 2.2. Saturator-Column Apparatus

regenerate the saturator column previously used for stability study sample preparation, as documented in the stability and testing plan. The column was then used to generate two 55-gal barrels of test solution on 8/2/03 to 8/3/03 and 8/19/03 to 8/21/03.

The 10 mL test concentration samples were collected into 20 mL culture tubes. Samples were collected 8/4/03 and 8/6/03 (both pretest), 8/7/03 (Day 0), 8/11/03, 8/13/03, 8/19/03, and 8/28/03 (termination) to verify the concentration of the test solutions in the testing aquaria.

The Battelle Sequim Chemical Repository directed the preparation the p,p'-DDE stock solution. Stock solutions were prepared 6/17/03 by weighing 1.0169 g p,p'-DDE (CAS 72-55-9, CF-1832, Aldrich, Lot #09020KU, expiration date 10/04) and adding it to a 50 mL amber glass vial with 50 mL acetone (CAS 67-64-1, Fisher Optima Lot #965882, no expiration date), which was capped, hand-shaken, and allowed to sit until the p,p'-DDE dissolved. Then the solution was

transferred into a prepared saturator column and the acetone evaporated off using an aspirator vacuum pump. The column was then connected to the Fluid Metering, Inc. (FMI) pump and tubing assembly, and the discharge line was placed in a 55-gal barrel of water. The first barrel was delivered for use and connected to the diluter system on 6/19/03. The column was then used to generate five additional 55-gal barrels, which were delivered for use on 6/23/03, 6/30/03, 7/5/03, 7/8/03, and 7/14/03 (the final barrel was 2/3 full).

Stock barrel No.5 was sampled 7/10/03, and stock barrel No. 6 was sampled on 7/14/03. Test concentration samples of 10 mL were collected to verify test concentrations from each of four replicate exposure tanks (C, L, H) on 6/23/04 (pretest), 6/26/03 (Day 0), 6/30/03, 7/2/03, 7/7/03, and 7/17/03 (termination). On 6/30/03, a tube sample, an M cell sample, and a sample from the FS cell:high chamber were also collected. The 10 mL samples were placed in 20 mL culture tubes and processed by adding 25 µL internal standard PP-1226 (2000 ng/mL PCB-198 in acetone) and 2.5 g sodium chloride (NaCl, EM Science Lot #3293B28). Blanks and blank spikes were prepared using 10 mL DI water, and adding 25 µL PP-1225 (400 ng/mL p,p'-DDE in acetone). 1 mL hexane (TJ Baker, Lot# 40E12) was added to all samples, then they were capped and hand-agitated for 3 min. After a 20 min settling period, the subsamples were analyzed.

The Battelle Sequim Chemical Repository directed the preparation of the di-n-butylphthalate stock solution (CF 1778, CAS 84-74-2, Sigma, Lot #080K1023, expiration date 07/04). The stock was prepared 8/11/03 by adding 0.2 mL of di-n-butylphthalate to 1 L well water in a 20 L carboy, and the solution was brought to a 19 L total volume and stirred overnight. This solution was labeled as Stock 1, sampled 8/13/03. A second carboy (Stock 2) was prepared 8/14/03 and sampled 8/15/03, and a third stock (Stock 3) was prepared 8/17/03 in exactly the same way, but was not analyzed prior to use; the contents of all three carboys were used in their entirety. The rotation of stock was based on stability of the chemical and the volume of stock needed for the test. On 8/18/03, the stock preparation approach was modified for the preparation of Stock 4; each of three 5-gal glass carboys labeled A, B, and C were spiked with 200 µL di-n-butylphthalate in 2 L fresh water, and then brought to 19 L with additional fresh water. Each carboy (A,B, and C) was stirred overnight. Carboys B and C were combined to create Stock 4, with 30 L total volume. Stock 4 was sampled on 8/19/03 and used for 1 day before the test was terminated on 8/20/03. Carboy A was kept as reserve stock material, but was not used.

The diluter system was started 8/12/03, and chemical from the first stock solution was connected. Prepared stock was connected to the diluter system, and a 10 mL pretest sample was collected 8/13/03. The test concentration samples were collected using 20 mL glass culture tubes to verify the test concentrations. Samples from the test aquaria were collected on 8/13/03 (pretest), 8/14/03 (Day 0), and 8/19/03. Termination of the test was noted as 08/20/03; the 21-day test was not completed, but rather, was terminated early by direction from EPA, because the exposure concentrations could not be maintained.

2.3. Analytical Procedures

After preparation of the stock solutions for each of the chemicals, determinations of concentrations were made using the methods described below. Details of the methods—that is, calibration curves, analytical dates, specific conditions of analysis (e.g., instrument settings)—are provided in Appendix A. The concentrations of the chemicals in the test aquaria were measured prior to the addition of fish to verify that test concentrations were within 30% of target concentrations. Samples were collected weekly from the test chambers and were hand-carried and delivered to the analytical laboratory. Chain-of-custody forms accompanied all samples. Sample collection and extraction and analysis dates are included in the in-life chemistry data (Appendix A).

2.3.1 Atrazine

Samples were analyzed by both gas chromatography with flame ion detection (GC-FID) and GC-mass spectrometry (MS). Stock samples were analyzed by quantification of atrazine using GC-FID due to their concentrations, which were relatively higher than those of the diluter samples, and by GC-MS for test concentration samples.

GC-FID: Four continuing calibration verification (CCV) samples were analyzed with a mean recovery of 99.3%, and recoveries ranged from 97.0% to 101.6%.

GC-MS: There were 13 CCVs analyzed with a mean recovery of 95.1%, ranging from 75.2% to 113%. The acceptable range for continuing standard recovery is 75% to 125%.

2.3.2 Bisphenol A

Samples of each of the test concentrations were collected from the test aquaria weekly and were analyzed by high-performance liquid chromatography (HPLC) using an absorbance detector at the 275 nm wave length with a flow rate of 1.5 mL/min of 60% acetonitrile (ACN; JT Baker, Lot #X44836) and house DI water mixed by the system pump. A Phenomenex Synergi (4u Hydro-RP 80 Å 250 mm X 4.6 mm 4u) HPLC column was used to quantitate bisphenol A. The HPLC data were stored on the computer, WV-4736 using Varian Star (V.4.51) software. Varian Star chromatography software was used to quantify the data. There were 16 CCVs analyzed with the data, with a mean recovery of 99.3%, ranging from 97% to 104%. The acceptable range for continuing standard recovery was 75% to 125%.

2.3.3 p,p'-DDE

Samples were analyzed by quantification of p,p'-DDE using GC with an electron capture detector (ECD) and a 60 m X 0.25 mm DB-5 column with a 0.1 film thickness. Calibration used dilutions (A-H) of analytical standard PP-1224. GC-FID runs were stored on the computer, WD02318, in room 223, MSL 5 using Varian Star (V. 4.5.1) software. All data were instrument-corrected for internal standard recoveries. There were 24 CCVs analyzed, with a mean recovery of 102%, ranging from 91% to 117%. The acceptable range for continuing standard recovery is 75% to 125%.

2.3.4 Perchlorate

Samples were analyzed by ion chromatography (IC) using the Dionex 4500i system with a conductivity detector. A 100 mM sodium hydroxide (Integra Lot #0628E21) solution was used as the eluent. Separation was attained using a Dionex AS 11 column. Data were recorded and processed using standard Dionex software AI-450 release Version 3.31. Standard PP-1218 (dilutions G-B) was used to quantitate sodium perchlorate, reported as perchlorate. There were 15 continuing calibration verification standards analyzed. The average recovery was 105%, ranging from 89.7% to 114%.

2.3.5 Cadmium Chloride

Stock and diluter samples were analyzed directly with no preparation other than preservation. Samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) by EPA Methods 200.8 and 6020, following Sequim Method MSL-I-022, *Determination of Elements in Aqueous and Digestate Samples by ICP/MS*. Five ICVs and 31 CCVs were analyzed in the sample batches. ICV/CCV values averaged 99% and ranged from 97% to 102%, well within the acceptance criteria for ICV and CCV samples.

2.3.6 Di-n-butylphthalate

Samples were hand-carried to the laboratory and delivered on the same day collected. Samples from replicate diluters provided a measure of precision.

Stock solution samples were processed differently than test aquarium samples due to their high concentrations. Stock solution samples were processed by taking 1.0 mL of sample and adding it to a 1.8 mL vial. Approximately 0.1 g NaCl was added to the vial along with 0.02 mL internal standard (PP-1219, 1000 µg/mL), 5a-androstane. Then 0.5 mL hexane (JT Baker, Lot #X40E12) was added, and the vial was capped and agitated by hand for 3 min. An aliquot of the hexane was transferred to an autosampler vial and injected on a GC-FID, property number WB73809.

Test concentration samples were extracted for analysis by adding 20 µL surrogate mix PP-1261 to each 10 mL sample in the 20 mL culture tube in which the sample was received. Then, 3 mL NaCl (EM Sci, lot# 3293B28) was added, followed by the solvent used for extraction, 1 mL methyl tertiary butyl ether (MTBE; EM Sci Lot #35153). The tube was capped and hand-agitated for 3 min, after which 0.5 mL MTBE extract was pipetted into an autosampler vial with the internal standard solution PP-1262. To attain greater method sensitivity, GC-MS was used for the analysis of diluter samples.

2.4 Animals and Husbandry

The requirements for age and size of the test species stated that the fathead minnows (*Pimephales promelas*) should be sexually dimorphic, first-time spawners, approximately 120 days old, and the minimum size must be 2.5 g for males and 1.5 g for females. These requirements were met by purchasing organisms from ABC Laboratories, Columbia, Missouri. Documentation of chain-of-custody, the condition of the animals when shipped and upon receipt, and environmental parameters (e.g., temperature) at the time of shipping for comparison with conditions encountered at the time of receipt, and verification of the taxonomy of the organisms (genus, species) and disease-free status were submitted with each batch of organisms. The aquatic supply vendor provided documentation along with the animal shipment stating that there was no reported incidence of disease during the care and maintenance of the minnows.

Approximately 1120, 30- to 60-day old *P. promelas* were purchased from ABC Laboratories and cultured to the stage of reproductive differentiation and sexual maturation following the guidance described in *Guidelines for the Culture of Fathead Minnows (Pimephales promelas) for Use in Toxicity Tests* (EPA 1987) and procedure Number EDSP.E-001-01.

Water conditions for both supplies of *P. promelas* were maintained at 24°C to 26°C. A flow-through system design provided adequate volume replacement for organism needs while maintaining the required constant temperature. A continuous, gentle aeration from an oil-free air supply was provided to the tanks. The minnows were housed in 30-gal tanks upon arrival and until sexually differentiated, whereupon they were separated by sex and transferred to clean, 10-gal aquaria until needed for testing. To establish breeding pairs, four females and two males were transferred at the time of assay to 5-gal containers, which contained spawning tiles made of terracotta. Table 2.2 provides the water-quality characteristics for culturing and testing.

Table 2.2. Recommended Ranges of Water-Quality Characteristics for Testing Fathead Minnows

Water Characteristic	Preferred Range
Temperature (^{o}C)	24°C - 26°C
Dissolved Oxygen (mg/L)	>4.9 mg/L (≥60% saturation)
pH	6.5 - 9.0 pH units
Total Alkalinity (mg/L as $CaCo_3$)	>20 mg/L
Total Organic Carbon (mg/L)	≤5 mg/L
Unionized Ammonia	≤35 mg/L

2.5 Study Schedule and Design

The design of this study was to evaluate the sensitivity of short-term reproduction to identify specific modes of action of endocrine disruptors using six model compounds.

For the assay, the *P. promelas* were first held under a pre-exposure phase with no chemical present for 7 to 14 days to establish a record of spawning success and to measure viability of

embryos. The assay units (5-gal aquaria with four females and two males) were then chosen for the 21-day chemical exposure.

2.6 Description of Study Protocol

The 21-day EPA assay began with a pre-exposure phase of 14 days, during which time breeding pairs were established and monitored at testing conditions but without the presence of any chemical. During the pre-exposure period, the daily fecundity measurements were made only during the last 7 days. The first 7 to 10 days of the spawning accumulation period, the presence or absence of eggs was noted but not quantified. The fecundity data collected the last 7 days of the pre exposure period were used. For the last 7 days, data were used to determine which spawning pairs were suitable for the 21-day chemical exposure. The aquaria chosen for chemical exposure were then transferred to the proportional diluter table in the same system/tanks as used for the pre-exposure phase. During the 21-day chemical-exposure period, the appearance, behavior, and fecundity of fish were assessed daily. Viability of resultant embryos (e.g., hatching success) was measured on or around Day 10. The results of that assessment were forwarded to EPA to determine whether further larval hatching experiments were required. The larval hatching study was completed by incubating 50 eggs per replicate in dilution water for 3 to 6 days until the majority of eggs hatched. At the conclusion of the assay, blood samples were collected for determination of sex steroids and VTG, and the gonads were sampled for measurement of the GSI and for histological analyses. In addition, fork length measurements and general gross morphological conditions were noted (appearance of adults).

2.6.1 Summary of Assay Endpoints

Survival: Daily assessment of survival was made to provide a basis for expression and interpretation of reproductive output: that is, number of eggs per female per day (Figure 2.3).

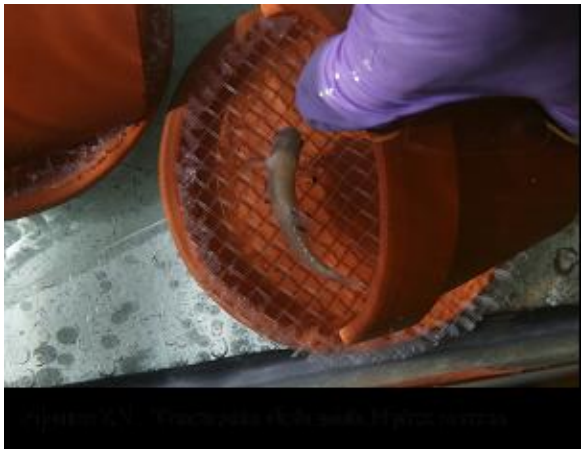

Figure 2.3. Terracotta dish with Nytex screen

Behavior of Adults: Abnormal behavior relative to controls, such as hyperventilation, loss of equilibrium, and feeding abstinence, was noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, were noted.

Fecundity/Fertilization Success: Egg production was determined daily (Figure 2.4). The terracotta spawning substrates were removed from the tanks, and the eggs were allowed to harden prior to enumeration of eggs. After hardening, the eggs were carefully rolled off the tile with a gentle circular motion of a gloved index finger and visually inspected under appropriate magnification. Some eggs could not be included in the evaluation of fertilization because of physical damage or the presence of fungal infection. If no embryos were present, the substrate was left in the aquarium. If spawning occurred that morning, embryos typically underwent late cleavage, and determination of the fertility rate (number of embryos/number of eggs x 100) was easily achieved. Infertile eggs were opaque or clear with a white dot where the yolk precipitated; viable

embryos remained clear for 36 to 48 hours until reaching the eyed stage. Fecundity was expressed on the basis of surviving females per reproductive (test) day per replicate.

Figure 2.4. Staff Members Count Eggs In Support Of Fecundity Analysis

Hatchability and Larval Appearance: This endpoint was assessed up to two times during the 21-day assay. Viability of resultant embryos (e.g., hatching success) was measured for all chemicals on or around Day 10. The results of that assessment were forwarded to EPA to determine whether further larval hatching experiments were required. A second embryo hatching/larval survival assessment was conducted only for atrazine and cadmium chloride.

Fifty (50) normal, healthy looking embryos were transferred to incubation chambers (1-L flow-through jars with gentle flow and aeration) and held at 25°C (Figure 2.5). The larvae typically hatched in 3 to 6 days. Daily observations were conducted and the number of embryos, newly hatched larvae, and any dead embryos or larvae were scored. The data from the hatching test were used to determine the percentage of eggs that hatched and number of normal appearing larvae.

Appearance of Adults: The external appearance of the adults was assessed as part of the daily observations, and any unusual changes were noted. These data were used only to evaluate the health of fish, but they were not included in the body or appendices of this report.[4] External features of particular importance included body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (dorsal nape pad, nuptial tubercles in males; ovipositor in females).

Figure 2.5. Flow-Through Embryo-Incubation Chambers

Blood Sampling: At the conclusion of the exposure, the fish were anesthetized by transfer to an oxygenated solution of tricane sulfate (MS-222) (250 mg/L buffered with 200 mg $NaHCO_3$/L). Blood was collected from the caudal vein with a heparinized microhematocrit capillary tube.[5] Depending on the size of the fathead minnow, which usually is gender-dependent, blood volumes generally ranged from 30 µL to 80 µL. Plasma was separated from the blood *via* centrifugation (2 min) and stored with protease inhibitors at -80ºC until analyzed for VTG and sex steroids. The blood sampling method is documented in the QAPP, and blood sample volumes are recorded in data files.

[4] These data are available in the project files.
[5] Four females were sampled from each aquarium; plasma from two females was combined to form each sample.

Morphology and Gonad Size: After blood was sampled, fish weights and lengths were collected and the gonads were removed and weighed to the nearest 0.1 mg to determine the GSI (GSI = 100 X gonad wt/body wt) (Figure 2.6). Typical GSI values for reproductively active fathead minnows range from 8% to 13% for females and from 1% to 2% for males (EPA MED 2002). Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes. After removal of the gonads, the remainder of the carcass of the fish was discarded.

Vitellogenin: The measurement of VTG plasma samples was performed using an enzyme-linked immunoabsorbant test (ELISA). Vitellogenin levels were quantitated in plasma samples from each individual fish, for all treatments, using commercially available research quality test kits (product number V01003401) procured from Biosense Laboratories (AS – HIB-Thormohlengsgt. 55 N-5008, Bergen, Norway). The analyses were conducted on a Bio-Tek Synergy HT microtiter plate reader interfaced to a Dell computer, employing the Bio-Tek KC4 test analysis software.

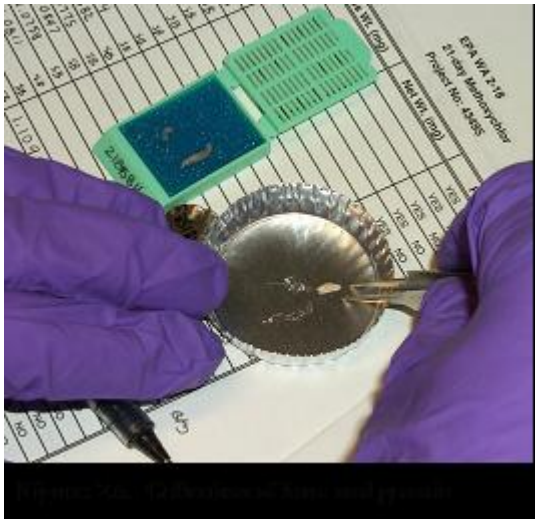

Figure 2.6. Collection of Liver and Gonads

Plasma samples were frozen in 5-µL aliquots and stored in 600-µL microcentrifuge tubes at -80°C until the day of analysis. For analyses, samples were removed from -80°C storage and placed on ice. The samples were rehydrated with 495 µL cold phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). Appropriate dilutions of the samples, beyond the initial 1:100 dilution, were carried out prior to the analysis. Dilutions ranged from 1:100 to $1:10^8$, determined by the sex of the fish and specific chemical exposure defined in the study. The test requires approximately 6 to 8 hours to complete, depending on the number of samples run and the number of dilutions of each sample required. A typical daily run would include 82 samples divided between two 96-well microtiter plates.

The assay has a calibration range of 0.24 ng/mL to 250 ng/mL, with a viable quantitation range of 5 ng/mL to 75 ng/mL. The mean CV for all 217 samples quantitated was 3.33%. For the quantitation range of 5 ng/mL to 75 ng/mL, the average of the CVs was less than 5%. Also, within this quantitation range, dilution recorvery was exceptionally good with matrix effects minimal at the levels of dilutions tested. For example, a sample that quantitated at 50 ng/mL would quantitate at 25 ng/mL when diluted in half. Precision was not affected by overall dilution; a sample that quantitated in the ideal calibration range with a 1:100 showed no improvement or loss in precision over a sample that required a 1:1,000,000 dilution to bring it into calibration range. For data analyses, a value of zero was assigned to samples in which VTG was not detected. Data were natural-log transformed (ln (concentration +1)) before analyses were conducted.

The test required three 1-hour incubations, each with a different antibody-based reagent required to capture the sample analyte and create the detectable sandwich. The sandwich was made up of

the capture antibody, the analyte, the detecting antibody, and the secondary antibody labeled with the horseradish peroxidase (HRP) enzyme. In the final step, the microtiter plate wells were thoroughly washed with a wash buffer, removing all test components, save the analyte captured within the bound sandwich. An HRP substrate was then added to the wells, and following a 30-min incubation, the absorbance of each well was read at 492 nm. Absorbance levels increased with increased calibrator or sample concentrations.

Sex Steroid: Plasma concentrations of β-estradiol, testosterone, and 11-ketotestosterone were measured using competitive enzyme immunoassays (EIAS) commercially available for each steroid of interest. The levels for 11-ketotestosterone, testosterone, and estradiol were quantitated in plasma samples, collected from *P. promelas*, by competitive EIA. Production quality assay kits were procured from Cayman Chemical Company (1180 E. Ellsworth Road, Ann Arbor, Michigan 48108). The tests were carried out on a Bio-Tek Synergy HT microtiter plate reader interfaced to a Dell computer, employing the Bio-Tek KC4 assay analysis software.

Plasma samples were frozen in 5 µL aliquots and stored in 600 µL microcentrifuge tubes at -80°C until the day of analysis. At testing, samples were removed from -80°C storage and placed on ice. The samples were diluted with an appropriate volume of cold assay buffer provided in the Cayman kits. The choice of dilution volumes for the samples was determined by referencing the literature for similar studies to determine a starting point. Dilutions ranged from 1:35 to 1:1500, determined by the sex of the fish and specific chemical exposure defined in the study. The assay requires approximately 7.5 hours to complete when running 4 plates and a total of 156 samples at a single dilution.

The protocols for all three assays are identical except for the specific levels of the calibrators. The calibration ranges for the three assays are as follows: 11-ketotestosterone – 7.8 pg/mL through 1000 pg/mL; testosterone – 3.9 pg/mL through 500 pg/mL, or 1.95 through 250 pg/mL; estradiol – 7.8 pg/mL through 1000 pg/mL. The tests are extremely simple to run and quantitated accurately across the entire dynamic ranges specified in the package inserts. The absorbance readings for all of the calibration points was below 1.5 OD, well within the linear range of the spectrophotometer. The average of duplicate CVs for all samples run for each of the steroid assays was below 5%. The appropriate sample dilution was determined in advance of sample quantitation for most of the runs. In cases where additional dilutions were required to bring a sample into the calibration range, recovery was consistent.

In the first step, sample and both reagents are added to each well, in 50 µL volumes, followed by a 1 hour incubation on a shaker table. In the second step, the plate is washed with a wash buffer, then 200 µL of substrate is added to each well and the plate is incubated for another 1.5 hours on the shaker table. Absorbance is then read at 412 nm on the Bio-Tek reader. The absorbance levels are inversely related to the concentration of analyte. The assay is based on competition between free analyte and a tracer labeled analyte (the tracer is the enzyme acetylcholinesterase), for a limited number of analyte-specific antibody binding sites. For data analyses, a value of zero was assigned to samples in which the sex steroid was not detected. Data were natural-log transformed (ln (concentration +1)) before analyses were conducted.

Histology: Routine histological procedures were used to assess the condition of testes and ovaries from the fish (EPA MED 2002, Appendices D and E). Gonads were placed in fixative (10% buffered formalin) and embedded in paraffin. Serial sections 4-to 5-µm thick were cut along the long axis of the gonad. At least two serial sections were collected from at least three steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six tissue sections per sample. Sections were stained with hematoxylin and eosin, and were submitted to and evaluated by Aqua Technics without prior knowledge of the treatment regime associated with specific samples. A summary of the methods used by the histologist follows.

The histology method was previously described in Ankley et al. (2001); methods, original references, and photomicrographs of normal developmental stages of the reproductive tract of both female and male fathead minnows were included. The methods were developed for the systematic assessment of fathead minnow reproductive tracts and reference to pathological changes that may occur upon exposure to endocrine disruptor chemicals (EDCs). These methods were followed. The paraffin-embedding technique was selected over the glycol methacrylate-embedding technique for these analyses.

The following is an outline summary of the procedures that were used to evaluate the histological sections. Multiple measurements from each individual were taken from a variety of locations on the tissue sections.

Females:

1. The ovary was staged, that is, given a number from 1A, 1B, 2, 3, 4, or 5, based on the most advanced stage present (Table 2.3). The explanation of the stage numbering was provided in Ankley et al. (2001). This staging was done in six locations on each of the three histological slides.

2. Oogonia and oocytes were typed, and 100 cells from each of three sections were rated according to developmental stage (stages noted above, including atretic follicles and post-ovulatory follicles).
3. Abnormalities in the ovary were noted.

Males:

1. Testes were staged, that is, given a number from 1, 2A, 2B, 3A, 3Bb, 4, or 5, based on the most advanced stage present (Table 2.4). This staging was done on four locations on each of the three slides.

2. Based on stages noted above, 100 spermatic cells were typed from each of the three slides, by counting the cells along a straight line on an ocular grid in a predetermined pattern. More than one line on the grid is used if required to obtain 100 cells.

3. The testicular lumen diameter was measured from six tubules on each of three slides.

4. Other changes were noted, including changes to the interstitial tissues (e.g., proliferation of Sertoli or Leydig cells). In addition, abnormal patterns of development were noted, such as premature shedding of spermatocytes into the tubule lumen or foci of necrotic spermatocytes. The presence of any ovatestes or patterns of testicular atrophy was noted.

Table 2.3. Histological Stages Of Fathead Minnow Ovarian Development

Stage	Characteristics
1. Primary Growth	Oogonia and primary oocytes
	1A. Oocytes in nests; small cytoplasmic volume
	1B. Oocytes larger, out of nests, surrounded by follicle cells; many pleiomorphic nucleoli bordering the nuclear envelope
2. Cortical Alveolus	Appearance of cortical alveoli and possibly small lipid droplets
3. Early Vitellogenic	Appearance of yolk bodies; initially few and small, ultimately many and variably-sized; centrally-located germinal vesicle is round to oval with several peripheral nucleoli
4. Late Vitellogenic	Germinal vesicle loses nucleoli, moves towards the periphery and breaks down; yolk bodies frequently fill the entire center of the oocyte and a germinal vesicle may not be evident
5. Mature/Spawning Oocytes	Germinal vesicle breakdown complete; yolk bodies fuse and may become larger than cortical alveoli

Table 2.4. Histological Stages Of Fathead Minnow Testicular Development

Stage	Characteristics
1. Resting Germ Cells	No development
2. Spermatogonia	2A. Primary Spermatogonia: Large cells near edges of tubule; have a lightly staining nucleus with a prominent nucleolus
	2B. Secondary Spermatogonia: Clusters of medium-sized cells with a round, lightly basophilic nucleus; cluster or cyst is the result of several mitotic divisions of primary spermatocyte
3. Spermatocytes	3A. Primary Spermatocytes: Smaller cells with smaller, more basophilic nuclei than Spermatogonia; will undergo meiosis I to produce secondary spermatocytes
	3B. Secondary Spermatocytes: Small cells with smaller, more basophilic nuclei than primary spermatocytes; will undergo meiosis II to produce spermatids
4. Spermatids and some spermatozoa in lumen of seminiferous tubules; small tubule lumen	Spermatids have a small, intensely basophilic nucleus; mature into spermatozoa
5. Abundant sperm in an expanded lumen	

Quality Assurance: Randomly picked slides were examined in detail for conformance with the descriptions provided in Ankley et al. (2001) prior to beginning the systematic examination of the histology slides. The raw data from systematic slide evaluation were recorded on hand-written sheets. These data were transferred to a Microsoft Excel spreadsheet. A second 100%-accuracy check of data transfer was conducted after the initial transfer of data to the spreadsheets. In addition, spot checks of comments and abnormal conditions were conducted by re-examining the slides for conformance with the original comments. In addition, a final examination of the spreadsheet for accuracy was made by the histology principal investigator.

2.6.2 General Water Chemistry

General water quality parameters measured as part of the assays included temperature, dissolved oxygen (D.O.), pH, hardness, alkalinity, and light intensity and photoperiod. Frequency of water quality monitoring is summarized in Table 2.5.

Temperature, pH, and D.O. were measured using a sonde 600 probe coupled with a YSI Environmental Monitoring System (either 610-DM or the 600 XL) and Data logger. Real time measurements of temperature, pH, and D.O. were collected using portable laptop. Data were downloaded to an Excel spreadsheet. Alkalinity, hardness, and total ammonia were monitored *via* commercial Hach kits. Light intensity measured using with a Licor 185-A or an Extech

Light Meter. A photoperiod of 16 hours light and 8 hours of dark was maintained by an automated lighting system, monitored nightly by Battelle Sequim security staff.

Table 2.5. Summary of the Testing Conditions for the 21-Day Assay

Parameters	EPA 21-day
Age of organisms	Reproductive adult fathead minnows (120 day minimum)
Holding Conditions	Temp: 25°C ±1°C
	D.O.[(a)] >4.9 mg/L
	Light: 16 h light:8 h dark at 400 – 500 lux
	Fed: live brine shrimp and ground salmon starter (automatic feeder) until able to eat frozen brine shrimp. Brine shrimp was given up to three times daily
Assay Conditions	Flow-through continuous dispersal of chemical concentrations using a proportional diluter
Duration	21-day
Dilution water	Clean artesian well water monitored yearly for drinking water standards
Material	EDC-chemical
Chamber size	18 L (40 x 20 x 20 cm)
Volume	10 L
# Exchanges/day	6 volumes
# Of conc./chemical	2
# Replicates	4
Weight of each fish	Not specified
# Fish/replicate	4 females and 2 males per test aquarium with nesting tiles
Feeding regime	Frozen brine shrimp, two to three times per day. Typically up to 1 mL per tank.
# Controls	Dilution water
# Replicates/control	4
# Fish/control	4 adult females and 2 adult males per replicate = 24
Conditions	
Photoperiod	16 h light:8 h dark
Temperature	25°C ±1°C- monitored continuously in one chamber and daily in one test replicate per concentration. Monitoring conducted using a min-max thermometer with readings recorded every 24 hours

Table 2.5. (cont'd)

Parameters	EPA 21-day
Light intensity	540-1080 lux, monitored at the start and end of assay
Aeration:	D.O. >4.9 mg/L
pH	Not Specified
Alkalinity:	>20 mg/L CaCO3
Hardness:	>140 mg/L CaCO3
Total ammonia	<0.5 mg/L
Monitoring	
Water Quality Frequency: (Minimum frequency)	Start and end of assay: temperature, D.O., pH, and total ammonia in one replicate of each treatment (control, low and high concentration)
	Daily: temperature, pH and D.O. one test replicate
	Weekly total ammonia: 20% of samples
	Once during assay out of the water head tank – hardness and alkalinity
Corrective Actions:	Temperature: adjust controller
	Total ammonia: increase water flow and clean tanks
	Photoperiod: adjust controller
	Light intensity: adjust bulbs over tables
	pH: no action
	Alkalinity: no action
	Hardness: no action
Biological endpoints:	Adult survival, reproductive behavior, fecundity, fertility, embryo hatch, secondary sexual characteristics, gross morphology (gonadosomatic index) and gonadal histology, plasma vitellogenin and sex steroids (β - estradiol, testosterone, 11- ketotestosterone) concentrations
Validity Criteria:	D.O. ≥60% saturation
	Mean temp. 25°C ±2°C should be maintained and corrective action taken if water quality is outside of these limits Also, 90% survival in the controls

a) D.O. Dissolved oxygen.

2.7 Statistical Analyses

The screening assays as described were designed to detect potential EDCs with high power (minimum of 80%) and not to produce a precise estimate of toxicity. The statistical considerations were restricted to the demands of the screening test. The amount of information obtained from the screening test was limited to detecting effects on reproductive traits when both genders were exposed or in determining whether or not gender-specific differences are detected when gender-selective exposure was used.

Descriptive statistics, including the mean, standard deviation, minimum, maximum, and quartiles, were used to characterize each endpoint measured in the three tests. All summary data reported in the results section tables are based on more significant figures than are shown in the summary tables. Hand calculations of means and coefficients of variance may not yield the exact calculation shown. Statistical significance for each endpoint and chemical was evaluated based on the difference in the mean characteristics between the treated and control groups using analysis of variance, Tukey's multiple comparisons test, and the nonparametric Kruskal-Wallis test. Chemical-dosing regimes were considered classifications of fixed effects (i.e., control, low dose, mid-dose, and high dose). Box plots were used to visually characterize the effect of each treatment.

Power analysis assuming a Type I error rate of α[alpha] = 0.05 was used to compare the sensitivity of selected endpoints. Power is the probability that a significant response will be detected at $\alpha = 0.05$ when a true difference of σ[delta]% exists. The achieved power, given the observed maximum difference between treatment means and the control, was calculated. Further, the achieved power given a 10%, 20%, and 50% difference from the control mean was calculated to evaluate the sensitivity of the endpoint. When a significant difference was not achieved between potentially biologically important differences in means, and the power was low (<50%), then there should be a concern that the null hypothesis was falsely concluded. In contrast, when the power was high (>80%) and a significant difference was not achieved, then it can be more certain that the null hypothesis was not falsely concluded.

Appropriate data transformations were applied to maintain homogeneity of the within-class variances (i.e., data expressed as a percentage may be arcsine-square-root or light transformed, counts may be square-root or log transformed, and continuous data may be transformed to the natural logarithm) (Snedecor and Cochran 1980). A rank transformation or nonparametric statistics were used when the common data transformation was not successful in controlling heterogeneity (Daniel 1978).

Analysis may have been conducted both with and without suspected outliers (Chapman et al. 1996). Potential outliers may have been identified by values that exceed the median plus three times the interquartile range (i.e., the difference between the 75th and 25th percentiles). If an explanation could not be made for the divergence of data, then both analyses were presented, assuming that the results differed. If there were no changes to the results, then the analysis including the outliers was presented. If differences occurred, then the implications of removing the outliers were carefully documented. If an explanation could be made for the existence of outliers, the analysis excluding outliers may have been sufficient.

2.8 Quality Assurance

2.8.1 Technical Systems Audits

The Battelle Sequim Quality Assurance (QA) Unit performed assessments on activities and operations affecting data quality, the raw data and final report. Any findings were reported to the Work Assignment Project Manager and management to ensure that the requirements in relevant Standard Operating Procedures (SOPs), WA protocol, Quality Assurance Project Plans (QAPP), and the Quality Management Plan (QMP) were met. The assessments for this study included technical systems audits (TSAs) and audits of data quality (ADQs) that included reviews of project notebooks, data base entry verifications from raw data sheets, and reviews of statistical analyses performed.

TSAs were performed at the start of the study, and for critical elements during the study such as the following:

- personnel training files for documentation of EDSP SOPs
- work plan and WA QAPP read and understood by WA personnel before startup
- calibration status of project instrumentation
- dosing and sample collection of dosing solutions, body weights, and clinical observations
- chemical analysis of test chemicals
- termination of each experiment.

During TSA activities, the Battelle Sequim QA Unit recorded observations to be used later in preparing the audit report. The Battelle Sequim QA Unit observed completion of permitting requirements, implementation of procedures, data recording and record keeping, and equipment maintenance and calibration procedures and/or documentation, noting whether or not the activities adhered to the work plan, and the QAPP, applicable SOPs, and the QMP. Any findings were communicated to the technical personnel at the completion of the WA activity unless an error could compromise the WA (e.g., misdosing an animal). If necessary, the EDSP QA team members immediately notified the WA leader/study director by telephone and/or e-mail of any adverse findings that could affect the conduct of the WA. This direct communication was also documented in the audit report.

2.8.2 Audits of Data Quality

Audits of data quality (ADQs) focused on the accuracy of data collection, recording, traceability and calculations to ensure that the reported results are documented, traceable, and of high quality that accurately reflects the raw data and that the report accurately describes the materials and methods used in the WA, and that conclusions are supported by the data. The assessment criteria for ADQs were that data collection, analysis, and reporting met the requirements of the applicable facility and program SOPs, the work plan and QAPP, and the EDSP QMP, and that deviations be documented according to the requirements of the procedure. Deviation reports relative to the work assignment were submitted to WA leader and included in the project records.

Direct and frequent communication between the project manager, laboratory staff, and the QA Unit manager was designed to provide for sufficient time to perform an ADQ so that the submission date of the audited final report met those specified in the work plan.

All data and records for review were submitted to the QA Unit manager or delegate who reviewed the data packages for completeness and, if incomplete, requested that the additional records needed for review be submitted. EDSP QA team members reviewed a minimum 10% of the raw data, depending on the level of prior technical review, the tabulated data, and WA records of performance and methods to ensure compliance with planning documents mentioned previously. All tables and graphs were reviewed for completeness and accuracy of titles, headers, and footnotes. EDSP QA team members checked all tabulated data designated as statistically significant. Findings were reported and corrective actions undertaken as described earlier. EDSP QA team members reviewed the report using the audited data and corrected tables to ensure that the reported results were of high quality; that they accurately reflected the raw data; and that the report accurately described the materials and methods used in the WA. Findings were reported and corrective actions undertaken as described earlier.

TSA and ADQ were conducted throughout the duration of this WA. Neither of these activities resulted in any major findings nor any stop work associated with the conduct of the experiments.

2.9. Storage of Records and Data Management

The data for this study were collected on preprinted data collection forms. The data forms included, as appropriate, the following items: study code, protocol number, tank number, treatment code, and others. The forms had preprinted dates for collection of data when possible. Otherwise, the dates for data collection were hand printed on the forms as needed prior to or on the day of collection of the data. Data forms were initialed and dated by the person collecting the data, and all forms received documented technical review and signature approval. Corrections to data entries were made by drawing a single line through the error and recording the correct entry, initials, date, and error code that explained the reason for the correction.

The datasheets were clearly divided by chemical and protocol and placed in a workbook. These workbooks were kept next to the tanks until data entry into the database. The data were entered into a Microsoft Access database. The database entry forms corresponded to the datasheets in the workbooks. Data entry included transferring information on the written form to the database form. These database forms and tables associated with the forms had data integrity such that deletions were not allowed by the data entry personnel. Also, there was a quality control (QC) process during data entry to identify and correct any obvious discrepancies in the data.

The original raw data collected on the data forms remain in the wet lab project file until there is a signed final report, at which time they are inventoried and archived on compact disks with read-only memory (CD-ROM) for at least 2 years (longer if required by study protocol or government regulations), unless the sponsor requests that they be transferred to an alternative archive location other than at Battelle.

All specimens and records remain the responsibility of Battelle Pacific Northwest Division (PNWD) and are retained for the length of time stipulated in the contract, which is typically 5 years. The archive is located at Battelle's facility in Richland, Washington, and is maintained according to a policy of limited access. The Battelle sample custodian is responsible for archiving and retrieving work assignment materials. An archive inventory is maintained, and storage capability is provided for the expedient retrieval of materials. Specimens and samples are disposed only after an assessment is made that they no longer afford evaluation.

3.0 RESULTS: ATRAZINE

The results of the EPA 21-day assay for atrazine are presented in this section. Total fecundity is reported as the total number of eggs laid during the assay, regardless of egg condition. However, some eggs could not be included in the evaluation of fertilization because of physical damage or the presence of a fungal infection. Summary tables are presented for the major endpoints. The values presented were calculated before being rounded for inclusion in the tables. Values reported include the mean for each treatment, the number of samples (N), the standard deviation (SD), and the coefficient of variation (CV). Values reported for the statistical analyses are the test statistic for the Kruskal-Wallis test used to determine the significance level (*H*), the probability that the observed result was due to chance (*p*), and the degrees of freedom associated with the test (df).

3.1 EPA 21-Day Assay for Atrazine

The EPA 21-day atrazine assay was conducted from July 29, 2003 to August 7, 2003 (pre-exposure assay), and from August 7, 2003 to August 28, 2003 (exposure assay).

3.1.1 Atrazine Concentrations

Atrazine was not detected at concentrations above the method detection limit (MDL; 2.50 µg/L) in the control at any time during the EPA 21-day atrazine assay. The mean (standard deviation) in the low concentration and high concentration were 24.5 (18.9) µg/L and 223.7 (156.6) µg/L, respectively (Figure 3.1).

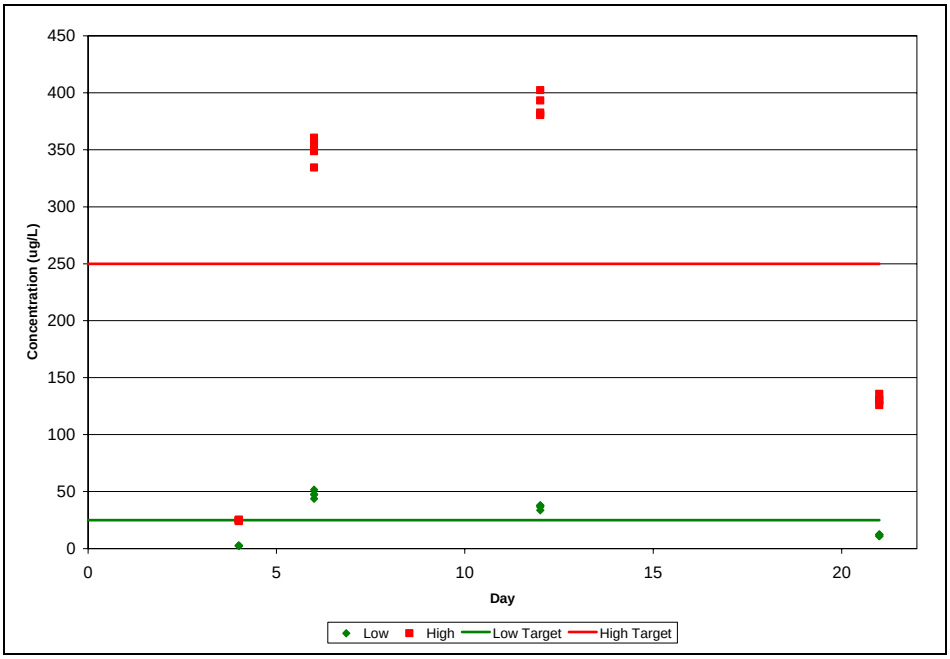

Figure 3.1. Atrazine Concentrations During The EPA 21-Day Atrazine Assay

3.1.2 Survival

One female in the control treatment died during the assay. All other males and females in all treatments survived the EPA 21-day atrazine assay.

3.1.3 Fecundity

Total Fecundity: A 9-day pre-exposure evaluation of total egg production was performed. Total 9-day counts among the three treatments in the exposure assay (individual tank values summed for each treatment) ranged from about 5700 eggs to 9900 eggs (Figure 3.2). No significant differences in the mean 9-day egg production among the groups of replicates evaluated during the pre-exposure assay were detected (Kruskal-Wallis, $H = 3.12$, $p = 0.211$, df = 2).

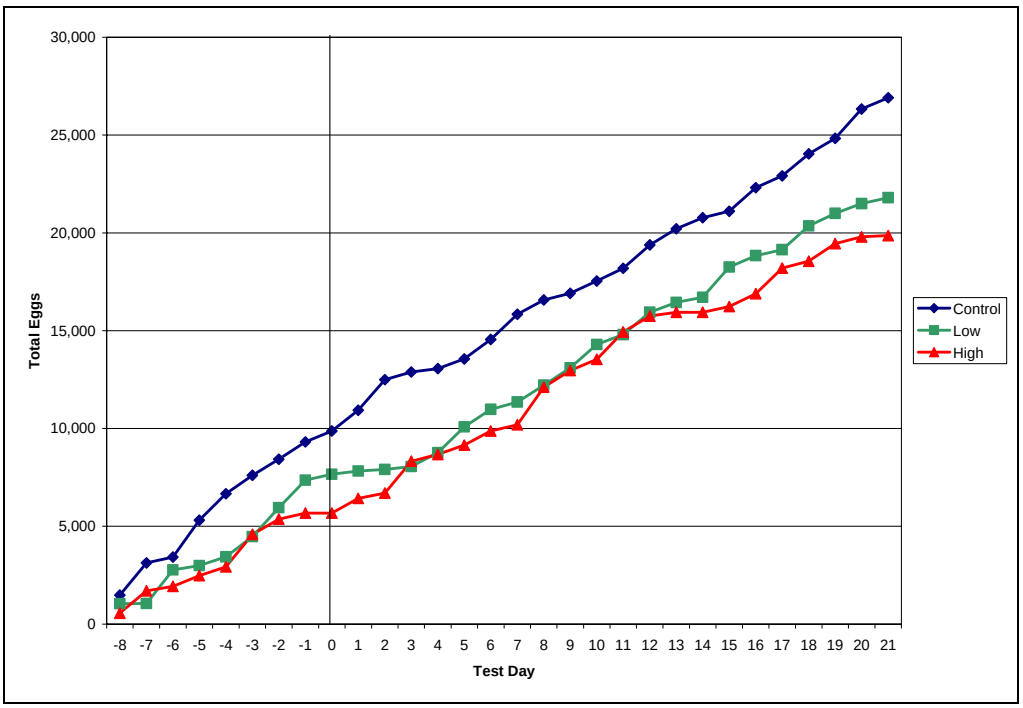

Figure 3.2. Total Egg Production Per Treatment for the Atrazine Assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to atrazine)

During the EPA 21-day atrazine assay, total eggs counts in the control varied from 3355 eggs to 4891 eggs (Figure 3.3). Total egg production among low-concentration replicates was similar, ranging from 2476 eggs to 4739 eggs. Total counts among the high-concentration replicates varied from 2021 eggs to 6688 eggs. Statistical analysis of square-root transformed egg counts showed no significant among-treatment differences (Kruskal-Wallis, $H = 1.88$, $p = 0.390$, df = 2) in mean total numbers of eggs produced (Table 3.1). The achieved power at the observed maximum difference from the control response for this assay was 10%. The probability of detecting as much as a 20% difference in total fecundity was low at 22%, based on the observed variability (Table 3.1).

