REVISED DRAFT FINAL REPORT

on

Fathead Minnow (*Pimephales promelas*) Fish Screening Assay OECD Phase 1 B Follow-Up

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1.0 INTRODUCTION

The U.S. Environmental Protection Agency (EPA) is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is a short-term screening assay designed to detect substances that interact with the estrogen and androgen systems of fish. It is thought that the inclusion of the fish screening assay in Tier 1 is important because estrogenic and androgenic controls on reproduction and development in fish may differ significantly enough from that of higher vertebrates that mammalian screening methods may not identify potential endocrine disrupting chemicals (EDCs) in this important class of animals. As an example, dihydrotestosterone is a potent androgen in mammals, but 11-ketotestosterone is generally the more prevalent androgen in fish.

U.S. EPA (2001) has described a short-term test with the fathead minnow *(Pimephales promelas)* 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 (Ankley et al., 2001) 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 is 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 and sex steroids (17ß-estradiol, testosterone, and 11-ketotestosterone).

The Organization for Economic Cooperation and Development (OECD) initiated a fish screening assay validation activity and has completed its Phase 1A and Phase 1B trials. Phase 1A evaluated a non-spawning version of a 21-day exposure assay with fathead minnow, medaka, and zebra fish. The results of the Phase 1A led to the Phase 1B trials where spawning was included in the method. The results of the Phase 1B trial raised questions regarding the spawning conditions utilized for the fathead minnow.

Previous work assignments under this contract (WA 2-18 and WA 2-29) were initiated to evaluate a short-term reproduction assay with fathead minnow and compare the EPA (2001) method to two other related assays to contribute to the optimization of the assay for use as a screen in the EDSP.

The laboratories that participated in the OECD Phase 1B work were ABC Laboratories, Springborn Smithers Laboratories, and Wildlife International, Ltd. Springborn Smithers performed the Phase 1B study with flutamide and 4-tert-pentylphenol (4-PP) using fathead minnows, ABC used 4-PP and prochloraz with fathead minnows, and Wildlife International used 4-PP and flutamide with medaka. Experimental Pathology Laboratories (EPL) performed the histopathology work under Phase 1B for the OECD effort.

The purpose of the work conducted under this assignment was to demonstrate the fish screening assay test method, based on the OECD Phase 1B study for short-term reproduction assay with the fathead minnow as described in EPA (2001). This follow-up study incorporated an increased number of replicates and used a semi-quantitative and quantitative egg-counting method.

The studies were conducted by three different laboratories plus a pathology laboratory, which performed the following chemical trials:

- ABC Laboratories – Potassium permanganate
- Wildlife International – Flutamide
- Springborn Smithers – Flutamide, Potassium permanganate, and Ketoconazole
- Experimental Pathology Laboratories (EPL) – Performed pathology on all tissues collected by the participating labs.

An overview of the experimental design is provided in Section 2.0. A description of each participating laboratory's methodology, analytical procedures and relevant test conditions is provided in Appendies A-C. The experimental phases of the study were conducted from June through November 2005. EPL, Sterling, Virginia, performed the histopathology work.

This document is a summary of the methods, results, and conclusions for WA 5-11. This document does not investigate the inter-laboratory variability. In addition, no power analysis was performed on measurement endpoints. The full text of each participating laboratory's report is found in Appendices A (ABC Laboratories), B (Springborn Smithers Laboratories), and C (Wildlife International Ltd.). Appendix D contains the results from purity and stability analysis performed by the Chemical Repository for requested test articles. Based upon the initial draft of this Summary Report it became apparent that each participating laboratory had a preferred statistical model that was used in reporting. The methods used varied among laboratories. A statistical method evaluation using data from participating laboratories was requested by EPA and subsequently performed by Battelle. The results of this statistical method evaluation are provided in this Revised Draft Final Report as Appendix E. A detailed synthesis of the results among the various participating laboratories (i.e., inter-laboratory and intra-laboratory) is beyond the scope of this WA-5-11 Report.

2.0 EXPERIMENTAL DESIGN: 21-DAY REPRODUCTIVE PERFORMANCE ASSAY WITH THE FATHEAD MINNOW

The experimental protocol for a short-term reproduction assay followed the protocol developed by Ankley et al. (2001) using the fathead minnow (*Pimephales promelas*). The assay measured the reproductive performance of groups of fathead minnows as the primary indicator for endocrine disruption. Additional measurements of morphology, histopathology, and biochemical endpoints were performed to aid identification of the specific toxicological mode of action of the test chemical.

The assays were initiated with mature male and female fish. During a 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics were observed while fecundity and fertilization success were monitored daily. At termination of the assay, measurements were made of a number of endpoints reflective of the status of the reproductive endocrine system, including the gonadosomatic Index (GSI), gonadal histology, and plasma concentrations of vitellogenin (VTG).

The assays were initiated with mature spawning adults. This was established during a "pre-exposure" period of 14 days. During the pre-exposure observation period, reproductive performance was monitored as described for the chemical exposure period.

The pre-exposure observations occurred in the same system/tanks as were utilized for the chemical test. An overview of the tests and relevant test conditions is provided in Table 1.

Table 1: Experimental Design for the Assay Method

Parameter	Assay Protocol
Test species:	Reproductively active fathead minnows (minimum 120 day old)
Fish husbandry conditions:	Temp:25°C ± 1°C; D.O. >5.0 mg/L; Light: 16 h light : 8 h dark with 540-1080 lux; Feed: frozen brine shrimp twice daily
Pre-exposure evaluation	Duration: 14 days; Data Collected: fecundity semi-quantitative (daily)
Dilution water	Clean, surface, well or reconstituted water
Test material	Chemicals listed in Table 3
Test chamber size	18 L (40 x20 x 20 cm)
Test volume:	10 L
# Exchanges/day	6 tank volume exchanges
Flow rate:	2.5 L / hr
# Concentration / chemical	3; identified in Table 3
# Replicates:	4
Weight of each fish	NS
# Fish/vessel	4 females and 2 males
Total # fish/concentration	16 females and 8 males
Feeding regime	Frozen brine shrimp, twice a day
# Controls	1; Dilution water control
# Fish/control	4 adult females and 2 adults males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)
Photo period:	Austro sunrise/sunset system, at time of testing the lighting was approximately 16 h light : 8 h dark with 540-1080 lux;
Temperature:	25°C ± 1°C
Light intensity	540 - 1080 lux
Aeration:	None unless D.O. <4.9 mg/L
pH	NS
Biological endpoints:	Adult survival, reproductive behavior, secondary sexual characteristics, GSI, gonadal histology, VTG, fecundity and fertility
Test validity criteria:	D.O. = 60% saturation; Mean temp. 25°C ± 2°C; 90% survival in the controls and successful egg production in controls. Typical spawning occurs every 3 to 4 days in controls, or approximately 15 eggs/female/day/test chamber.

NS = Not specified in procedure.

2.1 Description of the Method

Test Animals: The assays were started with newly mature fish (typically four to six months old), as opposed to older animals that have been actively reproducing for some period of time.

Water: It is well established that the fathead minnow can reproduce successfully over a wide range of water quality. Therefore, no specific water type was required for this test. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully would be acceptable. Minimal recommended water quality characteristics are listed in Table 1. The animals were tested using a flow-through water renewal system that maintained adequate water quality (temperature, dissolved oxygen, low ammonia, etc.) and ensured a consistent exposure to the parent chemical.

Assay System: Five-gallon glass exposure vessels were used for the test system. As recommended by Ankley et al. (2001), dimensions of the test chambers must be such that the animals would interact in a fashion conducive to successful spawning. The test chamber contained 10 L of test solution, which was renewed once every 4 hours. This particular animal loading/water renewal rate is within recommended guidelines and, in studies conducted according to this method, has maintained acceptable water quality while not utilizing an excessive amount of test material.

A randomized design was used for the reproductive assay. This design was intended to randomize out the effects associated with the local environment (i.e., light and water) and possible trends associated with the diluter during testing. All fish were transferred from the pre-validation tank and then randomly assigned a treatment within a block. The blocks were filled in a random order. Thus, when one evaluates the difference between treatment means, the variability associated with experimental environment, experimental containers, and organisms being treated is removed and only the effect of the treatment remains.

Range-finding tests for potassium permanganate were necessary. Guidance on test chemical concentrations for ketoconazole and flutamide was obtained based upon previous research. For the tests, the highest concentration of the chemicals used was selected to avoid causing significant mortality while still being sufficiently high to allow evaluation of any effects on reproduction. The low concentrations were reduced by a factor of 10, as suggested by U.S. EPA (2001). A 96-hour exposure to five test concentrations plus a control (six total), two replicates for each treatment of four females and two males per exposure tank (72 fish total) was

conducted. The number of mortalities that occurred were used to develop a dose response curve. Based upon the results, the highest concentration that did not result in increased mortality or signs of overt morbidity compared to controls served as the highest exposure concentration in the 21-day test.

2.2 Assay Initiation and Conduct

Pre-exposure: The pre-exposure phase was for 14 days. The assay used fish that were approximately 4 to 6 months old, previously maintained in communal culture tanks. Four females and two males were randomly assigned to the replicate exposure chambers at each treatment concentration. Additional exposure chambers were set up for pre-exposure to account for a lack of spawning in some chambers and/or mortality during the pre-exposure spawning. Any specimens whose gender could not be identified were excluded from the assay. It has been reported that, at 5 to 6 months of age, males are larger and darker and exhibit nuptial tubercles, while females possess an ovipositor.

The pre-exposure phase of the assay was conducted under conditions (temperature, photo-period, feeding, etc.) identical to those used during the chemical exposure. The animals were fed frozen *Artemia* twice daily.

The fish were monitored daily for any abnormal behavior (relative to controls), and any such behavior was noted; this included signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. Fecundity data were collected daily. For each assay, successful pre-exposure (suitability for testing) was defined as recommended in U.S. EPA guidelines (page 37, U.S. EPA 2001): establishment that regular spawning occurs in each test chamber every 3 to 4 days. For the test used in this work assignment, regular spawning was defined as a minimum average of 15 eggs/female/day per test chamber. If this criterion was established after 14 days, then the test chamber was deemed suitable for use in the chemical exposure test. If, after 21 days, regular spawning could not be established, then the test chamber was rejected from use.

Chemical Exposure: After successful spawning was verified during pre-exposure as per the requirements of the assay, the chemical exposure was initiated and continued for 21 days. The assays were conducted at three chemical concentrations (identified in Table 3), as well as a diluent water control, with four experimental units (replicates) per treatment. The test chemical was delivered to the exposure chamber using a proportional diluter (concentrated aqueous stock solutions were prepared without using carrier solvents). The exposure was conducted for 21 days, during which time the appearance of the fish, behavior, and fecundity were assessed daily. The plasma from each fish was analyzed for VTG. The gonads were also removed for GSI determination and later histological analyses. This exposure duration allowed for collection of sufficient data for assessments of fecundity and fertilization success (Ankley et al. 2001).

Gonad Histology: The first step of gonad histological analysis was necropsy and rapid gonad fixation to prevent autolysis and cellular deterioration. Immediately after humane killing of an individual fish (plus length and weight measurements and collection of fresh tissues, e.g., blood for VTG analysis, the abdomen was sectioned and the paired gonads fixed. Davidson's fixative was used for maintaining the structural integrity of the gonad. After gonads were fixed in place and then excised, material was placed in labeled histological cassettes and were sent to EPL for histological analysis.

2.3 Observations and Measurements

A number of endpoints were assessed over the course of and/or at conclusion of the assays. A description of these endpoints and their utility, particularly in the context of the assay as an EDC screen, is as follows:

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

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

Fecundity: Egg production was determined daily. Because fathead minnows spawn within a few hours after the lights are turned on, they were not disturbed (except for feeding) until late morning. This allowed time for spawning and fertilization to be completed and for eggs to water-harden. The spawning substrates were removed from the tanks to enumerate any eggs that were present. Based on the published report (and our past experience) of this protocol, it was expected that one spawn typically is composed of 50 to 250 eggs. If no embryos were present, the substrate was left in the tank; new substrates were added to replace any that were removed. Fecundity was expressed on the basis of surviving females per reproductive (test) day per replicate or cumulative eggs laid over the test. Semi-quantitative fecundity measurements were measured by visual estimation using a matrix [(i.e., 0 (none), 10, 25, 50, 100, 150, 200, 250, 300, and >300)].

Fertilization Success: After the spawning substrate was removed from the tank, the embryos were carefully rolled off with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically were undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100) was easily achieved. Infertile eggs were opaque or clear with a white dot where the yolk had precipitated; viable embryos remained clear for 36 to 48 hours until reaching the eyed stage.

Appearance of Adults: The external appearance of the adults was assessed over the course of the test and at the conclusion of the study, and any unusual changes were noted. 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 (size of dorsal nape pad, number of nuptial tubercles in males, ovipositor size in females). These observations were especially important for assessing endocrine active agents that were (anti)-androgenic.