Table 3.1. Summary Statistics and Power Estimates for Total Fecundity Data for the EPA 21-Day Atrazine Assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	4259	741	17%	11%	9%	22%	87%
low	4	3535	1045	30%				
high	4	3547	2129	60%				

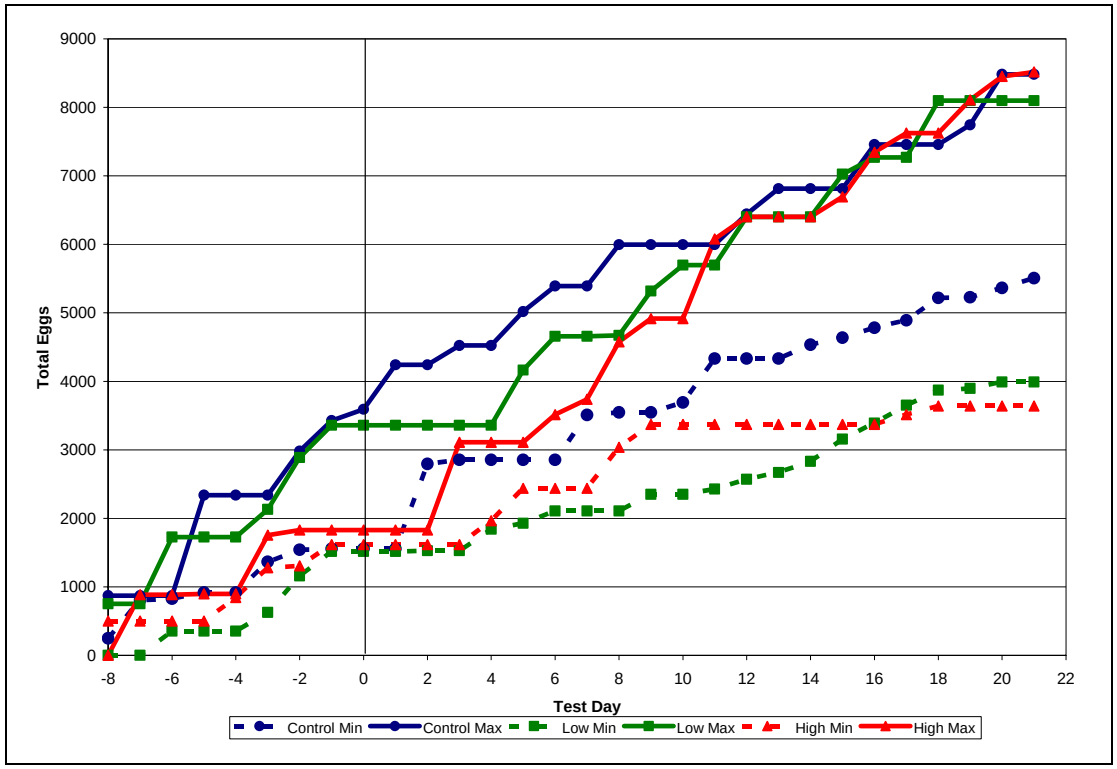

Figure 3.3. Range In Replicate Total Egg Production Per Treatment For The Atrazine Assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to atrazine)

Fecundity per Female Reproductive Day: During the 9-day pre-exposure evaluation, the mean number of eggs produced per female reproductive day ranged from 39.4 eggs/day for the tanks that would be used for the high concentration to 68.6 eggs/day for the tanks that would be used for the control during the 21-day exposure assay. There were no significant differences among treatments in the mean numbers of eggs produced per reproductive day during the pre-exposure period (Kruskal-Wallis, $H = 3.12$, $p = 0.211$, df = 2).

During the EPA 21-day atrazine assay, the maximum number of female reproductive days was achieved for the low and high treatments (Table 3.2). One control female died at Day 8 of the testing. The number of eggs produced per female reproductive day varied from 39.9 eggs to 69.9 eggs in the control and from 29.5 eggs to 56.4 in the low concentration (Figure 3.4). For the high concentration, the number of eggs produced per female reproductive day ranged from 24.1

eggs to 79.6 eggs. No significant differences among treatments in the mean number of eggs produced per day were detected (Kruskal-Wallis, $H = 1.88$, $p = 0.390$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 12%. The probability of detecting as much as a 20% difference in the fecundity per female reproductive day was low at 21%, based on the observed variability (Table 3.2).

Table 3.2. Summary Statistics And Power Estimates For Fecundity Per Female Reproductive Day For The EPA 21-Day Atrazine Assay

Level	Mean Number of Reproductive Days [(a)]	N	Mean Fecundity Per Female Reproductive Day	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	80.5	4	53.6	13.1	24%	13%	9%	21%	86%
low	84.0	4	42.1	12.4	30%				
high	84.0	4	42.2	25.3	60%				

a) Maximum number = 84.

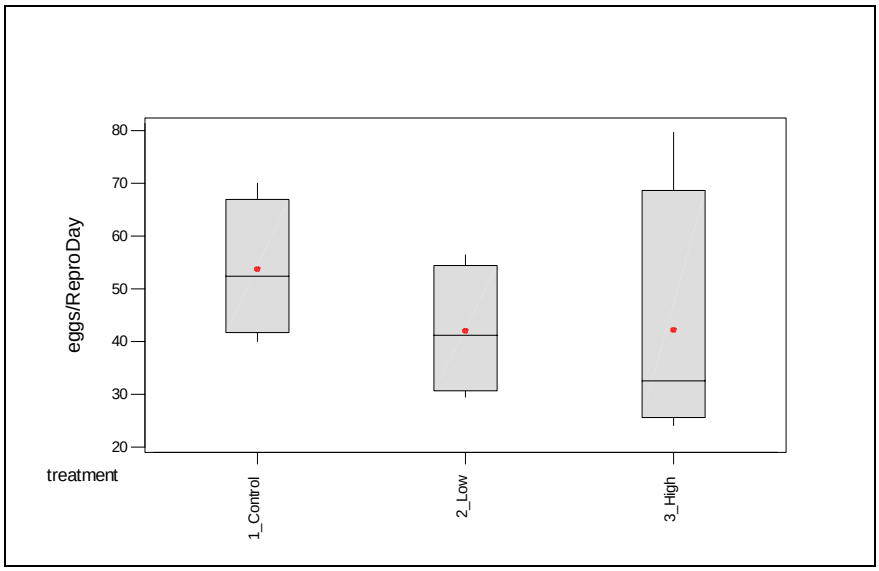

Figure 3.4. Box Plot of the Number of Eggs Produced Per Female Reproductive Day by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Eggs on Tiles/Dishes: The mean number of eggs laid on the tiles during the 9-day pre-exposure assay varied from 1160 eggs for the tanks that would be used for the high concentration to 2016 eggs for the tanks that would be used for the control. The mean number of eggs on dishes ranged from 259 eggs for the high concentration to 452 eggs for the control. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated. There

were no significant differences in the mean proportional difference among treatments during the 9-day pre-exposure assay (Kruskal-Wallis, $H = 0.01$, $p = 0.995$, df = 2).

The mean number of eggs laid on the tiles among the treatments during the EPA 21-day atrazine assay varied from 2521 eggs for the low concentration to 3064 eggs for the control (Appendix C, Table C.2). The mean number of eggs on dishes ranged from 533 eggs for the high concentration to 1195 eggs for the control. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated. There were significant differences in the mean proportional difference among treatments during the 21-day exposure assay (Kruskal-Wallis, $H = 7.42$, $p = 0.024$, df = 2). The proportion of eggs laid on tiles was greater for the high concentration than for the other two treatments.

3.1.4 Fertilization Success

Total Fertilization: Eggs were collected during the 9-day pre-exposure period for the evaluation of fertilization success rate. All undamaged eggs laid during this evaluation were fertilized.

All undamaged eggs laid in all replicates of the control and high concentration during the EPA 21-day atrazine assay were fertilized. All undamaged eggs laid in all but one replicate of the low concentration were fertilized. Mean fertilization rates among treatments were not significantly different (Kruskal-Wallis, $H = 2.00$, $p = 0.368$, df = 2).

Fertilization of Eggs on Tiles and Dishes: During the 9-day pre-exposure assay, all undamaged eggs laid on tiles or on dishes were fertilized. All undamaged eggs laid on tiles during the EPA 21-day atrazine assay were fertilized. All undamaged eggs laid in dishes in all treatment replicates, except one low concentration replicate, during the assay were fertilized.

3.1.5 Hatchability and Larval Development

Eggs were collected during the EPA 21-day atrazine assay for the evaluation of hatchability. The larval hatching study was completed by incubating 50 eggs per replicate in dilution water for 3 to 6 days until the majority of eggs hatched. The proportion of fertilized eggs that hatched ranged from 0.84 to 1.00 in the control and from 0.74 to 1.00 for the two atrazine concentrations. The widest range in values was from the high atrazine treatment (Figure 3.5). No significant differences were seen among treatments in the proportion of eggs that hatched (Table 3.3) were detected (Kruskal-Wallis, $H = 0.72$, $p = 0.697$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 8%. The probability of detecting as much as a 20% difference in proportion of fertile eggs that hatched was moderate at 73%, based on the observed variability (Table 3.3).

Table 3.3. Summary Statistics and Power Estimates for the Proportion of Fertile Eggs that Hatched for the EPA 21-Day Atrazine Assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.933	0.072	8%	4%	23%	73%	100%
low	8	0.974	0.018	2%				
high	8	0.906	0.110	12%				

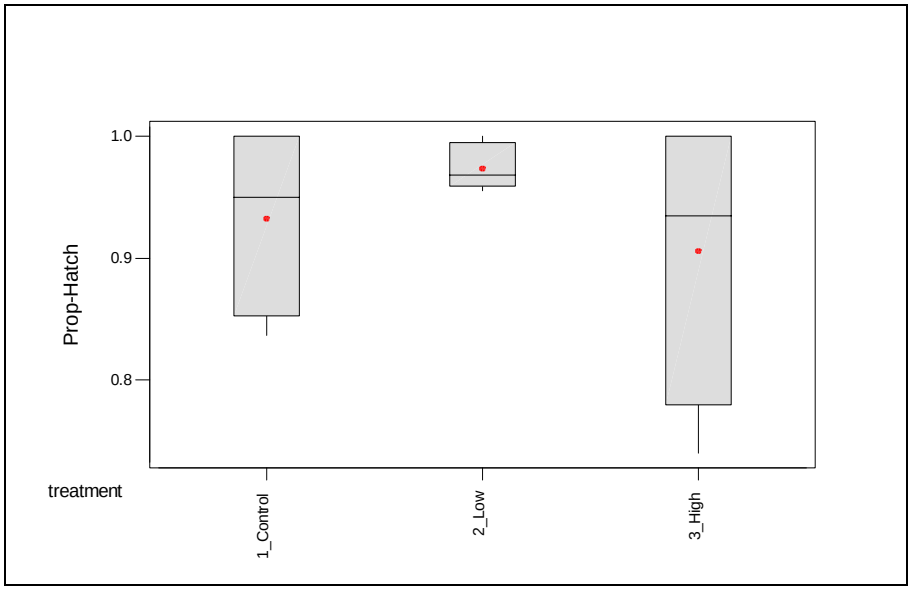

Figure 3.5. Box Plot of the Proportion of Fertile Eggs that Hatched by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Eggs were collected during the EPA 21-day atrazine assay for the evaluation of larval development. The proportion of larvae that developed normally (i.e., that showed no morphological abnormalities) ranged from 0.93 to 1.00 in the control and from 0.88 to 1.00 for the two atrazine concentrations (Figure 3.6). There were no significant differences among treatments in the proportion of larvae that developed normally (Kruskal-Wallis, $H = 1.95$, $p = 0.378$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 26%. The probability of detecting as much as a 20% difference in the proportion of normally developing larvae was high at 98%, based on the observed variability (Table 3.4).

Table 3.4. Summary Statistics and Power Estimates for the Proportion of Normal Larvae for the EPA 21-Day Atrazine Assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.972	0.032	3%	7%	47%	98%	100%
low	8	0.941	0.043	5%				
high	8	0.949	0.043	4%				

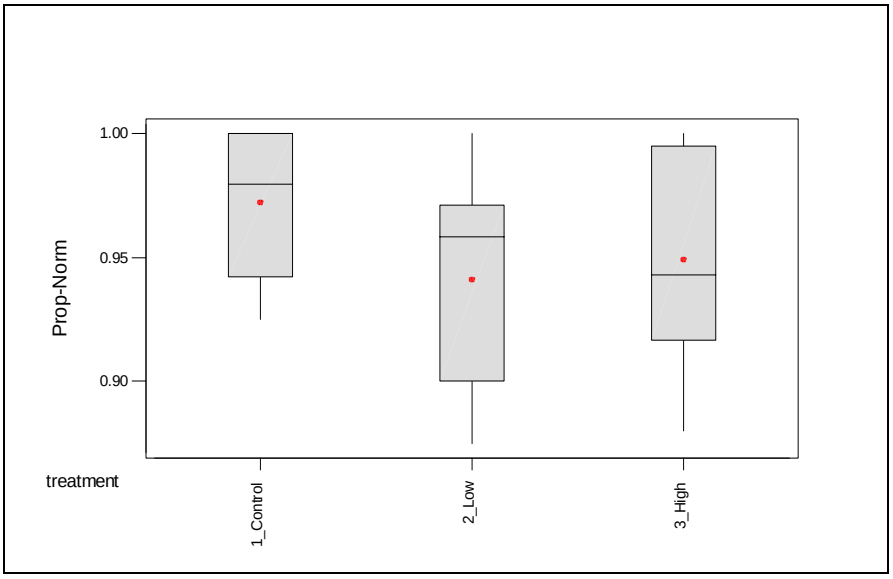

Figure 3.6. Box Plot of the Proportion of Normal Larvae by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

3.1.6 Body Weight

The body weight of females used in the EPA 21-day atrazine assay ranged from 1.5 g to 6.3 g. There were no significant differences in mean body weight among all treatments (Kruskal-Wallis, $H = 2.41$, $p = 0.299$, df = 2). The body weight of males used in the EPA 21-day atrazine assay ranged from 3.0 g to 8.3 g. There were no significant differences in mean body weight among treatments (Kruskal-Wallis, $H = 4.34$, $p = 0.114$, df = 2).

3.1.7 Gonadosomatic Index

The range of GSI values calculated for females in all treatments varied from three- to fourfold (Figure 3.7). The highest value (GSI = 32.0) was obtained for a female from the low concentration. One female exposed to the high concentration had a GSI value of 30.1. No significant differences in the mean GSI value per treatment (Table 3.5) were detected (Kruskal-

Wallis, $H = 0.38$, $p = 0.829$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 8%. The probability of detecting as much as a 20% difference in the female GSI was moderate at 74%, based on the observed variability (Table 3.5).

Table 3.5. Summary Statistics and Power Estimates for Female GSI Data for the EPA 21-Day Atrazine Assay

Level	N[(a)]	Mean[(b)]	SD[(c)]	CV[(d)]	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	15	13.8	3.9	28%	4%	23%	74%	100%
low	16	15.1	5.8	38%				
high	16	15.0	5.6	37%				

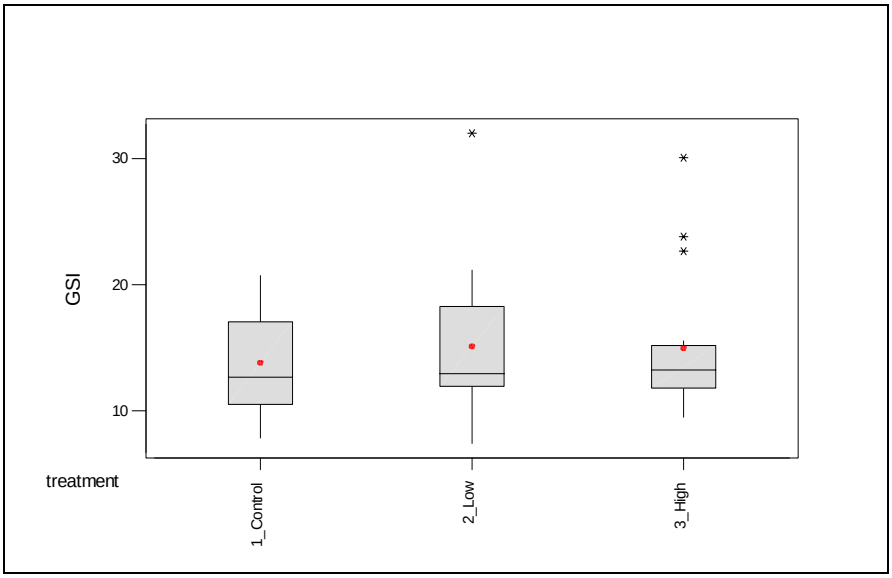

Figure 3.7. Box Plot of Female GSI by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

The range of most GSI values calculated for males during the EPA 21-day atrazine assay was small, ranging from 0.8 to 1.7 (Figure 3.8), which approximates the typical range for reproductively active male fathead minnows (Jensen et al. 2001). One male in the low concentration had a very low GSI value of 0.15, attributable to its very low gonad weight of 0.01 g and relatively large body size (7.6 g). Histological evaluation of this fish showed no gonadal development. This male was excluded from the statistical analysis. There were no significant differences in mean GSI values (Table 3.6) among treatments (Kruskal-Wallis, $H = 0.47$, $p = 0.791$, df = 2) (Figure 3.8). The achieved power at the observed maximum difference from the

control response for this assay was 10%. The probability of detecting as much as a 20% difference in the male GSI was low at 63%, based on the observed variability (Table 3.6).

Table 3.6. Summary Statistics and Power Estimates for Male GSI Data for the EPA 21-Day Atrazine Assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	1.19	0.32	27%	6.3%	19%	63%	100%
low	7	1.04	0.28	27%				
high	8	1.13	0.32	28%				

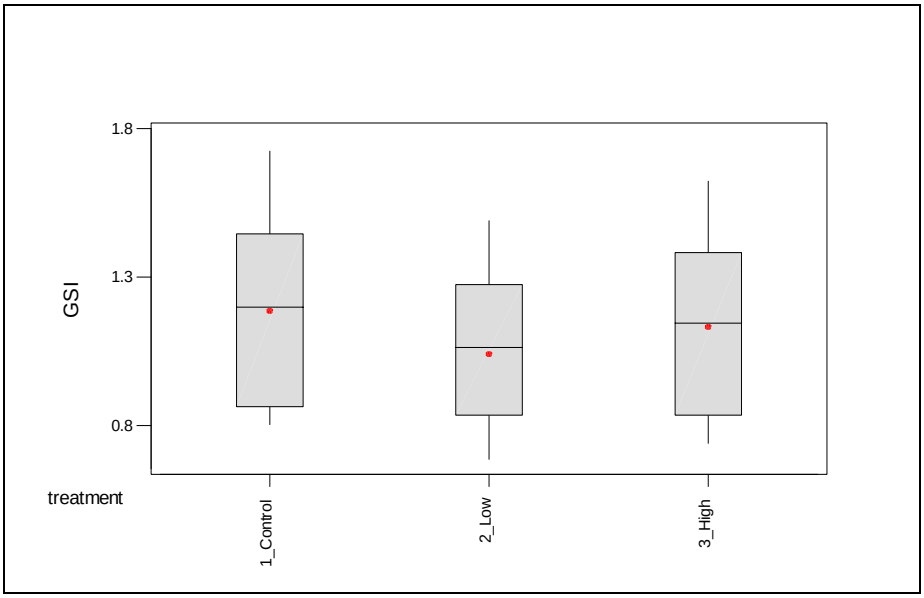

Figure 3.8. Box Plot of Male GSI by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

3.1.8 Female Gonad Histology

Histological analyses were conducted on the ovaries of 47 females exposed to atrazine during the EPA 21-day assay.

General Ovary Staging: Statistical analysis of the mean ovarian staging from 18 microscopic fields per female in the EPA 21-day atrazine assay revealed no significant differences among treatments (Kruskal-Wallis, $H = 4.81$, $p = 0.090$, df = 2).
Quantitative Ovarian Staging: One hundred cells in each of three sections per female were examined to quantitatively determine the developmental stage of the ovaries. Ova from fish from all treatments ranged from Stage 1A to Stage 5 (see Methods for a description of the stages)

(Figure 3.9). Statistical analyses showed that there were no significant differences among treatments in the proportion of cells occurring in any developmental stage (Table 3.7).

Table 3.7. Descriptive Statistics of the Proportion of Ovarian Cells in Each Developmental Stage for Females from the EPA 21-Day Atrazine Assay and Results of the Kruskal-Wallis Test (Df = 2) Comparing Treatments

	Control (N = 15)			Low (N = 16)			High (N = 16)			Kruskal-Wallis	
Stage	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	***H***	***p***
1A	0.048	0.020	42%	0.050	0.020	40%	0.043	0.027	63%	1.12	0.572
1B	0.214	0.062	29%	0.181	0.058	32%	0.198	0.088	45%	1.52	0.468
2	0.185	0.065	35%	0.188	0.065	34%	0.167	0.070	42%	0.60	0.739
3	0.175	0.064	36%	0.154	0.091	59%	0.173	0.084	49%	0.67	0.717
4	0.205	0.082	40%	0.219	0.116	53%	0.231	0.134	58%	0.26	0.879
5	0.027	0.039	144%	0.018	0.057	313%	0.038	0.121	318%	5.34	0.069

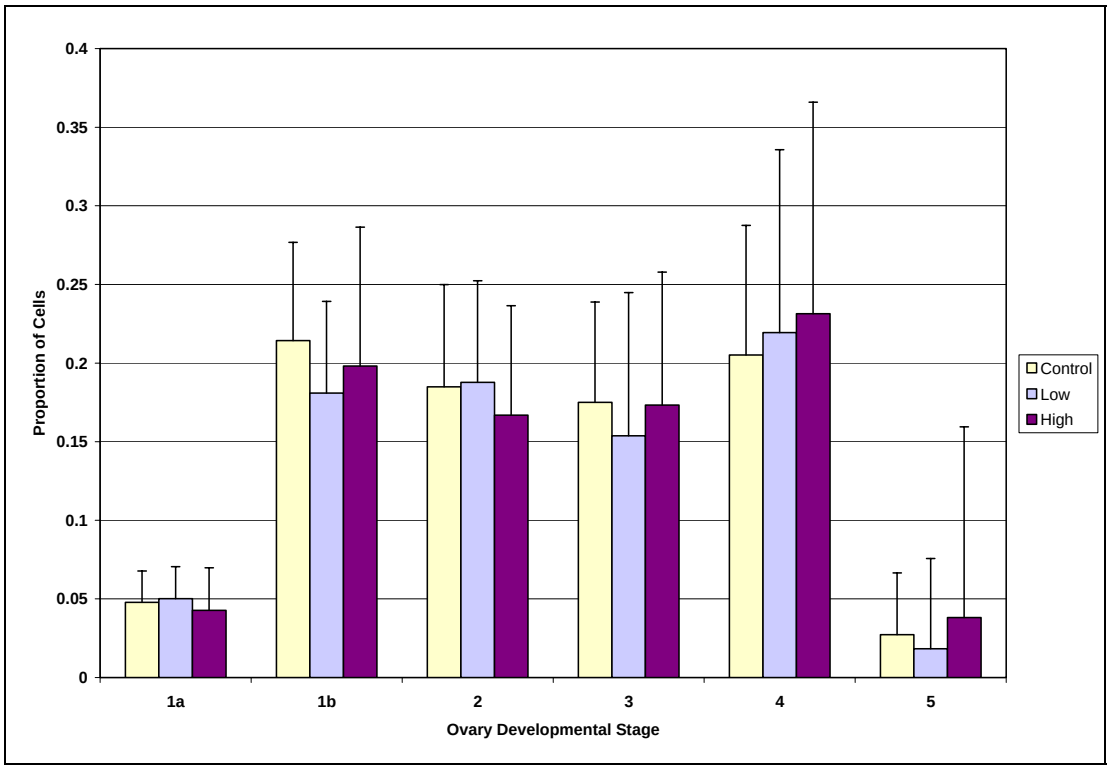

Figure 3.9. Frequency Histogram Showing the Quantitative Developmental Staging of Ovaries for Each Treatment of the EPA 21-Day Atrazine Assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

Atretic Follicles: The mean proportion of atretic follicles per 300 follicles (counted per fish) ranged from 0.139 for females from the control to 0.181 follicles for females from the low concentration (Figure 3.10). Several females in each treatment had high proportions of atretic

follicles, ranging to about 0.7. No significant differences in the proportions of atretic follicles among treatments was detected (Kruskal-Wallis, $H = 0.89$, $p = 0.640$, df = 2).

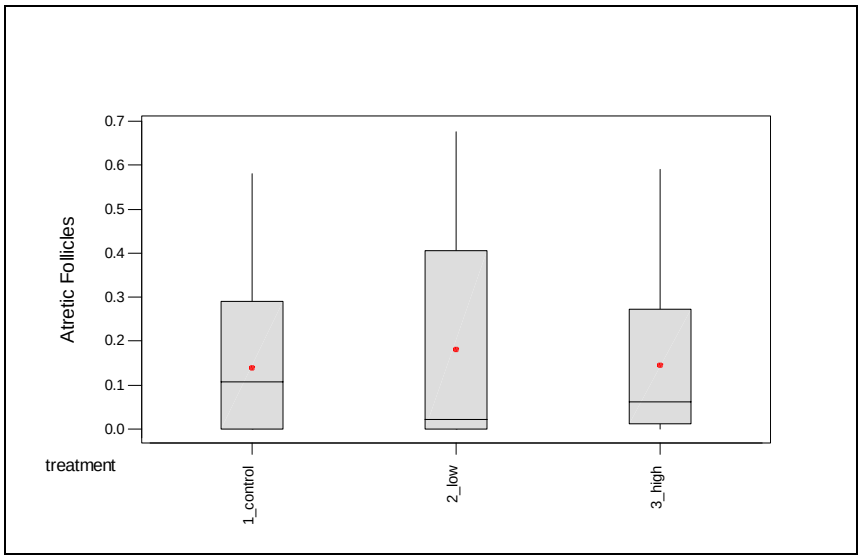

Figure 3.10. Box Plot of the Proportion of Atretic Follicles Per 300 Follicles by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Post-Ovulatory Follicles: The mean proportion of post-ovulatory follicles per 300 follicles (counted per fish) ranged from 0.004 for females from the high concentration to 0.009 for females from the low concentration (Figure 3.11). There were no significant differences in the mean proportion of post-ovulatory follicles among treatments (Kruskal-Wallis, $H = 0.84$, $p = 0.656$, df = 2).

Observations: The ovaries from several fish from each treatment were observed to have histological abnormalities (Table 3.8).

Table 3.8. Histological Observations for Females Exposed to Concentrations of Atrazine During the EPA 21-Day Assay

Fish ID	Treatment	Observations
229222	Control	Atretic follicles are stage 3 to 5; macrophages infiltrating ovary
229231	Control	Areas of coalesced atretic follicles in ovary, other areas nearly normal Some atretic follicles with fibrosis or granuloma type reaction surrounding follicle
229240	Control	Focal area of one ovary completely comprised of atretic follicles, other areas normal; macrophages infiltrating ovary
229244	Low	Extensive macrophage infiltration into ovary
229250	Low	Areas of coalesced atretic follicles in ovary, other areas nearly normal Inflammatory cell infiltration in more normal areas of ovary
229258	Low	Extensive macrophage infiltration into ovary
229262	Low	Multiple foci of macrophage clusters in ovary containing brown material
229270	High	Areas of coalesced atretic follicles in ovary, other areas nearly normal Spatially uneven distribution of follicle stages due to damaged follicles
229274	High	Atretic follicles are areas of coalesced lipid
229275	High	Macrophages infiltrating ovary
229285	High	Multiple foci of macrophage clusters in ovary containing brown material
229287	High	Multiple foci of macrophage clusters in ovary containing brown material

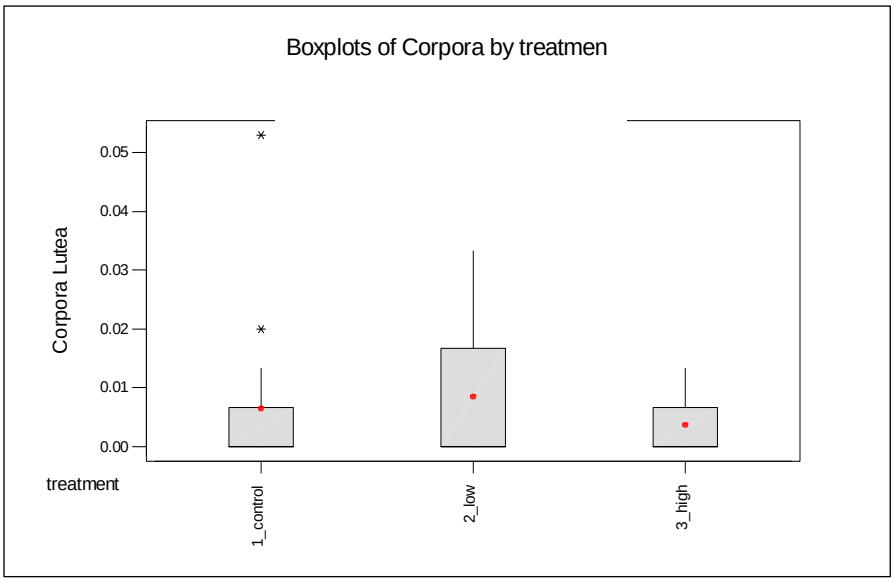

Figure 3.11. Box Plot of the Proportion of Post-Ovulatory Follicles Per 300 Follicles by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the median value for each treatment = 0, the circle is the mean value, and asterisks represent probable outliers)

3.1.9 Male Gonad Histology

General Testes Staging: Testes from 22 males exposed to atrazine during the EPA 21-day atrazine assay were examined to determine the general developmental condition. Males in all treatments had well-developed testes, with most showing Stage 4 and Stage 5 development (see Methods for description of developmental stages). All of the 84 microscopic fields examined in the 7 control males analyzed showed Stage 4 (70 fields) or Stage 5 (14 fields) development. One control male that had no gonadal development was excluded from all histological analyses. All of the 84 microscopic fields examined in the 7 low-concentration treatment males showed Stage 4 (61 fields) or Stage 5 (23 fields) development. All of the 96 microscopic fields examined in the 8 high-concentration treatment males showed Stage 4 (73 fields) or Stage 5 (23 fields) development. Statistical analysis of the mean staging from 12 microscopic fields per fish revealed no significant differences among treatments (Kruskal-Wallis, $H = 0.91$, $p = 0.635$, df = 2).

Quantitative Testicular Staging: One hundred cells in each of three sections per male were examined to quantitatively determine the developmental condition of the testes. The developmental stage for all treatment testes ranged from Stage 2A or 2B to Stage 5 (Figure 3.12). Statistical analyses showed that there were significant differences among treatments in the proportions of cells in developmental stages 2A and 2B (Table 3.9). The mean proportion of cells showing developmental stage 2A was greater in males from the low and high concentrations than from the control. The mean proportion of cells showing developmental stage 2B was lower in males from the low concentration than from the control.

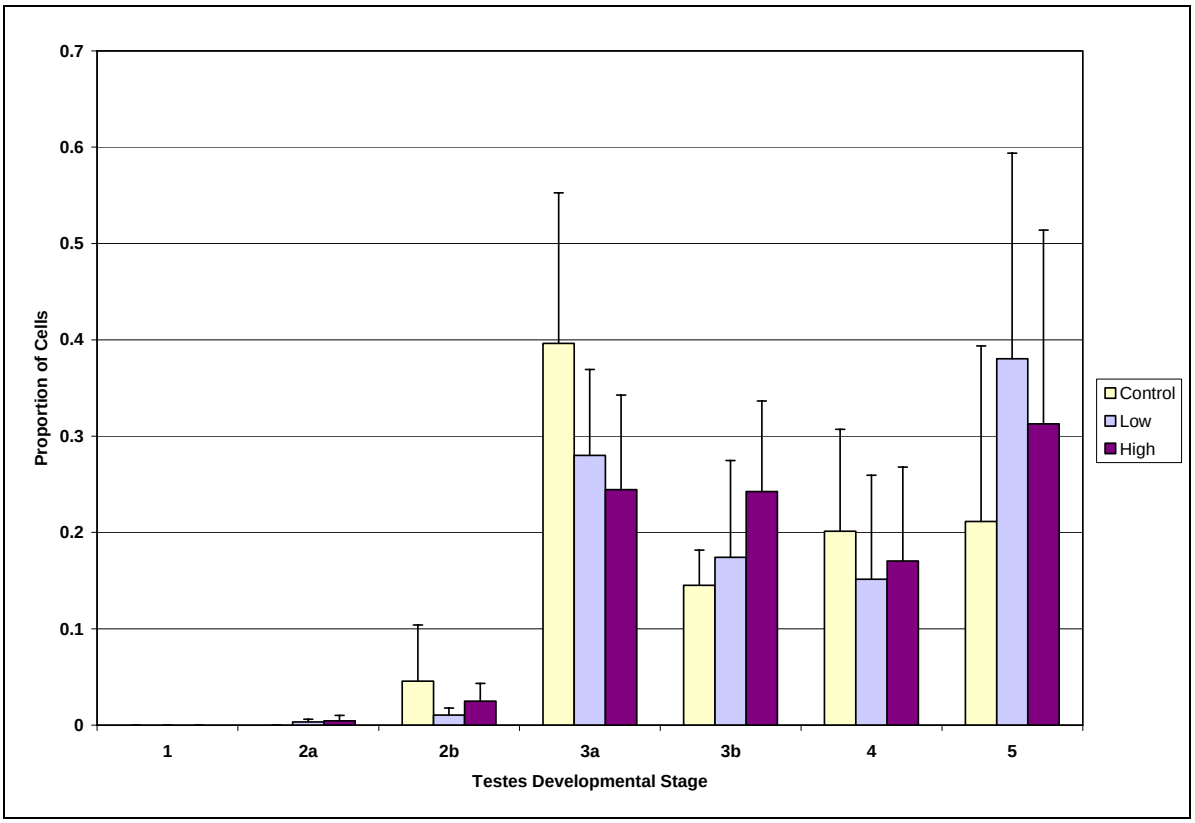

Figure 3.12. Frequency Histogram Showing the Quantitative Developmental Staging of Testes for Each Treatment of the EPA 21-Day Atrazine Assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

Table 3.9. Descriptive Statistics of the Proportion of Testes Cells in Each Developmental Stage for Males from the EPA 21-Day Atrazine Assay and Results of the Kruskal-Wallis Test (Df = 2) Comparing Treatments

	Control (N = 7)			Low (N = 7)			High (N = 8)			Kruskal-Wallis	
Stage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	*H*	*p*
1	0	0	0%	0	0	0%	0	0	0%	–	–
2A	0	0	0%	0.003	0.003	82%	0.005	0.006	123%	7.41	0.025*
2B	0.046	0.058	128%	0.010	0.007	70%	0.025	0.018	73%	7.80	0.020*
3A	0.396	0.156	39%	0.280	0.089	32%	0.245	0.098	40%	4.83	0.089
3B	0.145	0.036	25%	0.174	0.100	58%	0.243	0.094	39%	5.25	0.072
4	0.201	0.106	53%	0.151	0.108	71%	0.170	0.098	57%	0.65	0.724
5	0.211	0.182	86%	0.380	0.213	56%	0.313	0.201	64%	2.91	0.233

* $p<0.05$

Tubule Diameter: The average diameter of the seminiferous tubules of males from the control ranged from 132.2 μm to 182.5 μm (Figure 3.13). Tubule diameters of males from the two test concentrations ranged from 89.4 μm to 221.4 μm. Significant differences in the mean tubule diameter per treatment (Table 3.10) were detected (Kruskal-Wallis, $H = 7.81$, $p = 0.020$, df = 2). Tubule diameter in males from the high concentration were smaller than those in males from the

control. The achieved power at the observed maximum difference from the control response for this assay was 50%.

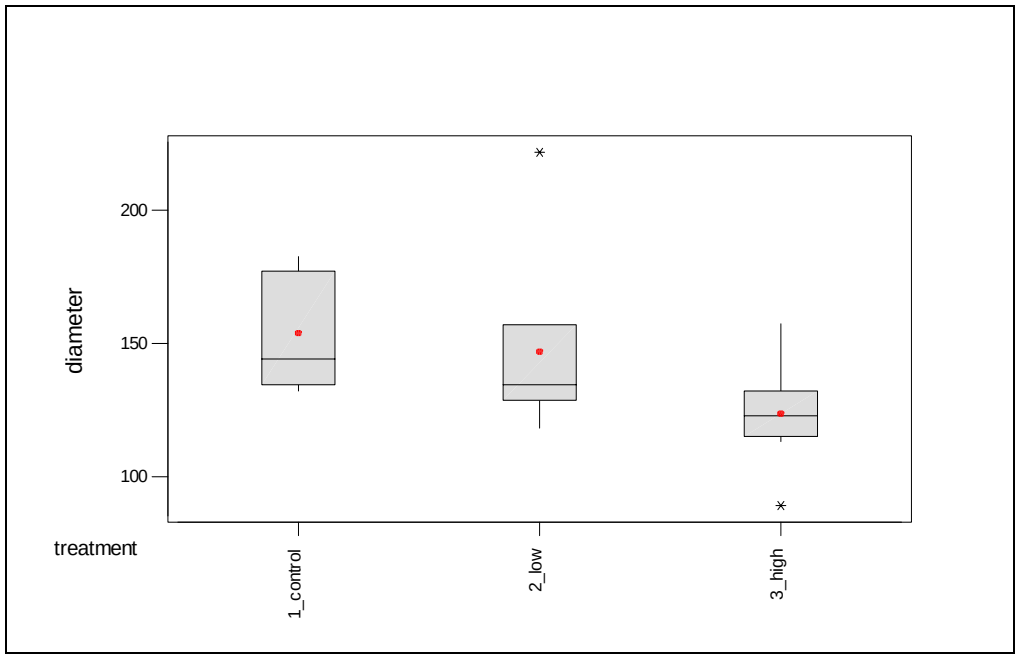

Figure 3-13. Box Plot of Male Seminiferous Tubule Diameter (µM) by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

Table 3.10. Summary Statistics and Power Estimates for Male Seminiferous Tubule Diameter Data for the EPA 21-Day Atrazine Assay

Level	N	Mean (µm)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	153.8	21.28	14%	4.3%	100%	100%	100%
low	7	146.8	34.90	24%				
high	8	123.8	19.09	15%				

Observations: Sertoli cell proliferation was observed among several males from all treatments (Table 3.11). Leydig cell proliferation also was observed among several males from all treatments. Several males in each treatment showed other histological abnormalities (Table 3.11). No testicular atrophy was recorded, and no ovatestes were observed for any treatment.

Table 3.11. Histological Observations for Males Exposed to Concentrations of Atrazine During the EPA 21-Day Assay

Fish ID	Treatment	Sertoli Cell Proliferation	Leydig Cell Proliferation	Observations
229218	Control	Yes	Yes	Diffuse to multifocal interstitial cell proliferation Necrotic stage 3B and 4 cells in tubule lumina
229224	Control	None	Yes	Multifocal proliferation of Leydig cells
229229	Control	None	Yes	Multifocal proliferation of Leydig cells
229230	Control	Yes	Yes	Severe diffuse interstitial cell proliferation Tubule contents disorganized, contains mixed cell stages
229236	Control	No	No	No development = definition of stage 1
229241	Low	None	None	Focal area with abnormal stage 3A cells
229248	Low	Yes	Yes	Mild multifocal proliferation of interstitial cells
229253	Low	Yes	Yes	Mild multifocal proliferation of interstitial cells
229254	Low	Yes	Yes	Mild multifocal proliferation of interstitial cells Empty, presumed spawned out, tubules contain basophilic cystic structures
229260	Low	Yes	Yes	Mild multifocal proliferation of interstitial cells
229265	High	Yes	Yes	Moderate interstitial cell proliferation, mostly Leydig cells Necrotic stage 3B and 4 cells in tubule lumina
229266	High	Yes	Yes	Mild multifocal proliferation of interstitial cells Multifocal basophilic cysts in tubules Premature release of clusters of stage 3B and stage 4 cells to tubule lumina
229271	High	None	Yes	Multifocal proliferation of Leydig cells
229272	High	None	Yes	Moderate interstitial cell proliferation, mostly Leydig cells Multifocal necrotic stage 3B cells
229277	High	Yes	Yes	Moderate to severe interstitial cell proliferation and sequestration of stage 4 and 5 cells Multifocal necrotic stage 3B and stage 4 cells
229278	High	Yes	Yes	Mild multifocal proliferation of interstitial cells
229283	High	None	Yes	Multifocal proliferation of Leydig cells
229284	High	None	Yes	Multifocal proliferation of Leydig cells

3.1.10 Vitellogenin

VTG concentrations in control females used during the EPA 21-day atrazine assay ranged from 3.4 mg/mL to 10.1 mg/mL (Figure 3.14). Among females exposed to the two atrazine concentrations, VTG concentrations ranged from 0 mg/mL (not detected) to 15.6 mg/mL. No significant differences in the mean VTG concentration per treatment (Table 3.12) were detected (Kruskal-Wallis, $H = 0.41$, $p = 0.813$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 7%. The probability of detecting as much as a 50% difference in the female VTG concentration was high at 89%, based on the observed variability (Table 3.12).

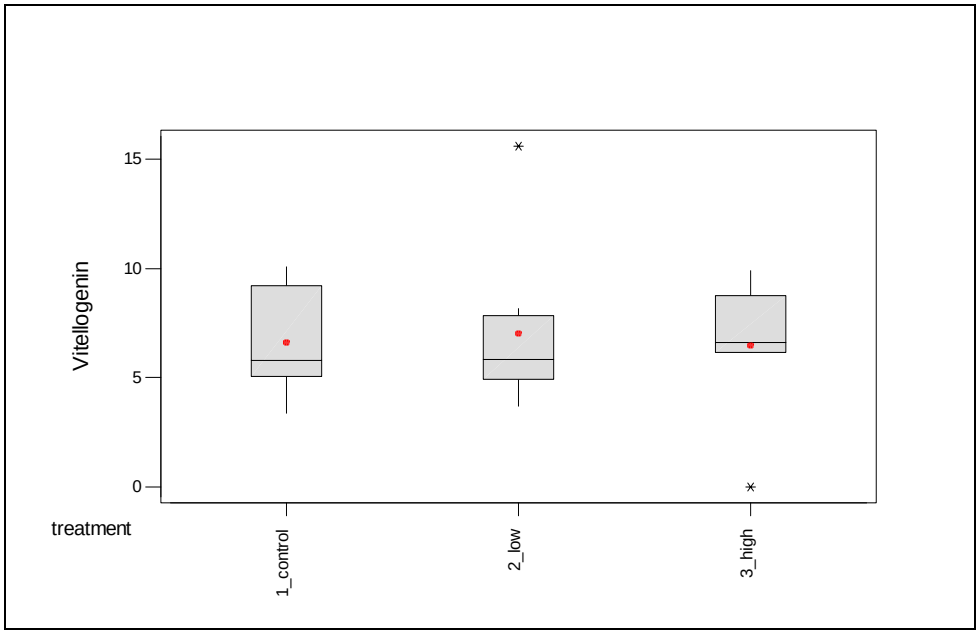

Figure 3.14. Box Plot of Female VTG Concentration (mg/mL) by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

Table 3.12. Summary Statistics and Power Estimates for Female VTG Concentrations for the EPA 21-Day Atrazine Assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	6.63	2.38	36%	7%	9%	22%	89%
low	8	7.02	3.72	53%				
high	8	6.46	2.99	46%				

VTG concentrations in control males used during the EPA 21-day atrazine assay ranged from 0 mg/mL (not detected) to 0.033 mg/mL (Figure 3.15). Among males exposed to the two atrazine concentrations, VTG concentrations ranged from 0 mg/mL (not detected) to 0.176 mg/mL. No significant differences in the mean VTG concentration per treatment (Table 3.13) were detected (Kruskal-Wallis, $H = 0.30$, $p = 0.860$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 9%. The probability of detecting as much as a 50% difference in the male VTG was low at 5%, based on the observed variability (Table 3.13).

Table 3.13. Summary Statistics and Power Estimates for Male VTG Concentrations for the EPA 21-Day Atrazine Assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.009	0.013	140%	141%	5%	5%	5%
low	8	0.002	0.002	144%				
high	8	0.024	0.062	263%				

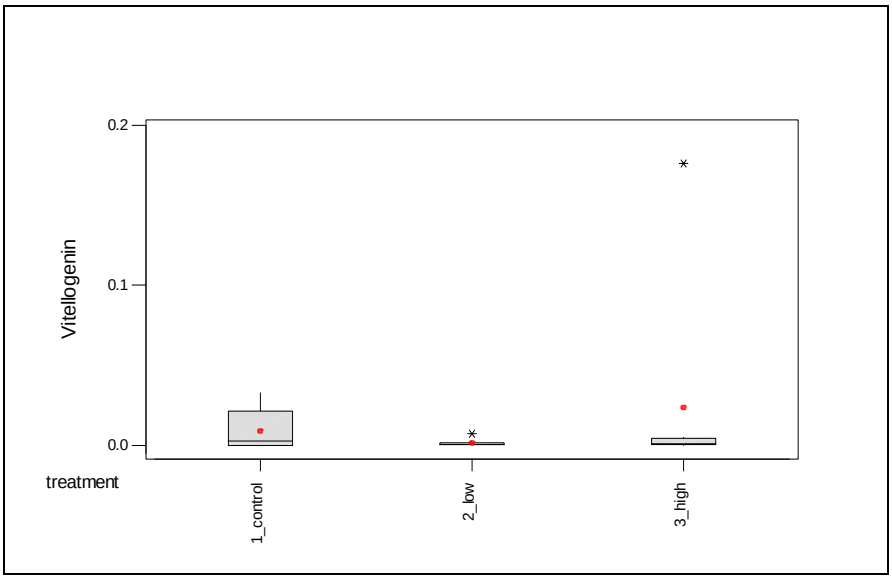

Figure 3.15. Box Plot of Male VTG Concentration (mg/mL) by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

3.1.11 Plasma Steroid Concentrations

Estradiol: Estradiol concentrations in control females used during the EPA 21-day atrazine assay ranged from 0.819 ng/mL to 5.14 ng/mL (Figure 3.16). Among females exposed to the two atrazine concentrations, estradiol concentrations ranged from 0 ng/mL (not detected) to 2.17 ng/mL. No significant differences in the mean estradiol concentration per treatment (Table 3.14) were detected (Kruskal-Wallis, $H = 3.89$, $p = 0.143$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 36%. The probability of detecting as much as a 50% difference in the female estradiol concentration was high at 82%, based on the observed variability (Table 3.14).