Blood Sampling: At the conclusion of the exposure, the fish were anesthetized by transfer to an oxygenated solution of MS-222 (100 mg/L buffered with 200 mg $NaHCO_3$/L). Blood was

collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule. Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally ranged from 30 to 80 µL. Plasma was separated from the blood via centrifugation (approximately 3 minutes at 15,000 x g) and stored with protease inhibitors at -75°C to -85°C until analyzed for VTG.

Body Weights: Relative to control animals.

Gonad Size and Morphology: After sampling the blood, fish were weighed and the "fixed" gonads removed and weighed (fixed weights were determined to the nearest 0.1 mg) to determine the GSI (GSI=100 x gonad wt/body wt). Typical GSI values for reproductively active fathead minnows range from 8 to 13% for females and from 1 to 2% for males. 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.

Routine histological procedures were used to assess the condition of testes and ovaries from the fish. Gonads were placed in Davidson's fixative and embedded in paraffin and 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/sample. Sections were stained with hematoxylin and eosin, and were evaluated by an experienced histologist without prior knowledge of the treatment regime associated with specific samples. Evaluation of the testis was based on the amount of germinal epithelium present and the degree of spermatogenic activity. The ovary was evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Vitellogenin: The measurement of VTG in plasma samples was performed using an enzyme-linked immunoabsorbant assay (ELISA). For the ELISA, polyclonal fathead minnow (*Pimephales promelas*) VTG antibody and purified VTG protein also from the fathead minnow were utilized.

A summary of the measurement endpoints and the criteria are presented in Table 2.

Table 2: Measurement Endpoints and Associated Criteria

Parameter	Units	Expected Results
Survival: Daily assessment of survival were made to provide a basis for expression and interpretation of reproductive output.	Not Applicable	90% or greater survival in controls. Mortality was expected to be low based on previous studies at these exposure rates.
Behavior of Adults: Abnormal behavior (relative to controls), during the daily observations were noted.	Not Applicable	Expected observations included: Hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and feeding abstinence. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males. Qualitative anecdotal observations.
Fecundity: Egg production were determined daily, but only during the morning. Both quantitative and semi-quantitative measurements were performed.	Fecundity will be expressed either on the basis of average number of eggs laid by surviving females per reproductive (test) day per replicate or as cumulative eggs laid over the test.	One spawn typically will be composed of 10 to 250 eggs. If no embryos were present, the substrate was left in the tank; new substrates were added to replace any that were removed.
Fertilization Success: If spawning occurred that morning, embryos typically were undergoing late cleavage, and determination of the fertility rate were easily achieved.	Number embryos/number of eggs x 100	Fertilized eggs are apparent within a few hours of fertilization. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated. Control fertilization should be ≥95%.
Appearance of Adults: The external appearance of the adults was assessed as part of the daily observations, and any unusual changes were noted. These observations are especially important for assessing endocrine active agents that are (anti)-androgenic.	Not Applicable	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 (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females).
Body Weights	Grams	Normal/increased/decreased weights relative to control animals.
Blood Samples: collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule and analyzed for VTG	Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 µL.	Plasma was separated from the blood sample via centrifugation (approx 3 minutes at 3,000 x g) and stored with protease inhibitors at -75°C to -85°C until analysis.
Vitellogenin (VTG) Concentration	pg/mL	The measurement of VTG in plasma samples was performed using an enzyme-linked immunosorbent assay (ELISA). For the ELISA fathead minnow Amersham VTG kits with monoclonal antibodies, VTG antibody and purified VTG protein also from the FHM were utilized.
Gonad Size: After sampling the blood, the fixed gonads were removed and weighed (to the nearest 0.1 mg) to determine the GSI (GSI=100 x gonad wt/body wt).	Not Applicable	Typical GSI values for reproductively active fathead minnows range from 8 to 13% for females and from 1 to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes.
Gonad Morphology: Routine histological procedures were used to assess the condition of testes and ovaries from the fish. Gonads were placed in fixative (Davidson's fixative). EPL performed histology procedures and followed the protocol from the OECD Phase 1B Study.	Not Applicable	Evaluation of the testis was based on the amount of germinal epithelium present and the degree of spermatogenic activity. The ovary was evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Not Applicable. No unit can be defined for this parameter.

2.4 Performance Criteria

Water quality characteristics were to remain within the limits of tolerance described in Table 1 (water temperature did not differ by more than ±1 °C between test vessels at any time during the exposure period and was maintained within a range of 2 °C within the temperature ranges specified for the test species).

There was to be more than 90% survival of control animals over the duration of the chemical exposure, and the control fish in each replicate will spawn, at a minimum, every 3 to 4 days. Typically, there were to be approximately 15 eggs/female/day/test chamber and there was to be greater than 95% fertility of eggs from the control animals.

2.5 Test Chemicals

The three compounds and exposure concentrations evaluated are provided in Table 3.

Table 3: Test Chemicals and Exposure Concentrations

Test Chemical	Testing Lab	Exposure Concentration (µg/L)		
		Low	Med	High
Flutamide	Springborn Smithers Wildlife International	100	500	1000
Potassium permanganate	Springborn Smithers ABC Laboratory	225	450	900
Ketoconazole	Springborn Smithers	25	100	400

After preparation of the stock solution, determinations of the concentration were made using the methods as described by each participating laboratory's report found in Appendices A - C. The concentrations of the test chemical in the exposure chambers were to be measured prior to adding fish to verify that target concentrations were reached. Additionally, water samples were removed weekly and analyzed for the test chemicals.

All test chemicals were directly measured using spectrophotometry, gas chromatography - electron capture detection (GC-ECD), or HPLC (high-performance liquid chromatography).

2.6 Biochemical Determinations

Vitellogenin (VTG): Enzyme-linked immunosorbent assay (ELISA) tests were conducted using commercially available test kits from Amersham Bioscience. The methods used for the bioanalytical measurements of VTG followed the manufacturer's specifications. According to the manufacturer, this kit could be stored 3 months before usage if stored at 4°C.

3.0 RESULTS

The summary results from the participating laboratories in the Fathead Minnow (*Pimephales promelas*) Fish Screening Assay OECD Phase 1 B Follow-Up are provided below in Tables 4 through 6. Provided in Tables 7 through 9 are the coefficients of variance (CVs) for each measurement endpoint and their average CV.

Table 4: Summary Results: Test Article – Flutamide, Participating Laboratories – Springborn Smithers and Wildlife International.

Measurement End Points↑	Flutamide					
	Springborn Smithers			Wildlife International		
	Exposure level (nominal range in ug/L)					
	Low (100)	Med (500)	High (1000)	Low (100)	Med (500)	High (1000)
Female Length	---	---	---	---	---	---
Male Length	---	---	---	---	---	---
Survival	---	---	---	---	---	---
Female Weight	---	---	---	---	---	---
Female GSI	---	---	---	---	---	---
Male Weight	---	---	---	---	Y↓	Y↓
Male GSI	---	---	---	---	Y↑	Y↑
Male Tubercle score	---	---	---	Y↓	Y↓	Y↓
Plasma Vitellogenin - Female	---	---	---	---	---	---
Plasma Vitellogenin - Male	---	---	---	---	---	---
# of Spawns	---	---	---	not reported		
# of Eggs - Estimated	---	---	---	nd	nd	nd
# of Eggs - Actual	---	---	---	---	---	Y↓
Fecundity per Female Reproductive Day	not reported			---	---	Y↓
# of infertile Eggs	---	---	---	---	---	---
% of fertile Eggs	---	---	Y↓	---	Y↓	Y↓
Male Fatpad Index	---	---	Y↓	---	---	Y↓
Male Fatpad Score	not reported			---	---	Y↓
Histopathology*	---	---	---	---	Y↑	Y↑

*Various endpoints consult full histology report for details Appendix A-C.

---	not significantly different from controls
Y	a significant effect observed
↑ or ↓	significantly greater or less than controls.
nd	not determined statistically

Table 5: Summary Results: Test Article – Potassium Permanganate, Participating Laboratories – Springborn Smithers and ABC Laboratories.

Measurement End Points	Potassium Permanganate					
	Springborn Smithers			ABC Laboratories		
	Exposure level (nominal range in ug/L)					
	Low (225)	Med (450)	High (900)	Low (225)	Med (450)	High (900)
Female Length	---	---	---	---	---	---
Male Length	---	---	---	---	---	---
Survival	---	---	Y ↓	---	Y ↓	Y ↓
Female Weight	---	---	---	---	Y ↓	---
Female GSI	---	---	---	Y ↓	Y ↓	Y ↓
Male Weight	---	---	---	---	---	---
Male GSI	---	---	---	---	---	---
Male Tubercle score	---	---	---	---	---	---
Plasma Vitellogenin - Female	---	---	---	---	---	---
Plasma Vitellogenin - Male	---	---	---	nd	nd	nd
# of Spawns	---	---	---	nd	nd	nd
# of Eggs - Estimated	---	---	---	nd	nd	nd
# of Eggs - Actual	---	---	---	---	Y ↓	Y ↓
Fecundity per Female Reproductive Day	---	---	---	---	Y ↓	Y ↓
# of infertile Eggs	---	---	---	nd	nd	nd
% or # of fertile Eggs	---	---	---	---	---	---
Male Fatpad Index	---	---	---	---	---	---
Male Fatpad Score	not reported			nd	nd	nd
Histopathology*	---	---	---	not reported		

*Various endpoints consult full histology report for details Appendix A-C.

---	not significantly different from controls
Y	a significant effect observed
↑ or ↓	significantly greater or less than controls.
nd	not determined statistically

Table 6: Summary Results: Test Article – Ketoconazole, Participating Laboratory – Springborn Smithers.

Measurement End Points	Ketoconazole		
	Springborn Smithers		
	Exposure level (nominal range in ug/L)		
	Low (25)	Med (100)	High (400)
Female Length	---	---	---
Male Length	---	---	---
Survival	---	---	---
Female Weight	---	---	---
Female GSI	---	---	---
Male Weight	---	---	---
Male GSI	---	---	---
Male Tubercle score	---	---	---
Plasma Vitellogenin - Female	---	---	---
Plasma Vitellogenin - Male	---	---	Y↑
# of Spawns	---	---	---
# of Eggs - Estimated	---	---	---
# of Eggs - Actual	---	---	---
Fecundity per Female Reproductive Day	not reported		
# of infertile Eggs	---	---	---
% of fertile Eggs	---	---	---
Male Fatpad Index	---	---	---
Male Fatpad Score	not reported		
Histopathology* Interstitial (Leydig) cells - testes	Y↑	Y↑	Y↑

*Various endpoints consult full histology report for details Appendix A-C.

---	not significantly different from controls
Y	a significant effect observed
or ↓	significantly greater or less than controls.
nd	not determined statistically

Table 7: ABC Laboratories Coefficient of Variance (% CV) Comparison for the Biological Endpoints Determined During the 21-Day Potassium Permanganate Assay

NA = Not Applicable

	Endpoint CV for Each Treatment				
Endpoint	**Control**	**Low**	**Middle**	**High**	**Mean of all treatments**
Survival	0	8.85	22.00	82.26	37.70
Female Length	3.42	4.72	5.01	4.95	6.03
Male Length	4.59	3.62	4.22	2.81	5.08
Female Weight	11.81	10.60	25.23	17.77	21.80
Male Weight	13.54	14.64	23.22	7.08	19.49
Female GSI	19.45	30.84	78.42	52.17	60.29
Male GSI	29.20	33.12	51.64	42.73	52.23
Male Fatpad Score	50.00	31.79	54.44	0	45.41
Male Fatpad Index	67.09	58.97	62.35	63.31	83.91
Tubercle Score (mapping)	49.92	41.49	72.69	46.67	70.26
Number of Spawns	25.38	11.75	116.00	128.00	93.71
Cumulative Number of Eggs	28.97	59.54	192.22	151.11	143.95
Number of Eggs/Female Reproductive Day	36.79	57.53	190.91	154.68	146.64
Number of Non-viable eggs	42.77	55.86	90.00	73.52	87.38
Percent Fertility	9.55	5.63	33.11	141.41	63.23
Female Vitellogenin (w/o outlier values 2,046 mg/mL)	76.11	41.86	78.63	130.55	109.05
Male Vitellogenin	NA	NA	NA	NA	NA

Table 8: Wildlife International - Coefficient of Variance (% CV) Comparison for the Endpoints Determined During the 21-Day Exposure of Fathead Minnow (*Pimephales promelas*) to Flutamide.

	Endpoint CV for Each Treatment				
Endpoint	**Control**	**Low Treatment**	**Medium Treatment**	**High Treatment**	**Mean of all Treatments**
Male Length	3.55	2.99	2.31	3.31	**2.87**
Female Length	1.45	2.57	3.06	3.64	**3.09**
Male Weight	3.54	4.12	3.56	12.0	**6.56**
Female Weight	5.71	5.01	6.6	14.66	**8.76**
Male GSI	19.3	8.4	14.1	17.2	**13.2**
Female GSI	11.7	12.4	30.2	14.6	**19.1**
Male Fatpad Score	20.1	13.6	39.3	20.2	**24.4**
Male Tubercle Count	22.4	9.62	8.52	59.1	**25.7**
Male Tubercle Score	15.81	15.77	17.44	62.0	**31.7**
Male Fatpad Index	25.9	37.1	85.2	53.5	**58.6**
Female VTG	24.5	54.3	80.7	50.7	**61.9**
Fertile Eggs per Female	62.4	21.0	151	118	**96.7**
Eggs per Female	83.5	19.0	101	177	**99.0**
Number of Fertile Eggs	54.4	21.0	164	118	**101**
Number of Eggs	69.3	19.1	111	177	**102**
Male VTG	80.3	95.7	94.0	155	**115**
Number of Infertile Eggs	150	53.4	156	199	**136**

Table 9: Springborn Smithers Coefficient of Variance (CV) for each Control Group and Their Average CV

Endpoint CV for Each Treatment

Endpoint	Flutamide (µg a.i./L)				Potassium Permanganate (µg a.i./L)				Ketoconazole (µg a.i./L)				Average
	Control	100	500	1000	Control	225	450	900	Control	25	100	400	
Percent Fertile Eggs	0.61%	0.51%	0.51%	8.13%	0.46%	0.63%	0.43%	1.02%	0.46%	5.49%	16.66%	1.81%	3.06%
Female Length	1.98%	4.19%	2.24%	1.22%	5.75%	5.99%	3.03%	6.26%	2.85%	0.83%	3.67%	4.69%	3.56%
Male Length	4.03%	2.91%	3.65%	1.69%	7.82%	7.01%	2.93%	4.29%	3.61%	5.73%	1.86%	1.17%	3.89%
Survival	8.88%	0.00%	8.88%	0.00%	0.00%	8.88%	0.00%	18.11%	0.00%	0.00%	8.88%	0.00%	4.47%
Female Weight	6.88%	7.82%	8.38%	11.29%	15.38%	19.53%	9.32%	22.03%	5.03%	2.90%	10.13%	13.61%	11.02%
Female GSI	11.64%	25.84%	9.87%	20.77%	13.56%	15.87%	13.46%	48.29%	10.53%	12.50%	17.60%	13.67%	17.80%
Male Weight	13.04%	5.30%	12.14%	7.76%	18.21%	27.19%	12.68%	18.03%	10.60%	16.58%	8.40%	3.61%	12.80%
Male GSI	19.38%	17.42%	16.42%	13.14%	4.95%	17.31%	12.00%	12.50%	20.79%	18.63%	11.59%	15.48%	14.97%
Tubercle score	7.57%	27.74%	16.87%	12.08%	19.26%	12.36%	16.86%	28.07%	25.62%	8.68%	7.99%	33.85%	18.08%
Plasma Vitellogenin - Female	49.58%	26.16%	70.31%	70.71%	35.49%	63.02%	55.99%	68.45%	84.18%	62.09%	32.28%	40.65%	54.91%
# of Spawns	42.76%	30.97%	19.26%	114.67%	67.69%	141.18%	38.57%	94.29%	84.00%	94.29%	97.78%	73.33%	74.90%
# of Eggs - Estimated	27.04%	35.96%	15.52%	181.53%	102.86%	160.22%	33.61%	124.69%	93.09%	97.41%	79.61%	80.40%	86.00%
# of Eggs - Actual	34.38%	22.12%	37.94%	145.44%	100.44%	164.95%	34.24%	141.20%	90.66%	93.75%	88.59%	77.82%	85.96%
Plasma Vitellogenin - Male	70.00%	16.66%	191.54%	88.88%	94.79%	193.88%	195.89%	246.81%	56.30%	69.89%	94.34%	97.50%	118.04%
# of Infertile Eggs	62.22%	74.67%	48.00%	153.94%	120.00%	172.97%	73.91%	145.86%	200.00%	66.67%	160.00%	124.80%	116.92%
Male Fatpad Index	80.95%	200.00%	205.26%	NA[a]	200.00%	47.85%	NA	303.23%	200.00%	200.00%	NA	NA	179.66%

[a] NA = Not applicable since no data to calculate CV.

3.1 Statistical Methods Evaluation Results

3.1.1 Parametric tests

The linear trend and the Williams ordered alternatives step down tests were more sensitive than the F and Dunnett's general alternatives tests. Thus step down tests should be used unless there is statistical or toxicological indication that the dose response trend is not monotonic. The linear trend test had equivalent sensitivity to the Williams test for the three Springborn Smithers data sets and greater sensitivity for the ABC and Wildlife International data sets and should be preferred.

3.1.2 Nonparametric tests

The linear trend and the Jonckheere-Terpstra step down tests were more sensitive than the Kruskal-Wallis and the Wilcoxon Mann Whitney (with Bonferroni's adjustment) general alternatives tests. Thus step down tests should be used unless there is statistical or toxicological indication that the dose response trend is not monotonic. The Wilcoxon-Mann-Whitney test with Bonferroni's adjustment was very insensitive and is not recommended for use with future data sets. The linear trend test had equivalent or greater sensitivity than the Jonkheere-Terpstra test for four of the five data sets. For the Springborn Smithers (flutamide) data set none of the statistical tests considered identified many significant effects. The linear trend test should be preferred.

The parametric and the nonparametric step down tests were about equally sensitive. In general:

a. Springborn Smithers found fewer significant results than ABC Laboratories or Wildlife International
b. Step down tests were more sensitive than general alternative tests, e.g. linear trend tests versus Dunnett's test or Wilcoxon-Mann-Whitney test)
c. Parametric and nonparametric tests had about the same sensitivity when parametric tests were appropriate.

d. Linear trend tests appear to be a bit more sensitive than the Williams test or Jonckheere-Terpstra test, but the difference is slight. This would need to be studied in greater detail.

The evaluation of significant results among methods across participating laboratories is provided in Table 10. Based upon this table the linear trend test analysis utility for evaluating these data is apparent. Tables 11 through 16 compare the results from participating laboratories employing their preferred method against Battelle's recommend statistical model the "linear trend" (parametric or non-parametric).

Table 10 Summary of Significant Results[1]

Testing Laboratory - *Chemical*	Linear-Parametric			Williams			Linear-Non-Parametric			Jonckheere			Dunnett			WMW-Bonferroni			F-Test	Kruskal Wallis Test
Treatment Level	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H		
Springborn Smithers - *Flutamide*	0/11	0/11	0/11	0/11	0/11	0/11	0/19	0/19	1/19	0/19	0/19	5/19	0/11	0/11	0/11	0/19	0/19	0/19	0/11	0/19
Springborn Smithers - *Ketoconazole*	0/10	2/10	2/10	0/10	2/10	2/10	0/19	1/19	5/19	0/19	1/19	4/19	0/10	0/10	2/10	0/19	0/19	0/19	2/10	3/19
Springborn Smithers - *Potassium Permanganate*	0/12	2/12	2/12	0/12	2/12	2/12	0/19	2/19	2/19	0/19	1/19	1/19	0/12	2/12	1/12	0/19	0/19	0/19	2/12	2/19
ABC Laboratories *Potassium Permanganate*	0/12	7/12	7/12	0/12	2/12	4/12	2/21	13/21	15/21	2/21	12/21	15/21	0/12	2/12	1/12	0/21	0/21	0/21	4/12	9/21
Wildlife International *Flutamide*	0/15	3/15	6/15	0/15	2/15	5/15	0/20	4/20	10/20	0/20	3/20	9/20	0/15	3/15	2/15	0/20	0/20	0/20	4/15	5/20

[1]Highlighted results summarize comparisons between the parametric step down procedures (linear and Williams) and between the nonparametric step own procedures (linear and Jonkheere-Terpstra). When one procedure identifies more significant effects (p=0.05) than its comparison procedure it is highlighted. When both procedures identify the same number of significant effects they are both highlighted.

Table 11. Comparison of Significant Results Using Method as Provided by Participating Laboratory for Flutamide and Results Obtained Using Battelle's Recommended Statistical Model "Linear Trend" Parametric And Non-Parametric.

DRAFT REPORT (Participating Laboratory Statistical Analysis)	Flutamide Exposure level (nominal range in ug/L)	Female Length	Male Length	Survival	Female Weight	Female GSI	Male Weight	Male GSI	Male Tubercle score	Plasma Vitellogenin - Female
Springborn Smithers Laboratories	Statistical Method	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'
	Low (100)	---	---	---	---	---	---	---	---	---
	Med (500)	---	---	---	---	---	---	---	---	---
	High (1000)	---	---	---	---	---	---	---	---	---
Wildlife International, Ltd.	Statistical Method	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [4]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts Jonckheere [2] Kruskal-Wallis [3]
	Low (100)	---	---	---	---	---	---	---	Y ↓ [2]	---
	Med (500)	---	---	---	---	---	Y ↓ [1, 2]	Y ↑ [1, 2]	Y ↓ [2]	---
	High (1000)	---	---	---	---	---	Y ↓ [2]	Y ↑ [2]	Y ↓ [1, 2]	---
REVISED FINAL REPORT Battelle Statistical Analysis	**Flutamide Exposure level (nominal range in ug/L)**	**Female Length**	**Male Length**	**Survival**	**Female Weight**	**Female GSI**	**Male Weight**	**Male GSI**	**Male Tubercle score**	**Plasma Vitellogenin - Female**
Springborn Smithers Laboratories	Statistical Method	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]
	Low (100)	---	---	---	---	---	---	---	---	---
	Med (500)	---	---	---	---	---	---	---	---	---
	High (1000)	---	---	---	---	---	---	---	---	---
Wildlife International, Ltd.	Statistical Method	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]
	Low (100)	---	---	---	---	---	---	---	Y ↓ [P]	---
	Med (500)	---	---	---	---	---	Y ↓ [P]	Y ↑ [P]	Y ↓ [P]	---
	High (1000)	---	---	---	---	Y ↓ [NP]	Y ↓ [P]	Y ↑ [P]	Y ↓ [P]	---

Table 12 Comparison of Significant Results Using Method as Provided by Participating Laboratory for Flutamide and Results Obtained Using Battelle's Recommended Statistical Model "Linear Trend" Parametric And Non-Parametric. (Continued).

DRAFT REPORT (Participating Laboratory Statistical Analysis)	Flutamide Exposure level (nominal range in ug/L)	Plasma Vitellogenin - Male	# of Spawns	# of Eggs - Estimated	# of Eggs - Actual	Fecundity per Female Reproductive Day	# of infertile Eggs	% or # of fertile Eggs	Male Fatpad Index	Male Fatpad Score
Springborn Smithers Laboratories	Statistical Method	Williams'	Williams'	Steel's	Steel's	Williams'	Steel's	Steel's	Williams'	
	Low (100)	---	---	---	---	not reported	---	---	---	not reported
	Med (500)	---	---	---	---	not reported	---	---	---	not reported
	High (1000)	---	---	---	---	not reported	---	Y ↓	Y ↓	not reported
Wildlife International, Ltd.	Statistical Method	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [4]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [5]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]	Dunnetts [1] Jonckheere [2] Kruskal-Wallis [3]
	Low (100)	---	not reported	nd	---	---	---	---	---	---
	Med (500)	---	not reported	nd	---	---	---	---	---	---
	High (1000)	---	not reported	nd	Y ↓ [2]	Y ↓ [2]	---	Y ↓ [1, 2]	Y ↓ [2]	Y ↓ [2]
REVISED FINAL REPORT Battelle Statistical Analysis	**Flutamide Exposure level (nominal range in ug/L)**	**Plasma Vitellogenin - Male**	**# of Spawns**	**# of Eggs - Estimated**	**# of Eggs - Actual**	**Fecundity per Female Reproductive Day**	**# of infertile Eggs**	**% or # of fertile Eggs**	**Male Fatpad Index**	**Male Fatpad Score**
Springborn Smithers Laboratories	Statistical Method	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]
	Low (100)	---	---	---	---	---	---	---	---	---
	Med (500)	---	---	---	---	---	---	---	---	---
	High (1000)	---	---	---	(Y ↓)	(Y ↓)	---	Y ↓	---	---
Wildlife International, Ltd.	Statistical Method	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]	Linear Trend [P, NP]
	Low (100)	---	---	---	---	---	---	---	---	---
	Med (500)	---	---	---	---	---	---	---	---	---
	High (1000)	---	---	---	Y ↓ [P]	Y ↓ [P]	---	Y ↓ [P]	Y ↓ [NP]	---

Table 13 **Comparison of Significant Results Using Method as Provided by Participating Laboratory for Potassium Permanganate and Results Obtained Using Battelle's Recommended Statistical Model "Linear Trend" Parametric and Non-Parametric.**