Table 3.14. Summary Statistics and Power Estimates for Female Estradiol Concentrations for the EPA 21-Day Atrazine Assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	2.52	1.46	58%	29%	8%	19%	82%
low	8	1.37	0.51	37%				
high	8	1.45	0.66	46%				

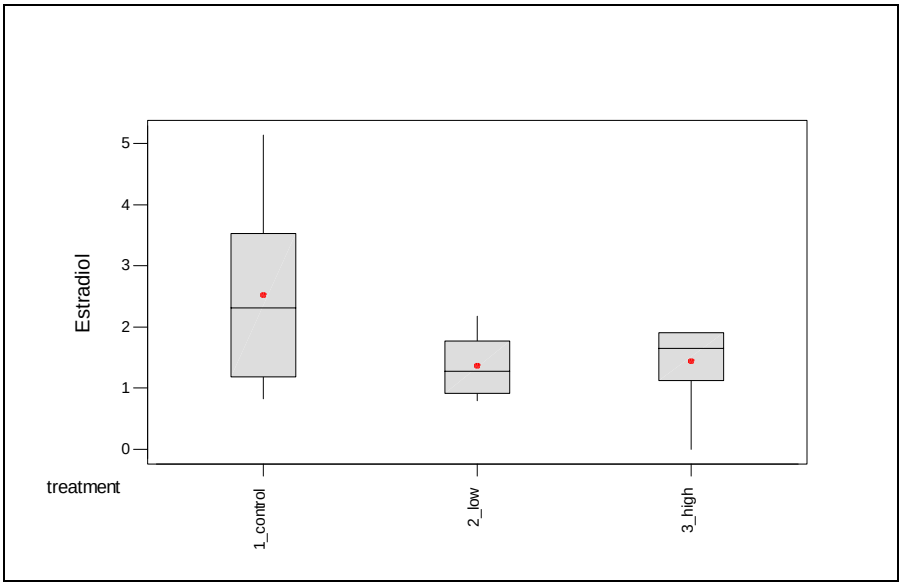

Figure 3.16. Box Plot of Female Estradiol Concentration (Ng/Ml) by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Estradiol concentrations in control males used during the EPA 21-day atrazine assay ranged from 0 ng/mL (not detected) to 0.391 ng/mL. Estradiol was not detected in males exposed to the low and high atrazine concentrations.

Testosterone: Testosterone concentrations in control females used during the EPA 21-day atrazine assay ranged from 0.515 ng/mL to 1.28 ng/mL (Figure 3.17). Among females exposed to the two atrazine concentrations, testosterone concentrations ranged from 0.414 ng/mL to 1.25 ng/mL. No significant differences in the mean testosterone concentration per treatment (Table 3.15) were detected (Kruskal-Wallis, $H = 1.67$, $p = 0.435$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 12%. The probability of detecting as much as a 50% difference in the female testosterone concentration was high at 97%, based on the observed variability (Table 3.15).

Table 3.15. Summary Statistics and Power Estimates for Female Testosterone Concentrations for the EPA 21-Day Atrazine Assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.902	0.294	33%	11%	10%	29%	97%
low	8	0.769	0.240	31%				
high	8	0.932	0.261	28%				

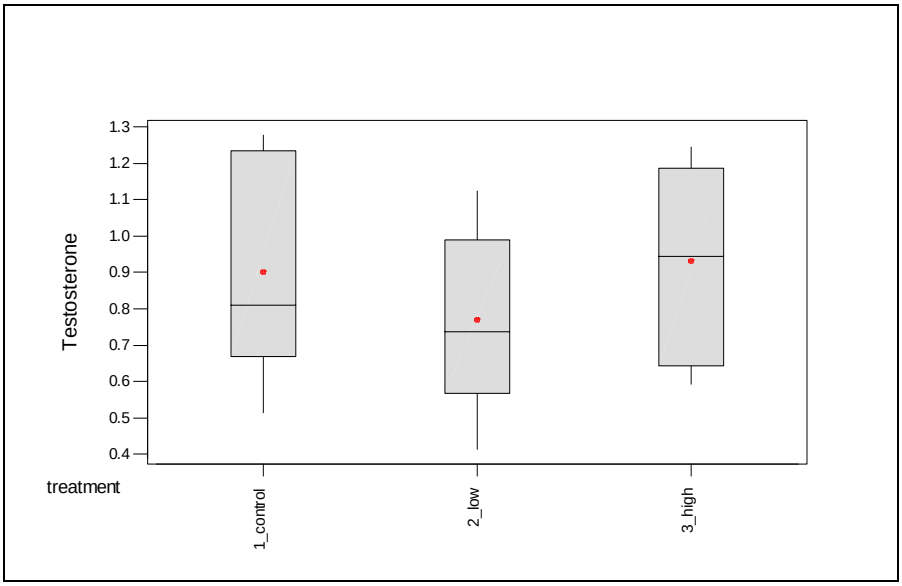

Figure 3.17. Box Plot of Female Testosterone Concentration (Ng/Ml) by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Testosterone concentrations in control males used during the EPA 21-day atrazine assay ranged from 1.37 ng/mL to 8.08 ng/mL (Figure 3.18). Among males exposed to the two atrazine concentrations, testosterone concentrations ranged from 0.924 ng/mL to 5.43 ng/mL. No significant differences in the mean testosterone concentration per treatment (Table 3.16) were detected (Kruskal-Wallis, $H = 2.05$, $p = 0.360$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 17%. The probability of detecting as much as a 50% difference in the male testosterone concentration was high, 82%, based on the observed variability (Table 3.16).

Table 3.16. Summary Statistics and Power Estimates for Male Testosterone Concentrations for the EPA 21-Day Atrazine Assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	3.31	2.14	65%	18%	8%	19%	82%
low	8	2.30	1.41	61%				
high	8	2.45	1.52	62%				

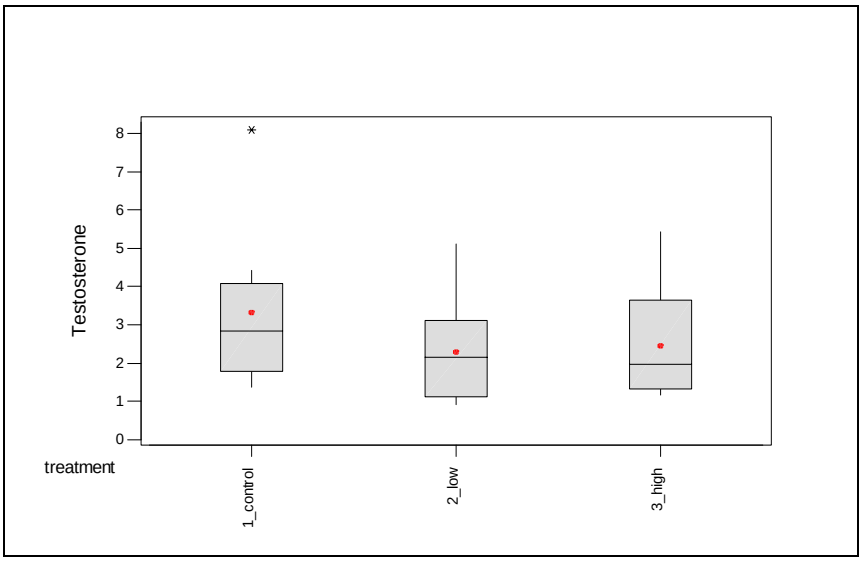

Figure 3.18. Box Plot of Male Testosterone Concentration (Ng/Ml) by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

11-ketotestosterone: 11-ketotestosterone was not detected in females from the control and the low concentration during the EPA 21-day atrazine assay. However, 11-ketotestosterone was detected in one of the eight female samples from the high concentration (7.02 ng/mL).

11-ketotestosterone concentrations in control males used during the EPA 21-day atrazine assay ranged from 6.2 ng/mL to 112 ng/mL (Figure 3.19). Among males exposed to the two atrazine concentrations, 11-ketotestosterone concentrations ranged from 1.31 ng/mL to 50.5 ng/mL. No significant differences in the mean 11-ketotestosterone concentration per treatment (Table 3.17) were detected (Kruskal-Wallis, $H = 1.86$, $p = 0.395$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 21%. The probability of detecting as much as a 50% difference in the male 11-ketotestosterone concentration was high at 80%, based on the observed variability (Table 3.17).

Table 3.17. Summary Statistics and Power Estimates for Male 11-Ketotestosterone Concentrations for the EPA 21-Day Atrazine Assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	26.8	35.3	132%	22%	8%	18%	80%
low	8	11.5	9.5	82%				
high	8	14.0	16.0	114%				

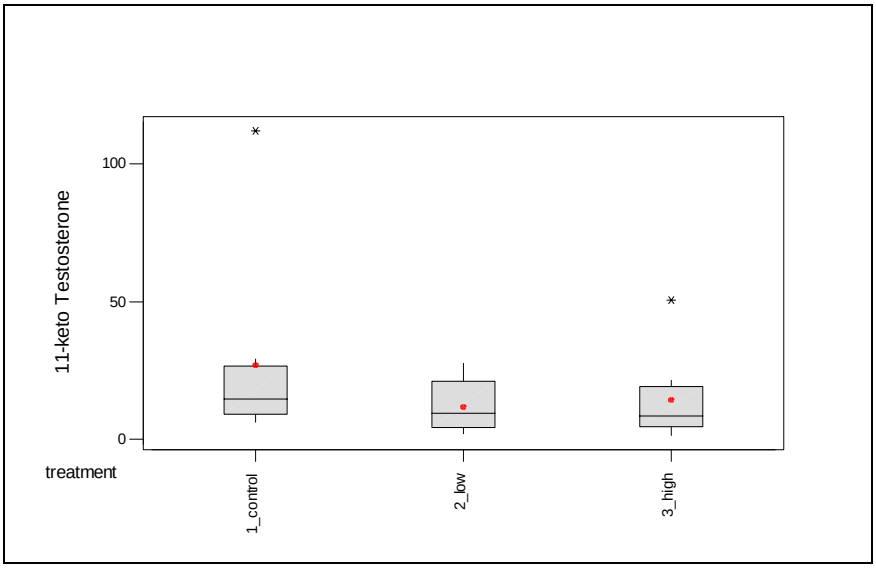

Figure 3.19. Box Plot of Male 11-Ketotestosterone Concentration (Ng/Ml) by Treatment for the EPA 21-Day Atrazine Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

4.0 RESULTS: BISPHENOL A

The results of the EPA 21-day assay for bisphenol A are presented in this section. Total fecundity is reported as the total number of eggs laid during the assay, regardless of egg condition. However, some eggs could not be included in the evaluation of fertilization because of physical damage or the presence of a fungal infection. Summary tables are presented for the major endpoints. The values presented were calculated before being rounded for inclusion in the tables. Values reported include the mean for each treatment, the number of samples (N), the standard deviation (SD), and the coefficient of variation (CV). Values reported for the statistical analyses are the test statistic for the Kruskal-Wallis test used to determine the significance level (*H*), the probability that the observed result was due to chance (*p*), and the degrees of freedom associated with the test (df).

4.1 EPA 21-Day Assay for Bisphenol A

The EPA 21-day bisphenol A assay was conducted from June 18, 2003 to June 25, 2003 (pre-exposure assay), and from June 25, 2003 to July 16, 2003 (exposure assay).

4.1.1 Bisphenol A Concentrations

Bisphenol A was not detected at concentrations above the MDL (20.19 μg/L) in the control at any time. The mean (standard deviation) in the low concentration and high concentration were 56.6 (21.7) μg/L and 344.1 (125.4) μg/L, respectively (Figure 4.1). While there was high variability in the high concentration replicates, it should be noted that for many of the important variables, the control CV was larger than, or as large as, the response from the dosed (low or high) treatments. It is unlikely that less variability in the diluter, or exposure concentrations would have affected the statistical significance of the observed results.

4.1.2 Survival

One female in the control treatment died during the EPA 21-day bisphenol A assay. Two males died during the assay, one each in the low and high concentrations.

4.1.3 Fecundity

***Total Fecundity*:** A 7-day pre-exposure evaluation of total egg production was performed. Total 7-day counts among the three treatments in the exposure assay (individual tank values summed for each treatment) ranged from about 7000 eggs to 8400 eggs (Figure 4.2). No significant differences in the mean 7-day egg production among the groups of replicates evaluated during the pre-exposure assay were detected (Kruskal-Wallis, $H = 2.35$, $p = 0.309$, df = 2).

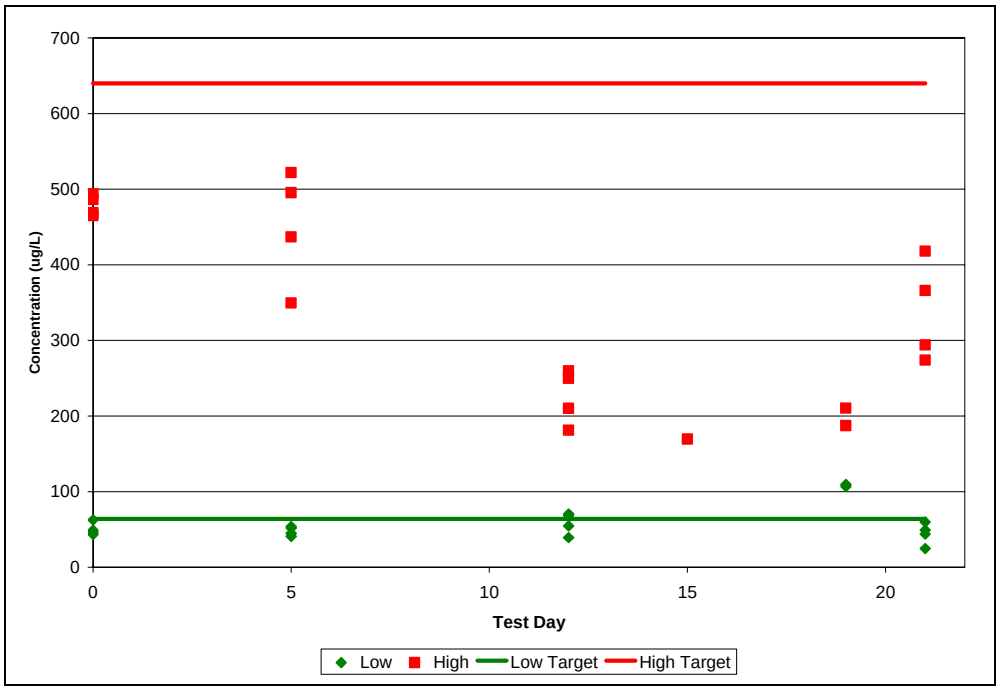

Figure 4.1. Bisphenol A concentrations during the EPA 21-day bisphenol A assay

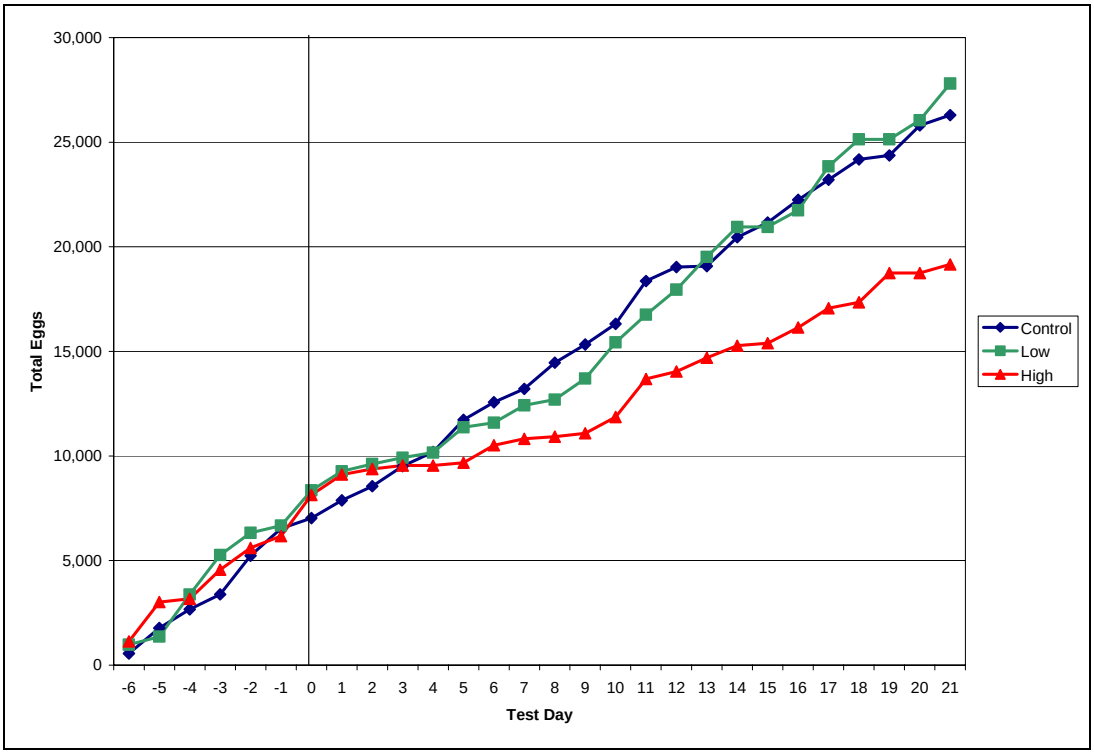

Figure 4.2. Total egg production per treatment for the bisphenol A assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to bisphenol A)

During the EPA 21-day bisphenol A assay, total counts in the control varied from 3959 eggs to 6114 eggs per replicate (Figure 4.3). Total egg production among low-concentration replicates was similar, ranging from 4500 eggs to 5196 eggs. Total counts among the high-concentration replicates varied from 2224 eggs to 3512 eggs. Statistical analysis of square-root transformed egg counts showed significant among-treatment differences (Kruskal-Wallis, $H = 7.54$, $p = 0.023$, df = 2) in mean total numbers of eggs produced (Table 4.1). The mean total number of eggs produced by females from the high concentration was significantly less than those for females from the control and the low concentration. The achieved power for this assay was 96%.

Table 4.1. Summary Statistics and Power Estimates for Total Fecundity Data for the EPA 21-Day Bisphenol A Assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	4818	932	19%	24%	29%	83%	100%
low	4	4863	385	8%				
high	4	2756	564	20%				

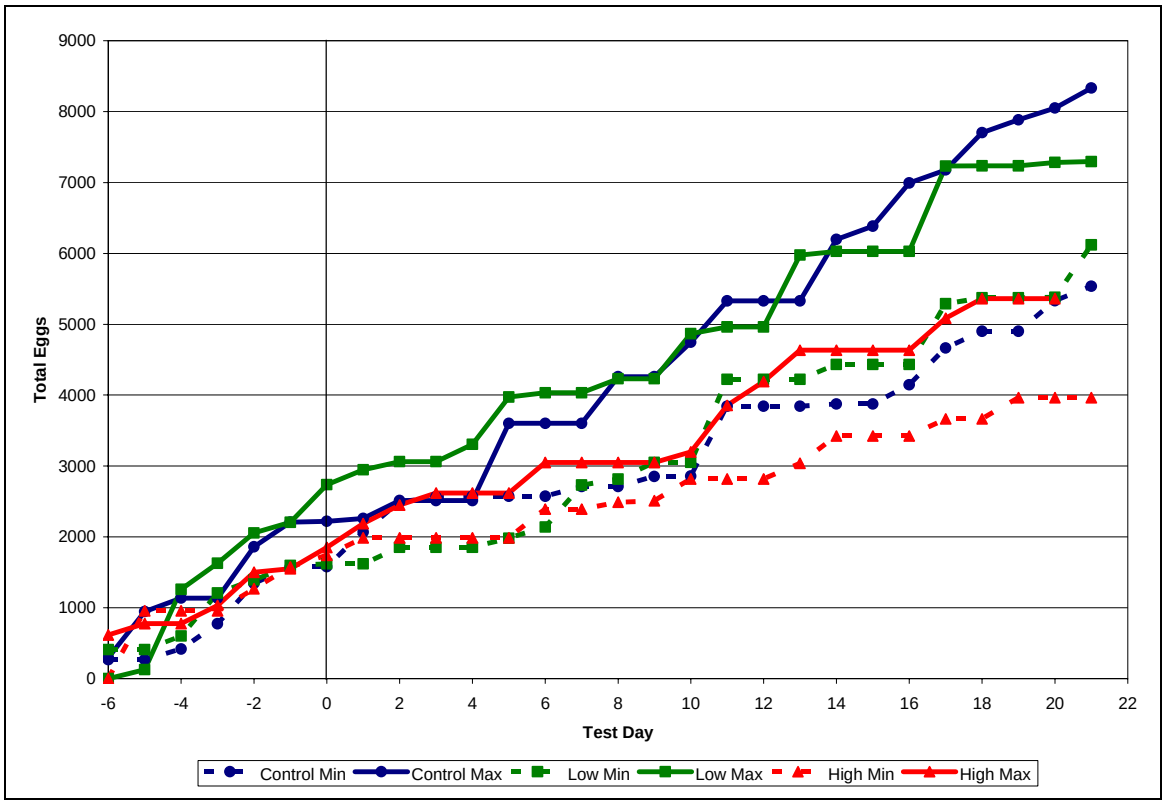

Figure 4.3. Range in replicate total egg production per treatment for the bisphenol A assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to bisphenol A)

Fecundity per Female Reproductive Day: During the 7-day pre-exposure evaluation, the mean number of eggs produced per female reproductive day ranged from 62.8 eggs/day for the tanks that would be used for the control to 74.6 eggs/day for the tanks that would be used for the low concentration during the 21-day exposure assay. There were no significant differences among treatments in the mean numbers of eggs produced per reproductive day during the pre-exposure period (Kruskal-Wallis, $H = 2.35$, $p = 0.309$, df = 2).

During the EPA 21-day bisphenol A assay, the maximum number of female reproductive days was achieved for the low and high treatments (Table 4.2). One control female died at day 19 of the testing. The number of eggs produced per female reproductive day varied from 48.9 eggs to 72.8 eggs in the control and from 53.6 eggs to 61.9 in the low concentration (Figure 4.4). For the high concentration, the number of eggs produced per female reproductive day ranged from 26.5 eggs to 41.8 eggs. The number of eggs produced per day by females in the high concentration was significantly less than the numbers produced by females in the control and the low concentration (Kruskal-Wallis, $H = 7.54$, $p = 0.023$, df = 2). The achieved power for this assay was 96%.

Table 4.2. Summary Statistics and Power Estimates for Fecundity Per Female Reproductive Day for the EPA 21-Day Bisphenol A Assay

Level	Mean Number of Reproductive Days [(a)]	N	Mean Fecundity Per Female Reproductive Day	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
Control	83.25	4	57.8	10.6	18%	25 %	30%	85%	100%
Low	84.0	4	57.9	4.6	8%				
High	84.0	4	32.8	6.7	20%				

a) Maximum number = 84.

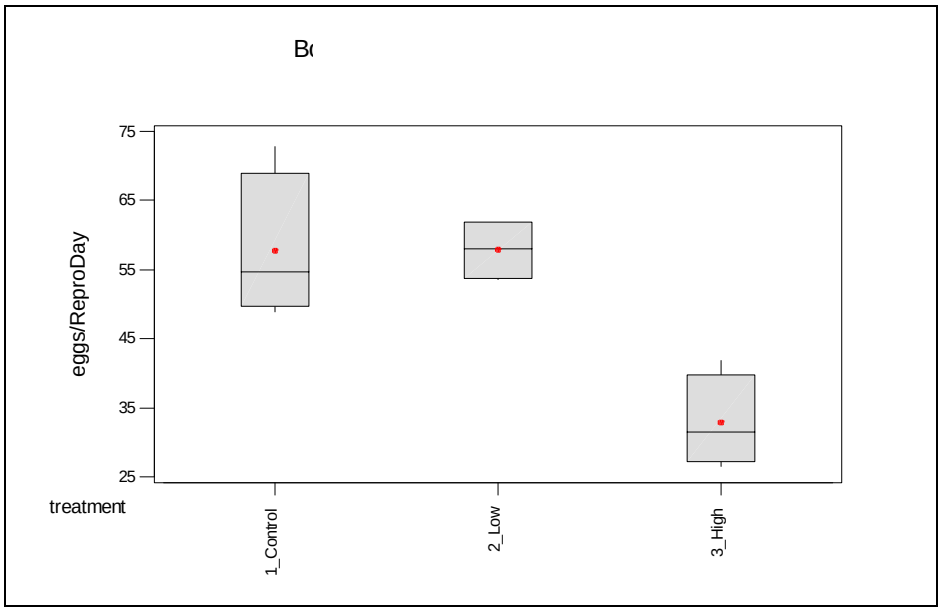

Figure 4.4. Box plot of the number of eggs produced per female reproductive day by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

***Eggs on Tiles/Dishes*:** The mean number of eggs laid on the tiles during the 7-day pre-exposure assay varied from 1305 eggs for the tanks that would be used for the control to 1682 eggs for the tanks that would be used for the low concentration. The mean number of eggs on dishes ranged from 407 eggs for the low concentration to 458 eggs for the high concentration. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated. There were no significant differences in the mean proportional difference among treatments during the 7-day pre-exposure assay (Kruskal-Wallis, $H = 0.88$, $p = 0.664$, df = 2).

The mean number of eggs laid on tiles among the treatments during the EPA 21-day bisphenol A assay ranged from 2148 eggs for the high concentration to 3943 eggs for the low concentration (Appendix D, Table D.2). The mean number of eggs laid on the dishes varied from 609 eggs for the high concentration to 944 eggs for the control treatment. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated. There were no significant differences in the mean proportional difference among treatments during the 21-day exposure assay (Kruskal-Wallis, $H = 0.59$, $p = 0.745$, df = 2).

4.1.4 Fertilization Success

Total Fertilization: Eggs were collected during the 7-day pre-exposure period for the evaluation of fertilization success rate. All undamaged eggs laid during the pre-exposure assay were fertilized. The total (tiles + dishes) fertilization success rates for all treatment replicates during the EPA 21-day bisphenol A assay ranged from 0.999 (control replicate) to 1.00 (replicates from

all treatments) (Figure 4.5). No significant differences in mean fertilization success rates (Table 4.3) among treatments were detected (Kruskal-Wallis, $H = 1.11$, $p = 0.573$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 10%. The probability of detecting as much as a 20% difference in the proportion of eggs fertilized was high, 100%, based on the observed variability (Table 4.3).

Table 4.3. Summary Statistics and Power Estimates for the Proportion of Undamaged Eggs Fertilized for the EPA 21-Day Bisphenol A Assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	1.000	0.001	0.1%	0.62%	100%	100%	100%
Low	4	1.000	0.0004	<0.1%				
High	4	1.000	0	0%				

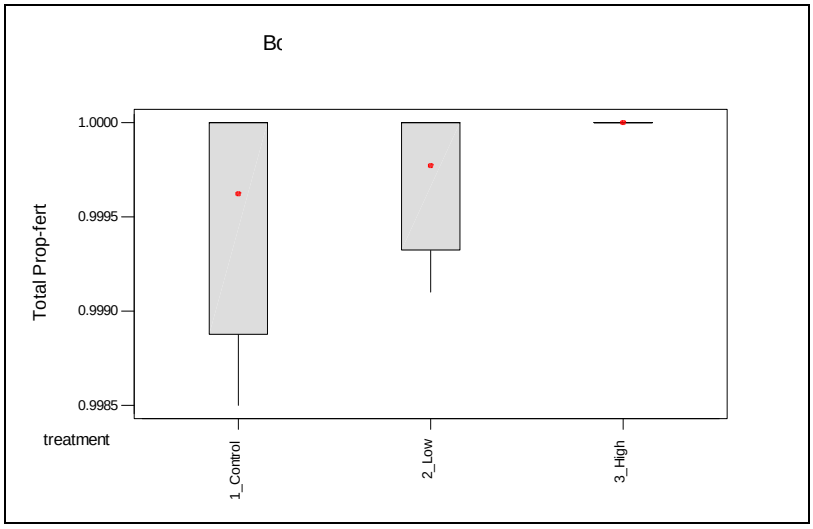

Figure 4.5. Box plot of the proportion of undamaged eggs fertilized by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Fertilization of Eggs on Tiles and Dishes: During the 7-day prevalidation assay, all undamaged eggs laid on tiles or on dishes were fertilized. The fertilization success rates for all treatment replicates for undamaged eggs laid on tiles during the EPA 21-day bisphenol A assay were high, ranging from 0.998 (control replicate) to 1.00 (replicates from all treatments). No significant differences (Kruskal-Wallis, $H = 1.11$, $p = 0.573$, df = 2) in mean fertilization success rates were detected among treatments (Appendix D, Table D.2). All undamaged eggs laid on dishes during the assay were fertilized.

4.1.5 Hatchability and Larval Development

Eggs were collected during the EPA 21-day bisphenol A assay for the evaluation of hatchability. The larval hatching study was completed by incubating 50 eggs per replicate in dilution water for 3 to 6 days until the majority of eggs hatched. One replicate from the high concentration was excluded from the analysis because of a fungal infection that prevented accurate counts. The proportion of fertilized eggs that hatched ranged from 0.98 to 1.00 in the control and from 0.55 to 0.98 for the two bisphenol A concentrations (Figure 4.6). Significant differences among treatments in the proportion of eggs that hatched (Table 4.4) were detected (Kruskal-Wallis, H = 7.55, p = 0.023, df = 2). The proportion of eggs that hatched in the control was greater than that for the low concentration. The achieved power at the observed maximum difference from the control response for this assay was 62%. The probability of detecting as much as a 20% difference in the proportion of fertile eggs that hatched was low, 38%, based on the observed variability (Table 4.4).

Table 4.4. Summary statistics and power estimates for the proportion of fertile eggs that hatched for the EPA 21-day bisphenol A assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	0.990	0.012	1%	27%	13%	38%	99%
low	3	0.766	0.200	26%				
high	3	0.960	0.021	2%				

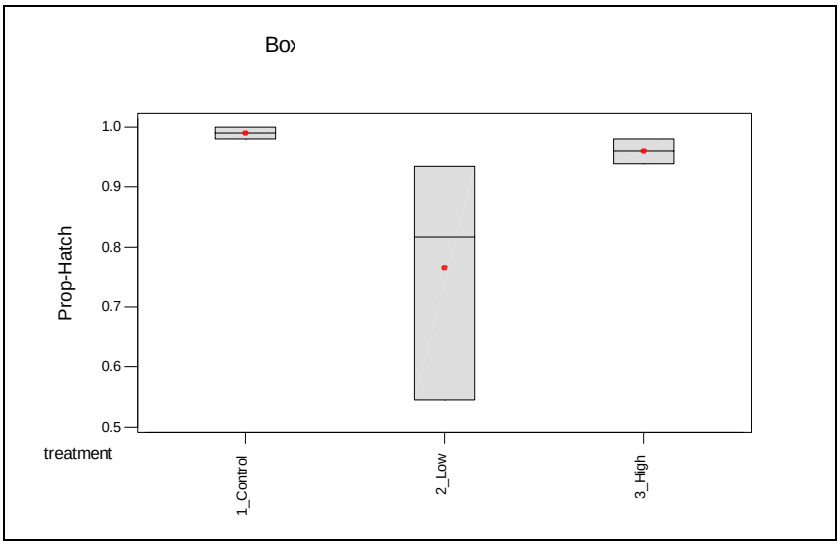

Figure 4.6. Box plot of the proportion of fertile eggs that hatched by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, the horizontal line is the median value, and the circle is the mean value)

Eggs were collected during the EPA 21-day bisphenol A assay for the evaluation of larval development. The proportion of larvae that developed normally (i.e., that showed no morphological abnormalities) ranged from 0 to 1.0 in the control and from 0.58 to 1.00 for the low and high concentrations (Figure 4.7). All larvae in one control replicate hatched, but were deformed, with slightly curved spines and a swollen area around the heart and gut. The proportion of normal development in the other three control replicates ranged from 0.92 to 1.00. There were no significant differences among treatments in the proportion of larvae that developed normally (Kruskal-Wallis, $H = 2.23$, $p = 0.327$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 8%. The probability of detecting as much as a 20% difference in the proportion of normal larvae was low, 7%, based on the observed variability (Table 4.5).

Table 4.5. Summary statistics and power estimates for the proportion of normal larvae for the EPA 21-day bisphenol A assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	0.724	0.484	67%	28%	5%	7%	16%
low	3	0.772	0.174	23%				
high	4	0.937	0.085	9%				

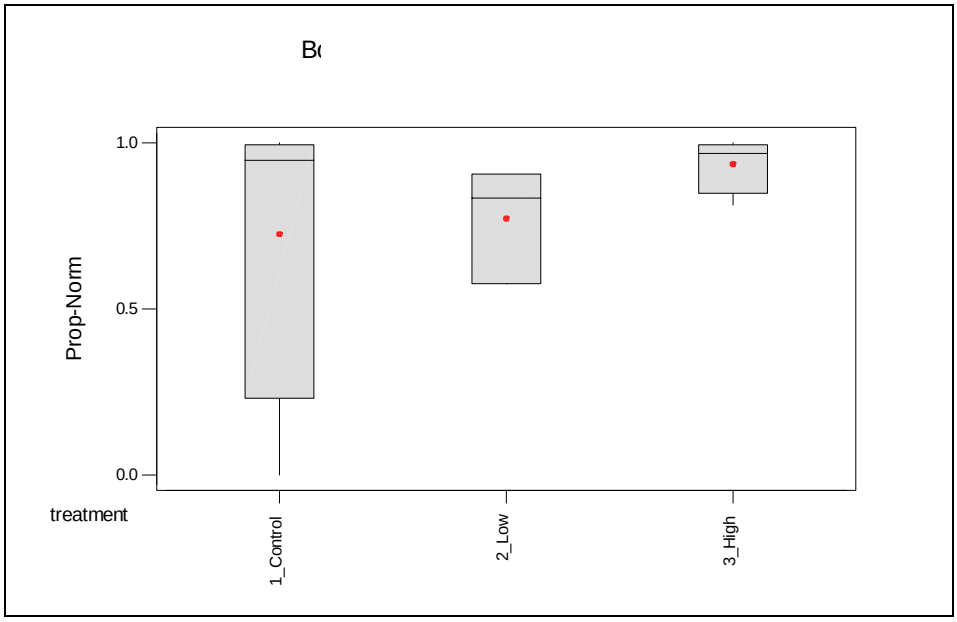

Figure 4.7. Box plot of the proportion of normal larvae by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

4.1.6 Body Weight

The body weight of females used in the EPA 21-day bisphenol A assay ranged from 1.5 g to 4.0 g. There were no significant differences in mean body weight among all treatments (Kruskal-Wallis, $H = 0.01$, $p = 0.994$, df = 2). The body weight of males used in the EPA 21-day bisphenol A assay ranged from 4.5 g to 11.1 g. There were no significant differences in mean body weight among treatments (Kruskal-Wallis, $H = 1.76$, $p = 0.414$, df = 2).

4.1.7 Gonadosomatic Index

The range of GSI values calculated for females in the all treatments varied from three- to six-fold (Figure 4.8). The highest value (GSI = 29.6) was obtained for a female from the control. One female exposed to the low-bisphenol A concentration had a GSI value of 20.5. No significant differences in the mean GSI value per treatment (Table 4.6) were detected (Kruskal-Wallis, $H = 1.58$, $p = 0.454$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 20%. The probability of detecting as much as a 20% difference in the female GSI was moderate, 73%, based on the observed variability (Table 4.6).

Table 4.6. Summary statistics and power estimates for female GSI data for the EPA 21-day bisphenol A assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	15	13.8	5.7	41%	9%	23%	73%	100%
low	16	13.1	4.6	35%				
high	16	11.4	3.2	28%				

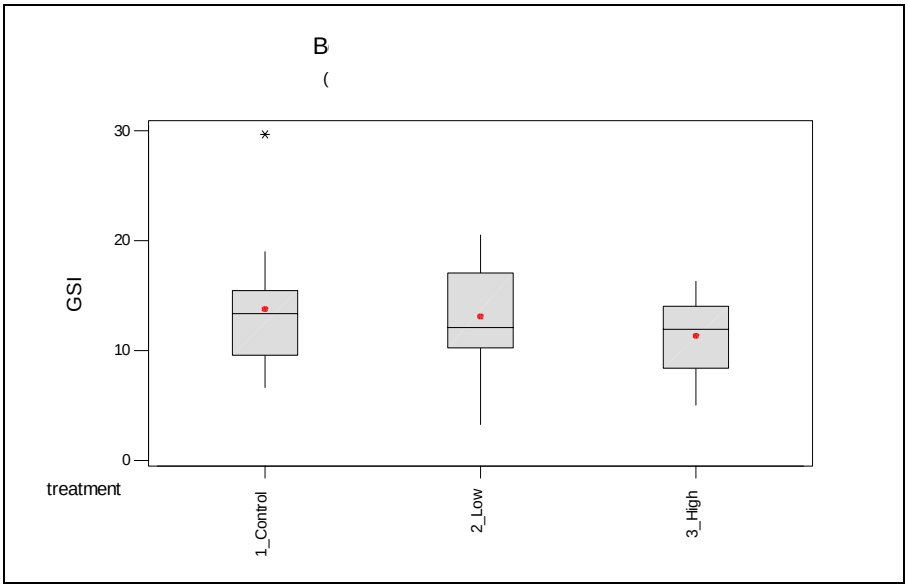

Figure 4.8. **Box plot of female GSI by treatment for the EPA 21-day bisphenol A** assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

The range of most GSI values calculated for males during the EPA 21-day bisphenol A assay was small, ranging from 1.4 to 1.9 (Figure 4.9), which approximates the typical range for reproductively active male fathead minnows. Two males in the control had low GSI values of 1.0, because of small gonad weights (<0.10 g) and relatively high body weights (8.1 g to 9.7 g). One male in the low concentration had a GSI value of 2.2, attributable to its relatively high gonad weight (0.10 g) and small body size (4.5 g). There were no significant differences in mean GSI values (Table 4.7) among treatments (Kruskal-Wallis, $H = 5.36$, $p = 0.069$, df = 2), although there was a trend for increasing GSI value with dose (Figure 4.9). The achieved power at the observed maximum difference from the control response for this assay was 46%. The probability of detecting as much as a 20% difference in the male GSI was high, 95%, based on the observed variability (Table 4.7).

Table 4.7. Summary statistics and power estimates for male GSI data for the EPA 21-day bisphenol A assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
Control	8	1.45	0.32	22%	11%	41%	95%	100%
Low	7	1.62	0.27	17%				
High	7	1.76	0.16	9%				

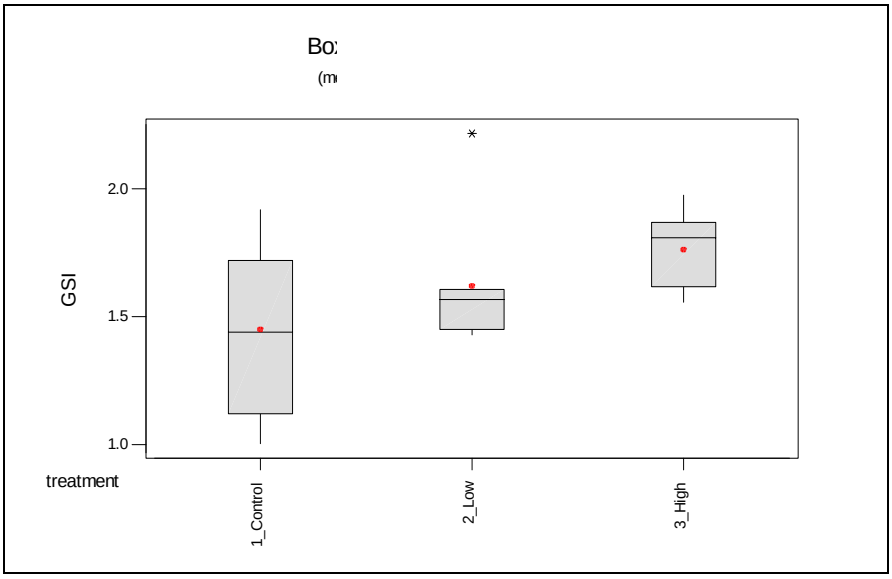

Figure 4.9. Box plot of male GSI by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

4.1.8 Female Gonad Histology

Histological analyses were conducted on the ovaries of 47 females exposed to bisphenol A during the EPA 21-day Assay.

General Ovary Staging: Statistical analysis of the mean ovarian staging from 18 microscopic fields per female in the EPA 21-day bisphenol A assay revealed no significant differences among treatments (Kruskal-Wallis, $H = 3.04$, $p = 0.219$, df = 2).

Quantitative Ovarian Staging: One hundred cells in each of three sections per female were examined to quantitatively determine the developmental stage of the ovaries. Ova from fish from all treatments ranged from Stage 1A to Stage 5 (see Methods for a description of the stages) (Figure 4.10). Statistical analyses showed that within developmental Stages 1A and 3 there were

significant differences among treatments in the proportion of cells occurring in the stage (Table 4.8). The proportion of ova in Stage 1A was significantly greater in the high-concentration females than in control females. The proportion of ova in Stage 3 was significantly lower in the high-concentration females than in control or low-concentration females.

Table 4.8. Descriptive statistics of the proportion of ovarian cells in each developmental stage for females from the EPA 21-day bisphenol A assay and results of the Kruskal-Wallis test (df = 2) comparing treatments

	Control (N = 15)			Low (N = 16)			high (N = 16)			Kruskal-Wallis	
Stage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	*H*	*p*
1A	0.055	0.026	47%	0.060	0.031	51%	0.085	0.036	43%	6.14	0.046*
1B	0.234	0.067	29%	0.231	0.070	30%	0.272	0.073	27%	1.75	0.417
2	0.212	0.056	26%	0.233	0.071	31%	0.212	0.060	28%	0.57	0.753
3	0.175	0.048	28%	0.182	0.058	32%	0.108	0.051	47%	14.34	0.001**
4	0.214	0.082	38%	0.203	0.120	59%	0.212	0.098	46%	0.73	0.696
5	0.013	0.029	218%	0.039	0.083	212%	0.049	0.061	126%	4.82	0.090

* $p < 0.05$.
** $p < 0.01$.

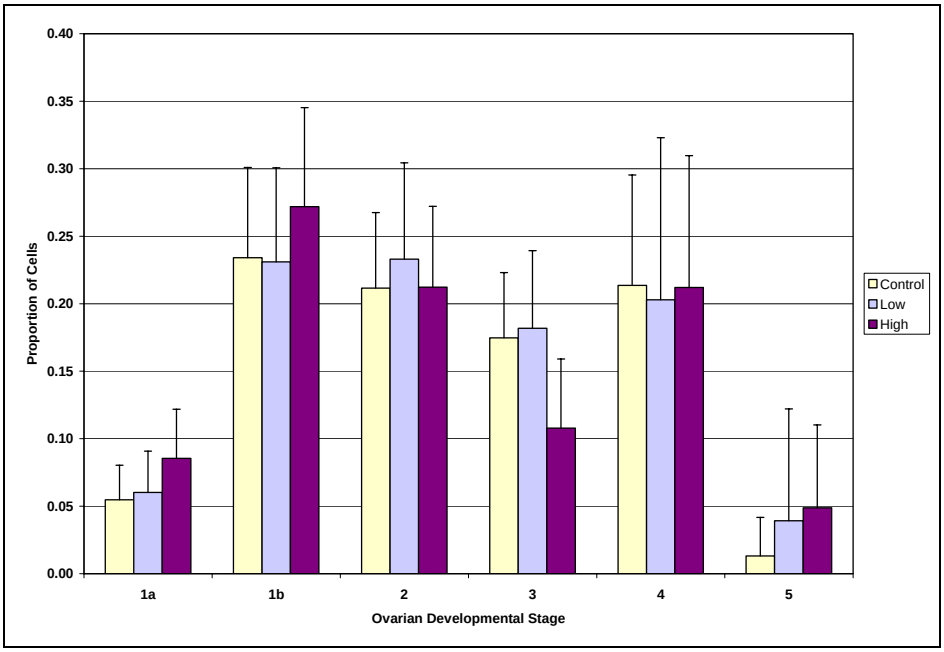

Figure 4.10. Frequency histogram showing the quantitative developmental staging of ovaries for each treatment of the EPA 21-day bisphenol A assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

Atretic Follicles: The mean proportion of atretic follicles per 300 follicles (counted per fish) ranged from 0.039 for females in the low concentration to 0.080 follicles for females in the control (Figure 4.11). Each treatment had at least one female with a high proportion of atretic follicles (>0.2). No significant differences in the proportions of atretic follicles among treatments was detected (Kruskal-Wallis, $H = 1.80$, p 0.406, df = 2).

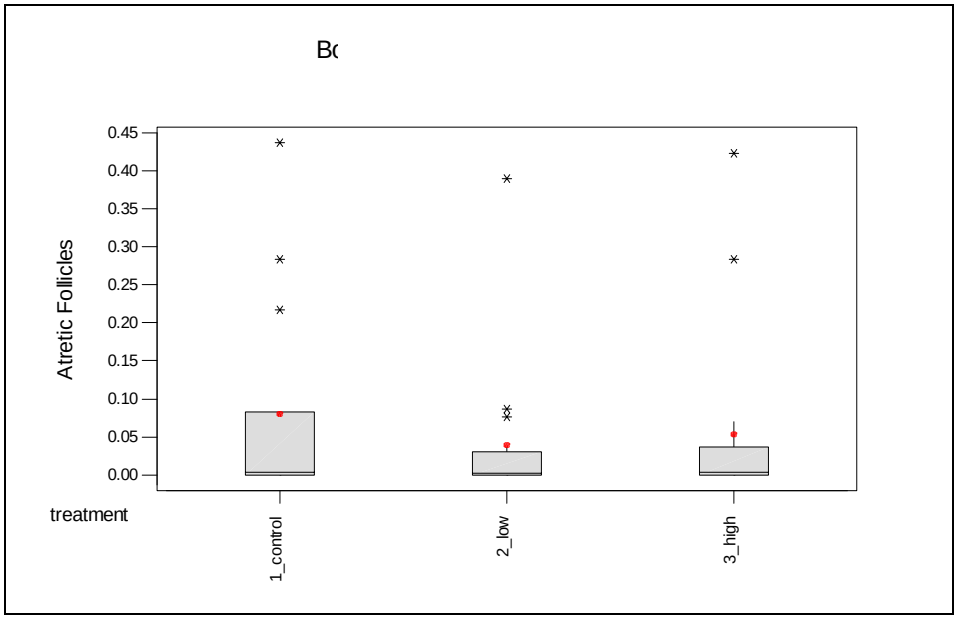

Figure 4.11. Box plot of the proportion of atretic follicles per 300 follicles by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and asterisks represent probable outliers)

***Post-Ovulatory Follicles*:** The mean proportion of post-ovulatory follicles per 300 follicles (counted per fish) ranged from 0.009 for females in the high concentration to 0.018 for females in the control (Figure 4.12). There were no significant differences in the mean proportion of post-ovulatory follicles among treatments (Kruskal-Wallis, $H = 4.04$, $p = 0.132$, df = 2).

Observations: The ovaries from several fish from each treatment were observed to have histological abnormalities (Table 4.9). One condition, multiple foci of macrophage clusters containing brown material, was more evident in females exposed to increased doses of bisphenol A.