DRAFT REPORT (Participating Laboratory Statistical Analysis)	**Potassium Permanganate Exposure level (nominal range in ug/L)**	**Female Length**	**Male Length**	**Survival**	**Female Weight**	**Female GSI**	**Male Weight**	**Male GSI**	**Male Tubercle score**	**Plasma Vitellogenin - Female**
Springborn Smithers Laboratories	Statistical Method	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'
	Low (225)	---	---	---	---	---	---	---	---	---
	Med (450)	---	---	---	---	---	---	Y↑ P	---	---
	High (900)	---	---	Y ↓	---	---	---	Y↑ P	---	---
ABC Laboratories, Inc.	Statistical Method	Kruskal-Wallis	Kruskal-Wallis	Kruskal-Wallis	Kruskal-Wallis	Kruskal-Wallis	Kruskal-Wallis	Kruskal-Wallis	Kruskal-Wallis	Kruskal-Wallis
	Low (225)	---	---	---	---	Y ↓	---	---	---	---
	Med (450)	---	---	Y ↓	Y ↓	Y ↓	---	---	---	---
	High (900)	---	---	Y ↓	---	Y ↓	---	---	---	---
REVISED FINAL REPORT Battelle Statistical Analysis	**Patassium Permanganate Exposure level (nominal range in ug/L)**	**Female Length**	**Male Length**	**Survival**	**Female Weight**	**Female GSI**	**Male Weight**	**Male GSI**	**Male Tubercle score**	**Plasma Vitellogenin - Female**
Springborn Smithers Laboratories	Statistical Method	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP
	Low (225)	---	---	---	---	---	---	---	---	---
	Med (450)	---	---	---	---	---	---	Y↑	---	---
	High (900)	---	---	---	---	---	---	Y↑	---	---
ABC Laboratories, Inc.	Statistical Method	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP
	Low (225)	---	---	---	---	Y ↓ P	---	---	---	---
	Med (450)	Y ↓ NP	---	Y ↓ NP	Y ↓ P	Y ↓ P	Y ↓ P	---	---	Y ↓ P
	High (900)	Y ↓ NP	---	Y ↓ NP	Y ↓ P	Y ↓ P	Y ↓ P	---	---	Y ↓ P

Table 14 Comparison of Significant Results Using Method as Provided by Participating Laboratory for Potassium Permanganate and Results Obtained Using Battelle's Recommended Statistical Model "Linear Trend" Parametric and Non-Parametric (Continued).

DRAFT REPORT (Participating Laboratory Statistical Analysis)	Patassium Permanganate Exposure level (nominal range in ug/L)	Plasma Vitellogenin - Male	# of Spawns	# of Eggs - Estimated	# of Eggs - Actual	Fecundity per Female Reproductive Day	# of infertile Eggs	% or # of fertile Eggs	Male Fatpad Index	Male Fatpad Score
Springborn Smithers Laboratories	Statistical Method	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'	Williams'	
	Low (225)	---	---	---	---	---	---	---	---	not reported
	Med (450)	---	---	---	---	---	---	---	---	not reported
	High (900)	---	---	---	---	---	---	---	---	not reported
ABC Laboratories, Inc.	Statistical Method		Kruskal-Wallis		Kruskal-Wallis	Kruskal-Wallis		Kruskal-Wallis	Kruskal-Wallis	
	Low (225)	nd	---	nd	---	---	nd	---	---	nd
	Med (450)	nd	Y ↓	nd	Y ↓	Y ↓	nd	---	---	nd
	High (900)	nd	Y ↓	nd	Y ↓	Y ↓	nd	---	---	nd
REVISED FINAL REPORT Battelle Statistical Analysis	**Patassium Permanganate Exposure level (nominal range in ug/L)**	**Plasma Vitellogenin - Male**	**# of Spawns**	**# of Eggs - Estimated**	**# of Eggs - Actual**	**Fecundity per Female Reproductive Day**	**# of infertile Eggs**	**% or # of fertile Eggs**	**Male Fatpad Index**	**Male Fatpad Score**
Springborn Smithers Laboratories	Statistical Method	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}
	Low (225)	---	---	---	---	---	---	---	---	---
	Med (450)	---	---	---	---	---	---	---	---	---
	High (900)	---	---	---	---	---	---	---	---	---
ABC Laboratories, Inc.	Statistical Method	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}	Linear Trend ^{P,NP}
	Low (225)	---	---	---	Y ↓ ^{NP}	---	---	---	---	---
	Med (450)	---	Y ↓ ^{NP}	Y ↓ ^{NP}	Y ↓ ^{NP}	Y ↓ ^{NP}	---	Y ↓ ^{P}	Y ↓ ^{P}	Y ↓ ^{P}
	High (900)	---	Y ↓ ^{NP}	Y ↓ ^{NP}	Y ↓ ^{NP}	Y ↓ ^{NP}	---	Y ↓ ^{P}	Y ↓ ^{P}	Y ↓ ^{P}

Table 15 Comparison of Significant Results Using Method as Provided by Participating Laboratory for Ketoconazole and Results Obtained Using Battelle's Recommended Statistical Model "Linear Trend" Parametric and Non-Parametric.

DRAFT REPORT (Participating Laboratory Statistical Analysis)	**Ketoconazole Exposure level (nominal range in ug/L)**	**Female Length**	**Male Length**	**Survival**	**Female Weight**	**Female GSI**	**Male Weight**	**Male GSI**	**Male Tubercle score**	**Plasma Vitellogenin - Female**
Springborn Smithers Laboratories	Statistical Method	Williams'	Williams'	Williams'	Williams'	Steel's	Williams'	Williams'	Williams'	Williams'
	Low (25)	---	---	---	---	---	---	---	---	---
	Med (100)	---	---	---	---	---	---	---	---	---
	High (400)	---	---	---	---	---	---	---	---	---
REVISED FINAL REPORT Battelle Statistical Analysis	**Ketoconazole Exposure level (nominal range in ug/L)**	**Female Length**	**Male Length**	**Survival**	**Female Weight**	**Female GSI**	**Male Weight**	**Male GSI**	**Male Tubercle score**	**Plasma Vitellogenin - Female**
Springborn Smithers Laboratories	Statistical Method	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP
	Low (25)	---	---	---	---	---	---	---	---	---
	Med (100)	---	---	---	---	---	---	Y↑ P	---	---
	High (400)	---	Y↑ NP	---	---	Y↑ NP	---	Y↑ P	---	---

Table 16 Comparison of Significant Results Using Method as Provided by Participating Laboratory for Ketoconazole and Results Obtained Using Battelle's Recommended Statistical Model "Linear Trend" Parametric and Non-Parametric (Continued).

DRAFT REPORT (Participating Laboratory Statistical Analysis)	**Ketoconazole Exposure level (nominal range in ug/L)**	**Plasma Vitellogenin - Male**	**# of Spawns**	**# of Eggs - Estimated**	**# of Eggs - Actual**	**Fecundity per Female Reproductive Day**	**# of infertile Eggs**	**% or # of fertile Eggs**	**Male Fatpad Index**	**Male Fatpad Score**
Springborn Smithers Laboratories	Statistical Method	Kruskal-Wallis'	Williams'	Williams'	Williams'	Williams'	Steel's	Wilcoxon's	Kruskalwallis/Steel's	NA
	Low (25)	---	---	---	---	not reported	---	---	---	not reported
	Med (100)	---	---	---	---	not reported	---	---	---	not reported
	High (400)	Y↑	---	---	---	not reported	---	---	---	not reported
REVISED FINAL REPORT Battelle Statistical Analysis	**Ketoconazole Exposure level (nominal range in ug/L)**	**Plasma Vitellogenin - Male**	**# of Spawns**	**# of Eggs - Estimated**	**# of Eggs - Actual**	**Fecundity per Female Reproductive Day**	**# of infertile Eggs**	**% or # of fertile Eggs**	**Male Fatpad Index**	**Male Fatpad Score**
Springborn Smithers Laboratories	Statistical Method	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP	Linear Trend P, NP
	Low (25)	---	---	---	---	---	---	---	---	---
	Med (100)	---	---	---	---	---	---	---	---	---
	High (400)	Y↑ NP	---	---	---	---	---	---	---	---

4.0 CONCLUSIONS

4.1 ABC Laboratory Study Conclusions – Potassium Permanganate

Exposure to potassium permanganate under the test conditions described above was acutely and chronically toxic to the test organisms. Overall fish survival and reproductive output (i.e., number of spawns, cumulative number of eggs, and eggs per reproductive day) were the parameters that were most severely affected by the potassium permanganate exposure. Statistically significant reductions in survival had occurred the middle (450 µg/L) and high (900 µg/L) treatment concentrations as compared to the control survival. The estimated LC_{50} value was 662 µg/L (95% confidence limits: 510 to 1,014 µg/L). Exposure to potassium permanganate shut down reproduction within the middle and high treatment levels. In the middle treatment, the only spawning activity (i.e., one spawn in replicates B and C) occurred during the first 24 hours of exposure. This same trend occurred in the D replicate of the high treatment level with only one spawn within the first 24 hours of exposure. Although the females in the high treatment A replicate did actually spawn on days 7 (4 eggs) and 9 (34 eggs) of the exposure, they were not fertile. Although not as dramatic, the mean female GSI values of all of the treatment groups displayed statistically significant reductions in GSI values as compared to the control value.

Length and weight values from the surviving fish showed the least effect of the exposure with a statistically significant reduction only in the female body weights of the surviving fish exposed to the middle treatment only. Also these parameters displayed the least variability over the exposure with %CV values of $\leq$ 5.01 and $\leq$ 25.23, respectively, which can be attributed to the fact that the exposure duration was relatively short and adult fish were being tested.

Secondary male sex characteristics, i.e., fatpad scores, fatpad index (i.e., FPI), and tubercle scoring, were reduced with increasing potassium permanganate concentrations, but these were not statistically significant reductions. These characteristics also displayed some of the highest %CV values in the control animals at 49.94% for the tubercle scoring to 67.09% for the control FPI values.

Female plasma vitellogenin concentrations displayed a downward trend with mean values of 65.3 (excluding 2,046 mg/mL value), 74.6, 9.5, and 27.3 mg/mL in the control, low, middle, and high treatments, respectively, but there were no statistically significant effects at $p < 0.050$. Even without the inclusion of a plasma VTG concentration of 2,046 mg/mL from one control female, this parameter displayed a high variability (i.e., 76.11% CV) between control females.

In a comparison of the semi-quantitative egg estimate versus the actual counts, the egg estimates were most accurate (i.e., number of spawns in which the estimate was within 20% of the actual count) for the 200 and 250 egg estimate categories with 85 to 92% accuracy. With the number of females that could spawn within a replicate chamber, it was common that the >350 egg category did not closely represent the actual egg values, many of which were much greater than 350 eggs.

4.2 Wildlife International Study Conclusions - Flutamide

Reproductive groups of fathead minnows (4 females and 2 males) were exposed to three flutamide concentrations (100, 500, and 1000 μg/L) and a negative control. There were no apparent effects on female length, weight, GSI, or vitellogenin observed in any of the test concentrations. There were apparent treatment-related effects on fertility and fecundity at the 1000 μg/L test concentration, the highest concentration tested, that were statistically significant. The effects observed were consistent with the pathology report, which indicated an increase in both the incidence and severity of oocyte atresia in the 1000 μg/L treatment group relative to the controls. In males, there were no apparent effects on length or vitellogenin. There were apparent treatment-related effects on male weight, GSI, and secondary sex characteristics (fatpad score, fatpad index, tubercle count, and tubercle score) at the 1000 μg/L test concentration, the highest concentration tested, that were statistically significant. Effects on weight, tubercle count and score, and GSI were also apparent and statistically significant at 500 μg/L, the middle test concentration. Only effects on tubercle score were apparent at the 100 μg/L test concentration, the lowest concentration tested. The effects observed were consistent with the pathology report, which indicated an increase in spermatogonia in both the 500 and 1000 μg/L treatment groups and an increase in testicular stage score in the 1000 μg/L treatment group relative to the controls.

4.3 Springborn Smithers Study Conclusions – Flutamide, Potassium Permanganate and Ketoconazole

The screening test was sensitive in identifying potential endocrine disrupting effects in both the flutamide and the ketoconazole exposures, where effects were expected. The screening test was also non-sensitive in identifying false positive effects in the potassium permanganate exposure, where endocrine-related effects were not expected.

In the potassium permanganate exposure, with the exception of fish survival, no significant adverse effects were observed on any of the endpoints evaluated in this screening test. Survival was significantly reduced at the highest treatment concentration.

Multiple endpoints were sensitive in detecting effects, suggesting that a successful screening study will need multiple endpoints to identify and corroborate endocrine-related effects. Percent fertile embryos and fatpad index were significantly reduced in the highest treatment level in the flutamide exposure. Additionally, three other observations, though not significant, corroborate the effects seen in percent fertile embryos and fatpad index. The incidence of increased interstitial cells was slightly higher in the males from 1000 µg a.i./L group as compared to controls in the flutamide exposure. The flutamide exposure suggests that there is a dose-related trend in the cumulative number of eggs produced at the two highest treatment levels. Ovarian developmental stage average scores were generally higher in the two highest dose groups (500 and 1000 µg a.i./L) as compared to controls.