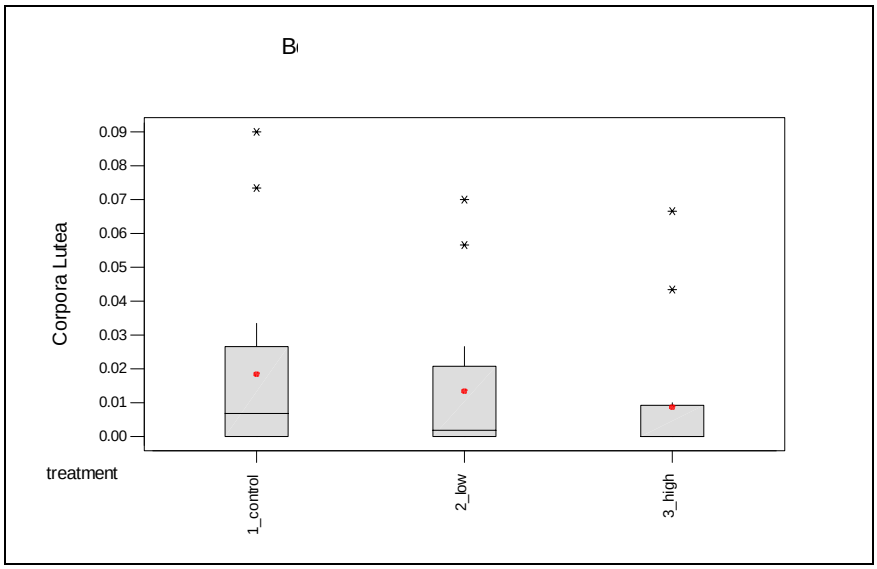

Figure 4.12. Box plot of the proportion of post-ovulatory follicles per 300 follicles by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

Table 4.9. Histological observations for females exposed to concentrations of bisphenol A during the EPA 21-day assay (individual fish may have more than one observed abnormality)

	Number of Fish		
Observation	**Control**	**Low**	**High**
Single focus of atretic follicles	1		
Mature follicles form syncytia (fusion of membranes and internal lipids)	1		
Foci of connective tissue (fibrocytes) in ovary	1		
A few focal testicular tubules in ovary	1		
Atretic follicles with infiltration of interstitial inflammatory cells	1		
Multifocal areas of atretic follicles			
Multiple foci of macrophage clusters in ovary containing brown material		4	10
Scattered necrotic epithelial cells in ovary		1	
Fibrotic reaction around stage 1a, 1b and 2 cells			1
Coalescence of ova lipid droplets and membranes			1
Mutifocal areas of stage 3, 4 and 5 cells abnormal and degenerating			1
Fibrosis and necrosis of interstitial cells			1
Focal extensive area of atretic follicle lipid coalescence			1

4.1.9 Male Gonad Histology

General Testes Staging: Testes from 22 males exposed to bisphenol A during the 21-day assay were examined to determine the general developmental condition. Males in all treatments had well-developed testes, with most showing Stage 4 and Stage 5 development (see Methods for description of developmental stages). All of the 96 microscopic fields examined in the eight control males showed Stage 4 (71 fields) or Stage 5 (25 fields) development. All of the 84 microscopic fields examined in the seven low-concentration treatment males showed Stage 4 (44 fields) or Stage 5 (40 fields) development. All of the 84 microscopic fields examined in the seven high-concentration treatment males showed Stage 4 (44 fields) or Stage 5 (40 fields) development. Statistical analysis of the mean staging from 12 microscopic fields per fish revealed no significant differences among treatments (Kruskal-Wallis, $H = 2.85$, $p = 0.241$, df = 2).

Quantitative Testicular Staging: One hundred cells in each of three sections per male were examined to quantitatively determine the developmental condition of the testes. The developmental stage of all treatment testes ranged from Stage 2A to Stage 5 (Figure 4.13). Statistical analyses showed that there were no significant differences among treatments in the proportion of cells in any of the developmental stages (Table 4.10). Therefore, there did not appear to be an effect on testicular developmental stage associated with bisphenol A dose.

Table 4.10. Descriptive statistics of the proportion of testes cells in each developmental stage for males from the EPA 21-day bisphenol A assay and results of the Kruskal-Wallis test (df = 2) comparing treatments

	Control (N = 8)			Low (N = 7)			High (N = 7)			Kruskal-Wallis	
Stage	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	***H***	***p***
1	0	0	–	0	0	–	0	0	–	–	–
2a	0.000	0.001	283%	0.001	0.003	184%	0.001	0.002	171%	0.81	0.668
2b	0.011	0.011	102%	0.007	0.005	71%	0.002	0.003	133%	4.39	0.111
3a	0.098	0.052	53%	0.131	0.094	71%	0.085	0.049	58%	1.86	0.394
3b	0.283	0.142	50%	0.196	0.125	64%	0.206	0.083	40%	2.06	0.358
4	0.208	0.101	49%	0.174	0.100	57%	0.167	0.084	50%	0.97	0.615
5	0.400	0.265	66%	0.490	0.302	61%	0.539	0.157	29%	1.90	0.388

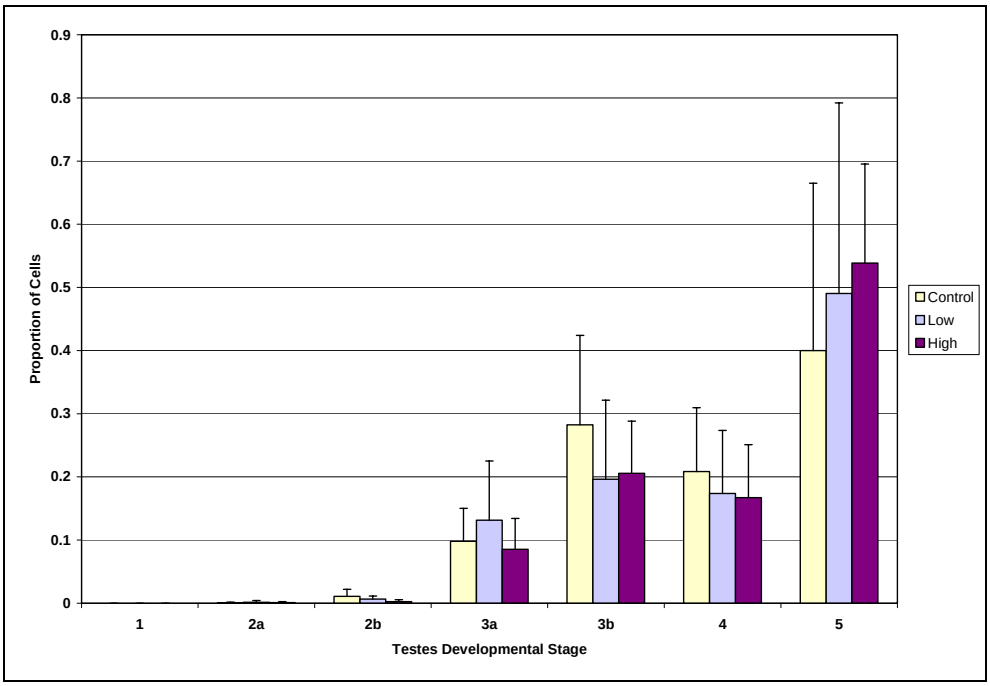

Figure 4.13. Frequency histogram showing the quantitative developmental staging of testes for each treatment of the EPA 21-day bisphenol A assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

Tubule Diameter: The average diameter of the seminiferous tubules of males from the control ranged from 126.1 μm to 257.5 μm (Figure 4.14). Tubule diameters of males from the two test concentrations ranged from 135.6 μm to 233.9 μm. No significant differences in the mean tubule diameter per treatment (Table 4.11) were detected (Kruskal-Wallis, $H = 2.41$, $p = 0.300$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 20%. The probability of detecting as much as a 20% difference in the male seminiferous tubule diameter was high, 100%, based on the observed variability (Table 4.11).

Table 4.11. Summary statistics and power estimates for male seminiferous tubule diameter data for the EPA 21-day bisphenol A assay

Level	N	Mean (μm)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	160.0	42.6	27%	3%	99%	100%	100%
low	7	183.8	35.0	19%				
high	7	166.0	25.1	15%				

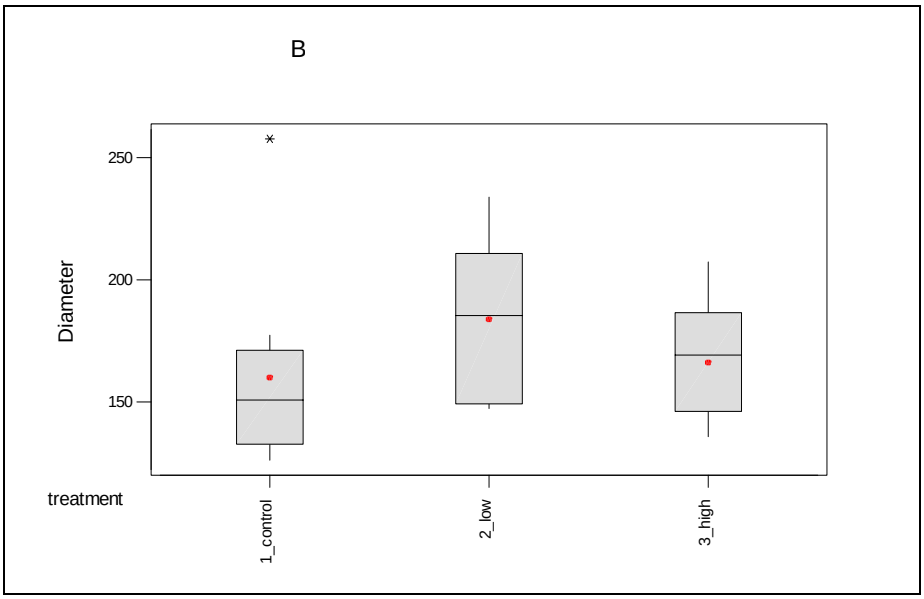

Figure 4.14. Box plot of male seminiferous tubule diameter (μm) by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

Observations: Several cases of Sertoli cell proliferation were observed among all treatments. Several cases of multifocal or focal proliferation of Leydig cells were observed among all treatments. Two males from the low concentration had focal proliferation of Leydig cells. Several males in each treatment showed other histological abnormalities (Table 4.12). No testicular atrophy was recorded and no ovatestes were observed for any treatment.

Table 4.12. Histological observations for males exposed to concentrations of bisphenol A during the EPA 21-day assay

Fish ID	Treatment	Sertoli Cell Proliferation	Leydig Cell Proliferation	Observations
22973	Control	Yes	Yes	Necrotic stage 5 cells in interstitial tissue (I.e. tissue between tubules) Some necrotic stage 3A and 3B cells in tubule lumina Foci of condensed stage 3A and 3B cells show beginning of encapsulation by interstitial cells
22974	Control	No	Yes	Multifocal proliferation of Leydig cells A few areas of stage 3B and stage 4 cell encapsulation
22979	Control	Yes	Yes	Tubular structure disorganized Large areas of sperm cell fusion (or also referred to as cell syncytia formation) Cystic inclusion of necrotic cells

Table 4.12 (cont'd)

Fish ID	Treatment	Sertoli Cell Proliferation	Leydig Cell Proliferation	Observations
22980	Control	No	Yes	Multifocal proliferation of Leydig cells Stage 4 ova in section - believed to be an artifact
22986	Control	No	Yes	Multifocal proliferation of Leydig cells Premature release of stage 3A, 3B cells to tubular lumen
22991	Control	No	Yes	Multifocal proliferation of Leydig cells
22992	Control	Yes	Yes	Multifocal proliferation of Leydig cells
22997	Low	No	Yes	Multifocal proliferation of Leydig cells One stage 4 ova in section, believed to be artifact
22998	Low	No	Yes	Focal proliferation of Leydig cells
229103	Low	Yes	Yes	Melanized cysts in some tubules
229104	Low	No	Yes	Focal proliferation of Leydig cells
229115	Low	Yes	Yes	Focal but extensive proliferation of interstitial cells. Sperm encysted in matrix of disorganized cells, unilateral (i.e. 1 of 2 testes)
229121	High	No	No	Vacuoles in stage 3A cells
229122	High	Yes	Yes	Mild multifocal proliferation of interstitial cells Hyalinization of interstitial areas Hemorrhage in some tubules
229127	High	No	Yes	Multifocal nests of necrotic stage 3B cells Multifocal fibrotic cysts in tubule lumina containing all developmental stages
229128	High	Yes	Yes	Extensive extravasation of plasma protein into tubule lumina Disorganized tubules and detached clusters of developing cells >50% of both testes affected as described above
229134	High	No	Yes	Mild multifocal interstitial cell proliferation A few necrotic cells in proliferative centers
229139	High	No	Yes	Multifocal interstitial cell proliferation Premature release of clusters of stage 3B and stage 4 cells to tubular lumina

4.1.10 Vitellogenin

VTG concentrations in control females used during the EPA 21-day bisphenol A assay ranged from 5.62 mg/mL to 15.1 mg/mL (Figure 4.15). Among females exposed to the two bisphenol A concentrations, VTG concentrations ranged from 5.95 mg/mL to 70.5 mg/mL. Significant differences in the mean VTG concentration per treatment (Table 4.13) were detected (Kruskal-Wallis, $H = 11.34$, $p = 0.003$, df = 2). VTG concentrations in high-concentration females were significantly greater than those in females from the control and those exposed to the low concentration. The achieved power at the observed maximum difference from the control response for this assay was 98%.

Table 4.13. Summary statistics and power estimates for female VTG concentration for the EPA 21-day bisphenol A assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
Control	8	11.1	3.4	31%	48%	12%	37%	99%
Low	8	14.0	7.4	53%				
High	8	42.0	20.2	48%				

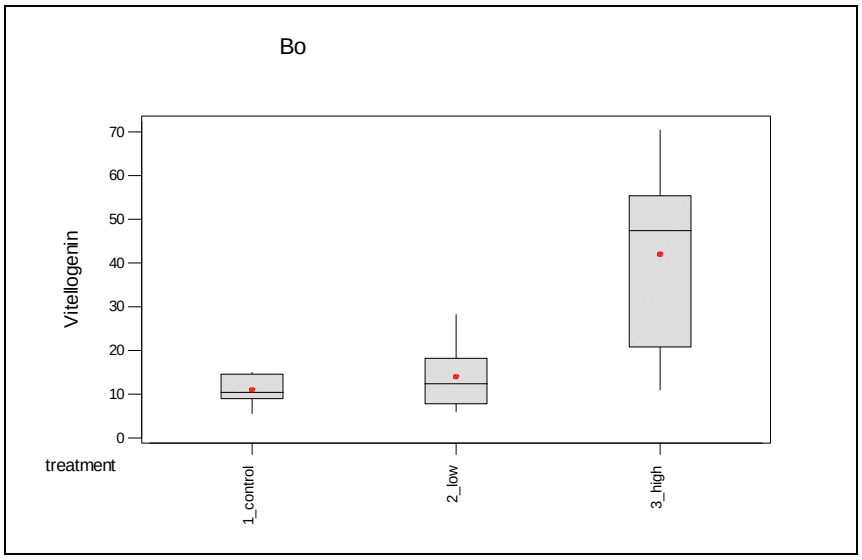

Figure 4.15. Box plot of female VTG concentration (mg/mL) by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

VTG concentrations in control males used during the EPA 21-day bisphenol A assay ranged from 0 mg/mL (not detected) to 0.185 mg/mL (Figure 4.16). Among males exposed to the two bisphenol A concentrations, VTG concentrations ranged from 0.258 mg/mL to 114 mg/mL. Significant differences in the mean VTG concentration per treatment (Table 4.14) were detected (Kruskal-Wallis, $H = 17.74$, $p = <0.001$, df = 2). VTG concentrations in males exposed to the low and high bisphenol A concentrations were greater than those in males from the control. VTG concentrations in males exposed to the high concentrations were also greater than VTG concentrations in males exposed to the low bisphenol A concentration. The achieved power at the observed maximum difference from the control response for this assay was 100%.

Table 4.14. Summary statistics and power estimates for male VTG concentrations for the EPA 21-day bisphenol A assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.025	0.065	254%	>1000%	5%	5%	5%
low	7	2.07	1.49	72%				
high	6	92.7	22.0	24%				

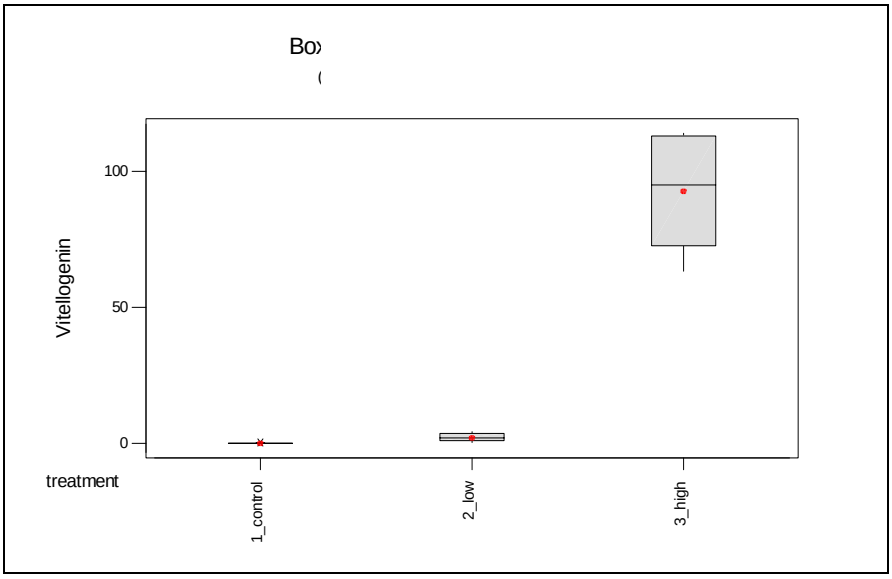

Figure 4.16. Box plot of male VTG concentration (mg/mL) by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

4.1.11 Plasma Steroid Concentrations

Estradiol: Estradiol concentrations in control females used during the EPA 21-day bisphenol A assay ranged from 0.836 ng/mL to 5.43 ng/mL (Figure 4.17). Among females exposed to the two bisphenol A concentrations, estradiol concentrations ranged from 0.327 ng/mL to 3.15 ng/mL. A significant difference in the mean estradiol concentration per treatment (Table 4.15) was detected (Kruskal-Wallis, $H = 8.66$, $p = 0.013$, df = 2). The mean estradiol concentration in females from the high concentration was less than that in females from the control and from the low concentration. The achieved power at the observed maximum difference from the control response for this assay was 83%.

Table 4.15. Summary statistics and power estimates for female estradiol concentrations for the EPA 21-day bisphenol A assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
Control	8	2.29	1.42	62%	46%	9%	22%	89%
Low	8	1.94	0.58	30%				
High	8	0.902	0.66	74%				

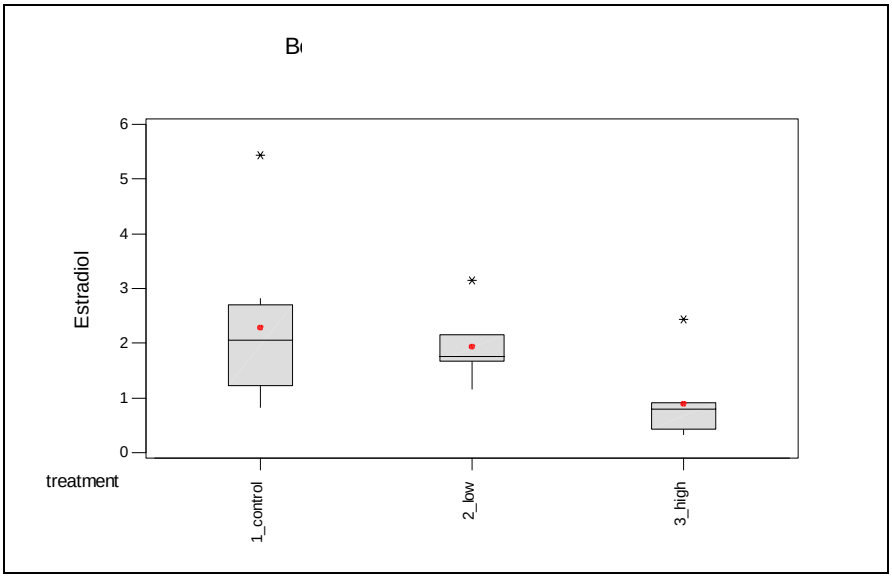

Figure 4.17. Box plot of female estradiol concentration (ng/mL) by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

Estradiol concentrations in control males used during the EPA 21-day bisphenol A assay ranged from 0 ng/mL (not detected) to 0.906 ng/mL (Figure 4.18). Estradiol was not detected in males exposed to the low concentration. Estradiol concentrations in males from the high concentration ranged from 0 ng/mL (not detected) to 0.274 ng/mL (Figure 4.18). Significant differences in the mean estradiol concentration per treatment (Table 4.16) were detected (Kruskal-Wallis, H = 13.99, p = 0.001, df = 2). The mean estradiol concentration in males from the control was greater than those in males from the low and high concentrations. The achieved power at the observed maximum difference from the control response for this assay was 98%.

Table 4.16. Summary statistics and power estimates for male estradiol concentrations for the EPA 21-day bisphenol A assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
Control	8	0.386	0.258	67%	100%	6%	11%	48%
Low	7	0	0	0%				
High	7	0.075	0.129	171%				

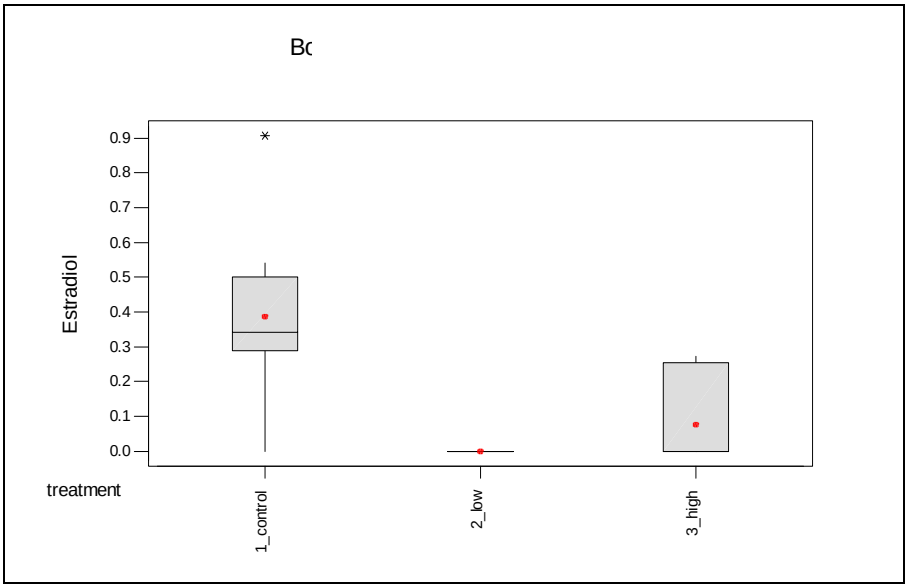

Figure 4.18. Box plot of male estradiol concentration (ng/mL) by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

Testosterone: Testosterone concentrations in control females used during the EPA 21-day bisphenol A assay ranged from 0 ng/mL (not detected) to 1.30 ng/mL (Figure 4.19). Among females exposed to the two bisphenol A concentrations, testosterone concentrations ranged from 0 ng/mL (not detected) to 1.65 ng/mL. No significant differences in the mean testosterone concentration per treatment (Table 4.17) were detected (Kruskal-Wallis, $H = 0.31$, $p = 0.857$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 6%. The probability of detecting as much as a 50% difference in the female testosterone concentration was low, 15%, based on the observed variability (Table 4.17).

Table 4.17. Summary statistics and power estimates for female testosterone concentrations for the EPA 21-day bisphenol A assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
Control	8	0.487	0.470	97%	20%	5%	6%	15%
Low	8	0.625	0.606	97%				
High	8	0.530	0.332	63%				

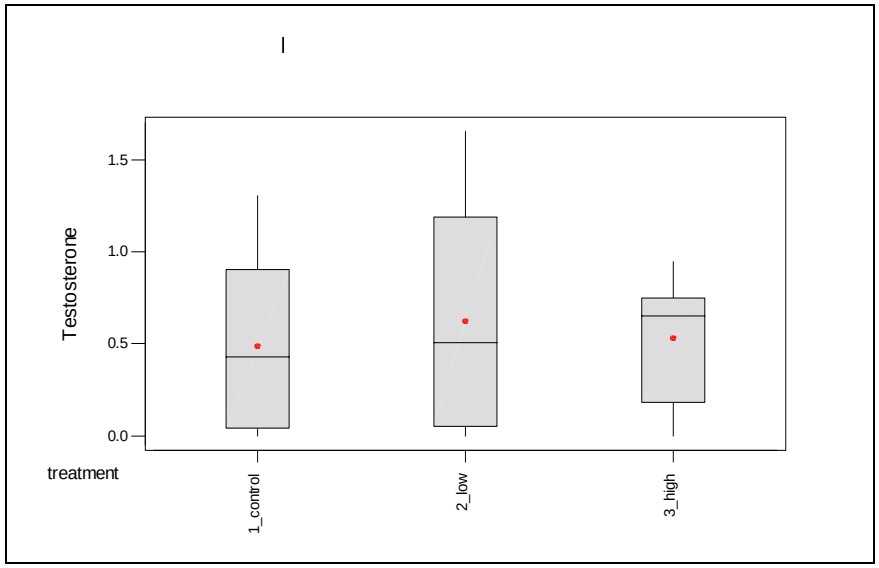

Figure 4.19. Box plot of female testosterone concentration (ng/mL) by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Testosterone concentrations in control males used during the EPA 21-day bisphenol A assay ranged from 1.57 ng/mL to 7.98 ng/mL (Figure 4.20). Among males exposed to the two bisphenol A concentrations, testosterone concentrations ranged from 0 ng/mL (not detected) to 8.48 ng/mL. Significant differences in the mean testosterone concentration per treatment (Table 4.18) were detected (Kruskal-Wallis, $H = 12.57$, $p = 0.002$, df = 2). The mean testosterone concentration in males from the high concentration was less than those in males from the control and the low concentration. The achieved power at the observed maximum difference from the control response for this assay was 100%.

Table 4.18. Summary statistics and power estimates for male testosterone concentrations for the EPA 21-day bisphenol A assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
Control	8	4.91	2.00	41%	78%	9%	21%	87%
Low	7	4.22	2.59	61%				
High	7	0.57	0.68	118%				

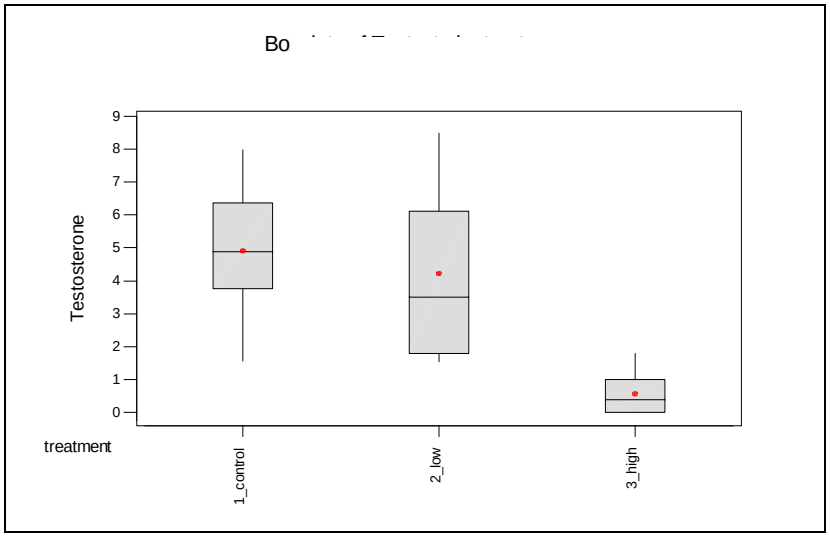

Figure 4.20. Box plot of male testosterone concentration (ng/mL) by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

11-ketotestosterone: 11-ketotestosterone was not detected in females from any treatment during the EPA 21-day bisphenol A assay.

11-ketotestosterone concentrations in control males used during the EPA 21-day bisphenol A assay ranged from 3.70 ng/mL to 32.1 ng/mL (Figure 4.21). Among males exposed to the two bisphenol A concentrations, 11-ketotestosterone concentrations ranged from 0 ng/mL (not detected) to 30.6 ng/mL. Significant differences in the mean 11-ketotestosterone concentration per treatment (Table 4.19) were detected (Kruskal-Wallis, $H = 13.12$, $p = 0.001$, df = 2). The mean 11-ketotestosterone concentration in males from the high concentration was less than those in males from the control and the low concentration. The achieved power at the observed maximum difference from the control response for this assay was 100%.

Table 4.19. Summary statistics and power estimates for male 11-ketotestosterone concentrations for the EPA 21-day bisphenol A assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
Control	8	19.8	9.3	47%	77%	9%	23%	90%
Low	7	14.5	11.1	77%				
High	7	1.35	1.61	120%				

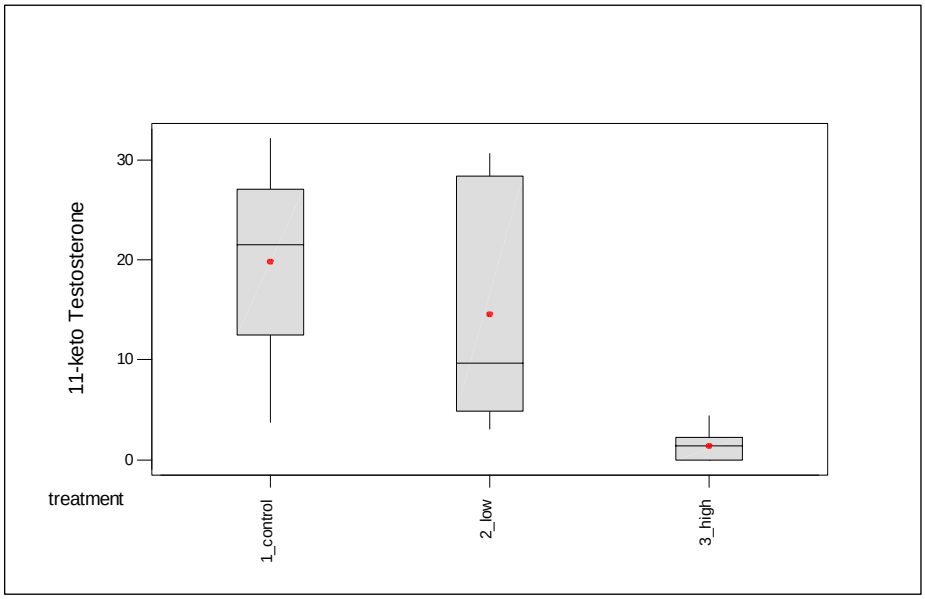

Figure 4.21. Box plot of male 11-ketotestosterone concentration (ng/mL) by treatment for the EPA 21-day bisphenol A assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

5.0 RESULTS: p,p'-DDE

The results of the EPA 21-day assay for p,p'-DDE are presented in this section. Total fecundity is reported as the total number of eggs laid during the assay, regardless of egg condition. However, some eggs could not be included in the evaluation of fertilization because of physical damage or the presence of a fungal infection. Summary tables are presented for the major endpoints. The values presented were calculated before being rounded for inclusion in the tables. Values reported include the mean for each treatment, the number of samples (N), the standard deviation (SD), and the coefficient of variation (CV). Values reported for the statistical analyses are the test statistic for the Kruskal-Wallis test used to determine the significance level (*H*), the probability that the observed result was due to chance (*p*), and the degrees of freedom associated with the test (df).

5.1 EPA 21-Day Assay for p,p'-DDE

The EPA 21-day p,p'-DDE assay was conducted from June 19, 2003 to June 26, 2003 (pre-exposure assay), and from June 26, 2003 to July 17, 2003 (exposure assay).

5.1.1 p,p'-DDE Concentrations

p,p'-DDE was not detected at concentrations above the MDL (0.022 µg/L) in the control at any time during the EPA 21-day p,p'-DDE assay. The mean (standard deviation) in the low concentration and high concentration were 0.022 (0.006) µg/L and 0.167 (0.058) µg/L, respectively (Figure 5.1).

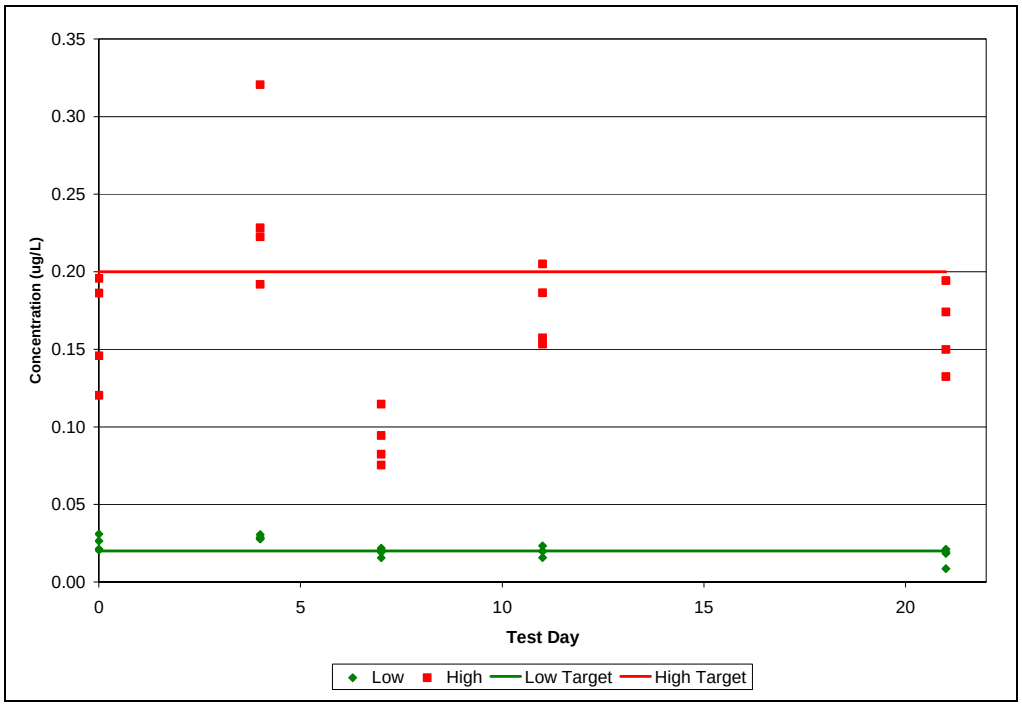

Figure 5.1. p,p'-DDE concentrations during the EPA 21-day p,p'-DDE assay

5.1.2 Survival

Three females, one from each treatment, died during the EPA 21-day p,p'-DDE assay. Two males, one each from the control and low concentration, died during the assay.

5.1.3 Fecundity

Total Fecundity: A 7-day pre-exposure evaluation of total egg production was performed. Total 7-day counts among the three treatments in the exposure assay (individual tank values summed for each treatment) ranged from about 8300 eggs to 10,200 eggs (Figure 5.2). No significant differences in the mean 7-day egg production among the groups of replicates evaluated during the pre-exposure assay were detected (Kruskal-Wallis, $H = 3.58$, $p = 0.167$, df = 2).

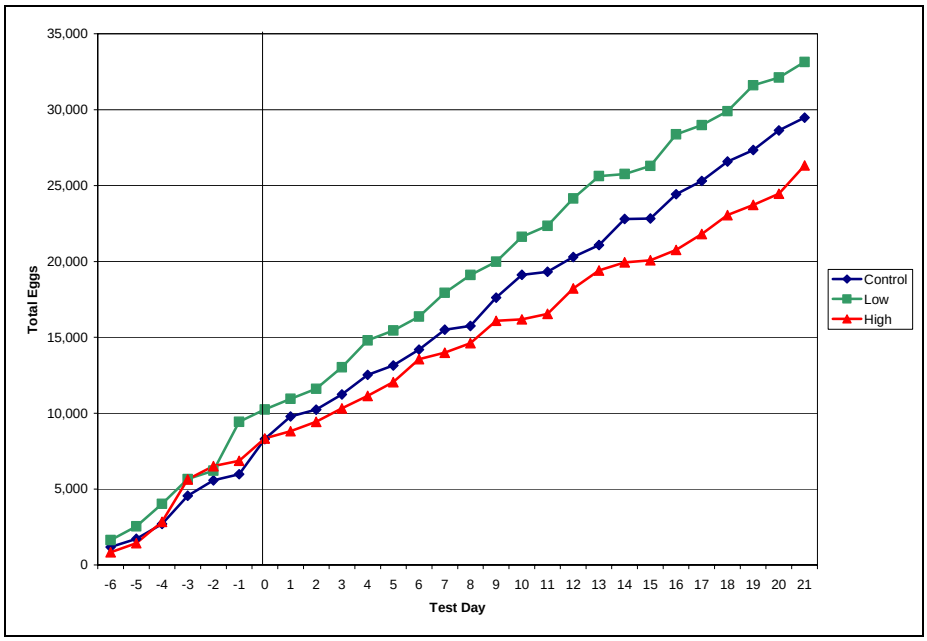

Figure 5.2. Total egg production per treatment for the p,p'-DDE assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to p,p'-DDE)

During the EPA 21-day p,p'-DDE assay, total counts in the control were reasonably consistent among replicates, varying from 4736 eggs to 6498 eggs (Figure 5.3). Total egg production among low-concentration replicates was similar, ranging from 5285 eggs to 6033 eggs. Total counts among the high-concentration replicates varied from 3886 eggs to 5192 eggs. Statistical analysis of square-root- transformed egg counts showed no significant among-treatment differences (Kruskal-Wallis, H=5.12, p= 0.077, df = 2) in mean total numbers of eggs produced (Table 5.1). The achieved power at the observed maximum difference from the control response for this assay was 26%. The probability of detecting as much as a 20% difference in total fecundity was high, 95%, based on the observed variability (Table 5.1).

Table 5.1. Summary statistics and power estimates for total fecundity data for the EPA 21-day p,p'-DDE assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	5,295	818	15%	8%	41%	95%	100%
low	4	5,724	366	6%				
high	4	4,500	624	14%				

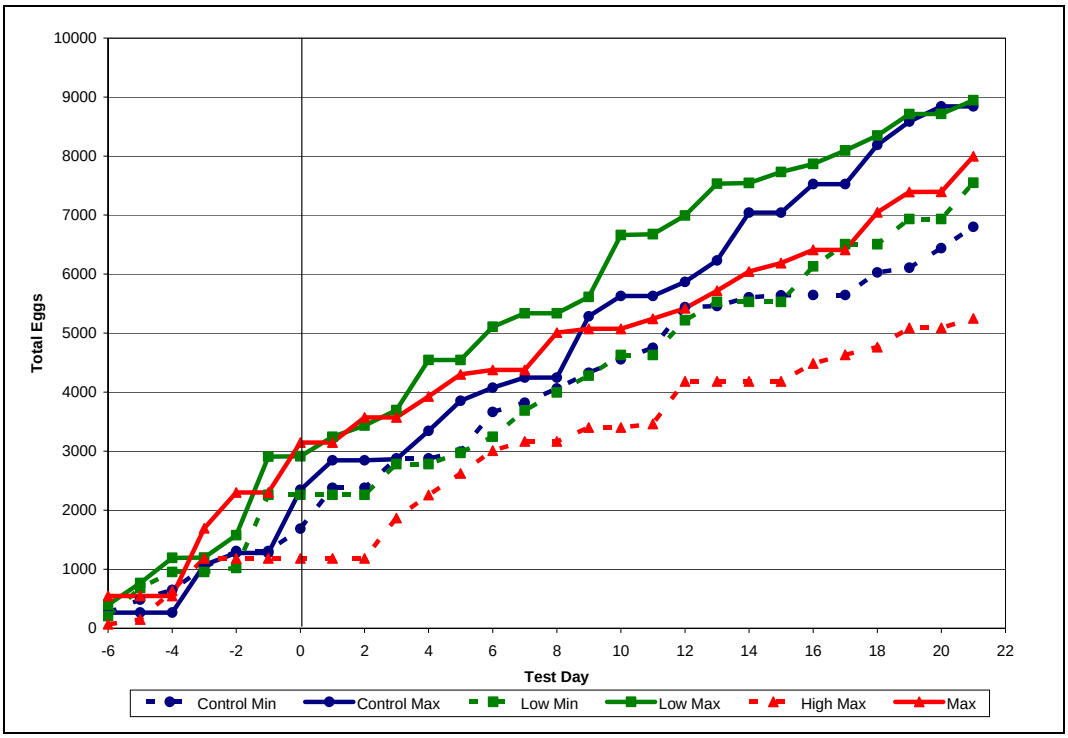

Figure 5.3. Range in replicate total egg production per treatment for the p,p'-DDE assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to p,p'-DDE)

Fecundity per Female Reproductive Day: During the 7-day pre-exposure evaluation, the mean number of eggs produced per female reproductive day ranged from 74.1 eggs/day for the tanks that would be used for the control to 91.5 eggs/day for the tanks that would be used for the low concentration during the 21-day exposure assay. There were no significant differences among treatments in the mean numbers of eggs produced per reproductive day during the pre-exposure period (Kruskal-Wallis, $H = 3.58$, $p = 0.167$, df = 2).

During the EPA 21-day p,p'-DDE assay, the maximum number of female reproductive days was not achieved for the any treatment (Table 5.2). One female died on each of the following: Day 10, Day 12, and Day 21 of the testing in the control, low concentration, and high concentration,

respectively. The number of eggs produced per female reproductive day varied from 56.4 eggs to 77.4 eggs in the control and from 66.2 eggs to 71.8 eggs in the low concentration (Figure 5.4). For the high concentration, 46.3 eggs to 61.8 eggs were produced per female reproductive day. There were no significant differences among treatments in the mean numbers of eggs produced per reproductive day during the EPA 21-day p,p'-DDE assay (Kruskal-Wallis, $H = 5.65$, $p = 0.059$, df = 2). The largest numerical difference in number of eggs produced per day was between the low concentration and the high concentration (Figure 5.4). The achieved power at the observed maximum difference from the control response for this assay was 44%. The probability of detecting as much as a 20% difference in fecundity per female reproductive day was high, 98%, based on the observed variability (Table 5.2).

Table 5.2. Summary statistics and power estimates for fecundity per female reproductive day for the EPA 21-day p,p'-DDE assay

Level	Mean Number of Reproductive Days[(a)]	N	Mean Fecundity Per Female Reproductive Day	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	81.0	4	65.5	9.1	14%	9%	49%	98%	100%
low	81.5	4	70.3	2.7	4%				
high	83.75	4	53.7	7.3	14%				

a) Maximum number = 84.

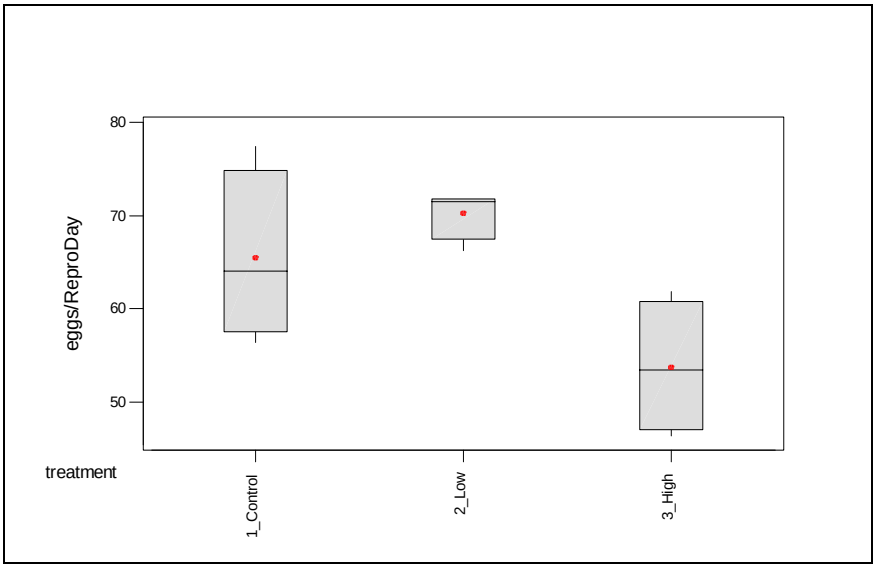

Figure 5.4. Box plot of the number of eggs produced per female reproductive day by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Eggs on Tiles/Dishes: The mean number of eggs laid on the tiles during the 7-day pre-exposure assay varied from 1337 eggs for the tanks that would be used for the control to 2157 eggs for the

tanks that would be used for the low concentration. The mean number of eggs on dishes ranged from 404 eggs for the low concentration to 737 eggs for the control. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated. There were significant differences in the mean proportional difference among treatments during the 7-day pre-exposure assay (Kruskal-Wallis, $H = 6.96$, $p = 0.031$, df = 2). The proportion of eggs laid on dishes was higher for the control than for the other two treatments.

The mean number of eggs laid on tiles among the treatments during the EPA 21-day p,p'-DDE assay ranged from 3493 eggs for the high concentration to 5019 eggs for the low concentration (Appendix E, Table E.2). The mean number of eggs laid on dishes varied from 650 eggs for the control to 1006 eggs for the high concentration. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated (Appendix E, Table E.2). There were no significant differences in the mean proportional difference among treatments during the 21-day exposure assay (Kruskal-Wallis, $H = 4.58$, $p = 0.101$, df = 2).

5.1.4 Fertilization Success

Total Fertilization: Eggs were collected during the 7-day pre-exposure period for the evaluation of fertilization success rate. All undamaged eggs laid during the pre-exposure assay were fertilized.

The total (tiles + dishes) fertilization success rates for all treatment replicates during the EPA 21-day p,p'-DDE assay ranged from 0.992 (control replicate) to 1.00 (replicates from all treatments) (Figure 5.5). No significant differences in mean fertilization success rates (Table 5.3) among treatments were detected (Kruskal-Wallis, $H = 3.60$, $p = 0.165$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 23%. The probability of detecting as small as a 10% difference in the proportion of eggs fertilized total fecundity was high at 100%, based on the observed variability (Table 5.3).