Two significant findings were attributable to ketoconazole exposure. There was an increased presence of interstitial (Leydig) cells in the testes of the 25, 100, and 400 µg a.i./L group males as compared to controls. This finding appeared to be somewhat dose-responsive in terms of incidence and severity. Vitellogenin production was induced in the male fish exposed to the 400 µg a.i./L treatment. There was also a dose-related trend (though not statistically significant) of increased male GSI.

A total of 16 endpoints were statistically evaluated in the three exposures. Table 9 compares the coefficient of variance (CV) for each control group and their average CV. Nine of the 16 endpoints have mean CV less than 20%. However, five of these endpoints (male and female length and weight and survival) may be not be sensitive to endocrine disruption. However, these endpoints are useful in demonstrating that the fish were of similar size, which is important when working to minimize the variability of spawning and fecundity endpoints.

The seven remaining endpoints have average CVs greater than 50%. The fecundity endpoints (number of eggs and number of spawns) are typically variable in fathead minnows. However,

they can be sensitive endpoints. Buikema (1992) found that the reproductive endpoints in fish full life cycle studies were the most variable. Eggs per female and spawns per female were the most variable endpoints with CVs of 106% and 80%, respectively.

Blood plasma vitellogenin concentration is also rather variable, but the three- to four-order of magnitude differences between control males and females and between affected and non-affected males may make the high CVs less of a concern. The ELISA kits used for analysis of plasma vitellogenin presented were problematic. The initial lot of kits supplied by the manufacturer was defective; therefore, the kits did not develop properly and samples were lost. In addition, due to the small size of some female fish, the blood plasma volumes collected were low. Due to these low volumes, archived samples were not available for reanalysis.

The number of infertile eggs is a highly variable endpoint, but integrating this observation into percent fertile embryos results in potentially the most sensitive apical endpoint of the screening assay. However, the biological relevance of this endpoint could be challenged. For example, in the flutamide exposure the percent of fertile embryos in the highest treatment level was 91%. A 9% reduction in fertilization is unlikely to have a biologically relevant impact on a fathead minnow population.

Male fatpad index may have been less variable if the skin region of the fatpads in male fish that scored a 1 had been dissected and weighed. Even with considerable variability, the male fatpad index was significantly reduced in the highest treatment level of the flutamide exposure. In all three chemical exposures, there were more male fish with fatpad scores greater than 1 in the control and lowest treatment level than in the two highest treatment levels.

The histopathological examination of the gonads, though qualitative, was important in identifying potential effects in the ketoconazole exposure. The histopathological examination of the gonads corroborated other apical and biochemical effects observed in the flutamide exposure.

4.5 Battelle's Statistical Methods Analysis Recommendations

The following steps are recommended for future evaluations:
Carry out preliminary outlier detection procedures based on a heterogeneous variance generalization of Grubbs screening test. Determine which screened values, if any, to delete. After outliers have been deleted, for all endpoints other than survival or percent (in) fertile eggs if the dose group means vary over more than an order of magnitude, then carry out a log transformation. For survival or percent (in) fertile eggs, carry out logit transformations.

For endpoints other than survival, first carry out the Shapiro-Wilk normality test and Levene's heterogeneity of variance test to determine whether there are departures from the normality and homogeneity of variance assumptions. If the data are compatible with both of these assumptions, then the parametric procedures will be used. If one or both of these assumptions are violated, then the nonparametric procedures will be used.

If parametric procedures are to be used, the F-test will first be carried out. The linear trend step down test procedure will be carried out irrespective of whether the F-test is significant since this is more sensitive than the F-test. If the linear trend test does not identify any significant differences from the control and the F-test is not significant then stop. If the linear trend test does not identify any significant differences from the control and the F-test is significant, then carry out Dunnett's general alternatives test to detect possible non-monotonic alternatives.

If nonparametric procedures are to be used, the Kruskal-Wallis test will first be carried out. The linear trend step down test procedure will be carried out irrespective of whether the Kruskal-Wallis test is significant since this is more sensitive than the Kruskal-Wallis test. If the linear trend test does not identify any significant differences from the control and the Kruskal-Wallis test is not significant then stop. If the linear trend test does not identify any significant differences from the control and the Kruskal-Wallis test is significant then carry out Steele's many one rank test or Dunnett's test on the rank transformation of the data to detect possible non- monotonic alternatives.

For the survival endpoint carry out tests based on males and females combined due to the small number of males per tank. Carry out a preliminary test of homogeneity of survival rates among tanks within dose groups by the Cochran Mantel Haenszel test. If the preliminary test is not significant combine data across tanks within dose groups. Test for dose response trends in survival by the exact Cochran-Armitage test in a step down fashion. If the preliminary test is significant, carry out logit transformations of the survival rates in each tank and treat the logit transformed survival rates in the same manner as the non survival endpoints.

4.6 Assay Lessons Learned

During the in-life portion of this study it became evident that contract laboratories performing this assay routinely should have their own in-house cultures. Fish shipped from offsite culture facilities experience stress and potentially high levels of ammonia during transit, especially when they are shipped at high densities. For this assay, adequate numbers of 20-week-old fish were required to be at the test facility, acclimated to the test facility conditions (e.g., water quality parameters) and in good health (disease-free) prior to spawning. The participating laboratories found that recently shipped fish had difficulty meeting the pre-exposure spawning success criteria within the suggested 14-day window. Because the fish experience increased stress during the spawning process, reducing or eliminating stress from shipping and acclimation is advisable. Stress in test fish would likely be significantly reduced if each facility reared the fish onsite.

In addition, the participating laboratories indicated that unnecessary stress in test fish may be further reduced by increasing the size of the test aquaria. The 5-gallon aquaria involved in this study housed four adult females and two adult males in 10 L of water. Study participants indicated that larger aquaria with greater water volume capacity may reduce fish aggression in the tanks. Additional laboratory space would be required to accommodate the larger tanks; however, all participating laboratories indicated that space was not a limiting factor.

5.0 REFERENCES

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Appendix A- ABC Report

STUDY TITLE

Potassium Permanganate: Fish Screening Assay Phase 1 B Follow-up
WA 5-11 Fish Study

AUTHOR

John Aufderheide

STUDY INITIATION DATE

October 18, 2005

EXPERIMENTAL COMPLETION DATE

November 21, 2005

SPONSOR

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1.0 INTRODUCTION

1.1 Purpose

The U.S. Environmental Protection Agency (EPA) is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is a short-term screening assay designed to detect substances that interact with the estrogen and androgen systems of fish. It is thought that the inclusion of the fish screening assay in Tier 1 is important because estrogenic and androgenic controls on reproduction and development in fish may differ significantly enough from that of higher vertebrates such that mammalian screening methods may not identify potential endocrine disrupting chemicals (EDCs) in this important class of animals. As an example, dihydrotestosterone is a potent androgen in mammals, but 11-ketotestosterone is generally the more prevalent androgen in fish.

EPA (2001) has described a short-term test with the fathead minnow *(Pimephales promelas)* 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 (Ankley et al. 2001 (1)) 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 is 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 gonadosomatic index (GSI), gonadal histology, and plasma concentrations of vitellogenin and sex steroids (17ß-estradiol, testosterone, and 11-ketotestosterone).

The Organization for Economic Cooperation and Development (OECD) initiated a fish screening assay validation activity and has completed its Phase 1A and Phase 1B trials. Phase 1A evaluated a non-spawning version of a 21-day exposure assay with fathead minnow, medaka, and zebrafish. The results of the Phase 1A led to the Phase 1B trials where spawning was included in the method. The results of the Phase 1B trial raised questions regarding the spawning conditions utilized for the fathead minnow.

The purpose of work to be conducted under this work assignment is to perform a follow-on study to the OECD Phase 1B study based on features in the short-term reproduction assay with the fathead minnow as described in EPA (2001(2)). Specifically, this incorporates an increased number of replicates and uses a semi-quantitative and quantitative egg-counting method. The studies will include three laboratories (Springborn Smithers Laboratories, ABC Laboratories, Inc., and Wildlife International, Ltd.).

This protocol describes an *in vivo* screening assay for identifying endocrine active chemicals in sexually dimorphic fish. It has been adapted from the OECD Phase 1B protocol with specific changes to various measurement endpoints.

2.0 MATERIALS AND METHODS

2.1 Test Material and Exposure Regime

The test chemical concentration series used for this project was determined by the Sponsor and is summarized in Table 2.1.

Table 2.1 Nominal test chemical concentrations used in the WA 5-11 fish screen study

Test Chemical	Low (µg/L)	Middle (µg/L)	High (µg/L)
Potassium permanganate	225	450	900

A test-grade aliquot of the test chemical, potassium permanganate (Lot No. 00310LC; CAS Number 7722-64-7) was shipped from the Battelle Sciences Laboratory Chemical Repository in Sequim, Washington and received by ABC Laboratories, Inc on October 10, 2005. The test substance was stored in an amber bottle and was assigned ABC Laboratories Reference Number TS-18481. The certificate of analysis reported the purity of the test substance as 99.5% (Appendix A) and described the test substance as dark black beads. The test substance was stored at ambient temperature. A copy of the chain-of-custody form accompanied the test substance.

2.2 Dilution Water and Preparation of Chemical Exposure Solutions

The dilution water was a moderately hard freshwater prepared by blending naturally hard well water with well water that was demineralized by reverse osmosis (RO). The well water and RO water were blended together to yield a dilution water with total hardness ranging from 130 to 150 mg/L as $CaCO_3$. As it entered the diluter system, the dilution water was passed through a particulate filter and an ultraviolet sterilizer. Chemical characterization of a representative sample of the dilution water is presented in Appendix B.

A 2-L proportional equal solvent diluter system similar to that described by Mount and Brungs (3), with a Hamilton Model 420 syringe dispenser, was used for the preparation and intermittent introduction of control and potassium permanganate test solutions into each test chamber during the screening test. The diluter was constructed from plate glass glued together with silicone adhesive. The diluter system mixing/flow-splitting cells delivered the dilution water control and each of the three test solutions to the test chambers. Each mixing/flow-splitting cell divided each 2-L volume four ways. This split resulted in a volume of approximately 500 mL being delivered to each test chamber with each cycle. The frequency of the 500-mL additions was maintained at a rate sufficient to provide at least six volume additions to each test chamber in a 24-hour period. The accuracy of the diluter was verified by volumetric measurement before initiation of the definitive test. The diluter was allowed to operate approximately eight days prior to initiating the definitive test. Proper operation of the proportional diluter and all mechanical systems was verified twice each day during the definitive test. Calibration checks were made periodically to determine that the diluter remained accurate in its dilution preparations (stayed within 0 to 5% of target volume) and delivery to the test chambers (stayed within 0 and 12% of target volume). The diluter system was labeled with the study number.

All-glass aquaria were used as the test chambers. Each test chamber measured 15 cm in width by 31 cm in length with a test solution depth of 22 cm, yielding a volume of approximately 10 L. All test chambers were individually drained through the side of the chamber to a floor drain, diluted with other laboratory wastewater, and onto a wastewater treatment system. Chamber drains were covered with stainless steel screen to prevent fish escape. Test chambers were labeled with treatment and replicate designation.

As needed, 1L diluter stock solutions were prepared at a nominal concentration of 3,140 mg/L by mixing approximately 3.155 g (equivalent to 3.14 g based upon purity of the test substance) with deionized water. When more volume was needed, diluter stock solutions were prepared by mixing approximately 6.31 g (equivalent to 6.28 g) into 2 L of deionized water. The diluter stock solutions were stored at room temperature and shielded from light with a black plastic cover. Diluter stock solution usage was monitored daily. The diluter system was equilibrated for eight days prior to initiation of the test substance exposure.

2.3 Analytical Procedures

2.3.1 Potassium Permanganate

Analytical samples were collected from two separate time periods prior to the addition of the test organisms to demonstrate that the diluter system had come to equilibrium (Day –N samples). Analytical samples were also collected at test initiation, and weekly thereafter until test termination. For each sampling period, fresh analytical standards were prepared at concentrations of approximately 125, 250, 500, 825, and 1,250 µg/L. A 10-mL sample was collected from each treatment replicate test chamber at each sampling period. A 1-mL sample of the diluter stock was also collected at each time period as a means to account for potential discrepancies in the test chamber recoveries from the nominal. Control dilution water was collected from the preparation of the quality control fortifications. Only the quality control fortification (QC) samples and the diluter stock solution needed further dilution such that the concentration of the analyte fell within the analytical standard curve.

The analysis of the samples was performed by a spectrophotometric analytical method that was provided by the Sponsor and checked prior to the initiation of the exposure phase of this study. The instrument conditions are described below:

Spectrophotometer (Perkin-Elmer Lambda UV/Vis)

Acquisition range:	190 to 1,100 nm
Interval:	2 nm
Integration Time:	0.5 seconds
Std. Deviation:	On

Data Analysis

Type:	Absorbance
Display Spectrum:	400 to 600 nm
Wavelengths in Use:	525 and 546 nm
Background Correction:	single reference wavelength at 590 nm

2.4 Animals and Husbandry

The fathead minnows, *Pimephales promelas*, were from in-house cultures utilizing either well water or dilution water distributed to the culture tanks as a single pass-through. The culture facility is illuminated with fluorescent lighting set to a 16-hour light to 8-hour dark photoperiod with two 30-minute transition periods to simulate dawn and dusk. The animals were adult males and females approximately 186 (182 to 190) days old at the initiation of the pre-exposure. The cultures were maintained at conditions conducive for this species. During the growth of these animals, they were fed *ad libitum* live brine shrimp nauplii (*Artemia* sp.) and a commercial fish feed (e.g., Rangen Salmon Starter granules) at least twice daily.

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

Water Characteristic	Preferred Range
Temperature (°C)	24 – 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 (mg/L)	≤35 mg/L

2.5 Study Schedule and Design

The experimental protocol for this short-term reproduction assay is based upon the protocol developed by Ankley et al. (2001 (1)) using the fathead minnow (*Pimephales promelas*). This assay measured the reproductive performance of groups of fathead minnows as the primary indicator for endocrine disruption. Additional measurements of morphology, histopathology, and biochemical endpoints were performed to aid identification of the specific toxicological mode of action of the test chemical.

2.6 Description of Study Protocol

The assay was initiated with mature male and female fish approximately 186 (182 to 190) days old at the initiation of the pre-exposure. A diluter system was set up with 28 individual replicate spawning chambers that were initiated with 2 male and 4 female adult fathead minnows on October 14, 2005. The pre-exposure test chambers were the same dimensions as those used in the exposure phase of this testing. The animals were randomly selected from the culture population based upon each fish meeting the required weight restrictions set by the protocol. The pre-exposure lasted for 17 days. A single stainless steel spawning substrate was provided to each test chamber for the first 12 days during the pre-exposure period. Three spawning tiles were provided for the remaining 5 days of pre-exposure. This inconsistency was based upon discrepancy in the protocol. Although the additional spawning substrates may have lessened intra-male competition and allowed for more spawning activity, the differences in the number of spawning tiles did not adversely affect the ability to assess or select active spawning units for the chemical exposure testing. During the pre-exposure period, survival, spawning frequency and semi-quantitative assessment of the number of embryos per spawn were performed on a daily basis. This

information was used to determine and select the spawning units (i.e., replicate) that were randomly placed in the exposure system.

Spawning units that had females with male-like coloration were removed from the pool of possible units for the testing. Also there were two units where one of the females was missing an eye (injured during the pre-exposure phase) and these were also removed from the pool. The four highest producers that were not rejected based upon the above mentioned criteria were randomly assigned a treatment level for replicate 1. This process was repeated for each of the four replicates.

The exposure diluter system was initiated on October 23, 2005 and was running and delivering toxicant to the replicate test chambers for eight days prior to initiation of the exposure phase (i.e., transfer of fish). The analytical verifications from the two day –N sampling events were used to demonstrate that the diluter system was at equilibrium prior to the initiation of the exposure phase of this study. Each test chamber contained three stainless steel spawning substrates. The transfer of fish was performed on October 31, 2005 (Day 0) and the chemical exposure was maintained until termination on November 21, 2005. The diluter cycle rate was increased on November 1, 2005 (Day 1) and November 7, 2005 (Day 7) in order to increase the dissolved oxygen concentration within the test chambers. During the 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics were observed while fecundity and fertilization success were monitored daily. At termination of the assay, measurements were made of a number of endpoints reflective of the animal morphology and status of the reproductive endocrine system, including the GSI, gonadal histology, and plasma concentrations of vitellogenin (VTG) (Section 2.6.1).

During the pre-exposure and exposure phases, the fish were fed frozen adult brine shrimp (*Artemia* sp.) and a commercial fish feed (Rangen Salmon Starter #2 granules) at least twice daily. The fish were not fed during the 24 hours immediately preceding termination of the exposure phase (i.e., necropsy) of the test. Contaminant analyses were conducted on representative samples of the types of food used in this study and the results are on file at ABC Laboratories, Inc. Historically, there have been no contaminants found above detection limits that would adversely affect the integrity of this study. The test chambers were cleaned periodically (at least every one to two days) during the test to remove waste material and uneaten food and to minimize biological growth on the sides and bottom of the test chamber.

After 21 days of exposure, surviving fish were carefully netted from each replicate chamber and anesthetized with tricaine methanesulfonate (MS-222; Western Chemical, Inc.). After the collection of the blood samples each fish was sacrificed with a cervical dislocation in accordance with ABC Laboratories, Inc. standard operating procedures and processed as described in section 2.6.1.

2.6.1 Summary of Assay Endpoints

Survival: Survival was assessed daily. Adult female survival was also used in the determination of the number of eggs per female per day.

Behavior of Adults: Abnormal behavior relative to controls, such as quiescence (lethargy), irregular respiration, and loss of equilibrium was noted during the daily observations.

Fecundity/Fertilization Success: Egg production was determined daily by both semi-quantitative estimates and direct quantitative counts. The stainless steel spawning substrates were removed from the tanks and eggs were enumerated (if present) or the substrate was returned to the test chamber if no spawn was present. If no spawn was present a 0 was recorded. If a spawn was present on any of the substrates, a semi-quantitative assessment (i.e., 10, 25, 50, 100, 150, 200, 250, 300, and >300) of the number of eggs was made and recorded as well as an actual count of the total number of eggs. The eggs were gently rolled off the substrate with a gentle circular motion of a gloved finger into a glass dish and visually assessed for fertilization under magnification. The number of viable eggs was recorded. The number of viable eggs versus the total number of eggs was used to calculate the percent fertilization.

Appearance of Adults: During the course of the exposure, secondary sexual characteristics, such as body coloration, presence of dorsal nap pad (fat pad of suspect or confirmed males), nuptial tubercles (all fish), general head shape, and ovipositor were observed and noted for all fish on a regular basis. During the course of the study, the position of the test chambers within the water bath did not always allow for fully detailed observations for all fish for all characteristics (e.g., presence of ovipositor, nuptial tubercles, etc.). Prior to termination, each fish was isolated and evaluated for all of the secondary sexual characteristics mentioned above. At the conclusion of the exposure, all individuals were anesthetized with tricaine methanesulfonate (MS-222; Western Chemical, Inc.), blotted dry with paper toweling, measured for standard length (measurement from tip of the snout to the caudal peduncle) using a millimeter scale, and weighed (blotted wet weight) on an electronic balance. The blotted wet weight was collected from the fish prior to the fixation of the gonads. The fat pads (dorsal nape pad) of all suspect or confirmed males (if present) were visually assessed and assigned a score (Table 2.3) prior to collection of the blood samples. After collection of the blood, the fat pad was carefully excised, weighed, and used to calculate the fat pad index (FPI = weight of fat pad/weight of fish*100).

Table 2.3 Fat Pad Score Criteria

Score	Description
1	No fat pad visible
2	Small fat pad evident
3	Fat pad is clearly visible and is just above body surface
4	Fat pad is prominent, and is clearly visible above the body surface, but not 'overhanging'
5	Fat pad is very prominent and is starting to 'overhang' the body surface

Blood Sampling: After scoring the fat pad of the males or immediately after anesthetization of the females, blood was collected from the caudal vein with a heparinized microhematocrit capillary tube. The collected blood was placed into an appropriate holding container and stored on ice until centrifugation. Once enough samples were available, the samples were centrifuged for 5 minutes, which separated the red blood cells and the plasma. The capillary tubes were

notched at the delineation of these two phases and broken at the notch. The plasma was expelled into a labeled 0.25-mL centrifuge tube containing 0.13 units of lyophilized aprotinin. The collected plasma was stored in a cooler with dry ice until transferred to a -80 °C cryogenic freezer.

Morphology and Gonad Size: After blood collections, the nuptial tubercles were evaluated. The abdominal cavity was opened and the gonads were fixed with Davidson's fixative (approximately 0.5 mL) *in situ* for 90 seconds, and excised along with the viscera. The gonads were weighed to the nearest 0.1 mg and the weights were used to calculate the gonadosomatic index (GSI = (gonad weight/body weight)*100). After weighing the gonads were placed within a tissue cassette and preserved along with the viscera. The remainder of the carcass was disposed of at this time.

Vitellogenin (Vtg): The measurement of the plasma samples for VtgG was performed using enzyme-linked immunosorbent test (ELISA) kits supplied by Amersham Biosciences. Where possible-if enough plasma was present-vitellogenin levels were quantitated in plasma from each fish. For analyses, samples were removed from the -80°C storage, thawed, and either a 5- or 10-µL volume of plasma was removed and diluted by a factor of 50, 5,000, or 500,000. After processing and incubation, the plates were read at 450 nm wavelength. The reported results were based on sample runs that had standard curve R^2 values of 0.995, 0.999, 0.996, and 0.995. The standard curves were generated from the results of duplicate analyses of standards at nominal concentrations of 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 ng Vtg/mL. Standards were eliminated from the standard curve if the %CV between the duplicate analyses for a given standard concentration exceeded 20% for a given run. The curves for the reported data all were based upon the equation: Log(Y) = A + B*Log (X). With only a few exceptions, all Vtg results were estimated for optical density values that were below or above the standard curve range, which is an issue that ABC Laboratories has had with this particular ELISA kit not only in this study but in past studies as well. Attempts were made to dilute plasma samples to an expected Vtg concentration that would fall within the standard curve; however, optical density values for the standards varied with each analysis. If the Vtg value for a particular sample was either above or below the standard curve, the result would be reported as either greater than or less than the dilution factor for that sample multiplied by the lowest or highest acceptable standard for a given run.

2.6.2. General Water Chemistry and Environmental Conditions

Illumination over the test vessels was provided by wide-spectrum fluorescent bulbs controlled by an electronic timer. A 16-hour light:8-hour dark photoperiod with a 30-minute simulated dawn and dusk transition period was provided. Light intensity, measured with a LI-COR Model LI-189 light meter equipped with a photometric sensor, ranged from 435 to 544 lux on Day 16 in the early afternoon and ranged from 654 to 829 on Day 16 in the early evening. The mean light intensity value of 650 lux fell within the range specified by the protocol. Curtains shielded the diluter system from surrounding laboratory activity. The test chambers were arranged in a temperature-controlled water bath using a computer-generated random number table to assign specific treatment location. The temperature of the water bath was controlled by an OPTO 22 computer controller and was set to maintain a target temperature of 25 ± 1 °C.

Temperature, pH, salinity, and dissolved oxygen concentration were measured in all replicates of the treatment groups at test initiation, weekly throughout the test, and at termination of the definitive test. Additional measurements were made for the temperature and dissolved oxygen to be able to modify the diluter delivery (increased diluter cycle rates) if needed to maintain acceptable water quality. Temperature and pH were measured with a WTW pH 330i pH meter. Dissolved oxygen concentration was measured with a WTW OXi 330 dissolved oxygen meter. No aeration was provided to any control or test substance chamber during the test. A continuous recording of temperature in a centrally located test chamber (vehicle control replicate C) was made using a data logger and thermistor probe. Alkalinity and total water hardness values were determined from samples collected from the control and the high treatment level. Total hardness and total alkalinity were measured using titrimetric methods adapted from Standard Methods (4).

2.7 Statistical Analyses

Descriptive statistics, including the mean, standard deviation, minimum, maximum, and quartiles, were determined for each endpoint measured in the tests. Statistical significance for each endpoint and chemical were evaluated based on the difference in the mean characteristics between the treated and control groups using the nonparametric Kruskal-Wallis test and the Mann-Whitney U ranks comparison's test. The median lethal concentration (LC_{50}) estimates and their 95% confidence limits were calculated using the Probit method. 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.

2.8 Quality Assurance

Procedure audits were performed for both the biological data collection and analytical phases of the exposure period. The in-life audits performed are presented in Table 2.4.

Table 2.4 Quality Assurance In-Life Audits

Date of Study-Based Inspection	Phase Inspected	Date Reported to Principal Investigator	Date Reported to Test Facility Management
10 Nov 05	Procedures: Egg Collection	10 Nov 05	10 Nov 05
22 Nov 05	Spiking Solution Preparation	22 Nov 05	22 Nov 05

Pertinent data and data calculations were reviewed for completeness and accuracy prior to inclusion in the final report.