Table 5.3. Summary statistics and power estimates for the proportion of eggs fertilized for the EPA 21-day p,p'-DDE assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	0.998	0.004	0.4%	2%	100%	100%	100%
low	4	1.000	0.000	0.0%				
high	4	0.9998	0.0003	0.03%				

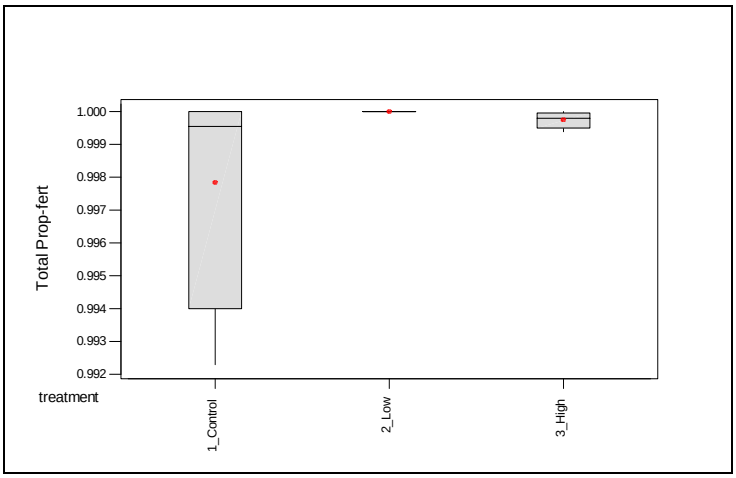

Figure 5.5. Box plot of the proportion of eggs fertilized by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Fertilization of Eggs on Tiles and Dishes: During the 7-day pre-exposure assay, all undamaged eggs laid on tiles or on dishes were fertilized. The fertilization success rates for all treatment replicates for undamaged eggs laid on tiles during the EPA 21-day p,p'-DDE assay ranged from 0.991 (control replicate) to 1.00 (replicates from all treatments). No significant differences in mean fertilization success rates among treatments were detected (Kruskal-Wallis, $H = 3.74$, $p = 0.155$, df = 2). The fertilization success rates for all treatment replicates for eggs laid on dishes during the EPA 21-day p,p'-DDE assay ranged from 0.993 (control replicate) to 1.00 (replicates from all treatments). No significant differences in mean fertilization success rates among treatments were detected (Kruskal-Wallis, $H = 2.00$, $p = 0.368$, df = 2).

5.1.5 Hatchability and Larval Development

Eggs were collected during the EPA 21-day p,p'-DDE assay for the evaluation of hatchability. The larval hatching study was completed by incubating 50 eggs per replicate in dilution water for 3 to 6 days until the majority of eggs hatched. One tank from the high concentration was excluded from the analysis because all eggs in it were infected by a fungus on Day 2. The proportion of fertilized eggs that hatched ranged from 0.75 to 0.98 in the control and from 0.94 to 1.00 for the low and high concentrations (Figure 5.6). No significant differences among treatments in the proportion of eggs that hatched (Table 5.4) were detected (Kruskal-Wallis, $H = 2.50$, $p = 0.286$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 33%. The probability of detecting as much as a 20% difference in the proportion of fertile eggs that hatched was low at 37%, based on the observed variability (Table 5.4).

Table 5.4. Summary statistics and power estimates for the proportion of fertile eggs that hatched for the EPA 21-day p,p'-DDE assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	0.898	0.109	12%	19%	12%	37%	99%
low	4	0.975	0.025	3%				
high	3	0.993	0.012	1%				

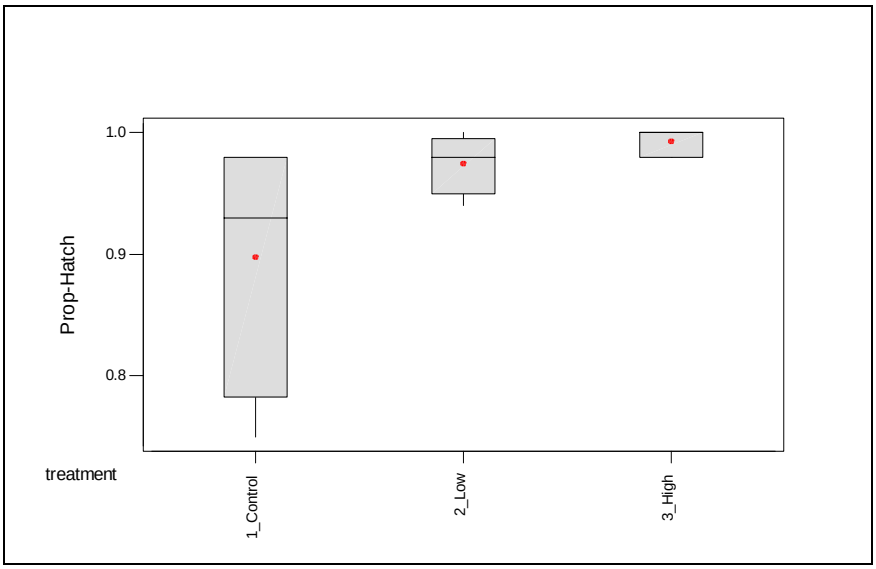

Figure 5.6. Box plot of the proportion of fertile eggs that hatched by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Eggs were collected during the EPA 21-day p,p'-DDE assay for the evaluation of larval development. The proportion of larvae that developed normally (i.e., that showed no morphological abnormalities) ranged from 0.87 to 1.0 in the control and from 0.74 to 1.00 for the low and high concentrations (Figure 5.7). There were no significant differences among treatments in the proportion of larvae that developed normally (Kruskal-Wallis, $H = 0.94$, $p = 0.625$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 6%. The probability of detecting as much as a 20% difference in the proportion of normal larvae was low at 17%, based on the observed variability (Table 5.5).

Table 5.5. Summary statistics and power estimates for the proportion of normal larvae for the EPA 21-day p,p'-DDE assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	0.945	0.065	7%	7%	8%	17%	74%
low	4	0.921	0.073	8%				
high	3	0.913	0.150	16%				

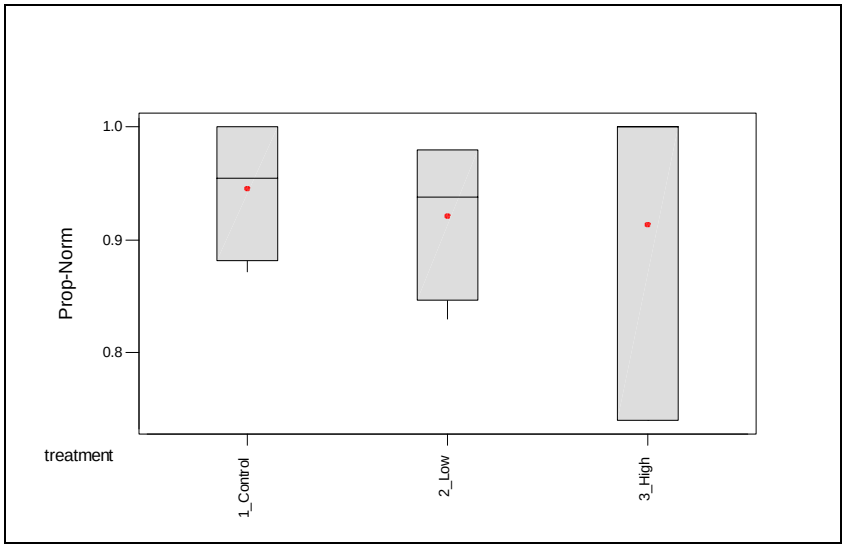

Figure 5.7. Box plot of the proportion of normal larvae by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

5.1.6 Body Weight

The body weight of females used in the EPA 21-day p,p'-DDE assay ranged from 1.5 g to 3.9 g. There were no significant differences in mean body weight among all treatments (Kruskal-Wallis, $H = 1.25$, $p = 0.534$, df = 2). The body weight of males used in the EPA 21-day p,p'-DDE assay ranged from 3.9 g to 9.1 g. There were no significant differences in mean body weight among treatments (Kruskal-Wallis, $H = 0.94$, $p = 0.625$, df = 2).

5.1.7 Gonadosomatic Index

The range of GSI values calculated for females in the all treatments varied from two- to fivefold (Figure 5.8). The highest value (GSI = 28.0) was obtained for a female from the control. One female exposed to the low-p,p'-DDE concentration had a GSI value of 20.7. No significant differences in the mean GSI value per treatment (Table 5.6) were detected (Kruskal-Wallis, $H = 0.35$, $p = 0.838$, df = 2). The achieved power at the observed maximum difference from the

control response for this assay was 7%. The probability of detecting as much as a 20% difference in female GSI was high, 92%, based on the observed variability (Table 5.6).

Table 5.6. Summary statistics and power estimates for female GSI data for the EPA 21-day p,p'-DDE assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	15	15.5	5.5	36%	3%	36%	92%	100%
low	15	15.2	3.0	19%				
high	15	14.5	3.4	23%				

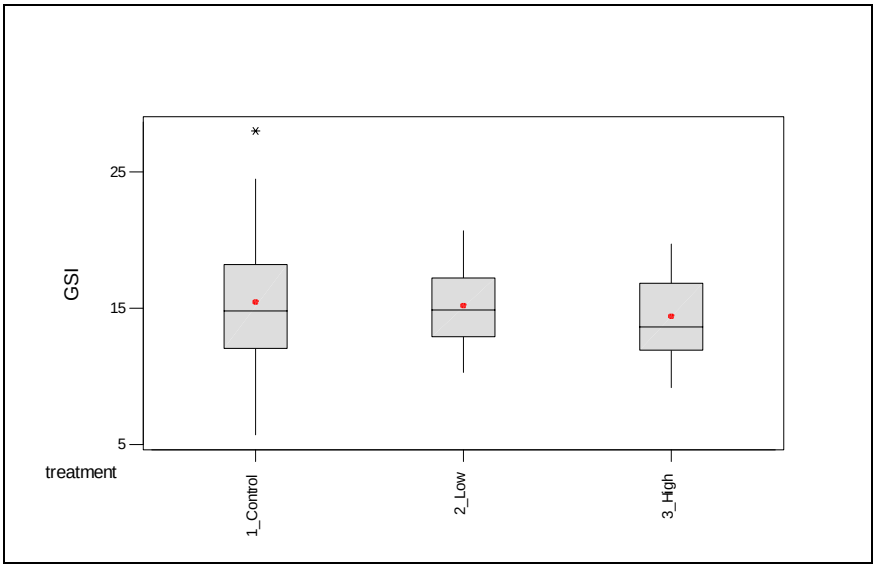

Figure 5.8. Box plot of female GSI by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

The range of most GSI values calculated for males during the EPA 21-day p,p'-DDE assay was small, ranging from 0.8 to 1.6 (Figure 5.9), which approximates the typical range for reproductively-active male fathead minnows. There were no significant differences in mean GSI values (Table 5.7) among treatments (Kruskal-Wallis, $H = 0.18$, $p = 0.915$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 6%. The probability of detecting as much as a 20% difference in male GSI was high, 95%, based on the observed variability (Table 5.7).

Table 5.7. Summary statistics and power estimates for male GSI data for the EPA 21-day p,p'-DDE assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	1.22	0.23	19%	2%	40%	95%	100%
low	7	1.22	0.13	11%				
high	8	1.18	0.23	20%				

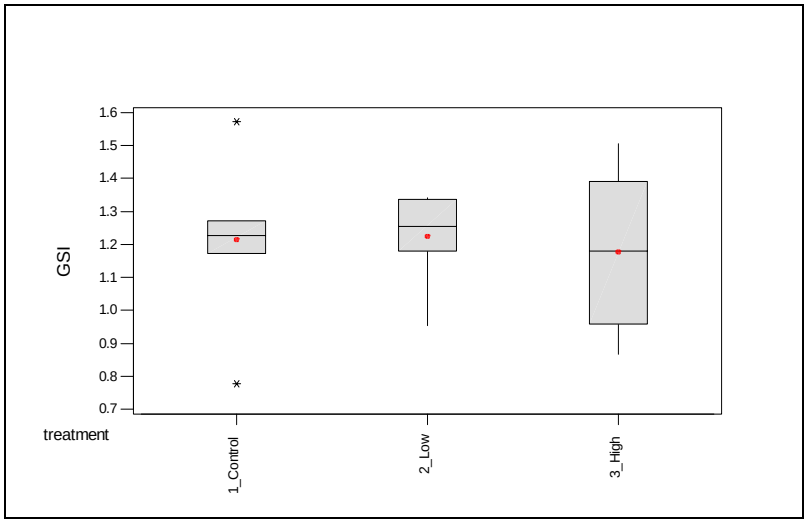

Figure 5.9. Box plot of male GSI by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

5.1.8 Female Gonad Histology

Histological analyses were conducted on the ovaries of 45 females exposed to p,p'-DDE during the EPA 21-day Assay.

General Ovary Staging: Statistical analysis of the mean ovarian staging from 18 microscopic fields per female in the EPA 21-day p,p'-DDE assay revealed no significant differences among treatments (Kruskal-Wallis, $H = 0.16$, $p = 0.922$, df = 2).

Quantitative Ovarian Staging: One hundred cells in each of three sections per female were examined to quantitatively determine the developmental stage of the ovaries. Ova from fish from all treatments ranged from Stage 1A to Stage 5 (see Methods for a description of the stages; Figure 5.10). Statistical analyses showed that there were no significant differences among treatments in the proportion of cells occurring in any developmental stage (Table 5.8).

Table 5.8. Descriptive statistics of the proportion of ovarian cells in each developmental stage for females from the EPA 21-day p,p'-DDE assay and results of the Kruskal-Wallis test (df = 2) comparing treatments

	Control (N = 15)			Low (N = 15)			High (N = 15)			Kruskal-Wallis	
Stage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	*H*	*p*
1a	0.063	0.020	31%	0.054	0.019	36%	0.069	0.026	38%	2.44	0.296
1b	0.248	0.045	18%	0.216	0.047	22%	0.238	0.056	23%	2.64	0.267
2	0.206	0.055	27%	0.181	0.040	22%	0.209	0.043	21%	3.72	0.156
3	0.174	0.058	33%	0.218	0.064	30%	0.191	0.053	28%	2.53	0.282
4	0.226	0.076	33%	0.241	0.115	48%	0.181	0.095	53%	2.52	0.283
5	0.040	0.065	161%	0.039	0.088	226%	0.015	0.042	281%	0.34	0.842

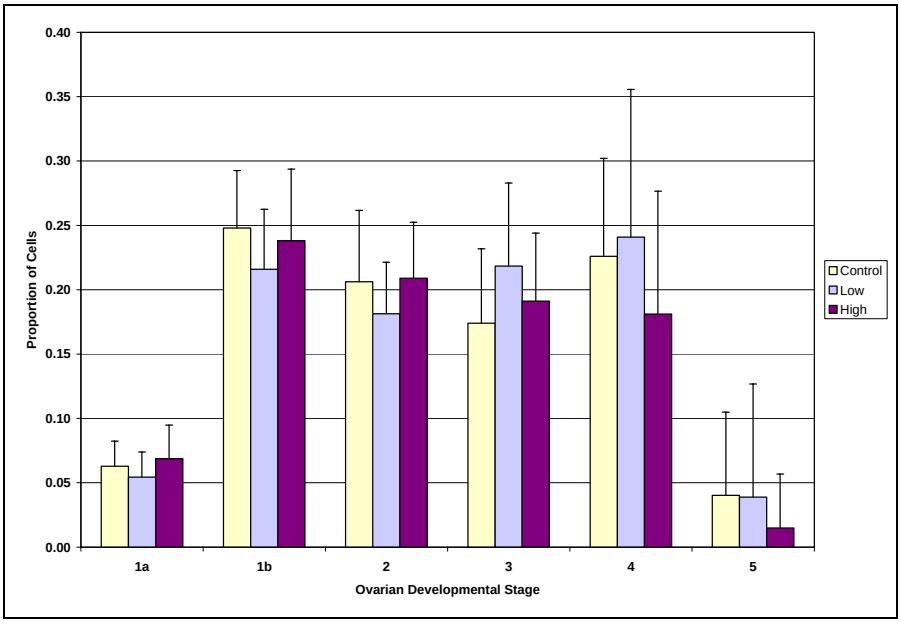

Figure 5.10. Frequency histogram showing the quantitative developmental staging of ovaries for each treatment of the EPA 21-day p,p'-DDE assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

Atretic Follicles: The mean proportion of atretic follicles per 300 follicles (counted per fish) ranged from 0.032 for females in the low concentration to 0.078 follicles for females in the high concentration (Figure 5.11). One female in the control had high proportion of atretic follicles (~0.5). A significant difference in the proportions of atretic follicles among treatments was detected (Kruskal-Wallis, $H = 6.25$, $p = 0.044$, df = 2). The mean proportion of atretic follicles in females from the high concentration was greater than that for females from the control. However, when the control female having the very high proportion of atretic follicles was excluded from the statistical analysis, the differences in mean values among treatments were more apparent (Kruskal-Wallis, $H = 8.97$, $p = 0.011$, df = 2) with the proportion of atretic follicles among females from the low concentration also greater than that for the control females.

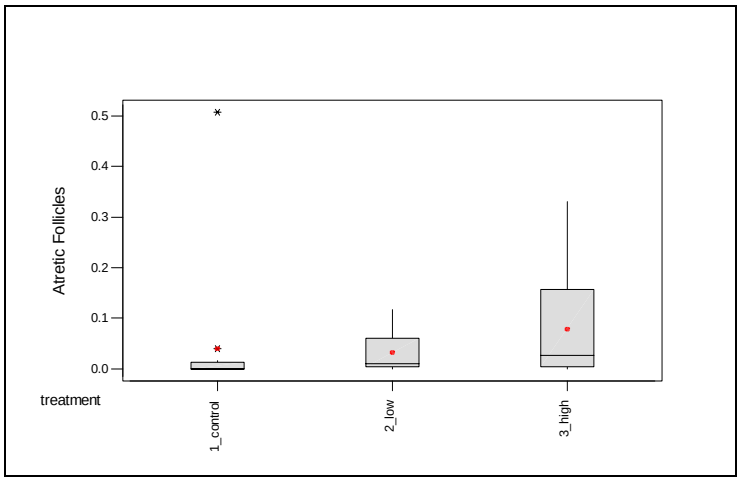

Figure 5.11. Box plot of the proportion of atretic follicles per 300 follicles by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

Post-Ovulatory Follicles: The mean proportion of post-ovulatory follicles per 300 follicles (counted per fish) ranged from 0.003 for females in the control to 0.020 for females in the high concentration (Figure 5.12). There were no significant differences in the mean proportion of post-ovulatory follicles among treatments (Kruskal-Wallis, $H = 1.83$, $p = 0.400$, df = 2).

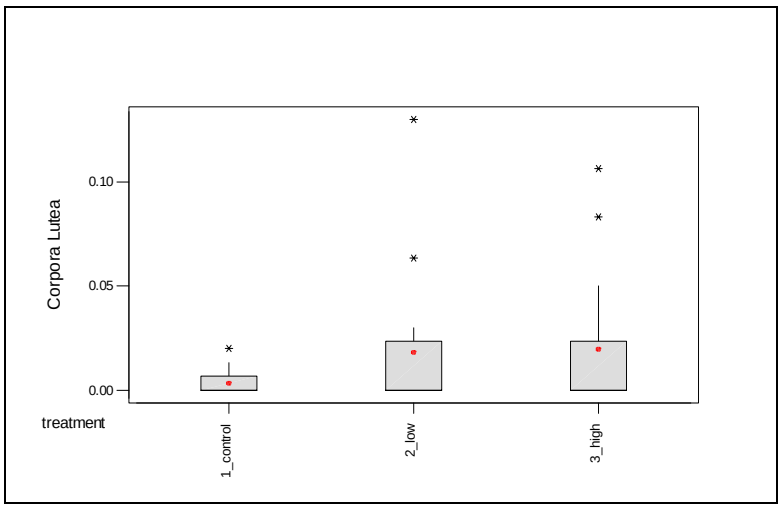

Figure 5.12. Box plot of the proportion of post-ovulatory follicles per 300 follicles by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

Observations: The ovaries from several female fish from each treatment were observed to have histological abnormalities (Appendix E, Table E.10). One condition, multiple foci of macrophage clusters containing brown material, was evident in females from the control and low concentration.

5.1.9 Male Gonad Histology

General Testes Staging: Testes from 22 males exposed to p,p'-DDE during the EPA 21-day p,p'-DDE assay were examined to determine the general developmental condition. Males in all treatments had well-developed testes, with most showing Stage 4 and Stage 5 development (see Methods for description of developmental stages). All of the 84 microscopic fields examined in the 7 control males showed Stage 4 (73 fields) or Stage 5 (11 fields) development. Seventy-eight of the 84 microscopic fields examined in the 7 low-concentration treatment males showed Stage 4 (71 fields) or Stage 5 (7 fields) development. One male from the low concentration had 6 fields showing Stage 1 development. All of the 96 microscopic fields examined in the 8 high-concentration treatment males showed Stage 4 (70 fields) or Stage 5 (26 fields) development. Statistical analysis of the mean staging from 12 microscopic fields per fish revealed no significant differences among treatments (Kruskal-Wallis, $H = 3.42$, $p = 0.181$, df = 2).

Quantitative Testicular Staging: One hundred cells in each of three sections per male were examined to quantitatively determine the developmental condition of the testes. The developmental stage for testes from all treatments ranged from Stage 2A to Stage 5 (Figure 5.13). Statistical analyses showed that there were no significant differences among treatments in the proportion of cells in any of the developmental stages (Table 5.9). Therefore, there did not appear to be an effect on testicular developmental stage associated with p,p'-DDE dose.

Table 5.9. Descriptive statistics of the proportion of testes cells in each developmental stage for males from the EPA 21-day p,p'-DDE assay and results of the Kruskal-Wallis test (df = 2) comparing treatments

	Control (N = 7)			Low (N = 7)			High (N = 8)			Kruskal-Wallis	
Stage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	*H*	*p*
1	0	0	–	0	0	–	0	0	–	–	–
2A	0.004	0.004	106%	0.003	0.002	81%	0.001	0.002	198%	2.68	0.261
2B	0.013	0.011	88%	0.016	0.009	60%	0.012	0.012	97%	0.58	0.748
3A	0.199	0.081	41%	0.186	0.070	38%	0.135	0.103	77%	1.93	0.382
3B	0.252	0.108	43%	0.318	0.102	32%	0.270	0.099	37%	1.86	0.395
4	0.234	0.073	31%	0.244	0.070	28%	0.209	0.100	48%	1.00	0.606
5	0.299	0.152	51%	0.234	0.108	46%	0.373	0.166	45%	2.87	0.238

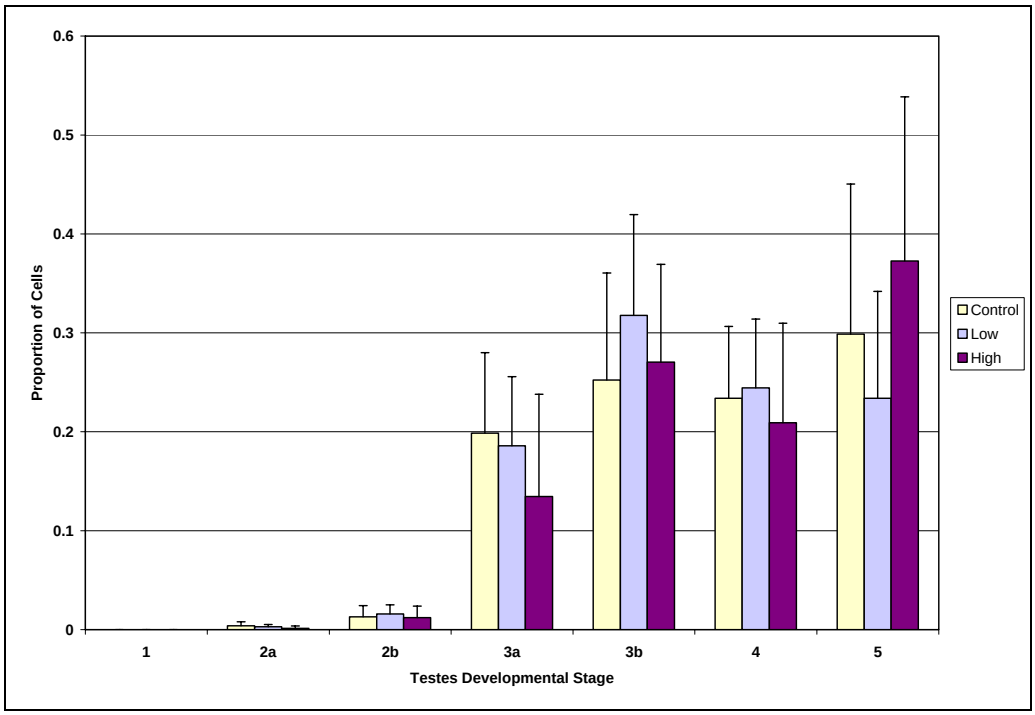

Figure 5.13. Frequency histogram showing the quantitative developmental staging of testes for each treatment of the EPA 21-day p,p'-DDE assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

Tubule Diameter: The average diameter of the seminiferous tubules of males from the control ranged from 96.7 µm to 178.1 µm (Figure 5.14). Tubule diameters of males from the two test concentrations ranged from 123.1 µm to 194.7 µm. No significant differences in the mean tubule diameter per treatment (Table 5.10) were detected (Kruskal-Wallis, $H = 2.34$, $p = 0.311$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 27%. The probability of detecting as much as a 20% difference in male seminiferous tubule diameter was high, 100%, based on the observed variability (Table 5.10).

Table 5.10. Summary statistics and power estimates for male seminiferous tubule diameter data for the EPA 21-day p,p'-DDE assay

Level	N	Mean (µm)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	137.3	25.2	18%	3%	100%	100%	100%
low	7	156.9	21.2	14%				
high	8	147.9	20.7	14%				

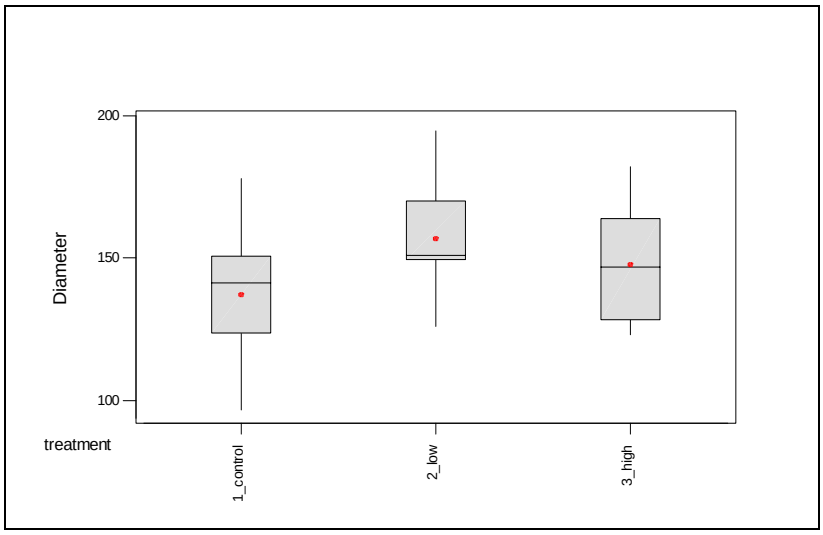

Figure 5.14. Box plot of male seminiferous tubule diameter (μm) by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Observations: No Sertoli cell proliferation was observed. Five males, three from the control, were found to have multifocal proliferation of Leydig cells. Several males in each treatment showed other histological abnormalities (Table 5.11). One male from the low concentration showed testicular atrophy. No ovatestes were observed for any treatment.

Table 5.11. Histological observations for males exposed to concentrations of p,p'-DDE during the EPA 21-day assay

Fish ID	Treatment	Leydig Cell Proliferation	Observations
229145	Control	No	Basophilic cysts in mature tubule lumina
229146	Control	Yes	Mild multifocal interstitial cell proliferation A few necrotic cells in proliferative centers
229151	Control	Yes	Multifocal interstitial cell proliferation
229163	Control	Yes	Multifocal proliferation of Leydig cells
229169	Low	No	One area of testes in stage 4, other area with no development Stage 4 tubules atrophied
229175	Low	Yes	Focal proliferation of Leydig cells Tubules with abnormal proliferation of stage 2b cells
229199	High	No	Multifocal basophilic cysts in tubules
229205	High	No	Basophilic cysts in spermatic ducts
229206	High	Yes	Mild multifocal interstitial cell proliferation A few necrotic cells in proliferative centers

5.1.10 Vitellogenin

VTG concentrations in control females used during the EPA 21-day p,p'-DDE assay ranged from 8.0 mg/mL to 15.1 mg/mL (Figure 5.15). Among females exposed to the two p,p'-DDE concentrations, VTG concentrations ranged from 7.8 mg/mL to 21.4 mg/mL. No significant differences in the mean VTG concentration per treatment (Table 5.12) were detected (Kruskal-Wallis, $H = 0.66$, $p = 0.718$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 11%. The probability of detecting as much as a 50% difference in the female VTG was high, 100% , based on the observed variability (Table 5.12).

Table 5.12. Summary statistics and power estimates for female VTG concentrations for the EPA 21-day p,p'-DDE assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	11.5	2.6	22%	5%	36%	92%	100%
low	7	12.1	3.0	24%				
high	8	13.2	4.0	30%				

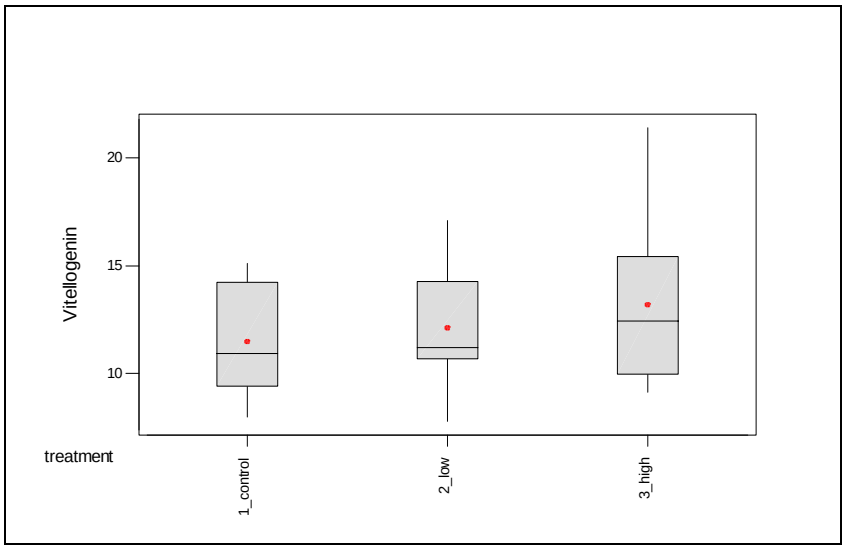

Figure 5.15. Box plot of female VTG concentration (mg/mL) by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

VTG concentrations in control males used during the EPA 21-day p,p'-DDE assay ranged from 0.001 mg/mL to 0.018 mg/mL (Figure 5.16). Among males exposed to the two p,p'-DDE concentrations, VTG concentrations ranged from 0.0001 mg/mL to 0.028 mg/mL. No significant differences in the mean VTG concentration per treatment (Table 5.13) were detected (Kruskal-

Wallis, $H = 2.54$, $p = 0.280$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 15%. The probability of detecting as much as a 50% difference in the male VTG was low, 7%, based on the observed variability (Table 5.13).

Table 5.13. Summary statistics and power estimates for male VTG concentrations for the EPA 21-day p,p'-DDE assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	0.004	0.006	139%	110%	5%	5%	7%
low	7	0.006	0.005	75%				
high	8	0.009	0.011	118%				

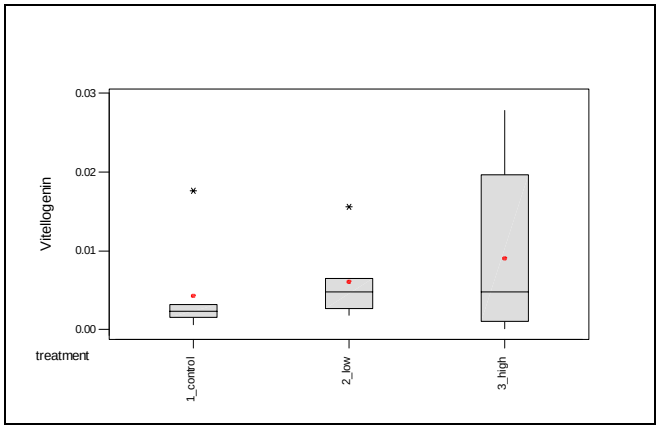

Figure 5.16. Box plot of male VTG concentration (mg/mL) by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

5.1.11 Plasma Steroid Concentrations

Estradiol: Estradiol concentrations in control females used during the EPA 21-day p,p'-DDE assay ranged from 1.53 ng/mL to 4.69 ng/mL (Figure 5.17). Among females exposed to the two-p,p'-DDE concentrations, estradiol concentrations ranged from 0.483 ng/mL to 3.78 ng/mL. No significant differences in the mean estradiol concentration per treatment (Table 5.14) were detected (Kruskal-Wallis, $H = 3.97$, $p = 0.138$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 35%. The probability of detecting as much as a 50% difference in female estradiol was high, 95%, based on the observed variability (Table 5.14).

Table 5.14. Summary statistics and power estimates for female estradiol concentrations for the EPA 21-day p,p'-DDE assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	2.92	1.00	34%	23%	10%	27%	95%
low	8	1.94	1.02	53%				
high	8	2.11	0.89	42%				

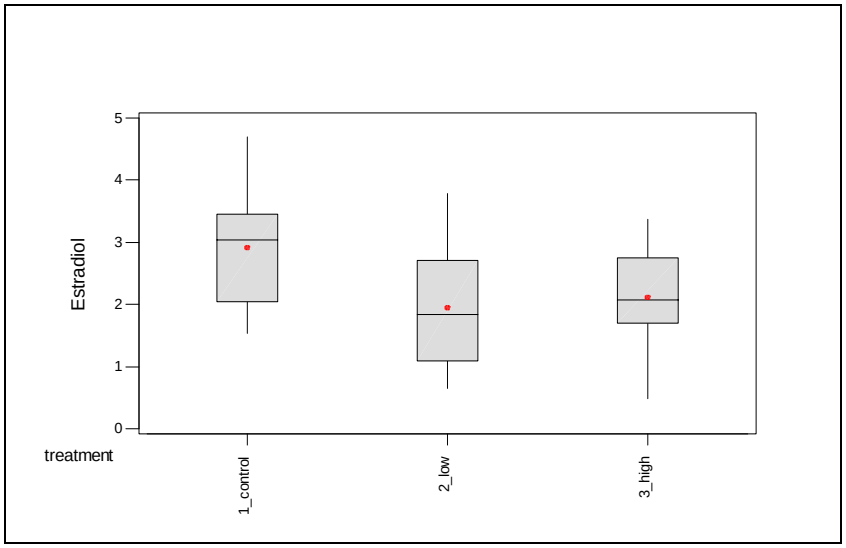

Figure 5.17. Box plot of female estradiol concentration (ng/mL) by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Estradiol concentrations in control males used during the EPA 21-day p,p'-DDE assay ranged from 0.326 ng/mL to 0.711 ng/mL (Figure 5.18). Estradiol concentrations in males from the two p,p'-DDE concentration ranged from 0 ng/mL (not detected) to 0.456 ng/mL (Figure 5.18). No significant differences in the mean estradiol concentration per treatment (Table 5.15) were detected (Kruskal-Wallis, $H = 5.16$, $p = 0.076$, df = 2). However, there seemed to be a trend for decreased estradiol concentrations at the two doses of p,p'-DDE (Figure 5.18). The achieved power at the observed maximum difference from the control response for this assay was 37%. The probability of detecting as much as a 50% difference in the male estradiol was moderate, 60%, based on the observed variability (Table 5.15).

Table 5.15. Summary statistics and power estimates for male estradiol concentrations for the EPA 21-day p,p'-DDE assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	0.436	0.147	34%	38%	7%	13%	60%
low	7	0.257	0.134	52%				
high	8	0.263	0.183	70%				

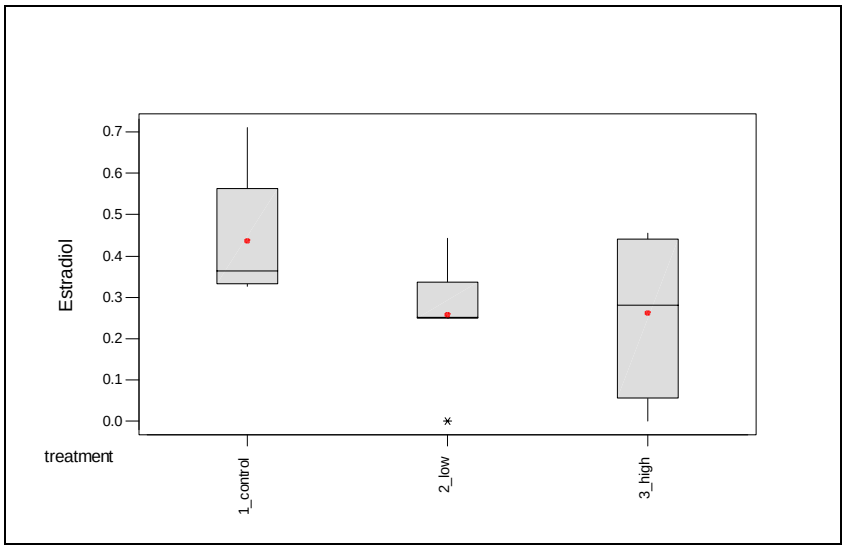

Figure 5.18 Box plot of male estradiol concentration (ng/mL) by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

Testosterone: Testosterone concentrations in control females used during the EPA 21-day p,p'-DDE assay ranged from 0.116 ng/mL to 1.07 ng/mL (Figure 5.19). Among females exposed to the two p,p'-DDE concentrations, testosterone concentrations ranged from 0 ng/mL (not detected) to 2.24 ng/mL. No significant differences in the mean testosterone concentration per treatment (Table 5.16) were detected (Kruskal-Wallis, $H = 0.52$, $p = 0.772$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 10%. The probability of detecting as much as a 50% difference in the female testosterone was low, 12%, based on the observed variability (Table 5.16).

Table 5.16. Summary statistics and power estimates for female testosterone concentrations for the EPA 21-day p,p'-DDE assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.407	0.312	77%	41%	5%	6%	12%
low	8	0.692	0.738	107%				
high	8	0.560	0.520	93%				

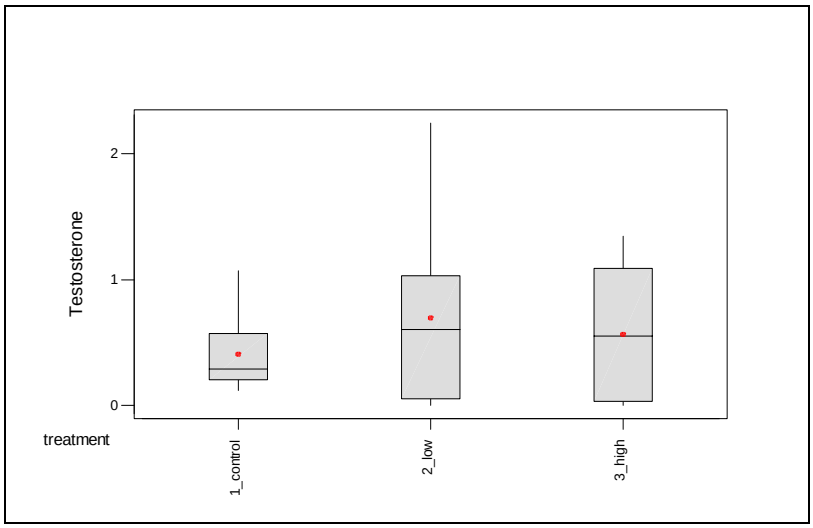

Figure 5.19. Box plot of female testosterone concentration (ng/mL) by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Testosterone concentrations in control males used during the EPA 21-day p,p'-DDE assay ranged from 2.46 ng/mL to 6.04 ng/mL (Figure 5.20). Among males exposed to the two p,p'-DDE concentrations, testosterone concentrations ranged from 1.15 ng/mL to 10.5 ng/mL. No significant differences in the mean testosterone concentration per treatment (Table 5.17) were detected (Kruskal-Wallis, $H = 1.11$, $p = 0.573$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 9%. The probability of detecting as much as a 50% difference in the male testosterone was moderate, 79%, based on the observed variability (Table 5.17).

Table 5.17. Summary statistics and power estimates for male testosterone concentrations for the EPA 21-day p,p'-DDE assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	4.05	1.43	35%	11%	8%	18%	79%
low	7	5.39	3.66	68%				
high	8	5.03	1.64	33%				

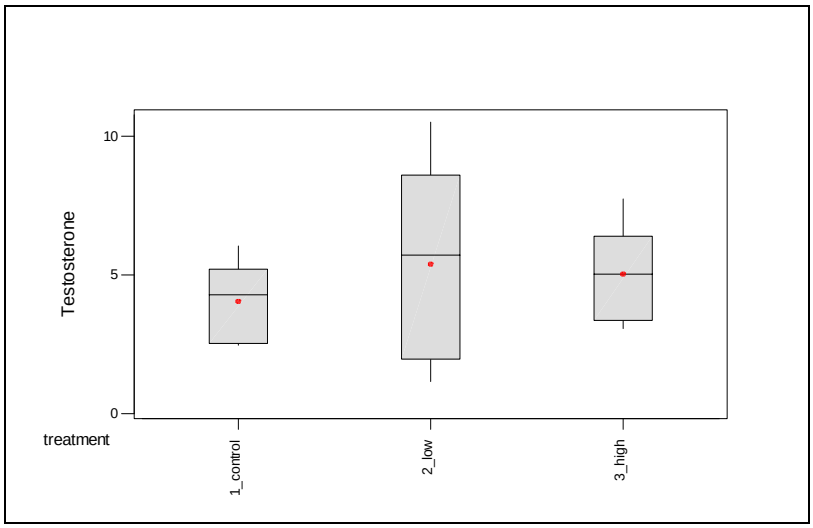

Figure 5.20. Box plot of male testosterone concentration (ng/mL) by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

11-ketotestosterone: 11-ketotestosterone was not detected in females from any treatment during the EPA 21-day p,p'-DDE assay.

11-ketotestosterone concentrations in control males used during the EPA 21-day p,p'-DDE assay ranged from 8.12 ng/mL to 25.5 ng/mL (Figure 5.21). Among males exposed to the two p,p'-DDE concentrations, 11-ketotestosterone concentrations ranged from 3.34 ng/mL to 47.2 ng/mL. No significant differences in the mean 11-ketotestosterone concentration per treatment (Table 5.18) were detected (Kruskal-Wallis, $H = 0.62$, $p = 0.732$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 8%. The probability of detecting as much as a 50% difference in the male 11-ketotestosterone was high, 88% , based on the observed variability (Table 5.18).

Table 5.18. Summary statistics and power estimates for male 11-ketotestosterone concentrations for the EPA 21-day p,p'-DDE assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	16.9	7.7	45%	9%	9%	22%	88%
low	7	22.2	17.6	79%				
high	8	23.0	13.3	58%				

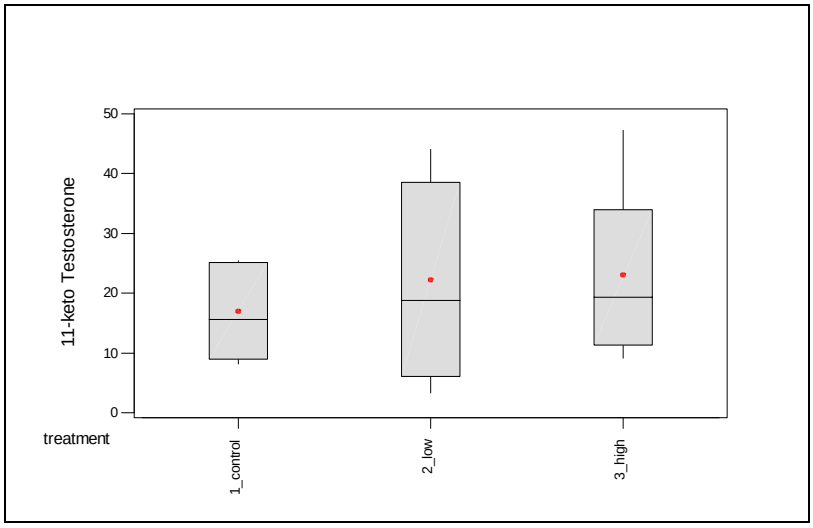

Figure 5.21. Box plot of male 11-ketotestosterone concentration (ng/mL) by treatment for the EPA 21-day p,p'-DDE assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

6.0 RESULTS: PERCHLORATE

The results of the EPA 21-day assay for perchlorate are presented in this section. Total fecundity is reported as the total number of eggs laid during the assay, regardless of egg condition. However, some eggs could not be included in the evaluation of fertilization because of physical damage or the presence of a fungal infection. Summary tables are presented for the major endpoints. The values presented were calculated before being rounded for inclusion in the tables. Values reported include the mean for each treatment, the number of samples (N), the standard deviation (SD), and the coefficient of variation (CV). Values reported for the statistical analyses are the test statistic for the Kruskal-Wallis test used to determine the significance level (*H*), the probability that the observed result was due to chance (*p*), and the degrees of freedom associated with the test (df).

6.1 EPA 21-Day Assay for Perchlorate

The EPA 21-day perchlorate assay was conducted from July 30, 2003 to August 6, 2003 (pre-exposure assay), and from August 6, 2003 to August 27, 2003 (exposure assay).

6.1.1 Perchlorate Concentrations

Perchlorate was not detected at concentrations above the MDL (116 μg/L) in the control at any time during the EPA 21-day perchlorate exposure period. The mean (standard deviation) in the low concentration and high concentration were 5638 (2081) μg/L and 43,540 (6503) μg/L, respectively (Figure 6.1).

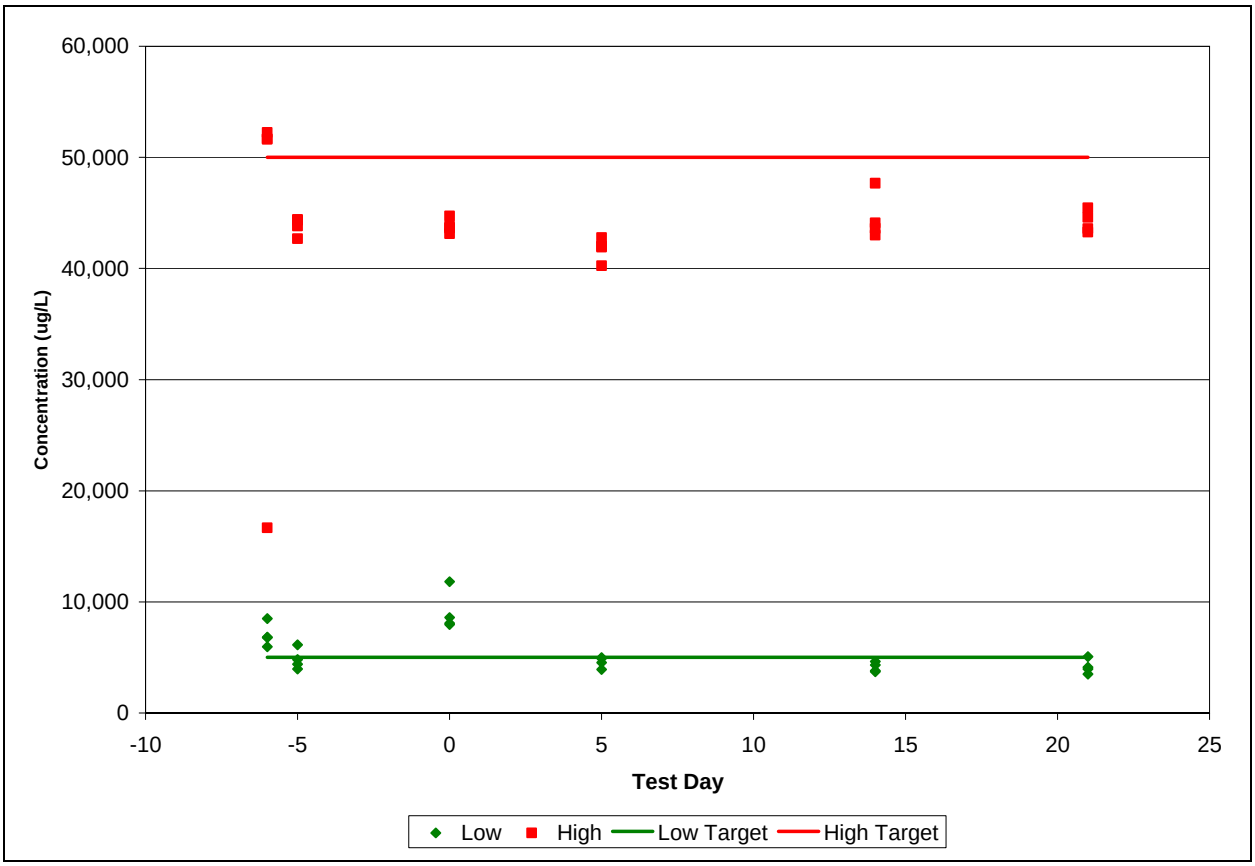

Figure 6.1. Perchlorate concentrations during the EPA 21-day perchlorate assay

6.1.2 Survival

Three females, two in the low concentration and one in the high concentration, died during the EPA 21-day perchlorate assay. Two males, one each in the control and high concentration, died during the assay.

6.1.3 Fecundity

Total Fecundity: A 7-day pre-exposure evaluation of total egg production was performed. Total 7-day counts among the three treatments in the exposure assay (individual tank values summed for each treatment) ranged from about 6400 eggs to 7800 eggs (Figure 6.2). No significant differences in the mean 7-day egg production among the groups of replicates evaluated during the pre-exposure assay were detected (Kruskal-Wallis, $H = 1.42$, $p = 0.491$, df = 2).

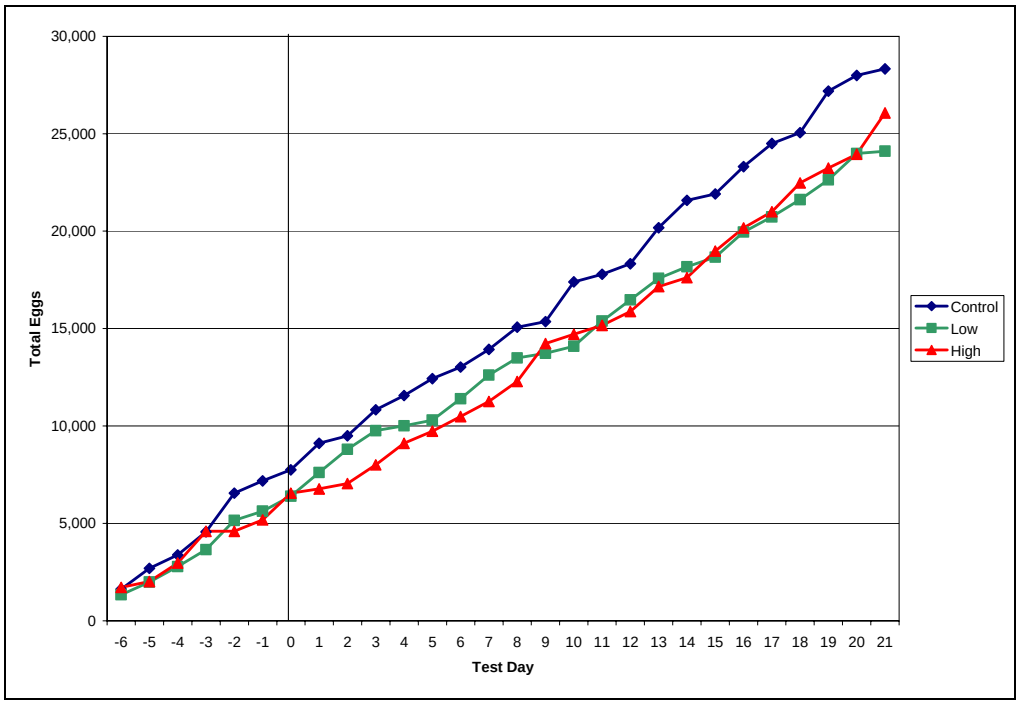

Figure 6.2. Total egg production per treatment for the perchlorate assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to perchlorate)

During the EPA 21-day perchlorate assay, total eggs counts in the control varied from 4382 eggs to 5835 eggs (Figure 6.3). Total egg production among low-concentration replicates was similar, ranging from 3413 eggs to 6200 eggs. Total counts among the high-concentration replicates varied from 4097 eggs to 6,100 eggs. Statistical analysis of square-root transformed egg counts showed no significant among-treatment differences (Kruskal-Wallis, $H = 1.65$, $p = 0.437$, df = 2) in mean total numbers of eggs produced (Table 6.1). The achieved power at the observed maximum difference from the control response for this assay was 13%. The probability of detecting as much as a 20% difference in the total fecundity was moderate, 59%, based on the observed variability (Table 6.1).

Table 6.1. Summary statistics and power estimates for total fecundity data for the EPA 21-day perchlorate assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	5144	695	14%	8%	18%	59%	100%
low	4	4426	1,224	28%				
high	4	4881	920	19%				

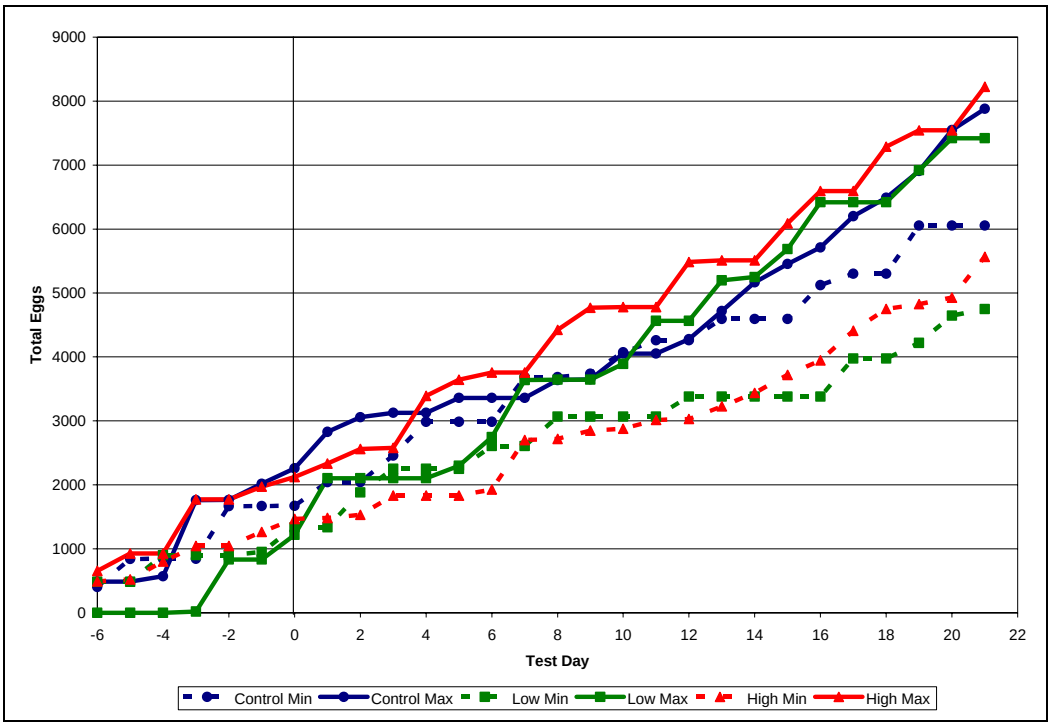

Figure 6.3. Range in replicate total egg production per treatment for the perchlorate assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to perchlorate)

***Fecundity per Female Reproductive Day*:** During the 7-day pre-exposure evaluation, the mean number of eggs produced per female reproductive day ranged from 57.2 eggs/day for the tanks that would be used for the low concentration to 69.2 eggs/day for the tanks that would be used for the control during the 21-day exposure assay. There were no significant differences among treatments in the mean numbers of eggs produced per reproductive day during the pre-exposure period (Kruskal-Wallis, $H = 1.42$, $p = 0.491$, df = 2).

During the EPA 21-day perchlorate assay, the maximum number of female reproductive days was achieved for the control (Table 6.2). Two females from one tank of the low concentration died during the assay, one at Day 8 and one at Day 13 of the testing. One female from the high concentration died at Day 5 of the assay. The number of eggs produced per female reproductive day varied from 52.2 eggs to 69.5 eggs in the control and from 46.7 eggs to 73.8 in the low concentration (Figure 6.4). For the high concentration, the number of eggs produced per female reproductive day ranged from 48.8 eggs to 91.0 eggs. No significant differences among treatments in the mean number of eggs produced per day were detected (Kruskal-Wallis, $H = 0.96$, $p = 0.618$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 7%. The probability of detecting as much as a 20% difference in the fecundity per female reproductive day was low, 47% , based on the observed variability (Table 6.2).

Table 6.2. Summary statistics and power estimates for fecundity per female reproductive day for the EPA 21-day perchlorate assay

Level	Mean Number of Reproductive Days[(a)]	N	Mean Fecundity Per Female Reproductive Day	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	84.0	4	61.3	8.3	12%	4%	15%	47%	100%
low	78.25	4	56.5	12.2	21%				
high	79.75	4	62.7	19.5	31%				

a) Maximum number = 84.

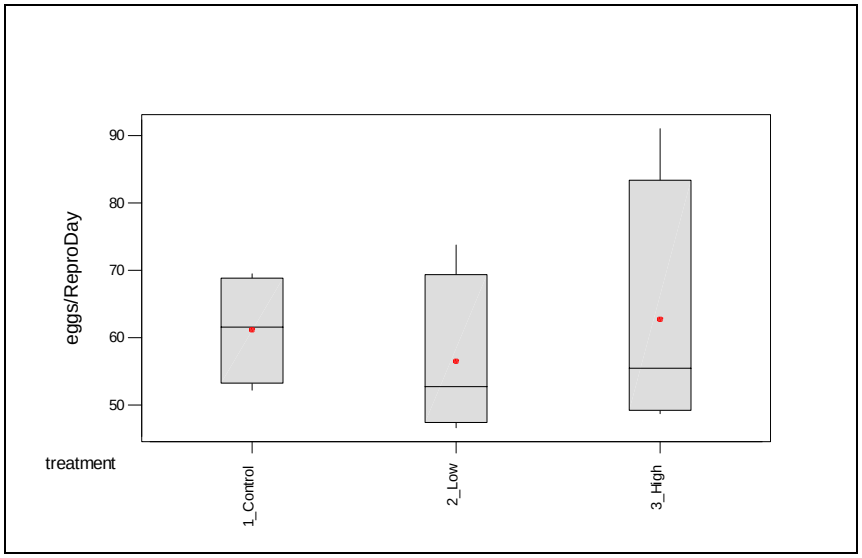

Figure 6.4. Box plot of the number of eggs produced per female reproductive day by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Eggs on Tiles/Dishes: The mean number of eggs laid on the tiles during the 7-day pre-exposure assay varied from 1048 eggs for the tanks that would be used for the low concentration to 1466 eggs for the tanks that would be used for the control. The mean number of eggs on dishes ranged from 424 eggs for the high concentration to 552 eggs for the low concentration. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated. There were no significant differences in the mean proportional difference among treatments during the 7-day pre-exposure assay (Kruskal-Wallis, $H = 2.20$, $p = 0.333$, df = 3).

The mean number of eggs on tiles among the treatments during the EPA 21-day perchlorate assay ranged from 3401 eggs for the low concentration to 4060 eggs for the control (Appendix F,

Table F.2). The mean number of eggs laid on the dishes varied from 904 eggs for the high concentration to 1084 eggs for the control. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated (Appendix F, Table F.2). There were no significant differences in the mean proportional difference among treatments during the 21-day exposure assay (Kruskal-Wallis, $H = 1.07$, $p = 0.585$, df = 2).

6.1.4 Fertilization Success

Total Fertilization: Eggs were collected during the 7-day pre-exposure period for the evaluation of fertilization success rate. All undamaged eggs laid during this evaluation, except one egg from a high-concentration tank, were fertilized.

All undamaged eggs laid in all treatments during the EPA 21-day perchlorate assay were fertilized.

Fertilization of Eggs on Tiles and Dishes: During the 7-day pre-exposure assay, the single unfertilized egg from a high-concentration tank was laid on a tile. All other eggs were fertilized. All eggs laid on tiles and dishes during the EPA 21-day perchlorate assay were fertilized.

6.1.5 Hatchability and Larval Development

Eggs were collected during the EPA 21-day perchlorate assay for the evaluation of hatchability. The larval hatching study was completed by incubating 50 eggs per replicate in dilution water for 3 to 6 days until the majority of eggs hatched. The proportion of fertilized eggs that hatched ranged from 0.70 to 0.96 in the control and from 0.50 to 1.00 for the low and high concentrations (Figure 6.5). No significant differences among treatments in the proportion of eggs that hatched (Table 6.3) were detected (Kruskal-Wallis, $H = 1.39$, $p = 0.499$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 11%. The probability of detecting as much as a 20% difference in the proportion of fertile eggs that hatched was low, 21%, based on the observed variability (Table 6.3).

Table 6.3. Summary statistics and power estimates for the proportion of fertile eggs that hatched for the EPA 21-day perchlorate assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	0.869	0.124	14%	12%	9%	21%	85%
low	4	0.948	0.055	6%				
high	4	0.833	0.227	27%				

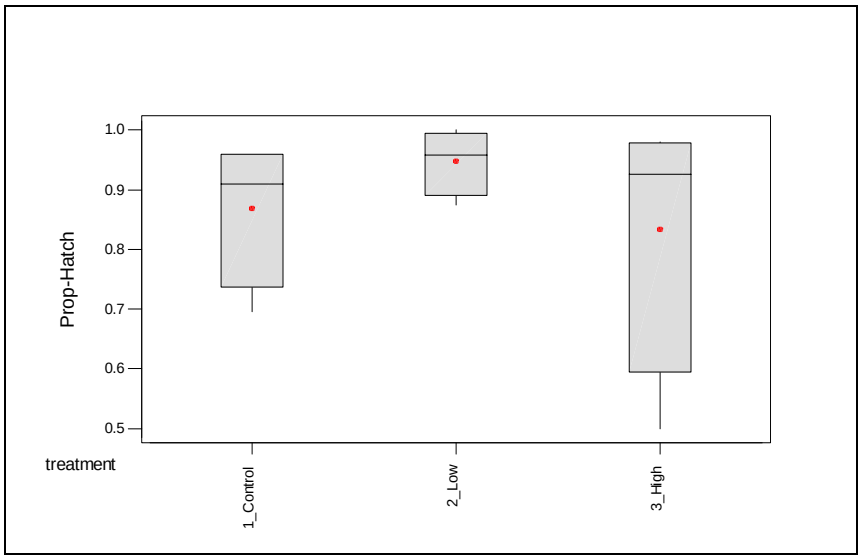

Figure 6.5. Box plot of the proportion of fertile eggs that hatched by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Eggs were collected during the EPA 21-day perchlorate assay for the evaluation of larval development. The proportion of larvae that developed normally (i.e., that showed no morphological abnormalities) ranged from 0.66 to 1.00 in the control and from 0.64 to 1.00 for the low and high concentrations (Figure 6.6). There were no significant differences among treatments in the proportion of larvae that developed normally (Kruskal-Wallis, $H = 3.76$, $p = 0.152$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 18%. The probability of detecting as much as a 20% difference in the proportion of normal larvae was low, 21%, based on the observed variability (Table 6.4).

Table 6.4. Summary statistics and power estimates for the proportion of normal larvae for the EPA 21-day perchlorate assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	0.851	0.176	21%	18%	9%	21%	85%
low	4	0.989	0.023	2%				
high	4	0.839	0.140	17%				

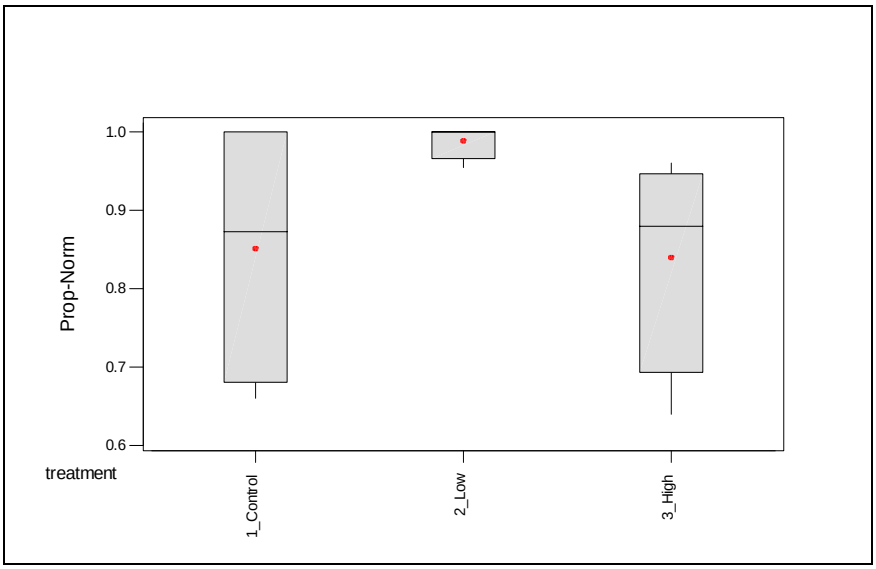

Figure 6.6. Box plot of the proportion of normal larvae by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

6.1.6 Body Weight

The body weight of females used in the EPA 21-day perchlorate assay ranged from 1.6 g to 5.3 g. There were no significant differences in mean body weight among all treatments (Kruskal-Wallis, $H = 4.47$, $p = 0.107$, df = 2). The body weight of males used in the EPA 21-day perchlorate assay ranged from 4.7 g to 11.7 g. There were no significant differences in mean body weight among treatments (Kruskal-Wallis, $H = 4.69$, $p = 0.096$, df = 2).

6.1.7 Gonadosomatic Index

The range of GSI values calculated for females in the all treatments varied from three- to sevenfold (Figure 6.7). The highest value (GSI = 26.0) was obtained for a female from the control. One female exposed to the low concentration had a GSI value of 24.5. No significant differences in the mean GSI value per treatment (Table 6.5) were detected (Kruskal-Wallis, $H = 1.17$, $p = 0.558$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 13%. The probability of detecting as much as a 20% difference in the female GSI was moderate, 58%, based on the observed variability (Table 6.5).

Table 6.5. Summary statistics and power estimates for female GSI data for the EPA 21-day perchlorate assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	16	13.4	5.4	40%	8%	18%	58%	100%
low	14	15.6	6.1	39%				
high	15	13.4	3.7	28%				

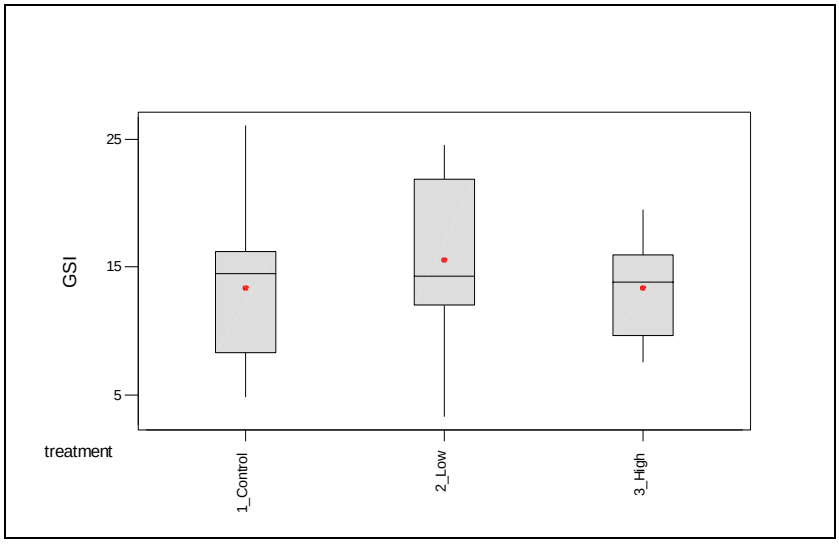

Figure 6.7. Box plot of female GSI by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

The range of most GSI values calculated for males during the EPA 21-day perchlorate assay was small, ranging from 0.8 to 1.8 (Figure 6.8), which approximates the typical range for reproductively-active male fathead minnows. There were no significant differences in mean GSI values (Table 6.6) among treatments (Kruskal-Wallis, $H = 4.65$, $p = 0.098$, df = 2) (Figure 6.8). The achieved power at the observed maximum difference from the control response for this assay was 19%. The probability of detecting as much as a 20% difference in the male GSI was low, 40%, based on the observed variability (Table 6.6).

Table 6.6. Summary statistics and power estimates for male GSI data for the EPA 21-day perchlorate assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	1.16	0.33	28%	13%	13%	40%	99%
low	8	1.47	0.19	13%				
high	7	1.06	0.60	56%				

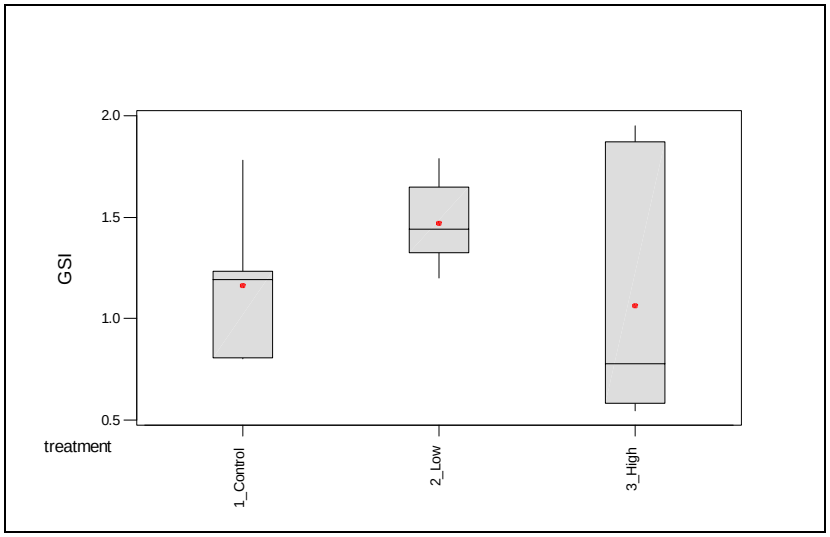

Figure 6.8. Box plot of male GSI by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

6.1.8 Female Gonad Histology

Histological analyses were conducted on the ovaries of 45 females exposed to perchlorate during the EPA 21-day assay.

General Ovary Staging: Statistical analysis of the mean ovarian staging from 18 microscopic fields per female in the EPA 21-day perchlorate assay revealed no significant differences among treatments (Kruskal-Wallis, $H = 3.78$, $p = 0.151$, df = 2).

Quantitative Ovarian Staging: One hundred cells in each of three sections per female were examined to quantitatively determine the developmental stage of the ovaries. Ova from fish from all treatments ranged from Stage 1A to Stage 5 (see Methods for a description of the stages; Figure 6.9). Statistical analyses showed that there was a significant differences among treatments in the proportion of cells occurring in developmental Stage 1A (Table 6.7). The proportion of cells showing this stage of development was higher for females from the control

than for those from the low and high concentrations. No other statistically significant differences in developmental stages were detected.

Table 6.7. Descriptive statistics of the proportion of ovarian cells in each developmental stage for females from the EPA 21-day perchlorate assay and results of the Kruskal-Wallis test (df = 2) comparing treatments

	Control (N = 16)			Low (N= 14)			High (N= 15)			Kruskal-Wallis	
Stage	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	***H***	***p***
1A	0.071	0.022	31%	0.042	0.019	46%	0.048	0.016	34%	12.78	0.002**
1B	0.244	0.062	25%	0.218	0.058	27%	0.226	0.040	18%	1.81	0.405
2	0.234	0.069	30%	0.239	0.059	25%	0.230	0.047	20%	0.80	0.670
3	0.175	0.034	20%	0.173	0.076	44%	0.197	0.064	33%	3.00	0.223
4	0.250	0.108	43%	0.164	0.111	68%	0.179	0.112	62%	4.77	0.092
5	0.002	0.006	275%	0.044	0.083	190%	0.022	0.060	270%	2.85	0.241

** $p < 0.01$.

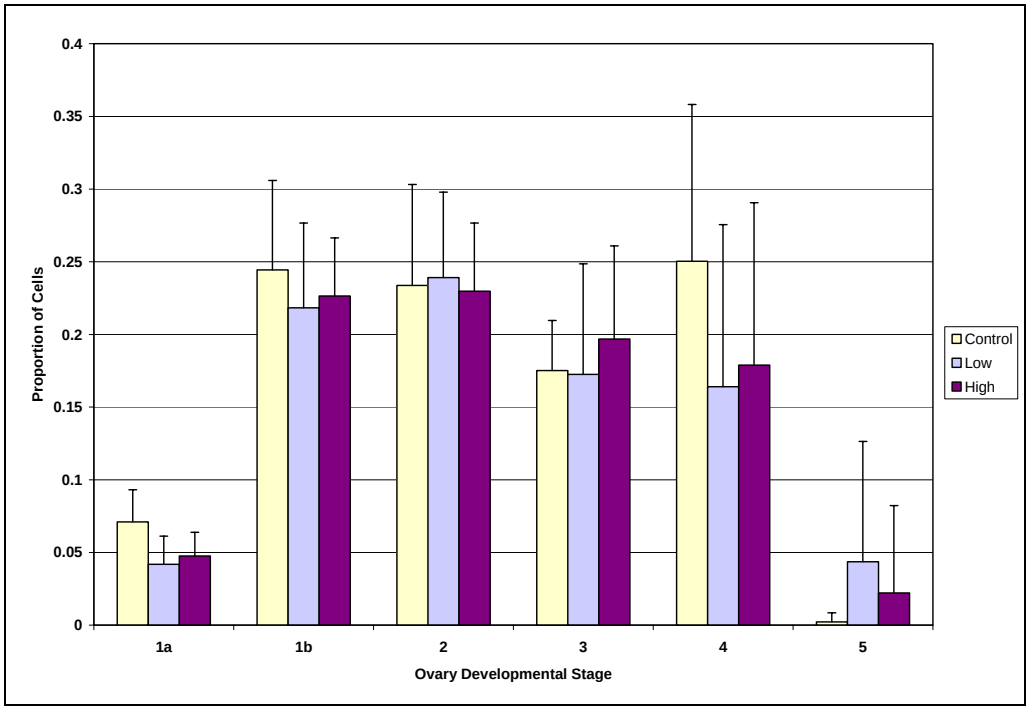

Figure 6.9. Frequency histogram showing the quantitative developmental staging of ovaries for each treatment of the EPA 21-day perchlorate assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

Atretic Follicles: The mean proportion of atretic follicles per 300 follicles (counted per fish) ranged from 0.015 for females from the control to 0.120 follicles for females from the low concentration (Figure 6.10). Some females from the low and high concentrations had high proportions of atretic follicles, ranging to about 0.4. Significant differences in the proportions of

atretic follicles among treatments was detected (Kruskal-Wallis, $H = 14.91$, $p = 0.001$, df = 2). The proportions of atretic follicles in females from the low and high concentrations were greater than those in females from the control.

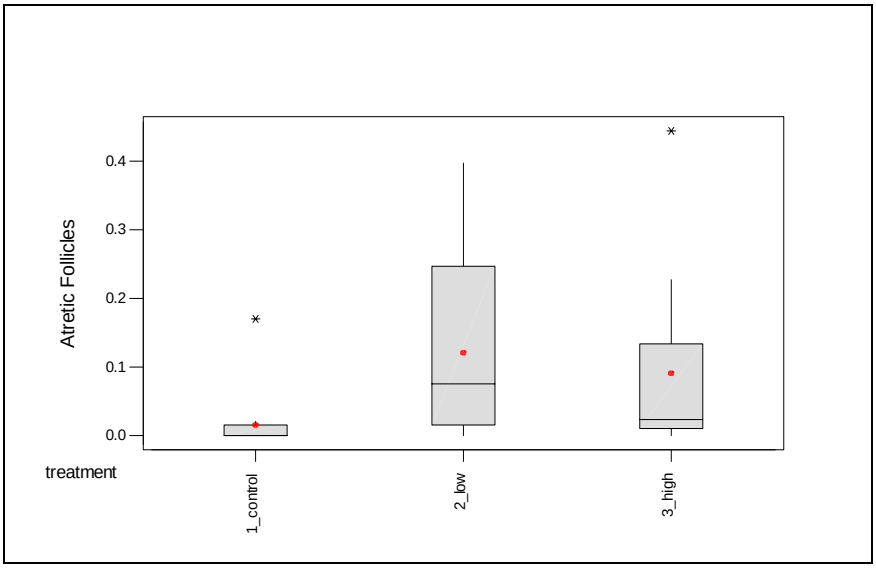

Figure 6.10. Box plot of the proportion of atretic follicles per 300 follicles by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and asterisks represent probable outliers)

Post-Ovulatory Follicles: The mean proportion of post-ovulatory follicles per 300 follicles (counted per fish) ranged from 0.0005 for females from the low concentration to 0.008 for females from the high concentration and the control (Figure 6.11). There were significant differences in the mean proportion of post-ovulatory follicles among treatments (Kruskal-Wallis, $H = 6.06$, $p = 0.048$, df = 2). The proportion of post-ovulatory follicles in females from the low concentration was less than that in females from the control and high concentration. Therefore, this endpoint likely did not show a response to perchlorate dose.

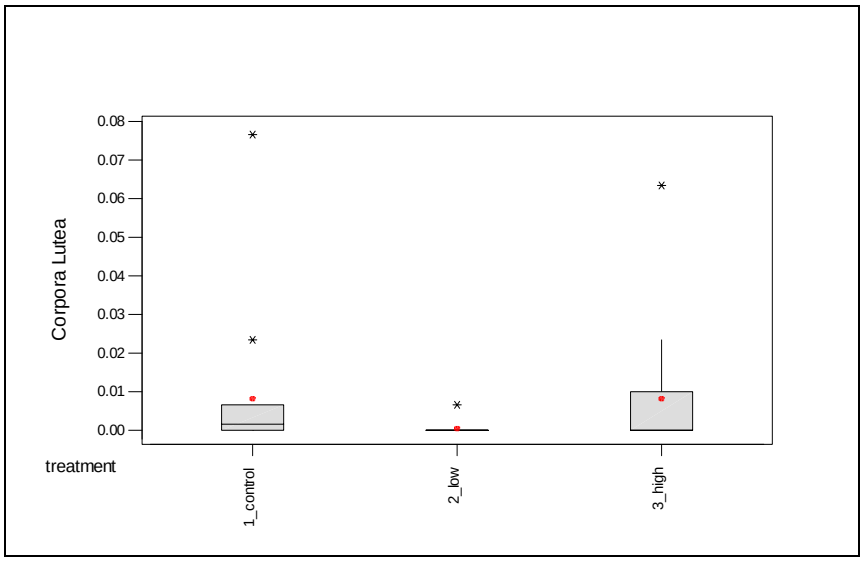

Figure 6.11. Box plot of the proportion of post-ovulatory follicles per 300 follicles by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and asterisks represent probable outliers)

Observations: The ovaries from several fish from each treatment were observed to have histological abnormalities (Table 6.8).

Table 6.8. Histological observations for females exposed to concentrations of perchlorate during the EPA 21-day assay

Fish ID	Treatment	Observations
229294	Control	Multiple foci of macrophage clusters in ovary containing brown material Foci of connective tissue (fibrocytes) in ovary
229298	Control	Multiple foci of macrophage clusters in ovary containing brown material
229304	Control	Stage 2 cells have very tiny cortical alveoli
229312	Control	Multiple foci of macrophage clusters in ovary containing brown material Foci of connective tissue (fibrocytes) in ovary Diffuse macrophage infiltration in ovary
229315	Low	Areas of coalesced atretic follicles in ovary, other areas nearly normal Diffuse macrophage infiltration in ovary
229318	Low	Focal area of one ovary completely comprised of atretic follicles, other areas normal Necrotic cells appear to be unspawned cycle of development while second cycle of development is also present
229322	Low	Multiple foci of macrophage clusters in ovary containing brown material Extensive macrophage infiltration into ovary

Table 6.8. (cont'd)

Fish ID	Treatment	Observations
229335	Low	Focal macrophage infiltration in ovary
229346	High	Multiple foci of macrophage clusters in ovary containing brown material
229352	High	Multiple foci of macrophage clusters in ovary containing brown material One ovary more affected with atretic follicles than other ovary
229357	High	Multiple foci of macrophage clusters in ovary containing brown material
229358	High	Areas of coalesced atretic follicles in ovary, other areas nearly normal
229359	High	Areas of coalesced atretic follicles in ovary, other areas nearly normal
229360	High	Multiple foci of macrophage clusters in ovary containing brown material

6.1.9 Male Gonad Histology

General Testes Staging: Testes from 22 males exposed to perchlorate during the EPA 21-day perchlorate assay were examined to determine the general developmental condition. Males in all treatments had well-developed testes, with most showing Stage 4 and Stage 5 development (see Methods for description of developmental stages). All of the 84 microscopic fields examined in the seven control males analyzed showed Stage 4 (71 fields) or Stage 5 (13 fields) development. All of the 96 microscopic fields examined in the eight low-concentration treatment males showed Stage 4 (72 fields) or Stage 5 (24 fields) development. All of the 84 microscopic fields examined in the seven high-concentration treatment males showed Stage 4 (72 fields) or Stage 5 (12 fields) development. Statistical analysis of the mean staging from 12 microscopic fields per fish revealed no significant differences among treatments (Kruskal-Wallis, $H = 1.76$, $p = 0.414$, df = 2).

Quantitative Testicular Staging: One hundred cells in each of three sections per male were examined to quantitatively determine the developmental condition of the testes. The developmental stage all treatment testes ranged from Stage 2A or 2B to Stage 5 (Figure 6.12). Statistical analyses showed that there were significant differences among treatments in the proportions of cells in developmental Stage 2B (Table 6.9). The mean proportion of cells showing developmental Stage 2B was greater in males from the high concentration than from the low concentration, but not from the control. Therefore, this response was not related to perchlorate dose.

Table 6.9. Descriptive statistics of the proportion of testes cells in each developmental stage for males from the EPA 21-day perchlorate assay and results of the Kruskal-Wallis test (df = 2) comparing treatments

	Control (N= 7)			Low (N= 8)			High (N= 7)			Kruskal-Wallis	
Stage	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	***H***	***p***
1	0	0	0%	0	0	0%	0	0	0%	–	–
2a	0.006	0.005	87%	0.008	0.004	48%	0.009	0.006	67%	1.36	0.505
2b	0.013	0.007	52%	0.004	0.007	187%	0.027	0.024	89%	7.41	0.025*
3a	0.169	0.090	53%	0.117	0.080	68%	0.228	0.121	53%	3.68	0.159
3b	0.268	0.094	35%	0.195	0.114	58%	0.220	0.126	57%	1.79	0.408
4	0.227	0.073	32%	0.180	0.093	52%	0.198	0.099	50%	0.88	0.643
5	0.317	0.213	67%	0.496	0.223	45%	0.319	0.278	87%	5.11	0.078

* $p < 0.05$.

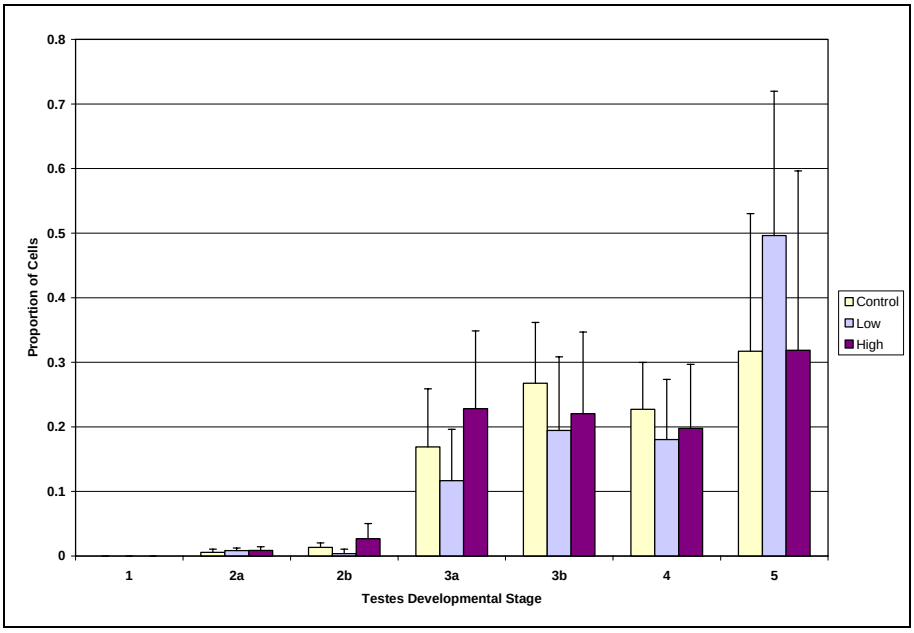

Figure 6.12. Frequency histogram showing the quantitative developmental staging of testes for each treatment of the EPA 21-day perchlorate assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

Tubule Diameter: The average diameter of the seminiferous tubules of males from the control ranged from 118.6 µm to 150.8 µm (Figure 6.13). Tubule diameters of males from the two test concentrations ranged from 92.2 µm to 256.4 µm. No significant differences in the mean tubule diameter per treatment (Table 6.10) were detected (Kruskal-Wallis, $H = 2.77$, $p = 0.251$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 18%. The probability of detecting as much as a 20% difference in the male seminiferous tubule diameter was high, 100%, based on the observed variability (Table 6.10).

Table 6.10. Summary statistics and power estimates for male seminiferous tubule diameter data for the EPA 21-day perchlorate assay

Level	N	Mean (μm)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	134.9	12.3	9%	3%	96%	100%	100%
low	8	161.5	46.6	29%				
high	7	144.2	31.7	22%				

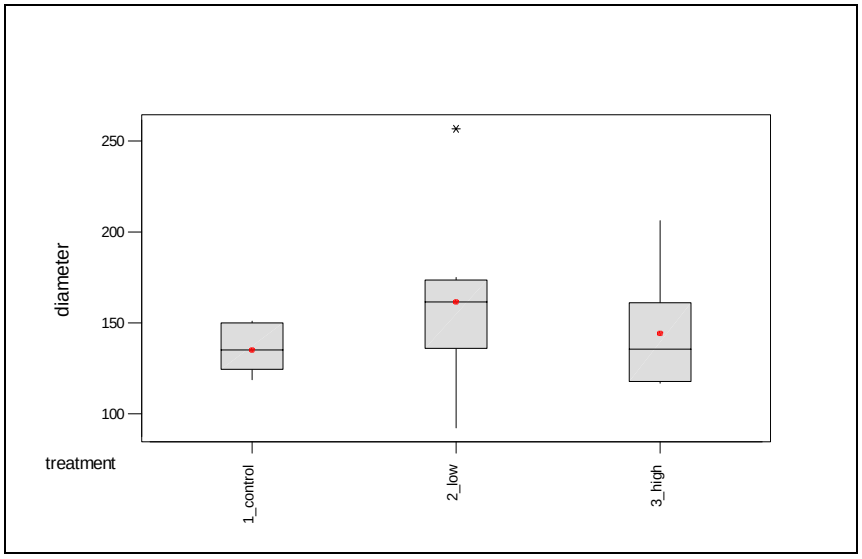

Figure 6.13. Box plot of male seminiferous tubule diameter (μm) by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

Observations: Sertoli cell proliferation was observed among two males from the control (Table 6.11). Leydig cell proliferation also was observed among at least one male from all treatments. Some males in each treatment showed other histological abnormalities (Table 6.11). No testicular atrophy was recorded and no ovatestes were observed for any treatment.

Table 6.11. Histological observations for males exposed to concentrations of perchlorate during the EPA 21-day assay

Fish ID	Treatment	Sertoli Cell Proliferation	Leydig Cell Proliferation	Observations
229290	Control	None	None	Basophilic cysts in ducts
229295	Control	Yes	Yes	Mild multifocal proliferation of interstitial cells, but confined to one area of testis
229307	Control	Yes	Yes	Mild multifocal proliferation of interstitial cells A few clusters of necrotic stage 3B cells
229325	Low	None	None	One testis and one kidney in each section
229326	Low	None	None	Areas of interstitial cell cysts with sequestered Stage 5 cells
229332	Low	None	Yes	Multifocal proliferation of Leydig cells Multifocal but sparse clusters of necrotic Stage 4 cells A few foci of light staining nonspermatic cells in tubules
229338	High	None	Yes	Multifocal proliferation of Leydig cells
229343	High	None	Yes	Multifocal proliferation of Leydig cells
229349	High	None	Yes	Multifocal proliferation of Leydig cells
229356	High	None	Yes	Multifocal proliferation of Leydig cells

6.1.10 Vitellogenin

VTG concentrations in control females used during the EPA 21-day perchlorate assay ranged from 5.5 mg/mL to 11.6 mg/mL (Figure 6.14). Among females exposed to the two perchlorate concentrations, VTG concentrations ranged from 4.2 mg/mL to 13.4 mg/mL. No significant differences in the mean VTG concentration per treatment (Table 6.12) were detected (Kruskal-Wallis, $H = 0.56$, $p = 0.755$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 8%. The probability of detecting as much as a 50% difference in the female VTG concentrations was high, 100%, based on the observed variability (Table 6.12).

Table 6.12. Summary statistics and power estimates for female VTG concentrations for the EPA 21-day perchlorate assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	8.28	1.97	24%	5%	21%	67%	100%
low	7	8.44	3.39	40%				
high	8	7.53	2.54	34%				

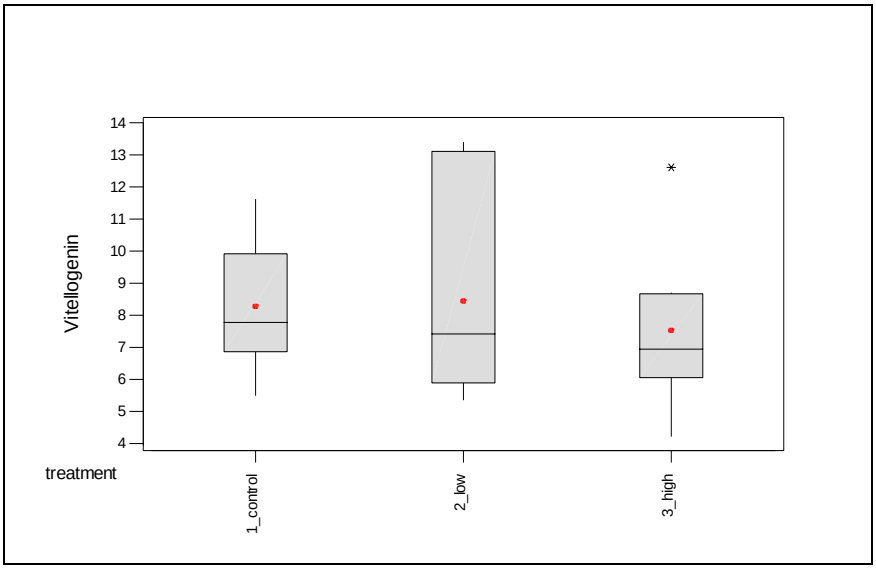

Figure 6.14. Box plot of female VTG concentration (mg/mL) by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

VTG concentrations in control males used during the EPA 21-day perchlorate assay ranged from 0.001 mg/mL to 0.060 mg/mL (Figure 6.15). Among males exposed to the two perchlorate concentrations, VTG concentrations ranged from 0 mg/mL (not detected) to 0.375 mg/mL. No significant differences in the mean VTG concentration per treatment (Table 6.13) were detected (Kruskal-Wallis, $H = 0.29$, $p = 0.864$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 13%. The probability of detecting as much as a 50% difference in the male VTG concentrations was low, 5%, based on the observed variability (Table 6.13).

Table 6.13. Summary statistics and power estimates for male VTG concentrations for the EPA 21-day perchlorate assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	0.012	0.022	175%	335%	5%	5%	5%
low	8	0.060	0.130	217%				
high	7	0.028	0.047	170%				

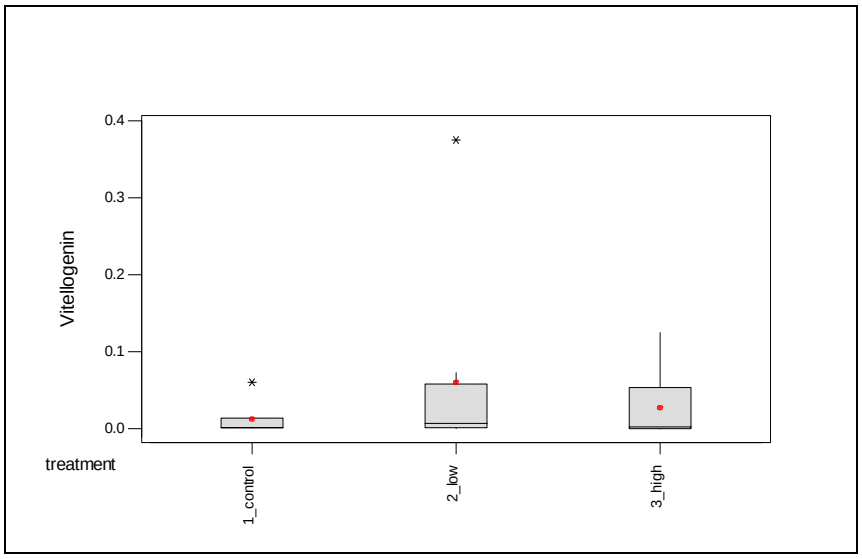

Figure 6.15. Box plot of male VTG concentration (mg/mL) by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

6.1.11 Plasma Steroid Concentrations

Estradiol: Estradiol concentrations in control females used during the EPA 21-day perchlorate assay ranged from 1.26 ng/mL to 2.47 ng/mL (Figure 6.16). Among females exposed to the two-perchlorate concentrations, estradiol concentrations ranged from 0 ng/mL (not detected) to 4.77 ng/mL. No significant differences in the mean estradiol concentration per treatment (Table 6.14) were detected (Kruskal-Wallis, $H = 0.00$, $p = 0.998$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 5%. The probability of detecting as much as a 50% difference in the female estradiol concentrations was low, 40%, based on the observed variability (Table 6.14).