3.0 RESULTS

3.1 Analytical Confirmation

Potassium permanganate concentrations were measured in test solutions collected from the control and each treatment solutions at test day 0, test day 8, test day 16, and test day 21. Prior to test initiation, samples were collected at two time periods (October 25 and October 28, 2005) and the recoveries ranged from 98 to 114% of the nominal concentrations, which demonstrated that

the diluter had come to equilibrium prior to test initiation (Table 3.1). Measured concentrations of potassium permanganate in the test solutions at test initiation were <MQL (control), 260, 468, and 924 µg/L, which represented recoveries of 103 to 116% of the nominal treatment concentrations. The analytical recovery values from the diluter stock solution samples ranged from 84 to 106% of the nominal concentration, 314 mg/L. Except for a low value on day 16 in the 450 µg/L treatment, the exposure concentrations for the 450 and 900 µg/L treatments were considered stable (within 20% of Day 0 measured concentrations) throughout the exposure. Although the mean measured concentration for the 225 µg/L treatment was 82% of the nominal concentration, the daily measurements were not maintained within 20% of the day 0 measured value for this treatment level. The decrease in the measured concentrations must be due to the fish (e.g., increased respiration due to stress) within the test chambers since it cannot be accounted for by changes in the diluter stock or a problem with the diluter system. The recoveries of the low quality control fortifications ranged from 69 to 110% of the nominal concentrations (193 to 218 µg/L). The recoveries of the high quality control fortifications ranged from 89 to 98% of the nominal concentrations (967 to 1,090 µg/L). The quality control fortifications were utilized to test effectiveness of the analytical methodology.

Table 3.1 Summary of the Exposure Analytical Data for the 21-Day Potassium permanganate Assay

Nominal Conc. (µg/L)	Measured Concentration as µg/L (Percent Nominal)						
	Day –N [a]	Day –N [b]	Day 0	Day 8	Day 16	Day 21	Mean [e]
0	<MQL [c]	<MQL [c]	<MQL [d]	<MQL [d]	<MQL [d]	<MQL [d]	<MQL
225	231 (103)	256 (114)	260 (116)	188 (84)	146 (65)	143 (64)	184 (82)
450	440 (98)	474 (105)	468 (104)	395 (88)	320 (71)	372 (83)	389 (86)
900	934 (104)	948 (105)	924 (103)	890 (99)	740 (82)	847 (94)	850 (94)

[a] First equilibration check (Day – N) was collected and analyzed on October 25, 2005 – 5 days prior to initiation.
[b] Second equilibration check (Day – N) was collected and analyzed on October 28, 2005 – 2 days prior to initiation.
[c] Minimum Quantifiable Limit (MQL) = 120 µg/L, Based upon the following equation:

$$MQL = \frac{(\text{low standard concentration } (120\ \mu g/L))\ \ (\text{analysis volume } (10\ mL))}{(\text{sample volume } (10\ mL))} = 120 \mu g / L$$

[d] MQL = 122 µg/L.
[e] MQL = 126 µg/L.
[e] The day –N values were not included in the mean calculations.

3.2 Survival

Adult survival was adversely affected by potassium permanganate exposure to the middle (450 µg/L) and high (900 µg/L) treatment levels, with mean percent survival values of 75 and 38%, respectively (Table 3.2; Figure 3.1). There were statistically significant differences in the percent survival values among treatments during the 21-day assay (Kruskal-Wallis, $H = 10.92$, $p = 0.012$, df = 3). The estimated LC_{50} value was 662 µg/L (95% confidence limits: 510 to 1,014 µg/L). There were no mortalities recorded for the control animals. Therefore the control survival of 100% met the acceptability criteria (% survival >90%) for this assay.

Table 3.2 Summary Statistics for Adult Survival Data for the 21-Day Potassium permanganate Assay

Level	N	Mean % Survival	SD	CV
CTRL	4	100	0.00	0
Low	4	96	8.50	8.85
Middle	4	75	16.50	22.00
High	4	38	31.26	82.26

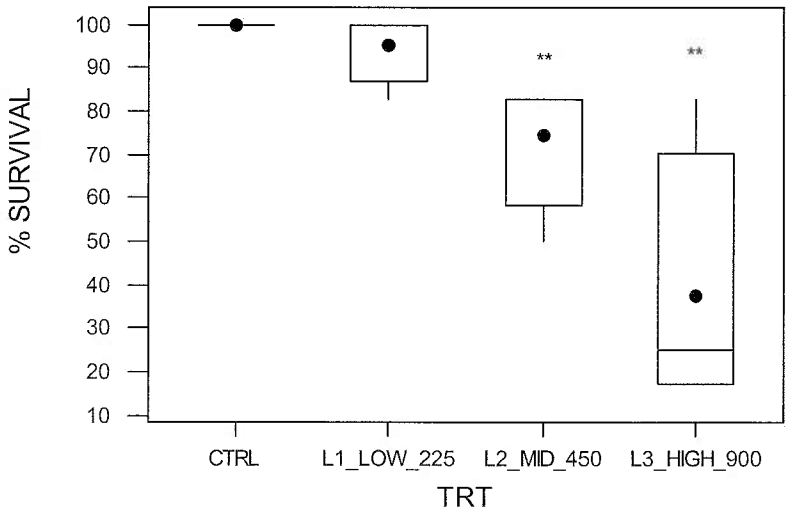

Figure 3.1 Box Plot of Adult Percent Survival by Treatment for the 21-Day Potassium permanganate Assay

(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 double asterisks represent values that are significantly ($p<0.05$) different from the control)

3.3 Secondary Sexual Characteristics

At test termination each surviving fish was visually examined for secondary sexual characteristics, including body coloration, body and head shape, presence of fat pad and score (suspected males), tubercle score (all fish), presence absence of ovipositor (suspected females only). One of the fish in the control and middle treatment was initially described as a female that did not have a prominent ovipositor or a distended abdomen (i.e., gravid), but did have normal female coloration and there was no fat pad present. These fish were determined to be male based upon gonadal confirmation of gender. There were no true females that displayed tubercles at test termination.

3.4 Body Length

Body lengths of individual females used in the 21-day screening assay ranged from 41 to 48 mm in standard length as measured from the tip of the snout to the caudal peduncle and the mean standard lengths ranged from 42.9 to 44.3 mm (Table 3.3, Figure 3.2). There were no statistically significant differences in mean length values among treatments (Kruskal-Wallis, $H = 4.29$, $p = 0.232$, df = 3).

Table 3.3 Summary Statistics for Female Body Length Data for the 21-Day Potassium permanganate Assay

Level	N	Mean [a] Length (mm)	SD	CV
CTRL	4	44.1	1.51	3.42
Low	4	44.3	2.09	4.72
Middle	4	42.9	2.15	5.01
High	4	43.8	2.17	4.95

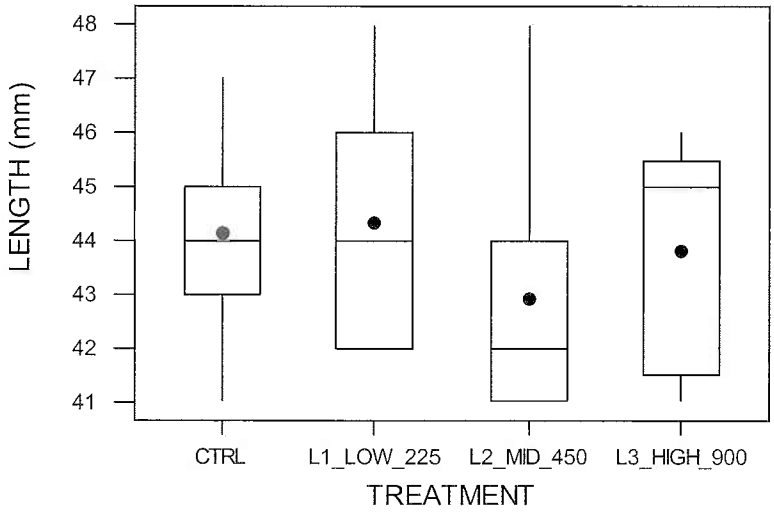

Figure 3.2 Box Plot of Female Body Length by Treatment for the 21-Day Potassium permanganate Assay

(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 body lengths of individual males used in the 21-day screening assay ranged from 50 to 58 mm in standard length and the mean standard lengths ranged from 53.2 to 54.1 mm

(Table 3.4, Figure 3.3). There were no statistically significant differences in mean length values among all treatments (Kruskal-Wallis, $H = 1.03$, $p = 0.793$, df = 3).

Table 3.4 Summary Statistics for Male Body Length Data for the 21-Day Potassium permanganate Assay

Level	N	Mean Length (mm)	SD	CV
CTRL	4	53.2	2.44	4.59
Low	4	54.1	1.96	3.62
Middle	4	53.3	2.25	4.22
High	4	53.3	1.50	2.81

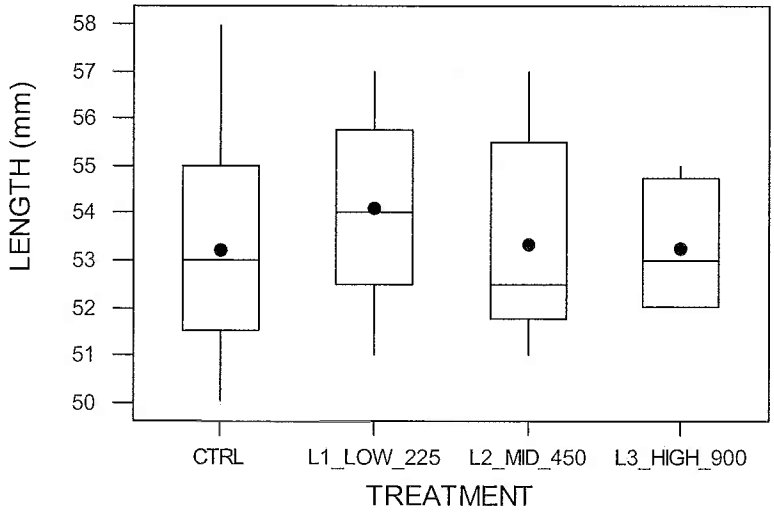

Figure 3.3 Box Plot of Male Body Length by Treatment for the 21-Day Potassium permanganate Assay

(box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

3.5 Body Weight

Body weight of individual females used in the 21-day screening assay ranged from 1.283 to 2.717 g and the mean blotted wet weights ranged from 1.704 to 2.075 g (Table 3.5, Figure 3.4). There was a statistically significant difference in mean body weight between the control (2.032 g) and the middle treatment (1.704 g)(Kruskal-Wallis, $H = 10.28$, $p = 0.016$, df = 3; Mann-Whitney U, $p = 0.0104$).

Table 3.5 Summary Statistics for Female Body Weight Data for the 21-Day Potassium permanganate Assay

Level	N	Mean [a] Weight (g)	SD	CV
CTRL	4	2.032	0.24	11.81
Low	4	2.075	0.22	10.60
Middle	4	1.704	0.43	25.23
High	4	1.857	0.33	17.77

[a] The mean value is inclusive of possible outliers as determined by the box plot analysis.

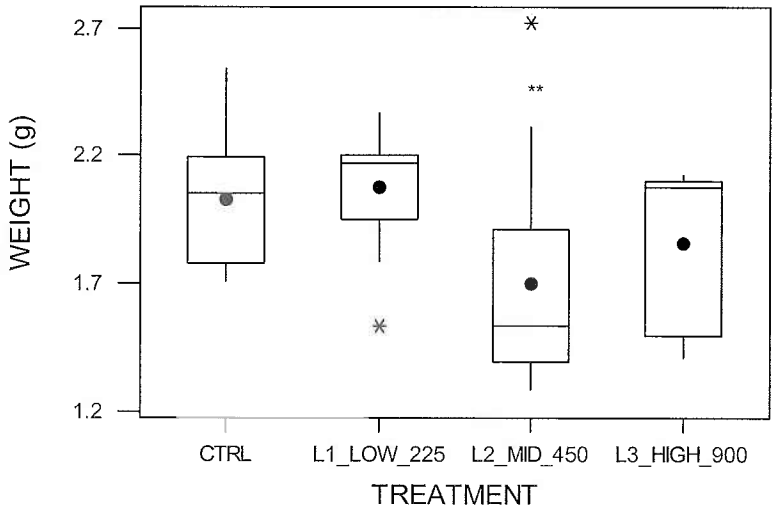

Figure 3. 4 Box Plot of Female Body Weight by Treatment for the 21-Day Potassium permanganate Assay

(box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, the asterisks represent possible outliers that have not been removed from the mean and median calculations value, and the double asterisks represent values that are significantly (p<0.05) different from the control)

The body weights of individual males used in the 21-day screening assay ranged from 2.751 to 5.066 g and the mean blotted wet weights ranged from 3.392 to 4.234 g (Table 3.6, Figure 3.5). There were no statistically significant differences in mean male body weight among all treatments (Kruskal-Wallis, $H = 5.83$, $p = 0.120$, df = 3).