Table 6.14. Summary statistics and power estimates for female estradiol concentrations for the EPA 21-day perchlorate assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	1.79	0.41	23%	4%	6%	10%	40%
low	7	1.86	1.20	65%				
high	7	2.15	1.79	83%				

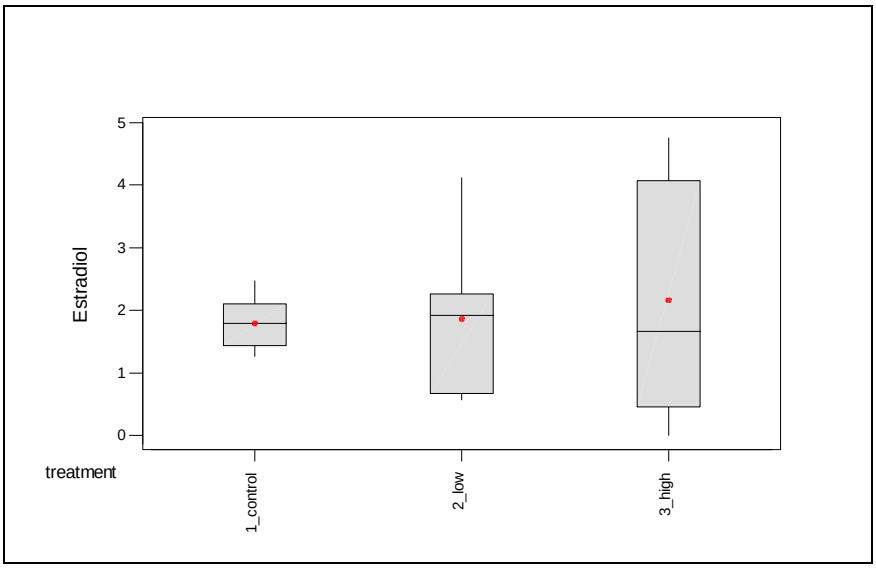

Figure 6.16. Box plot of female estradiol concentration (ng/mL) by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Estradiol was detected in only 5 of the 22 male plasma samples from the EPA 21-day perchlorate assay. One control male had an estradiol concentration of 0.442 ng/mL; two low-concentration males had estradiol concentrations of 0.323 ng/mL and 0.410 ng/mL; and two high-concentration males had estradiol concentrations of 0.253 ng/mL and 0.351 ng/mL.

Testosterone: Testosterone concentrations in control females used during the EPA 21-day perchlorate assay ranged from 0.545 ng/mL to 1.31 ng/mL (Figure 6.17). Among females exposed to the two perchlorate concentrations, testosterone concentrations ranged from 0 ng/mL (not detected) to 1.38 ng/mL. No significant differences in the mean testosterone concentration per treatment (Table 6.15) were detected (Kruskal-Wallis, $H = 0.12$, $p = 0.940$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 8%. The probability of detecting as much as a 50% difference in female testosterone concentration was moderate, 61%, based on the observed variability (Table 6.15).

Table 6.15. Summary statistics and power estimates for female testosterone concentrations for the EPA 21-day perchlorate assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.822	0.240	29%	11%	7%	13%	61%
low	7	0.786	0.307	39%				
high	7	0.748	0.452	60%				

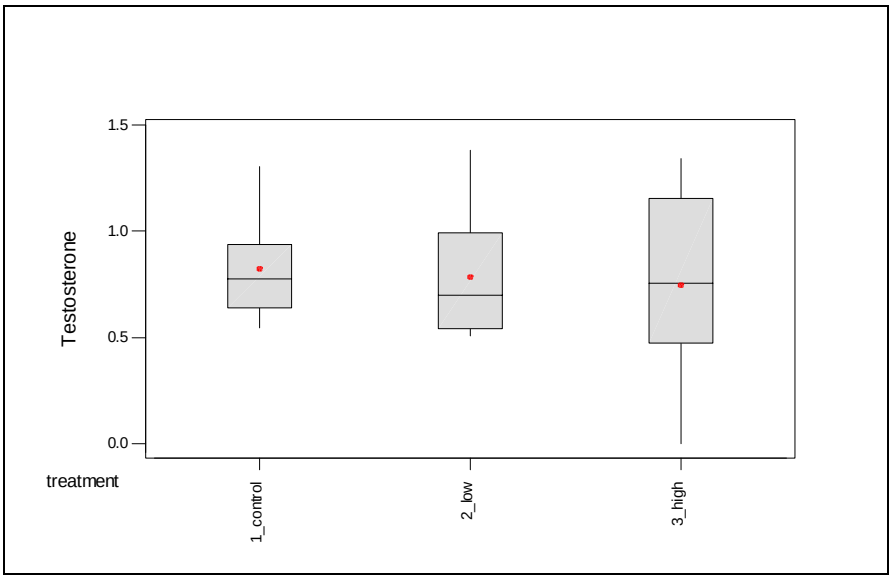

Figure 6.17. Box plot of female testosterone concentration (ng/mL) by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Testosterone concentrations in control males used during the EPA 21-day perchlorate assay ranged from 1.14 ng/mL to 4.93 ng/mL (Figure 6.18). Among males exposed to the two perchlorate concentrations, testosterone concentrations ranged from 1.14 ng/mL to 7.75 ng/mL. Significant differences in the mean testosterone concentration per treatment (Table 6.16) were detected (Kruskal-Wallis, $H = 7.46$, $p = 0.024$, df = 2). The mean testosterone concentration in males from the low concentration was greater than that in males from the control. The achieved power at the observed maximum difference from the control response for this assay was 71%.

Table 6.16. Summary statistics and power estimates for male testosterone concentrations for the EPA 21-day perchlorate assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	2.38	1.30	55%	53%	7%	14%	65%
low	8	5.19	1.87	36%				
high	7	3.55	1.99	56%				

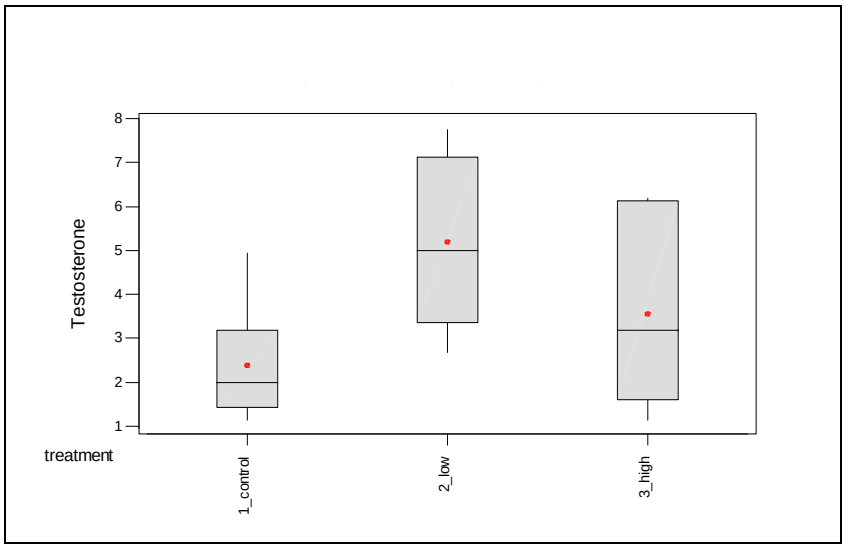

Figure 6.18. Box plot of male testosterone concentration (ng/mL) by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

11-ketotestosterone: 11-ketotestosterone was not detected in females from the control and the low concentration during the EPA 21-day perchlorate assay. However, 11-ketotestosterone was detected in two of the eight female samples from the high concentration (0.797 ng/mL, 0.532 ng/mL).

11-ketotestosterone concentrations in control males used during the EPA 21-day perchlorate assay ranged from 4.54 ng/mL to 39.9 ng/mL (Figure 6.19). Among males exposed to the two perchlorate concentrations, 11-ketotestosterone concentrations ranged from 3.19 ng/mL to 65.0 ng/mL. A significant difference in the mean 11-ketotestosterone concentration per treatment (Table 6.17) was detected (Kruskal-Wallis, $H = 6.05$, $p = 0.049$, df = 2). The mean 11-testosterone concentration in males from the low concentration was greater than that in males from the control. The achieved power at the observed maximum difference from the control response for this assay was 58%.

Table 6.17. Summary statistics and power estimates for male 11-ketotestosterone concentrations for the EPA 21-day perchlorate assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	13.5	12.6	94%	47%	7%	14%	64%
low	8	40.7	19.9	49%				
high	7	22.4	21.9	98%				

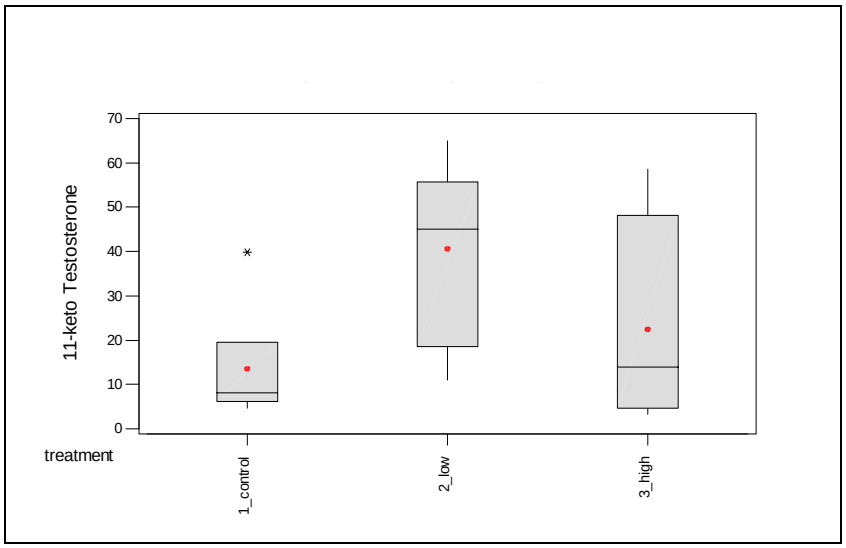

Figure 6.19. Box plot of male 11-ketotestosterone concentration (ng/mL) by treatment for the EPA 21-day perchlorate assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

7.0 RESULTS: CADMIUM CHLORIDE

The results of the EPA 21-day assay for cadmium chloride are presented in this section. Total fecundity is reported as the total number of eggs laid during the assay, regardless of egg condition. However, some eggs could not be included in the evaluation of fertilization because of physical damage or the presence of a fungal infection. Summary tables are presented for the major endpoints. The values presented were calculated before being rounded for inclusion in the tables. Values reported include the mean for each treatment, the number of samples (N), the standard deviation (SD), and the coefficient of variation (CV). Values reported for the statistical analyses are the test statistic for the Kruskal-Wallis test used to determine the significance level (*H*), the probability that the observed result was due to chance (*p*), and the degrees of freedom associated with the test (df).

7.1 EPA 21-Day Assay for Cadmium Chloride

The EPA 21-day cadmium chloride assay was conducted from June 17, 2003 to June 24, 2003 (pre-exposure assay), and from June 24, 2003 to July 15, 2003 (exposure assay).

7.1.1 Cadmium Chloride Concentrations

Cadmium chloride was detected at concentrations above the MDL (0.023 µg/L) in one control tank (tank 18) throughout the EPA 21-day cadmium chloride exposure period. For no greater than 6 days, this replicate had 755 of the low target dose. The concentration in that tank was 0.750 µg/L on Day 0, declined to about 0.56 µg/L and 0.54 µg/L on Days 6 and 13, and was slightly greater than the MDL on Day 21 (0.028 µg/L). Two other control tanks had cadmium chloride concentrations greater than the MDL on Day 0 (0.067 µg/L in tank 14; 0.031 µg/L in tank 1). Concentrations in these two tanks were below the MDL on the remaining sampling days. The mean (standard deviation) in the low concentration and high concentration were 1.2 (0.2) µg/L and 11.0 (0.3) µg/L, respectively (Figure 7.1).

Statistical analysis did not detect a low or high dose effect for any of the variables measured. The control CVs were greater than the low dose CVs for three variables (total eggs, total eggs/reproductive day, and female GSI). Only two p-values were less than 0.1 (ovarian staging and proportion of post-ovulatory follicles). The analysis for post-ovulatory follicles was redone and was not significant with or without a potential control dose outlier.

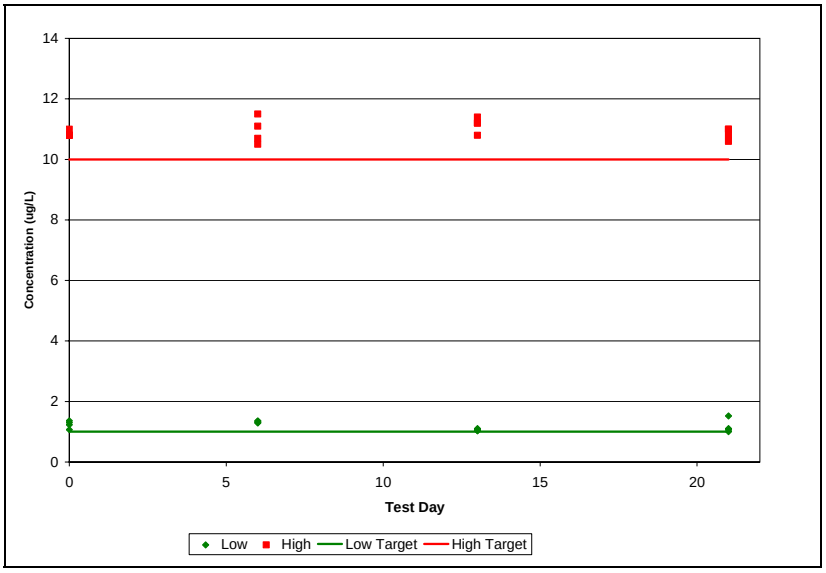

Figure 7.1. Cadmium chloride concentrations during the EPA 21-day cadmium chloride assay

7.1.2 Survival

There was no death in the control tanks. One female in the low concentration and one female and one male in the high concentration died during the assay. All other males and females in all treatments survived the EPA 21-day cadmium chloride assay.

7.1.3 Fecundity

Total Fecundity: A 7-day pre-exposure evaluation of total egg production was performed. Total 7-day counts among the three treatments in the exposure assay (individual tank values summed for each treatment) ranged from about 7700 eggs to 9100 eggs (Figure 7.2). No significant differences in the mean 7-day egg production among the groups of replicates evaluated during the pre-exposure assay were detected (Kruskal-Wallis, $H = 1.38$, $p = 0.500$, df = 2).

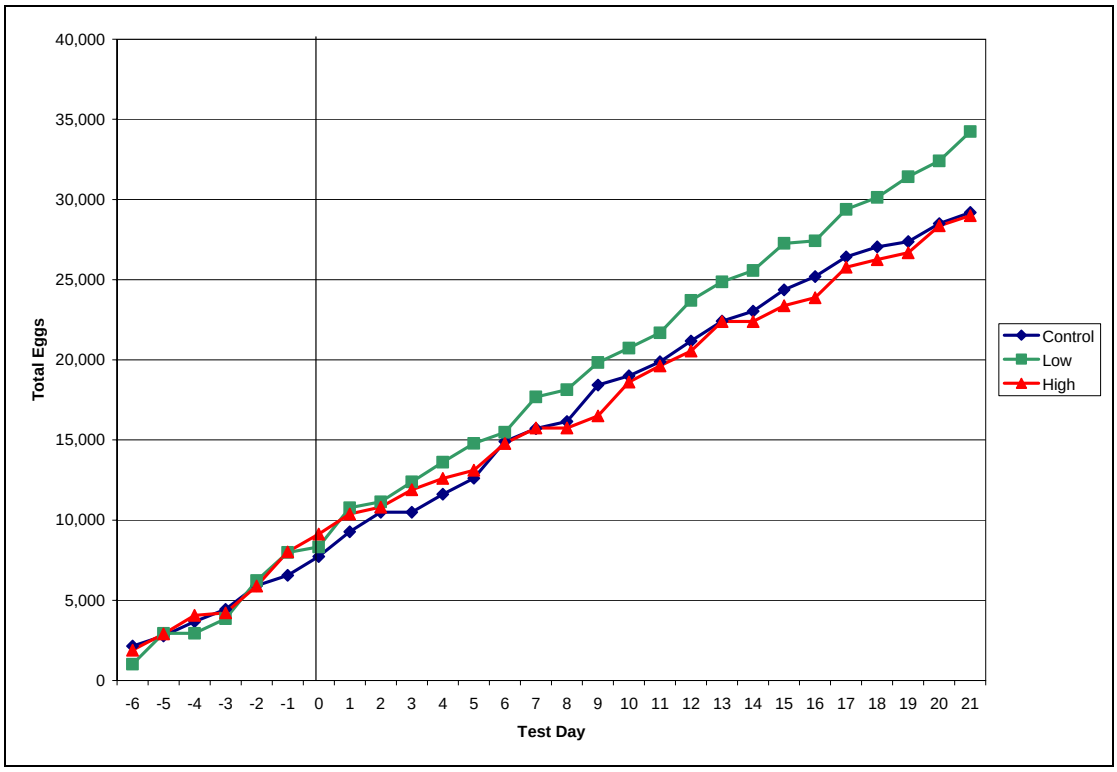

Figure 7.2. Total egg production per treatment for the cadmium chloride assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to cadmium chloride)

During the EPA 21-day cadmium chloride assay, total eggs counts in the control varied from 3088 eggs to 6313 eggs (Figure 7.3). Total egg production among low-concentration replicates was similar, ranging from 5349 eggs to 7350 eggs. Total counts among the high-concentration replicates varied from 2400 eggs to 8249 eggs. Statistical analysis of square-root transformed egg counts showed no significant among-treatment differences (Kruskal-Wallis, $H = 1.85$, $p = 0.397$, df = 2) in mean total numbers of eggs produced (Table 7.1). The achieved power at the observed maximum difference from the control response for this assay was 9%. The probability of detecting as much as a 20% difference in the total fecundity was low, 21%, based on the observed variability (Table 7.1).

Table 7.1. Summary statistics and power estimates for total fecundity data for the EPA 21-day cadmium chloride assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	4	5365	1533	29%	11%	9%	21%	85%
low	4	6478	831	13%				
high	4	4967	2633	53%				

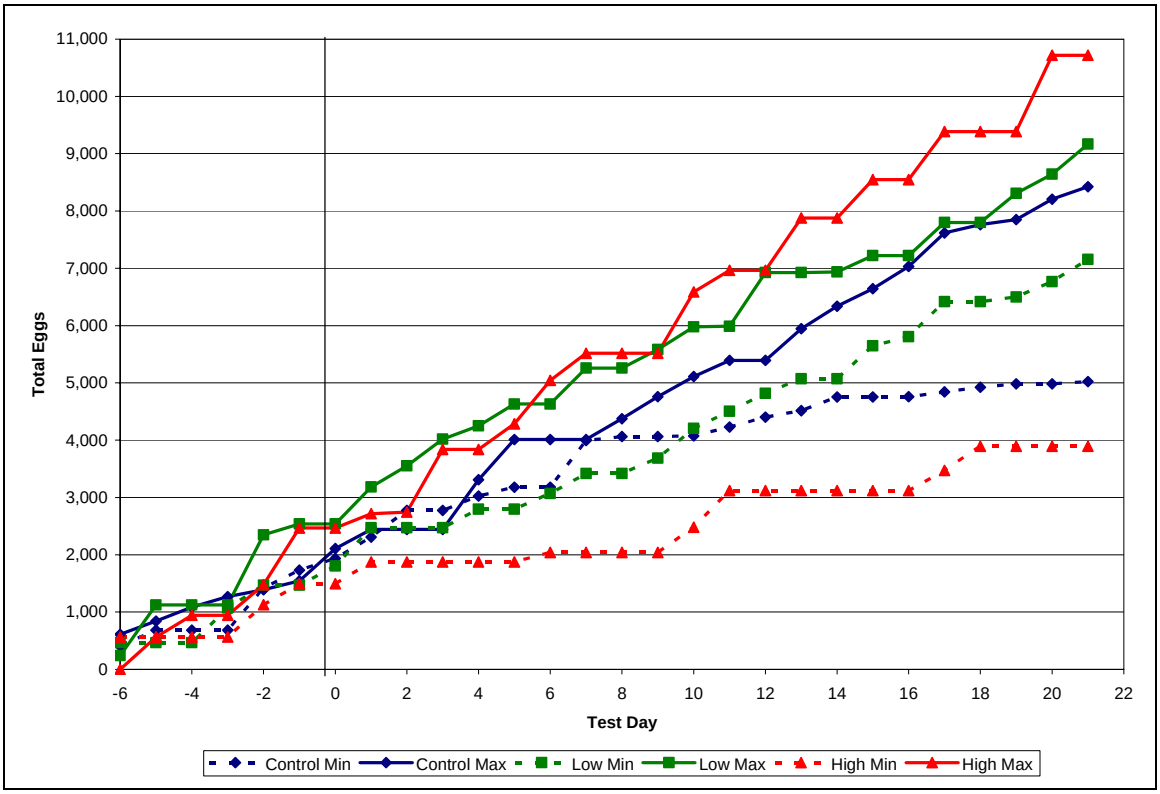

Figure 7.3. Range in replicate total egg production per treatment for the cadmium chloride assay (negative test days are prior to the exposure; Day 0 marks the initiation of the 21-day exposure to cadmium chloride)

Fecundity per Female Reproductive Day: During the 7-day pre-exposure evaluation, the mean number of eggs produced per female reproductive day ranged from 69.0 eggs/day for the tanks that would be used for the control to 81.5 eggs/day for the tanks that would be used for the high concentration during the 21-day exposure assay. There were no significant differences among treatments in the mean numbers of eggs produced per reproductive day during the pre-exposure period (Kruskal-Wallis, $H = 1.38$, $p = 0.500$, df = 2).

During the EPA 21-day cadmium chloride assay, the maximum number of female reproductive days was achieved for controls (Table 7.2). One female in the low concentration died at day 21 of the testing. The number of reproductive days achieved for the high concentration was substantially less than the maximum possible, because one female died at Day 17 of the testing, and two individuals that were thought to be females at the time of test initiation were subsequently determined to be males (fish 22959 from tank 7; fish 22965 from tank 13). The number of eggs produced per female reproductive day varied from 36.8 eggs to 75.2 eggs in the control and from 63.7 eggs to 88.6 in the low concentration (Figure 7.4). For the high concentration, the number of eggs produced per female reproductive day ranged from 38.1 eggs to 98.2 eggs. No significant differences among treatments in the mean number of eggs produced per day were detected (Kruskal-Wallis, $H = 1.94$, $p = 0.379$, df = 2). The achieved power at the

observed maximum difference from the control response for this assay was 11%. The probability of detecting as much as a 20% change in the eggs per female reproductive day was low, 26%, based on the observed variability (Table 7.2).

Table 7.2. Summary statistics and power estimates for fecundity per female reproductive day for the EPA 21-day cadmium chloride assay

Level	Mean Number of Reproductive Days[(a)]	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	84.0	4	63.9	18.3	29%	11%	10%	26%	94%
low	83.75	4	77.4	10.3	13%				
high	72.25	4	65.9	26.1	40%				

a) Maximum number = 84.

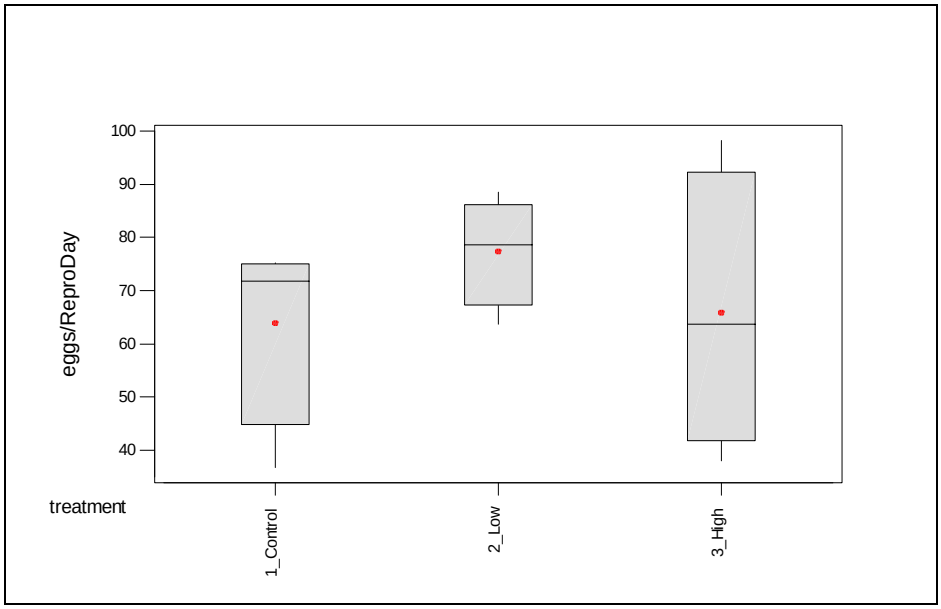

Figure 7.4. Box plot of the number of eggs produced per female reproductive day by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Eggs on Tiles/Dishes: The mean number of eggs laid on the tiles during the 7-day pre-exposure assay varied from 1218 eggs for the tanks that would be used for the control to 1813 eggs for the tanks that would be used for the high concentration. The mean number of eggs on dishes ranged from 468 eggs for the low concentration to 715 eggs for the control. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated. There were

significant differences in the mean proportional difference among treatments during the 7-day pre-exposure assay (Kruskal-Wallis, $H = 6.73$, $p = 0.035$, df = 2). Proportionally more eggs occurred on tiles in tanks that would be used for the low and high concentrations than in tanks that would be used for the control.

The mean number of eggs laid on tiles among the treatments during the EPA 21-day cadmium chloride assay ranged from 579 eggs for the high concentration to 1200 eggs for the control (Appendix G, Table G.2). The mean number of eggs laid on the dishes varied from 4165 eggs for the control to 5583 eggs for the low concentration. Because of the variability in the total number of eggs laid per treatment, the proportional difference in the number of eggs on dishes versus those on tiles [1–(# eggs on dishes ÷ # eggs on tiles)] was calculated . There were no significant differences in the mean proportional difference among treatments during the 21-day exposure assay (Kruskal-Wallis, $H = 4.19$, $p = 0.123$, df = 2).

7.1.4 Fertilization Success

Total Fertilization: Eggs were collected during the 7-day pre-exposure period for the evaluation of fertilization success rate. All undamaged eggs laid during this evaluation were fertilized, except for one egg from the low concentration and two eggs from the high concentration.

All undamaged eggs in all replicates of all treatments during the EPA 21-day cadmium chloride assay were fertilized.

Fertilization of Eggs on Tiles and Dishes: During the 7-day pre-exposure assay, all undamaged eggs laid on dishes were fertilized. The three eggs that were not fertilized were on tiles. All undamaged eggs laid on tiles or on dishes during the EPA 21-day cadmium chloride assay were fertilized.

7.1.5 Hatchability and Larval Development

Eggs were collected during the EPA 21-day cadmium chloride assay for the evaluation of hatchability. The larval hatching study was completed by incubating 50 eggs per replicate in dilution water for 3 to 6 days until the majority of eggs hatched. The proportion of fertilized eggs that hatched ranged from 0.92 to 1.00 in the control and from 0.82 to 1.00 for the low and high concentrations (Figure 7.5). No significant differences among treatments in the proportion of eggs that hatched (Table 7.3) were detected (Kruskal-Wallis, $H = 1.34$, $p = 0.512$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 22%. The probability of detecting as much as a 20% difference in the proportion of fertile eggs that hatched was high, 96%, based on the observed variability (Table 7.3).

Table 7.3. Summary statistics and power estimates for the proportion of fertile eggs that hatched for the EPA 21-day cadmium chloride assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	0.960	0.039	4%	7%	42%	96%	100%
low	8	0.947	0.039	4%				
high	8	0.917	0.062	7%				

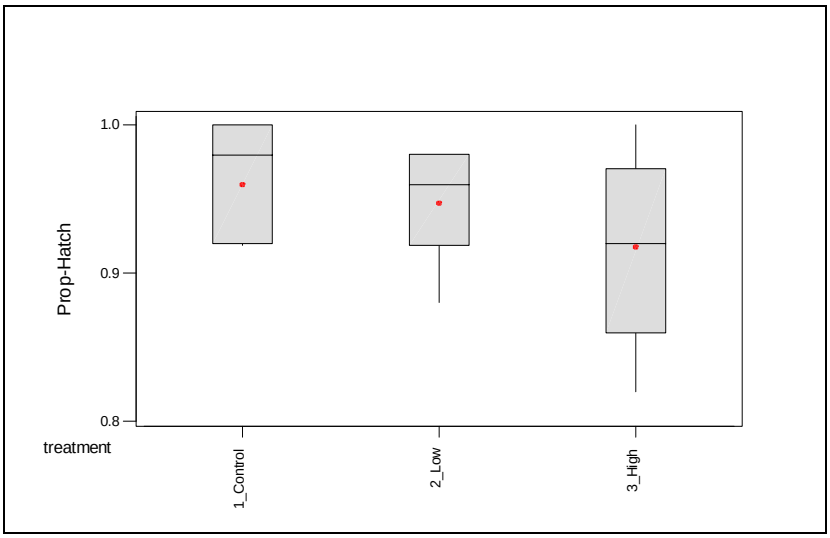

Figure 7.5. Box plot of the proportion of fertile eggs that hatched by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Eggs were collected during the EPA 21-day cadmium chloride assay for the evaluation of larval development. The proportion of larvae that developed normally (i.e., that showed no morphological abnormalities) ranged from 0.91 to 1.00 in the control and from 0.67 to 1.00 for the low and high concentrations (Figure 7.6). There were no significant differences among treatments in the proportion of larvae that developed normally (Kruskal-Wallis, $H = 1.01$, $p = 0.605$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 12%. The probability of detecting as much as a 20% difference in the proportion of normal larvae was moderate, 66%, based on the observed variability (Table 7.4).

Table 7.4. Summary statistics and power estimates for the proportion of normal larvae for the EPA 21-day cadmium chloride assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	7	0.972	0.037	4%	7%	21%	66%	100%
low	8	0.909	0.129	14%				
high	8	0.943	0.058	6%				

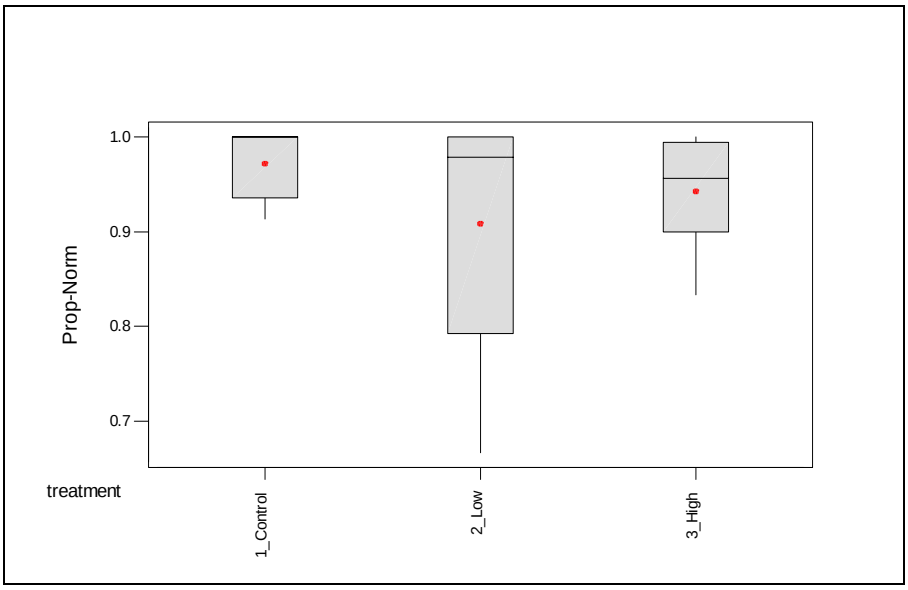

Figure 7.6. Box plot of the proportion of normal larvae by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

7.1.6 Body Weight

The body weight of females used in the EPA 21-day cadmium chloride assay ranged from 2.0 g to 6.5 g. There were no significant differences in mean body weight among all treatments (Kruskal-Wallis, H = 0.68, p = 0.710, df = 2). The body weight of males used in the EPA 21-day cadmium chloride assay ranged from 3.2 g to 11.0 g. There were no significant differences in mean body weight among treatments (Kruskal-Wallis, H = 1.68, p = 0.432, df = 2).

7.1.7 Gonadosomatic Index

The range of GSI values calculated for females in the all treatments varied about threefold (Figure 7.7). The highest value (GSI = 23.3) was obtained for a female from the high concentration. No significant differences in the mean GSI value per treatment (Table 7.5) were detected (Kruskal-Wallis, H = 3.04, p = 0.218, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 25%. The probability of

detecting as much as a 20% difference in the female GSI was moderate, 72%, based on the observed variability (Table 7.5).

Table 7.5. Summary statistics and power estimates for female GSI data for the EPA 21-day cadmium chloride assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	16	10.5	3.4	32%	11%	23%	72%	100%
low	15	10.5	3.1	29%				
high	13	12.8	4.4	35%				

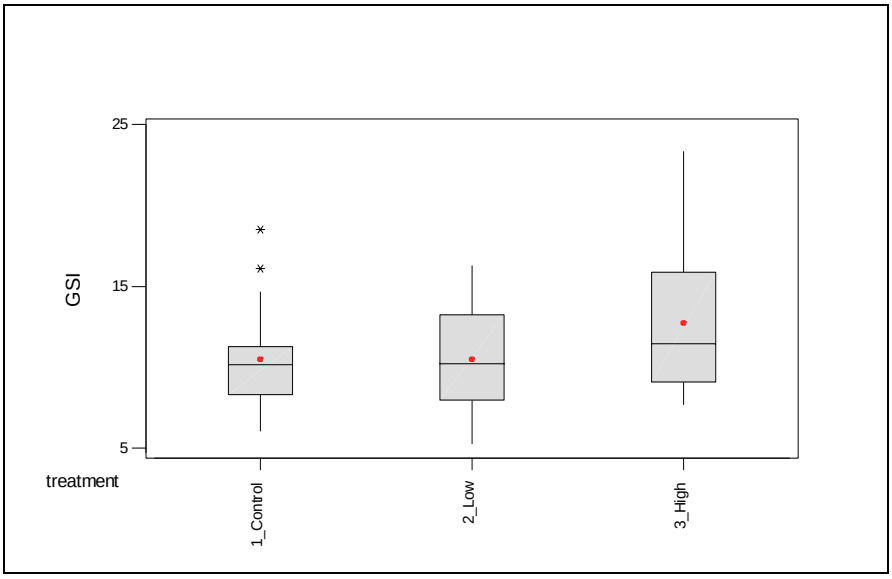

Figure 7.7. Box plot of female GSI by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represents probable outliers)

The range of most GSI values calculated for males during the EPA 21-day cadmium chloride assay, was small, ranging from 0.8 to 1.9 (Figure 7.8), which approximates the typical range for reproductively-active male fathead minnows. There were no significant differences in mean GSI values (Table 7.6) among treatments (Kruskal-Wallis, $H = 1.91$, $p = 0.384$, df = 2) (Figure 7.8). The achieved power at the observed maximum difference from the control response for this assay was 15%. The probability of detecting as much as a 20% difference in male GSI was low, 39%, based on the observed variability (Table 7.6).

Table 7.6. Summary statistics and power estimates for male GSI data for the EPA 21-day cadmium chloride assay

Level	N	Mean	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	1.17	0.35	30%	11%	13%	39%	99%
low	8	1.45	0.44	31%				
high	9	1.18	0.52	44%				

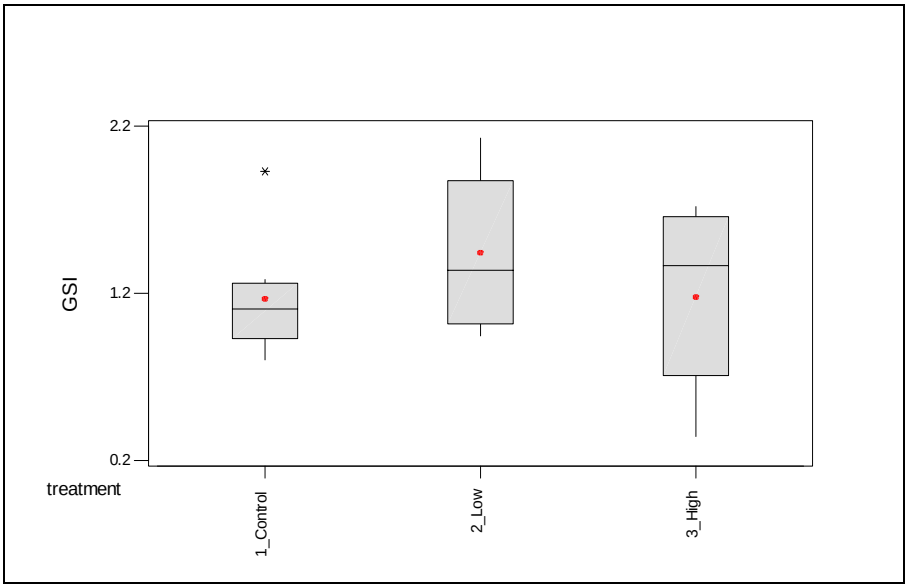

Figure 7.8. Box plot of male GSI by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

7.1.8 Female Gonad Histology

Histological analyses were conducted on the ovaries of 44 females exposed to cadmium chloride during the EPA 21-day assay.

General Ovary Staging: Statistical analysis of the mean ovarian staging from 12 microscopic fields per female in the EPA 21-day cadmium chloride assay revealed no significant differences among treatments (Kruskal-Wallis, $H = 4.76$, $p = 0.093$, df = 2).

Quantitative Ovarian Staging: One hundred cells in each of three sections per female were examined to quantitatively determine the developmental stage of the ovaries. Ova from fish from all treatments ranged from Stage 1A to Stage 5 (Figure 7.9) (see Methods for a description

of the stages). Statistical analyses showed that there were no significant differences among treatments in the proportion of cells occurring in any developmental stage (Table 7.7).

Table 7.7. Descriptive statistics of the proportion of ovarian cells in each developmental stage for females from the EPA 21-day cadmium chloride assay and results of the Kruskal-Wallis test (df = 2) comparing treatments

	Control (N = 16)			Low (N = 15)			High (N = 13)			Kruskal-Wallis	
Stage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	*H*	*p*
1A	0.063	0.038	61%	0.060	0.026	43%	0.048	0.027	56%	1.29	0.526
1B	0.210	0.059	28%	0.272	0.084	31%	0.235	0.090	38%	4.09	0.129
2	0.193	0.058	30%	0.213	0.052	25%	0.181	0.039	22%	2.07	0.356
3	0.187	0.054	29%	0.182	0.055	30%	0.193	0.061	31%	0.09	0.956
4	0.229	0.103	45%	0.228	0.051	22%	0.277	0.082	30%	2.57	0.276
5	0.071	0.126	176%	0.003	0.005	165%	0.037	0.132	361%	5.02	0.081

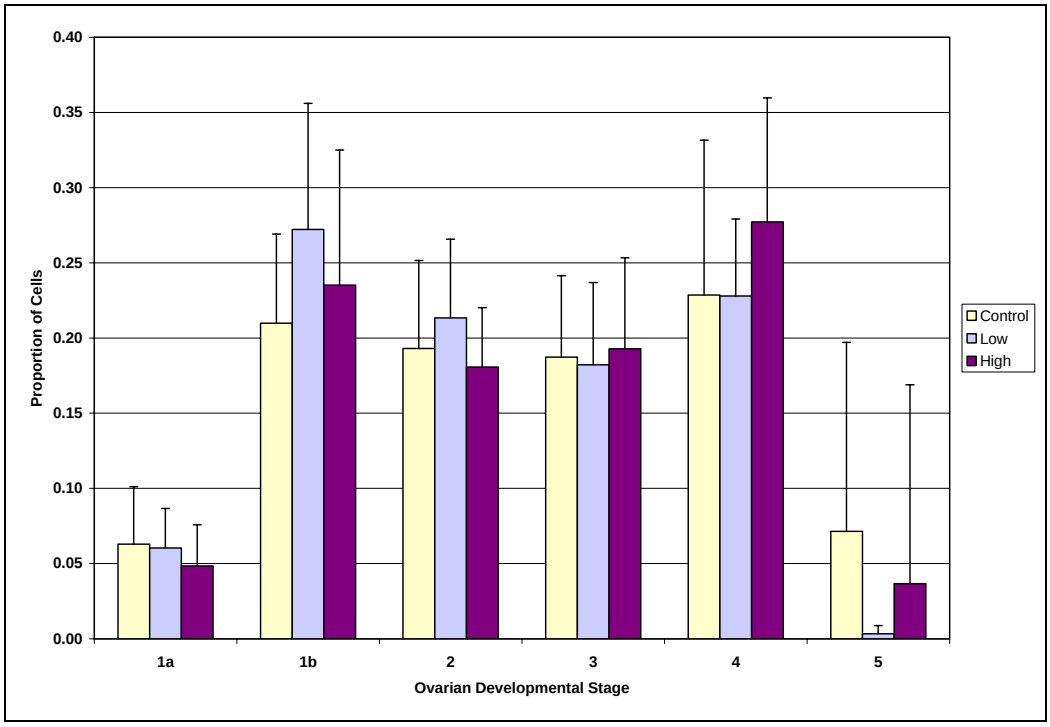

Figure 7.9. Frequency histogram showing the quantitative developmental staging of ovaries for each treatment of the EPA 21-day cadmium chloride assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

***Atretic Follicles*:** The mean proportion of atretic follicles per 300 follicles (counted per fish) ranged from 0.015 for females from the control to 0.033 follicles for females from the low concentration (Figure 7.10). No significant differences in the proportions of atretic follicles among treatments was detected (Kruskal-Wallis, $H = 4.24$, p 0.120, df = 2).

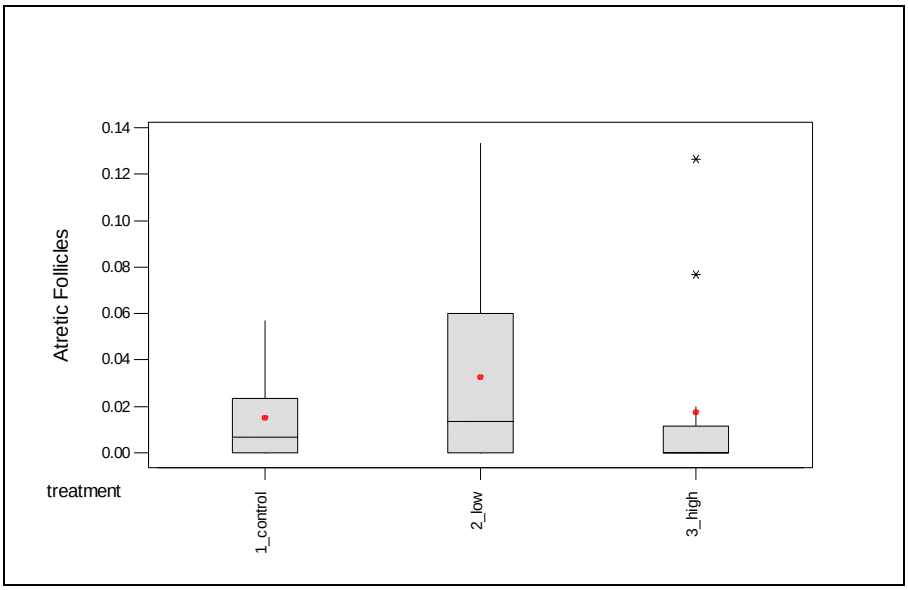

Figure 7.10. Box plot of the proportion of atretic follicles per 300 follicles by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent probable outliers)

Post-Ovulatory Follicles: The mean proportion of post-ovulatory follicles per 300 follicles (counted per fish) ranged from 0.008 for females from the low concentration to 0.032 for females from the control (Figure 7.11). There were no significant differences in the mean proportion of post-ovulatory follicles among treatments (Kruskal-Wallis, $H = 5.85$, $p = 0.054$, df = 2). However, one female from the control had a high proportion of post-ovulatory follicles (0.16) per 300 follicles. When this female is excluded from the analyses, the probability that any differences among three treatment means reflect chance variation increased (Kruskal-Wallis, $H = 4.68$, $p = 0.096$, df = 2).

Observations: The ovaries from two fish were observed to have histological abnormalities. One fish from the control retained fragmented Stage 5 ova. One fish from the high concentration had atretic follicles with retained post-ovulatory follicles.

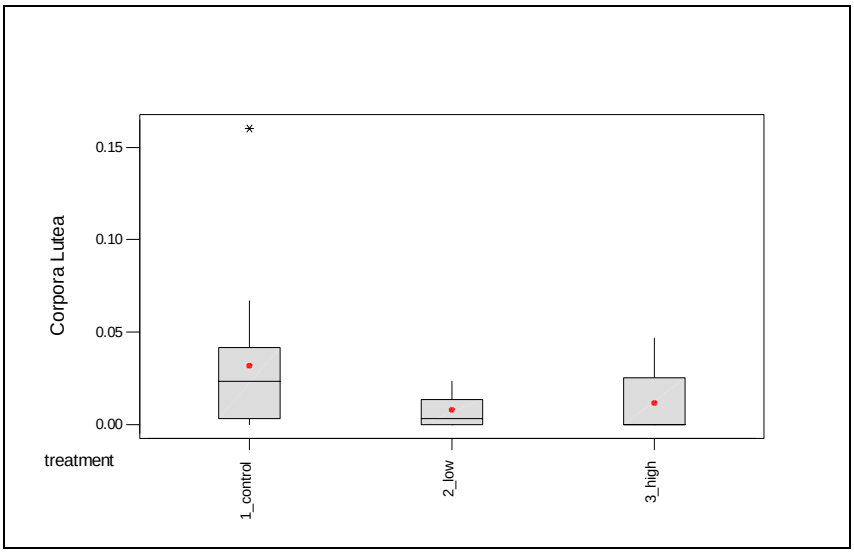

Figure 7.11. Box plot of the proportion of post-ovulatory follicles per 300 follicles by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

7.1.9 Male Gonad Histology

General Testes Staging: Testes from 24 males exposed to cadmium chloride during the EPA 21-day cadmium chloride assay were examined to determine the general developmental condition. The remaining males in all treatments had well-developed testes, with most showing Stage 4 and Stage 5 development (see Methods for description of developmental stages). Ninety-five of the 96 microscopic fields examined in the eight control males analyzed showed Stage 4 (49 fields) or Stage 5 (46 fields) development; one field showed Stage 1 development. All of the 96 microscopic fields examined in the eight low-concentration treatment males showed Stage 4 (70 fields) or Stage 5 (26 fields) development. Seventy-two of the 96 microscopic fields examined in the eight high-concentration treatment males showed Stage 4 (40 fields) or Stage 5 (32 fields) development. Two males from the high concentration showed no gonadal development (Stage 1) in all 24 fields examined. Because it was likely that the lack of development was not a treatment effect, statistical analyses were run with and without these two males. The results that included the males are reported. Excluding these males did not change the results reported, except that generally the probability that the observed differences among treatments represented chance variation increased. Statistical analysis of the mean staging from 12 microscopic fields per fish revealed no significant differences among treatments (Kruskal-Wallis, $H = 0.99$, $p = 0.609$, df = 2).

Quantitative Testicular Staging: One hundred cells in each of three sections per male were examined to quantitatively determine the developmental condition of the testes. The developmental stage of all treatment testes ranged from Stage 1 or 2A to Stage 5 (Figure 7.12). Statistical analyses showed that there were no significant differences among treatments in the proportions of cells in any of the developmental stages (Table 7.8).

Table 7.8. Descriptive statistics of the proportion of testes cells in each developmental stage for males from the EPA 21-day cadmium chloride assay and results of the Kruskal-Wallis test (df = 2) comparing treatments

	Control (N = 8)			Low (N = 8)			High (N = 8)			Kruskal-Wallis	
Stage	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	*H*	*p*
1	0	0	0%	0	0	0%	0.250	0.463	185%	4.18	0.124
2A	0.001	0.002	283%	0.001	0.002	138%	0.000	0.001	283%	1.59	0.451
2B	0.010	0.011	106%	0.012	0.009	71%	0.010	0.015	153%	0.99	0.608
3A	0.116	0.094	81%	0.175	0.119	68%	0.060	0.062	104%	4.73	0.094
3B	0.157	0.130	83%	0.234	0.091	39%	0.135	0.133	99%	2.37	0.305
4	0.168	0.129	77%	0.257	0.125	49%	0.115	0.088	76%	5.51	0.064
5	0.548	0.337	61%	0.320	0.331	103%	0.430	0.331	77%	1.68	0.432

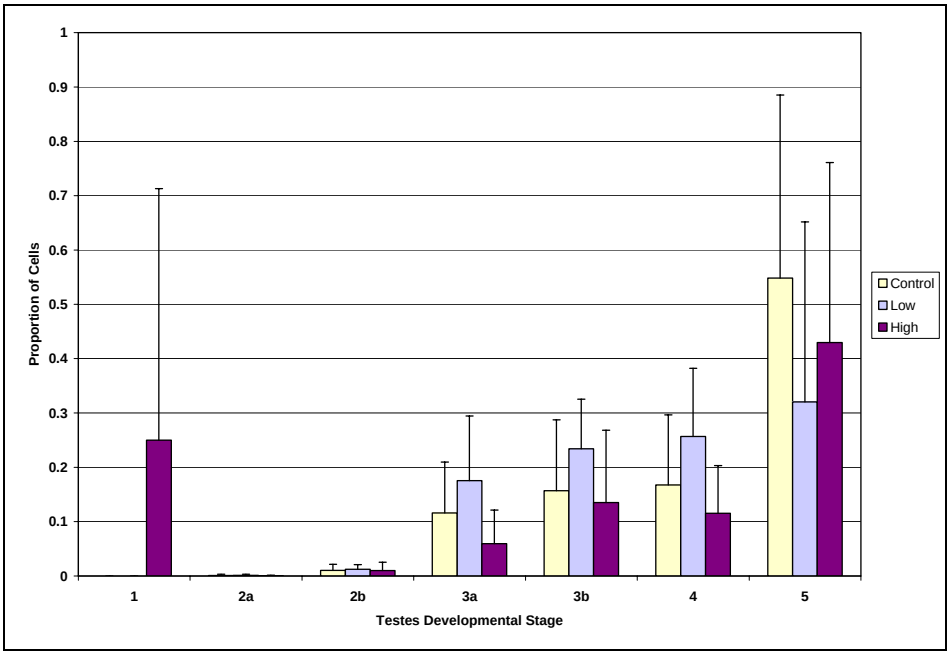

Figure 7.12. Frequency histogram showing the quantitative developmental staging of testes for each treatment of the EPA 21-day cadmium chloride assay (for each treatment, the columns represent the grand mean proportion of cells in each stage and the bars represent the standard deviation)

Tubule Diameter: The average diameter of the seminiferous tubules of males from the control ranged from 104.7 µm to 184.2 µm (Figure 7.13). Tubule diameters of males from the two test concentrations ranged from 98.9 µm to 211.9 µm. No significant differences in the mean tubule diameter per treatment (Table 7.9) were detected (Kruskal-Wallis, $H = 0.12$, $p = 0.943$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 6%. The probability of detecting as much as a 20% difference in male seminiferous tubule diameter was high, 100%, based on the observed variability (Table 7.9).

Table 7.9. Summary statistics and power estimates for male seminiferous tubule diameter data for the EPA 21-day cadmium chloride assay

Level	N	Mean (μm)	SD (μm)	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	142.1	27.3	19%	0.8%	89%	100%	100%
low	8	148.2	38.5	26%				
high	6	148.2	28.7	19%				

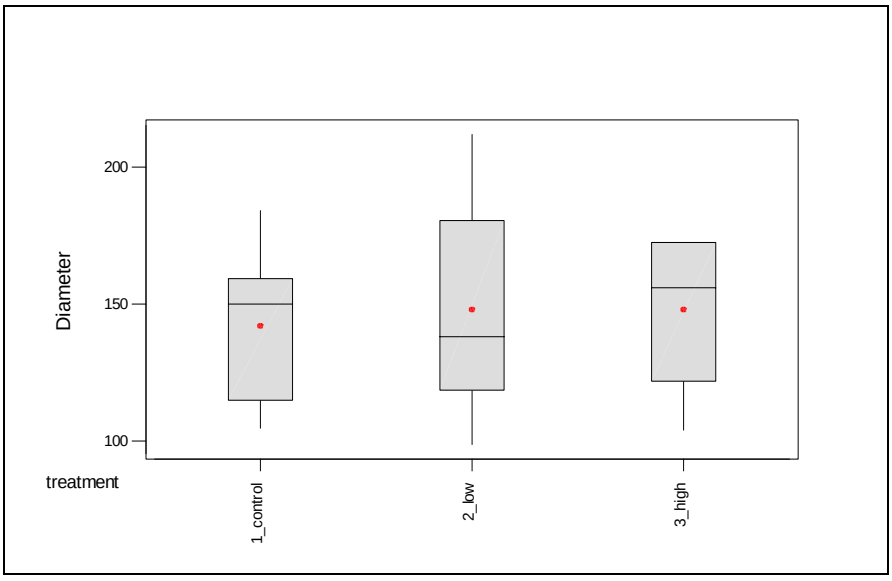

Figure 7.13. Box plot of male seminiferous tubule diameter (μm) by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Observations: No Sertoli cell proliferation or Leydig cell proliferation was observed among males from any treatment. Several males in each treatment showed other histological abnormalities (Appendix G, Table G.10). No testicular atrophy was recorded and no ovatestes were observed for any treatment.

7.1.10 Vitellogenin

VTG concentrations in control females used during the EPA 21-day cadmium chloride assay ranged from 6.75 mg/mL to 13.7 mg/mL (Figure 7.14). Among females exposed to the two cadmium chloride concentrations, VTG concentrations ranged from 5.38 mg/mL to 18.3 mg/mL. No significant differences in the mean VTG concentration per treatment (Table 7.10) were detected (Kruskal-Wallis, $H = 1.18$, $p = 0.553$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 9%. The probability of

detecting as much as a 50% difference in female VTG concentration was high, 100%, based on the observed variability (Table 7.10).

Table 7.10. Summary statistics and power estimates for female VTG concentrations for the EPA 21-day cadmium chloride assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	10.7	2.5	23%	5%	23%	73%	100%
low	8	9.34	2.3	25%				
high	6	11.1	4.2	37%				

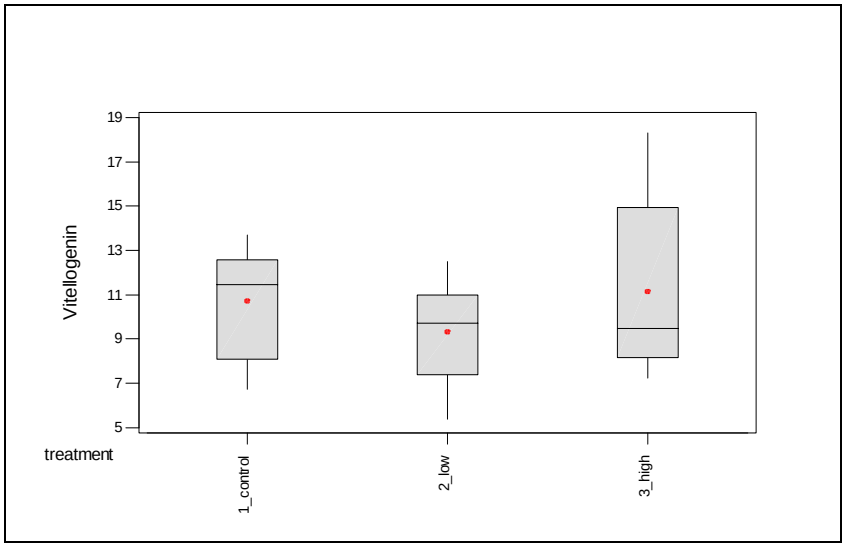

Figure 7.14. Box plot of female VTG concentration (mg/mL) by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

VTG concentrations in control males used during the EPA 21-day cadmium chloride assay ranged from 0.0001 mg/mL to 0.012 mg/mL (Figure 7.15). Among males exposed to the two cadmium chloride concentrations, VTG concentrations ranged from 0 mg/mL (not detected) to 0.068 mg/mL. No significant differences in the mean VTG concentration per treatment (Table 7.11) were detected (Kruskal-Wallis, $H = 1.19$, $p = 0.553$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 15%. The probability of detecting as much as a 20% difference in male VTG concentration was low, 5%, based on the observed variability (Table 7.11).

Table 7.11. Summary statistics and power estimates for male VTG concentrations for the EPA 21-day cadmium chloride assay

Level	N	Mean (mg/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.003	0.004	142%	277%	5%	5%	5%
low	8	0.012	0.023	197%				
high	7	0.004	0.003	72%				

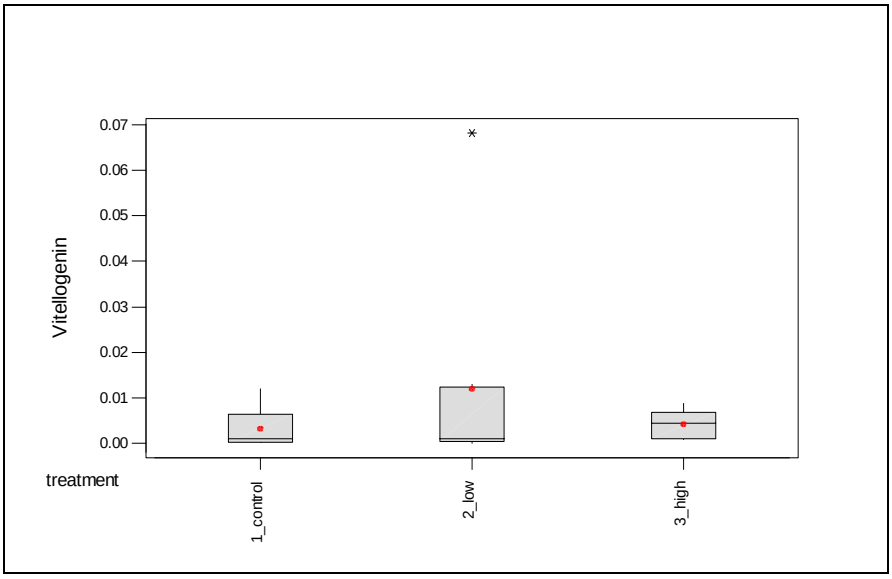

Figure 7.15. Box plot of male VTG concentration (mg/mL) by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

7.1.11 Plasma Steroid Concentrations

Estradiol: Estradiol concentrations in control females used during the EPA 21-day cadmium chloride assay ranged from 1.04 ng/mL to 3.65 ng/mL (Figure 7.16). Among females exposed to the two cadmium chloride concentrations, estradiol concentrations ranged from 0.293 ng/mL to 4.71 ng/mL. No significant differences in the mean estradiol concentration per treatment (Table 7.12) were detected (Kruskal-Wallis, $H = 2.47$, $p = 0.290$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 7%. The probability of detecting as much as a 50% difference in female estradiol concentration was moderate, 53%, based on the observed variability (Table 7.12).

Table 7.12. Summary statistics and power estimates for female estradiol concentrations for the EPA 21-day cadmium chloride assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	2.36	0.84	35%	11%	7%	12%	53%
low	8	3.00	1.29	43%				
high	6	2.20	1.26	57%				

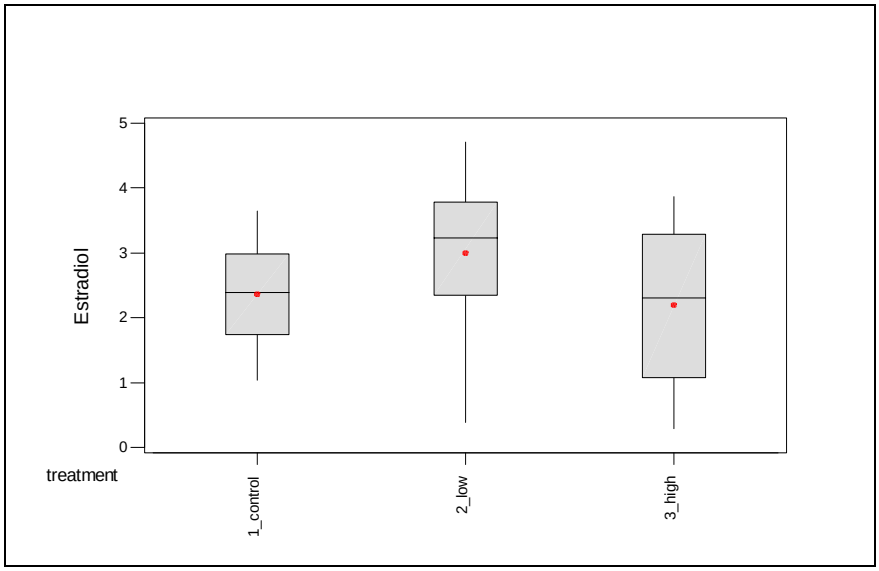

Figure 7.16. Box plot of female estradiol concentration (ng/mL) by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

Estradiol concentrations in control males used during the EPA 21-day cadmium chloride assay ranged from 0 ng/mL (not detected) to 0.457 ng/mL (Figure 7.17). Among males exposed to the two cadmium chloride concentrations, estradiol concentrations ranged from 0 ng/mL (not detected) to 0.473 ng/mL. No significant differences in the mean estradiol concentration per treatment (Table 7.13) were detected (Kruskal-Wallis, $H = 1.17$, $p = 0.556$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 12%. The probability of detecting as much as a 50% difference in male estradiol concentration was low, 13%, based on the observed variability (Table 7.13).

Table 7.13. Summary statistics and power estimates for male estradiol concentrations for the EPA 21-day cadmium chloride assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.214	0.189	88%	46%	5%	6%	13%
low	8	0.112	0.157	140%				
high	7	0.168	0.216	128%				

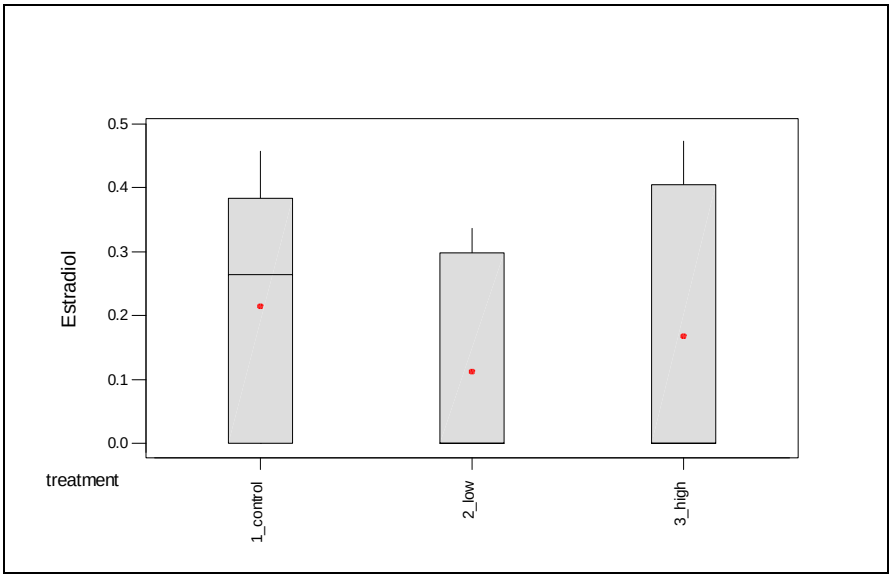

Figure 7.17. Box plot of male estradiol concentration (ng/mL) by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

***Testosterone*:** Testosterone concentrations in control females used during the EPA 21-day cadmium chloride assay ranged from 0.505 ng/mL to 2.59 ng/mL (Figure 7.18). Among females exposed to the two cadmium chloride concentrations, testosterone concentrations ranged from 0 ng/mL (not detected) to 2.65 ng/mL. No significant differences in the mean testosterone concentration per treatment (Table 7.14) were detected (Kruskal-Wallis, $H = 1.35$, $p = 0.510$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 10%. The probability of detecting as much as a 50% difference in female testosterone concentration was low, 27%, based on the observed variability (Table 7.14).

Table 7.14. Summary statistics and power estimates for female testosterone concentrations for the EPA 21-day cadmium chloride assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	0.99	0.68	69%	26%	6%	8%	27%
low	8	1.11	0.80	72%				
high	6	0.67	0.45	67%				

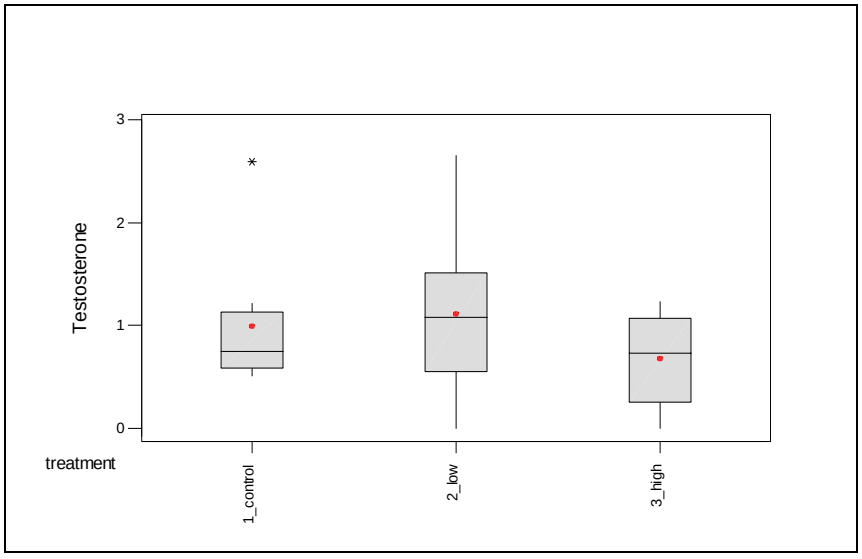

Figure 7.18. Box plot of female testosterone concentration (ng/mL) by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisk represents a probable outlier)

Testosterone concentrations in control males used during the EPA 21-day cadmium chloride assay ranged from 3.06 ng/mL to 8.80 ng/mL (Figure 7.19). Among males exposed to the two cadmium chloride concentrations, testosterone concentrations ranged from 2.03 ng/mL to 15.0 ng/mL. No significant differences in the mean testosterone concentration per treatment (Table 7.15) were detected (Kruskal-Wallis, $H = 2.18$, $p = 0.337$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 17%. The probability of detecting as much as a 50% difference in male testosterone concentration was high, 96%, based on the observed variability (Table 7.15).

Table 7.15. Summary statistics and power estimates for male testosterone concentrations for the EPA 21-day cadmium chloride assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	6.05	2.04	34%	14%	10%	29%	96%
low	8	8.58	3.67	43%				
high	7	6.14	2.71	44%				

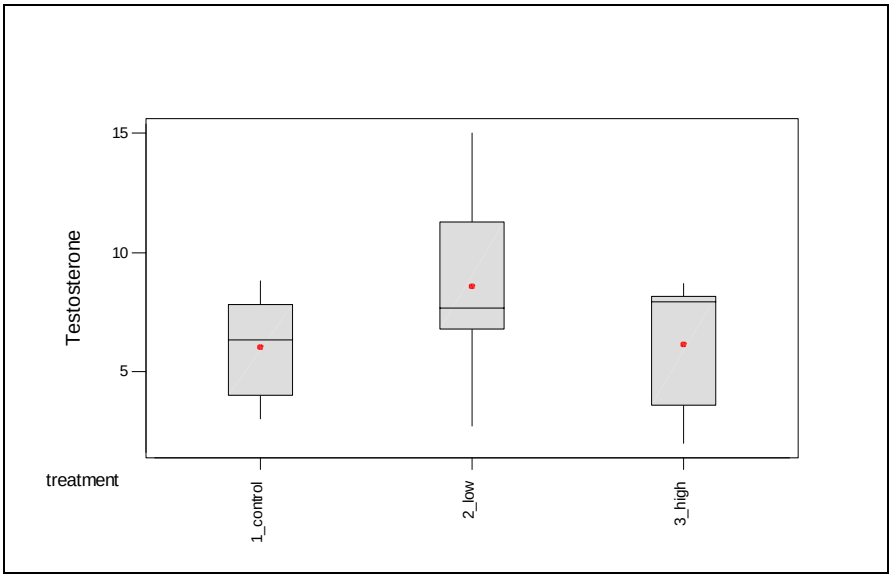

Figure 7.19. Box plot of male testosterone concentration (ng/mL) by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

11-ketotestosterone: 11-ketotestosterone was not detected in females from any treatment during the EPA 21-day cadmium chloride assay.

11-ketotestosterone concentrations in control males used during the EPA 21-day cadmium chloride assay ranged from 9.22 ng/mL to 45.6 ng/mL (Figure 7.20). Among males exposed to the two cadmium chloride concentrations, 11-ketotestosterone concentrations ranged from 3.90 ng/mL to 61.9 ng/mL. No significant differences in the mean 11-ketotestosterone concentration per treatment (Table 7.16) were detected (Kruskal-Wallis, $H = 0.42$, $p = 0.811$, df = 2). The achieved power at the observed maximum difference from the control response for this assay was 6%. The probability of detecting as much as a 50% difference in male 11-ketotestosterone concentration was high, 92%, based on the observed variability (Table 7.16).

Table 7.16. Summary statistics and power estimates for male 11-ketotestosterone concentrations for the EPA 21-day cadmium chloride assay

Level	N	Mean (ng/mL)	SD	CV	Maximum Observed Percentage Difference	Power at 10% Delta	Power at 20% Delta	Power at 50% Delta
control	8	26.8	13.4	50%	4%	9%	24%	92%
low	8	31.3	19.7	63%				
high	7	27.2	17.8	65%				

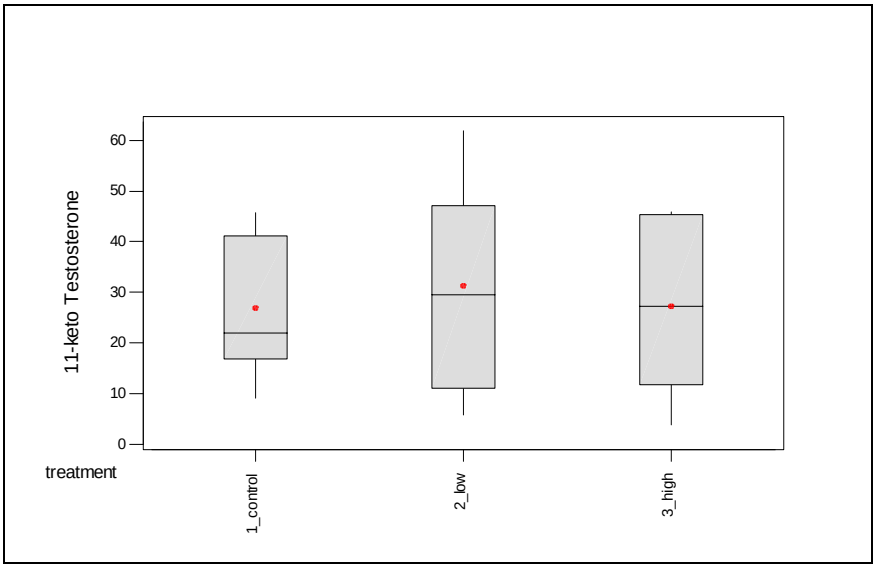

Figure 7.20. Box plot of male 11-ketotestosterone concentration (ng/mL) by treatment for the EPA 21-day cadmium chloride assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

8.0 DISCUSSION

8.1 Introduction and Summary

In this study, we evaluated six different contaminants for their effects on reproduction in the fathead minnow using the short-term reproduction assay described in EPA Mid-Continent Ecology Division (EPA MED 2002). This effort is intended to complement previous work conducted under WA 2-18 (EPA 2003), which evaluated three different fathead minnow reproduction protocols after exposure to four different endocrine disruptive chemicals. In the previous study, three of the test chemicals were pharmaceutical agents with well-established modes of action, previously established to reduce fecundity and in some cases, to significantly alter circulating sex steroid levels in fathead minnows. In the current study, the test compounds are all environmental pollutants with suspected modes of action that in most cases have not been evaluated in the fathead minnow. The five pollutants and their suspected mode of action are as follows:

1 - Atrazine (alters neuroendocrine activity)
2 - Bisphenol A (weak estrogen receptor agonist)
3 - p,p-DDE (weak androgen receptor antagonist)
4 - Perchlorate (iodine uptake inhibition; antithyroid)
5 - Cadmium chloride (altered steroidogenesis).

A sixth compound, di-n-butylphthalate, a weak antiandrogen, was also considered; however, the 21-day test of this compound was not completed, but rather, was terminated early by direction from EPA, because the exposure concentrations could not be maintained.

8.2 Preliminary Comparisons

Before the results from the various contaminant treatments are discussed, a comparison is made of fathead minnow body weights among all study fish and values for selected endpoints among control fish used during this study. This is intended to establish consistency in the measurement of key assay endpoints and to provide guidance on the normal range of values observed in control fish in our laboratory. With respect to body weight, mean values for females ranged between 2.2 g and 3.3 g and those for males from 5.4 g to 8.3 g (Table 8.1). Within-treatment variation was typically 21%-31% of the mean, which indicates that fish used in this study were of consistent body weight. There was no observable trend in the data for body weight and treatment group (Table 8.1).

Similarly, gonadosomatic index (GSI), fecundity, and seminiferous tubule diameter were consistent among control fish throughout the study (Tables 8.2, 8.3). In male fish, GSI averaged 1.24% of body weight with standard deviations that were 19%-30% of mean values. Seminiferous tubule diameter averaged 145.9 µm and varied little among male fish (Table 8.2). Among female fish, GSI averaged 13.3% of body weight, varying between 28% and 41% among the different groups (Table 8.3). Fecundity in control females was also consistent throughout the study, ranging from 53.6 to 65.5 eggs/female/day. Variation in fecundity was normally low, with a CV that was frequently less than 20% of the mean (Table 8.3). The latter observation is

Table 8.1 Body Weight

		CONTROL			LOW			HIGH		
		Mean	**SD[a]**	**CV[b]**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**
Females	**Cadmium chloride**	2.9	0.6	21%	3.1	1.1	35%	2.9	0.4	14%
	Bisphenol-A	2.6	0.7	26%	2.6	0.5	20%	2.6	0.5	20%
	p,p-DDE	2.2	0.5	22%	2.4	0.6	25%	2.2	0.4	17%
	Perchlorate	2.5	0.6	25%	3.1	0.8	27%	2.6	0.7	26%
	Atrazine	2.8	0.6	21%	3.3	1.1	33%	3.2	0.9	29%
	Study Average	2.6	0.6	25%	2.9	0.9	31%	2.7	0.7	26%
Males	**Cadmium chloride**	7.3	2.1	28%	6.2	1.4	23%	5.9	1.6	28%
	Bisphenol-A	8.2	1.2	15%	7.3	1.5	21%	8.3	1.4	17%
	p,p-DDE	6.4	0.3	5%	6.1	1.5	25%	6.2	1.4	23%
	Perchlorate	8.1	2.1	26%	7.5	1.9	25%	6.1	1.0	16%
	Atrazine	6.7	1.3	20%	6.3	1.2	20%	5.4	1.2	23%
	Study Average	7.4	1.6	21%	6.7	1.6	23%	6.3	1.6	26%

a) SD Standard deviation.
b) CV Coefficient of variation.

significant, because results from WA 2-18 (EPA 2003) indicated that fecundity was a sensitive endpoint to endocrine active compounds, and that the relatively low variation in this measurement during the study should enhance its power as an assay endpoint. Values for circulating sex steroids in control male and female fish are also presented in Tables 8.2 and 8.3. The two most important male sex steroids, testosterone and 11-ketotestosterone, ranged in value from 2.38-6.05 ng/mL and 13.5-26.8 ng/mL, respectively. With the exception of control male fish used in the atrazine exposures, variation within groups was usually less than 60% for these measurements. These values are consistent with results obtained from WA 2-18 (EPA 2003), where values ranged from 1.41 ng/mL to 5.21 ng/mL and 12.9 ng/mL to 35.2 ng/mL for testosterone and 11-keto-testosterone, respectively. As expected, values for estradiol in male fish were low, varying between 0.06 ng/mL and 0.44 ng/mL (Table 8.2). The greater variation in this sex steroid measurement can be attributed to values below or near assay sensitivity limits. Vitellogenin (VTG) levels were also consistently at or near MDLs in male fish (Table 8.2), indicating that no unintentional sources of xenoestrogens were present in the exposure system.

In female fathead minnows, the biochemical measurements of highest importance are estrogen, testosterone, and VTG. Among control fish, average estradiol levels ranged from 1.79-2.92 ng/mL, with CVs ranging from 23% to 62% of the mean. These values are consistent with our previous results obtained in WA 2-18 (EPA 2003), which are also near values reported by other investigators (Ankley et al. 2001). The average VTG concentrations in females ranged from 6.63-11.5 mg/mL and exhibited only moderate variation among individuals (CV≤ 36 %;

Table 8.2. Mean, standard deviation, and coefficient of variation for male fathead minnows used in controls

	Tubule Diameter (µm)			Vitellogenin (mg/mL)			Estradiol (ng/mL)			Testosterone (ng/mL)			11-keto-testosterone (ng/mL)			Gonadosomatic Index (%)		
	Mean	**SD[(b)]**	**CV[(c)]**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**
Atrazine	153.8	21.3	14%	0.009	0.013	144%	0.079	0.151	192%	3.31	2.14	65%	26.8	35.3	132%	1.19	0.32	27%
Bisphenol-A	160.0	42.6	27%	0.025	0.065	260%	0.386	0.258	67%	4.91	2.00	41%	19.8	9.3	47%	1.45	0.32	22%
Cadmium chloride	142.1	27.3	19%	0.003	0.004	142%	0.214	0.189	88%	6.05	2.04	34%	26.8	13.4	50%	1.17	0.35	30%
p,p-DDE	137.3	25.2	18%	0.004	0.006	139%	0.436	0.147	34%	4.05	1.43	35%	16.9	7.7	46%	1.22	0.23	19%
Perchlorate	134.9	12.3	9%	0.012	0.022	175%	0.063	0.167	265%	2.38	1.30	55%	13.5	12.6	93%	1.16	0.33	28%
Study Mean	145.9	47.2	32%	0.011	0.031	285%	0.325	0.152	47%	4.14	2.16	52%	21.0	18.7	89%	1.24	0.32	26%

a) SD Standard deviation.
b) CV Coefficient of variation.

Table 8.3. Mean, standard deviation, and coefficient of variation for female fathead minnows used in controls

	Fecundity Eggs/♀/day			Vitellogenin (mg/mL)			Estradiol (ng/mL)		
	Mean	**SD[a]**	**CV[b]**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**
Atrazine	53.6	13.1	24%	6.63	2.38	36%	2.52	1.46	58%
Bisphenol A	57.8	10.6	18%	11.1	3.39	31%	2.29	1.42	62%
Cadmium chloride	63.9	18.3	29%	10.7	2.48	23%	2.36	0.84	36%
p,p-DDE	65.5	9.1	14%	11.5	2.56	22%	2.92	1.00	34%
Perchlorate	61.3	8.3	14%	8.28	1.97	24%	1.79	0.41	23%
Study Mean	60.4	11.8	20%	9.64	3.11	32%	2.38	1.10	46%

a)SD Standard deviation.
b)CV Coefficient of variation.

Table 8.3). The values for VTG are approximately two to three times higher than those observed in WA 2-18 (EPA 2003), which showed a range of 1.29-5.99 mg/mL, but are very similar to values reported in past EPA MED studies (Ankley et al. 2001, 2002, 2003; Jensen et al. 2001). The difference in VTG values between those reported in WA 2-18 (EPA 2003) and in the present study may reflect subtle refinements in reagent stability and handling that are incorporated into the commercial fathead minnow VTG kit used in both studies.

Finally, histological examination of control male and female fathead minnows in this study is consistent with observations reported in WA 2-18 (EPA 2003). However, in some control fish, an unusually high number of atretic follicles was observed, which on some occasions (atrazine controls) approached 50%. This appeared to be a random occurrence, and suitable explanation is not available. Despite the observation, fecundity was always consistently high, as noted before in control fathead minnows.

8.3 Test Results

As described above for the control values, emphasis is placed on assay endpoints that are considered most important for identifying reproductive and endocrine effects. These endpoints include fecundity, plasma concentrations of VTG and sex steroids, GSI, and histology. Other potentially important biological endpoints, such as fertilization success, hatchability, and larval survivability/development, were found to be relatively insensitive endpoints, because consistently high values (>90%) were recorded for all chemical exposures, with the exception of bisphenol A. Less specific endpoints, such as body weight, can provide some indication of the general health status of the fish. For some chemical treatments, such as cadmium, perchlorate, and atrazine, there was a trend toward decreasing body weight for males in the low and high treatment groups (Table 8.1), although these differences were not statistically significant.

The combined results from the present study are notable in that with the exception of bisphenol A, most chemical treatments caused little or no effect on the measured endpoints. Occasionally, some histological abnormalities were observed in the gonads and are described below, but for the most part, endocrine effects or changes consistent with a specific neuroendocrine, antiandrogenic, or antithyroid mode of action were not observed. In the ensuing discussion of the results for each treatment, a table is included that summarizes whether a significant change occurred (higher [↑], lower [↓]), or no difference [—]) in important assay endpoints relative to values measured in control fish.

8.3.1 Atrazine

Atrazine is a widely used chlorotriaize herbicide. Because of its worldwide agricultural usage, the toxicity of atrazine has received considerable study. The suspected endocrine mode of action is considered to be at the neuroendocrine level (brain-pituitary axis), although this is based primarily on mammalian studies (Cooper et al. 1999). Recent studies in amphibians suggest that atrazine may alter steroidogenesis at the gonad level in males, decreasing circulating testosterone levels (Hayes et al. 2002). Thus, atrazine exposure could potentially cause a variety of changes in circulating sex steroids and perhaps in maturation of the gonads. However, the results from this study indicate that atrazine exposure at levels as high as 223 μg/L had no significant effect

on important assay endpoints (Table 8.4). Despite the lack of statistically significant changes, some trends in the data suggested that atrazine exposure did cause more subtle effects. In females, atrazine exposure at both the low and high treatments caused a slight decrease in estradiol levels (Table 3.14). In males, atrazine treatment at both the low and high exposure levels lowered circulating testosterone and 11-keto-testosterone levels by approximately 30%-50%. In males, atrazine exposure did cause some effects on testicular histology, such as increases in developmental Stage 2A (Figure 3.12) and decreases in seminiferous tubule diameter (Table 3.10). However, the biological relevance of these histological changes is unclear considering that no other histological abnormalities were noted.

Table 8.4. Summary of effects of atrazine on endpoints measured for the fathead minnow

	Endpoint	Low Dose	High Dose
Females	**Fecundity**	—[(a)]	—
	Proportion hatch	—	—
	Vitellogenin	—	—
	Estradiol	—	—
	Testosterone	—	—
	11-ketotestosterone	—	—
	Gonadosomatic Index	—	—
	Histology	—	—
Males	**Vitellogenin**	—	—
	Estradiol	—	—
	Testosterone	—	—
	11-ketotestosterone	—	—
	Gonadosomatic Index	—	—
	Histology	~↓[(b)]	↓[(c)]

a)— no significant change occurred in assay endpoint relative to values measured in control fish.
b)Seminiferous tubule diameter smaller than those in control males, but difference not statistically significant.
c)Seminiferous tubule diameter smaller than those of control males.

8.3.2 Bisphenol A

Bisphenol A is a commonly used plasticizer that is considered to have affinity for the vertebrate estrogen receptor that is approximately 2000 times less than that of estrogen (Routledge and Sumpter 1996). A previous study in which fathead minnows were exposed to various concentrations of bisphenol A for up to 164 days reported VTG induction in males at exposure levels down to 160 μg/L (Sohoni et al. 2001). Higher exposure levels (640 μg/L) also significantly raised VTG levels in females (Sohoni et al. 2001). Additional effects reported were a decrease in fecundity at exposure levels of 1280 μg/L and a diminished hatching success at exposure levels above 640 μg/L (Sohoni et al. 2001). A more recent study of medaka reported that 21-day bisphenol A exposures of 1720 μg/L induced VTG in males, but had no effect on fecundity or fertilization success (Kang et al. 2002). The results of the bisphenol A tests conducted in the present study and discussed below are consistent with an estrogenic mode of action and with results from past studies in fish (Table 8.5). At the high exposure concentration (344 μg/L; Figure 4.4), fecundity was significantly reduced to a level nearly half that of the

Table 8.5. Summary of effects of bisphenol A on endpoints measured for the fathead minnow

	Endpoint	Low Dose	High Dose
Females	**Fecundity**	—[a]	↓[b]
	Proportion hatch	↓	↓
	Vitellogenin	—	↑[c]
	Estradiol	—	↓
	Testosterone	—	—
	11-ketotestosterone	ND[d]	ND
	Gonadosomatic Index	—	—
	Histology	—	↓[e]
Males	**Vitellogenin**	↑	↑
	Estradiol	↓	↓
	Testosterone	—	↓
	11-ketotestosterone	—	↓
	Gonadosomatic Index	—	—
	Histology	—	—

a) — no significant change occurred in assay endpoint relative to values measured in control fish.
b) ↓ with no other marking means value below that of control.
c) ↑ with no other marking means value above that of control.
d) ND not detected.
e) Greater proportion of follicles in Stage 1A than in control; lower proportion of follicles in Stage 3 than in control; many fish with multiple foci of macrophage clusters in ovary.

control and low treatment groups (Figure 4.4; Table 4.2). With regard to the biochemical endpoints, the most striking result was the significant induction of VTG in males at both treatment doses and in females at the high treatment dose (Tables 4.13, 4.14). In males, mean VTG levels were more than 2 mg/mL in the low exposure and 92.7 mg/mL in the high treatment group, a value higher than that observed for female fathead minnows. Bisphenol A treatment also affected circulating sex steroid levels. In both genders, estradiol levels were reduced, and in females, the reduction was dose-dependent: levels in the high exposure group exhibited levels half the value of those of the control-group females (Tables 4.15, 4.16). Testosterone levels in females was unaffected by treatment, but in males, the high exposure group had levels that were roughly 15% of those of controls (Table 4.18). Similarly, 11-ketotestosterone levels in males from the high treatment were 5% to 10% of the values observed in control fish (Table 4.19). The reduced estrogen levels in females is consistent with histological changes, in which quantitative staging of the ovaries indicated an increase in early staging (1A) and a decrease in Stage 3 oocytes (Figure 4.10). However in males, no significant changes in testes histology were observed despite the large decreases in circulating androgen levels that occurred during the bisphenol A treatment.

8.3.3 p,p-DDE

p,p-DDE is a metabolite of the pesticide, dichlorodiphenyltrichloroethane (DDT). Past studies in rodents suggest that p,p-DDE may act via an antiandrogenic mode of action, based on competitive inhibition of testosterone binding to androgen receptors (Gray et al. 1999). In fish, conflicting results have been reported for the antiandrogenic effects of p,p-DDE. In male guppies (*Poecilia reticulata*), 26-week dietary exposure to p,p-DDE caused changes in secondary sex characteristics and decreased sperm counts (Bayley et al. 2002). In contrast, summer flounder (*Paralichthys dentatus*) given multiple injections of p,p-DDE and sampled at selected times up to 8 weeks later, had normal circulating sex steroid and VTG levels (Mills et al. 2001). In the present study, p,p-DDE exposure had little or no effect on most of the assay endpoints (Table 8.6). In females, p,p-DDE exposure did not alter fecundity and in both sexes, no significant biochemical changes were observed, although slight decreases in estrogen and increases in testosterone and 11-ketotestosterone levels were reported (Figures 5.17-5.19). The only significant histological change observed was an increase in the numbers of atretic follicles in the low and high treatment groups (Figure 5.11). These results suggest that weak antiandrogens may produce only subtle changes that can be difficult to detect if the statistical power of the sex steroid measurements is low. Although not statistically significant, the trend in the changes of sex steroid levels are consistent with those obtained in flutamide tests conducted under WA 2-18 (EPA 2003) as a model antiandrogenic chemical. Flutamide significantly decreased fecundity in females in addition to causing significant decreases in estrogen, increased 11-ketotestosterone levels in males, and elevated testosterone in females. Thus, decreased plasma estrogen and increased androgen levels may be a characteristic of competitive androgen antagonists in fathead minnows.

8.3.4 Perchlorate

Perchlorate is a by-product of rocket fuel production widely found in groundwater; it is known to competitively inhibit iodine uptake by the thyroid gland (Wolfe 1998). Decreased iodine uptake can alter thyroid gland function and lower secretion of thyroid hormones. In fish, normal thyroid function is essential for maintaining growth and normal reproductive performance (Cyr and Eales 1996). In the present study, perchlorate exposure at levels up to 43.5 mg/L had little or no effect on most of the assay endpoints (Table 8.7). However, histological examination in females identified a significant decrease in stage 1A oocytes and increased numbers of atretic follicles in the low and high perchlorate treatment groups. In males, no changes in testes histology were observed. In general, these findings are consistent with a recent study of thyroid and reproductive effects of perchlorate in zebrafish (*Danio rerio*; Patino et al. 2003). In Patino et al. (2003), 8-week aqueous exposure to perchlorate at 18 mg/L had no effect on packed egg volume (a measure of fertilization rate). A higher treatment level of 677 mg/L did decrease fecundity, but also caused significant mortality (Patino et al. 2003). The 18 mg/L treatment did cause measurable changes in the thyroid gland histology in zebrafish (Patino et al. 2003). Histological examination of the thyroid gland was not part of the experimental protocol in the present study.

8.3.5 Cadmium Chloride

Cadmium is considered to potentially alter sex steroid synthesis in both males and females. In fish, cadmium has been suggested to reduce testosterone and estrogen synthesis via a mechanism that inhibits cholesterol uptake by mitochondria (Karels et al. 2003). However in medaka, *in ovo* (maternal) exposure alone or in combination with subsequent short-term exposure as mature adults tended to increase estrogen levels in females (Foran et al. 2002). In the present study, exposure to cadmium was remarkable in that no effects on fecundity, gonad histology or biochemical parameters were observed (Table 8.8), even though the high cadmium treatment rate of 11.0 μg/L in this study is within the reported range of effects observed by Foran et al. (2002). The contrast in response is likely due to species differences and perhaps to exposure protocol.

Table 8.6. Summary of effects of p,p-DDE on endpoints measured for the fathead minnow

	Endpoint	Low Dose	High Dose
Females	**Fecundity**	—[(a)]	—
	Proportion hatch	—	—
	Vitellogenin	—	—
	Estradiol	—	—
	Testosterone	—	—
	11-ketotestosterone	ND[(b)]	ND
	Gonadosomatic Index	—	—
	Histology	↑[(c)]	↑[(c)]
Males	**Vitellogenin**	—	—
	Estradiol	?[(d)]	?[(d)]
	Testosterone	—	—
	11-ketotestosterone	—	—
	Gonadosomatic Index	—	—
	Histology	—	—

a) — no significant change occurred in assay endpoint relative to values measured in control fish.
b) ND Not detected.
c) ↑ Proportion of atretic follicles greater than that in control.
d) ? Means lower than that of control, but differences not statistically significant (p=0.076).

Table 8.7. Summary of effects of perchlorate on endpoints measured for the fathead minnow

	Endpoint	Low Dose	High Dose
Females	**Fecundity**	—[a]	—
	Proportion hatch	—	—
	Vitellogenin	—	—
	Estradiol	—	—
	Testosterone	—	—
	11-ketotestosterone	N.D.	[b]
	Gonadosomatic Index	—	—
	Histology	↑[c] ↓[d]	↑[c] ↓[d]
Males	**Vitellogenin**	—	—
	Estradiol	—	—
	Testosterone	↑[e]	—
	11-ketotestosterone	↑[f]	—
	Gonadosomatic Index	—	—
	Histology	—	—

(a) — No significant change occurred in assay endpoint relative to values measured in control fish.
(b) Detected in only 2 of 8 fish.
(c) Proportion of atretic follicles greater than that in control.
(d) Proportion of Stage 1A oocytes lower than that in control.
(e) Testosterone level greater than that in control.
(f) 11-ketotestosterone level greater than that in control.
N.D. Not detected.

Table 8.8. Summary of effects of cadmium chloride on endpoints measured for the fathead minnow

	Endpoint	Low Dose	High Dose
Females	**Fecundity**	—[(a)]	—
	Proportion hatch	—	—
	Vitellogenin	—	—
	Estradiol	—	—
	Testosterone	—	—
	11-ketotestosterone	ND [(b)]	ND
	Gonadosomatic Index	—	—
	Histology	—	—
Males	**Vitellogenin**	—	—
	Estradiol	—	—
	Testosterone	—	—
	11-ketotestosterone	—	—
	Gonadosomatic Index	—	—
	Histology	—	—

a) — no significant change occurred in assay endpoint relative to values measured in control fish.
b) ND Not detected.

9.0 REFERENCES

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