Table 3.6 **Summary Statistics for Male Body Weight Data for the 21-Day Potassium permanganate Assay**

Level	N	Mean Weight (g)	SD	CV
CTRL	4	4.061	0.55	13.54
Low	4	4.234	0.62	14.64
Middle	4	3.445	0.80	23.22
High	4	3.392	0.24	7.08

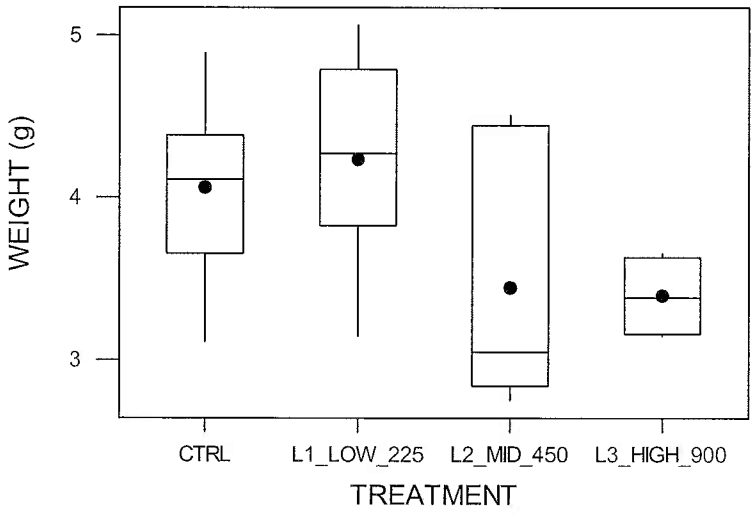

Figure 3.5 **Box Plot of Male Body Weight by Treatment for the 21-Day Potassium permanganate Assay**

(box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

3.5.1 Gonadosomatic Index

The range of GSI values calculated for individual females in all treatments ranged from 0.350 to 18.2 and the mean GSI values ranged from 4.82 to 13.78 (Table 3.7, Figure 3.6). The highest value (GSI 18.2) was obtained for a female from the control treatment. All treatment levels displayed statistically significant reductions in the mean GSI values (Kruskal-Wallis, $H = 22.00$, $p = < 0.001$, df = 3; Mann-Whitney U, $p = 0.0144$ control versus low treatment).

Table 3.7 **Summary Statistics for Female GSI Data for the 21-Day Potassium permanganate Assay**

Level	N	Mean [a] GSI Values	SD	CV
CTRL	4	13.78	2.68	19.45
Low	4	10.83	3.34	30.84
Middle	4	4.82	3.78	78.42
High	4	8.53	4.45	52.17

[a] The mean value is inclusive of possible outliers as determined by the box plot analysis.

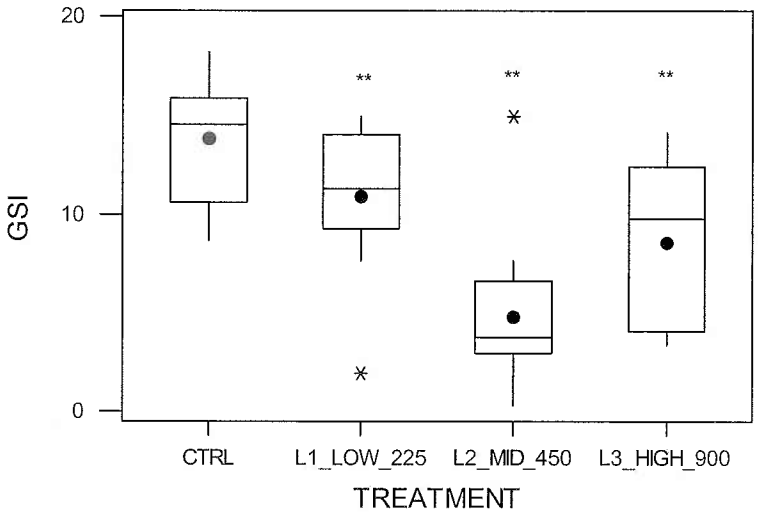

Figure 3.6 **Box Plot of Female GSI by Treatment for the Potassium permanganate Assay**

(box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, the asterisks represent possible outliers that have not been removed from the mean and median calculations value, and the double asterisks represent values that are significantly ($p<0.05$) different from the control)

The range of GSI values calculated for individual males in all treatments ranged from 0.414 to 2.41 and the mean GSI values ranged from 1.10 to 1.54 (Table 3.8, Figure 3.7). The highest value (GSI = 2.41) was obtained for a male from the low treatment. There were no statistically significant differences in mean GSI values among treatments (Kruskal-Wallis, $H = 2.93$, $p = 0.403$, df = 3).

Table 3.8 **Summary Statistics for Male GSI Data for the 21-Day Potassium permanganate Assay**

Level	N	Mean GSI Value	SD	CV
CTRL	4	1.13	0.33	29.2
Low	4	1.54	0.51	33.12
Middle	4	1.22	0.63	51.64
High	4	1.10	0.47	42.73

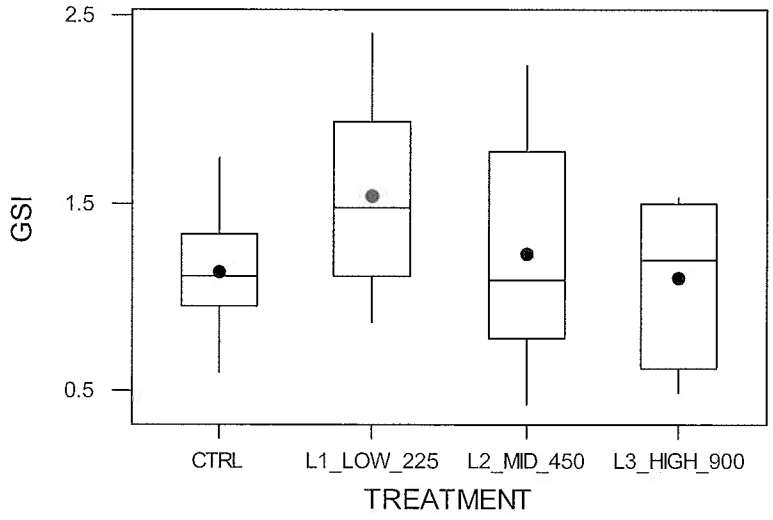

Figure 3.7 **Box Plot of Male GSI by Treatment for the Potassium permanganate Assay**

(box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

3.5.2 Fat Pad Score and Fat Pad Index

There were no female fish that exhibited a noticeable fat pad. The range of fat pad score for individual males in all treatments ranged from 0 (no visible fat pad) to 5 (very prominent) and the mean fat pad scores ranged from 1.8 to 3.0 (Table 3.9, Figure 3.8). The highest value (5) was obtained for a male from the control treatment. The lowest fat pad scores (0) were assessed for the two fish that were originally identified as females in the control and middle treatments. The fish exposed to the high treatment level all had fat pad scores of 2; a small fat pad was evident for these fish.

Table 3.9 Summary Statistics for Male Fat Pad Score Data for the 21-Day Potassium permanganate Assay

Level	N	Mean [a] Fat pad Score	SD	CV
CTRL	4	3.0	1.50	50.00
Low	4	2.8	0.89	31.79
Middle	4	1.8	0.98	54.44
High	4	2.0	0	0

[a] The mean value is inclusive of possible outliers as determined by the box plot analysis.

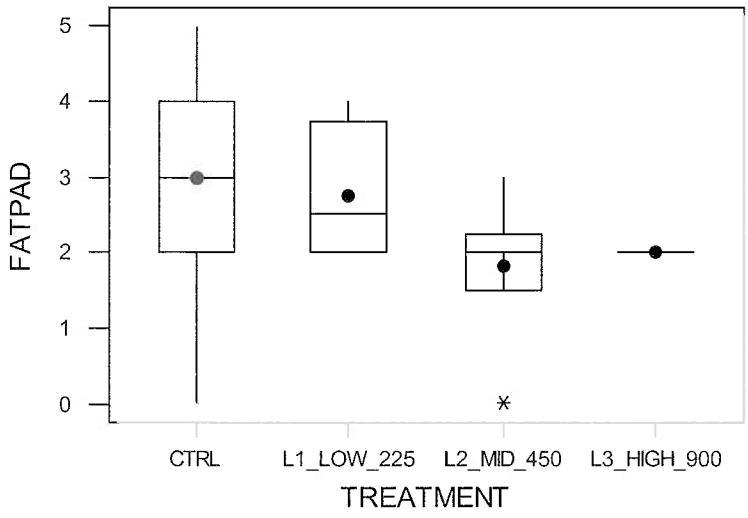

Figure 3.8 Box Plot of Male Fat Pad Score by Treatment for the Potassium permanganate Assay

(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 possible outliers that have not been removed from the mean and median calculations)

The range of FPI values calculated for individual males in all treatments ranged from 0 to 10.8 and the mean FPI values ranged from 1.69 to 4.71 (Table 3.10, Figure 3.9). The highest value (FPI = 10.8) was obtained for a male from the control treatment. The lowest FPI (FPI = 0) was calculated for the two fish that were originally identified as females in the control and 450 µg/L treatments. There were no statistically significant differences ($p < 0.05$) in mean FPI values among treatments (Kruskal-Wallis, $H = 4.93$, $p = 0.177$, df = 3).

Table 3.10 Summary Statistics for Male Fat Pad Index (FPI) Data for the 21-Day Potassium permanganate Assay

Level	N	Mean FPI Value	SD	CV
CTRL	4	4.71	3.16	67.09
Low	4	3.90	2.30	58.97
Middle	4	2.47	1.54	62.35
High	4	1.69	1.07	63.31

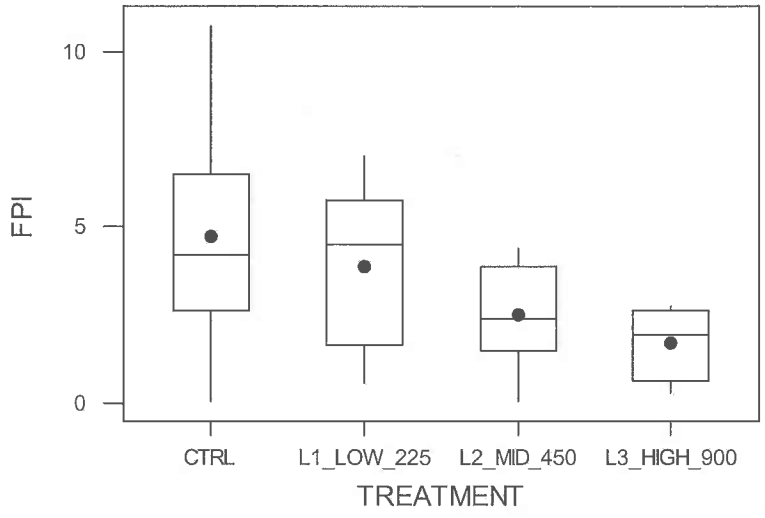

Figure 3.9 Box Plot of Male FPI by Treatment for the Potassium permanganate Assay

(box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, , and the circle is the mean value)

3.5.3 Tubercle Score

The range of tubercle scores for individual males in all treatments ranged from 0 to 36 and the mean tubercle scores ranged from 14.25 to 24.56 (Table 3.11, Figure 3.10). There were no true females that displayed tubercles at test termination. The highest value (36) was obtained for a male from the control and the low treatment. The lowest tubercle score (0) was obtained from the fish that based upon external characters was originally identified as a female in the control treatment. There were no statistically significant differences ($p < 0.05$) in mean tubercle scores among treatments (Kruskal-Wallis, $H = 3.67$, $p = 0.299$, df = 3).

Table 3.11 Summary Statistics for Male Tubercle Data for the 21-Day Potassium permanganate Assay

Level	N	Mean Tubercle Score	SD	CV
CTRL	4	24.56	12.26	49.92
Low	4	22.75	9.44	41.49
Middle	4	17.50	12.72	72.69
High	4	14.25	6.65	46.67

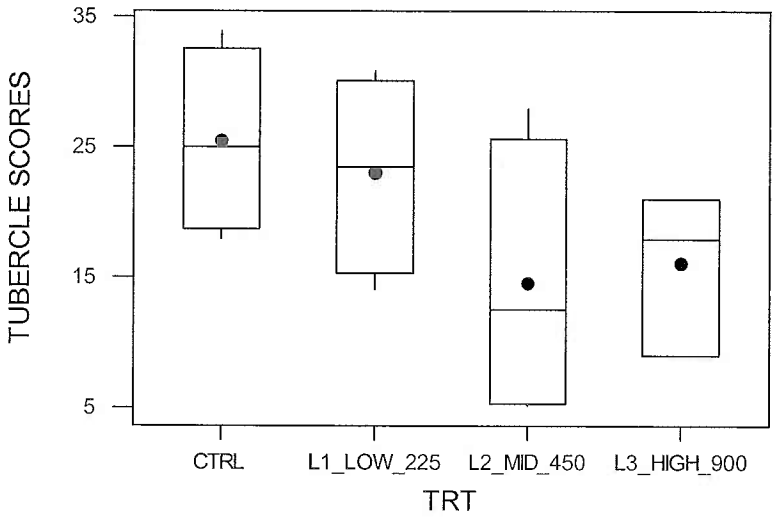

Figure 3.10 Box Plot of Male Tubercle Scores by Treatment for the Potassium permanganate Assay

(box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

3.6 Fecundity

Total Fecundity: The cumulative fecundity of each treatment including the semi-quantitative estimates are presented in Figure 3.11, the trends in the cumulative estimated number of eggs for each treatment level are similar to one another during the pre-exposure phase of this test. Since these values were only estimated, statistical analysis of these data was not performed to confirm the apparent similarities during the pre-exposure phase.

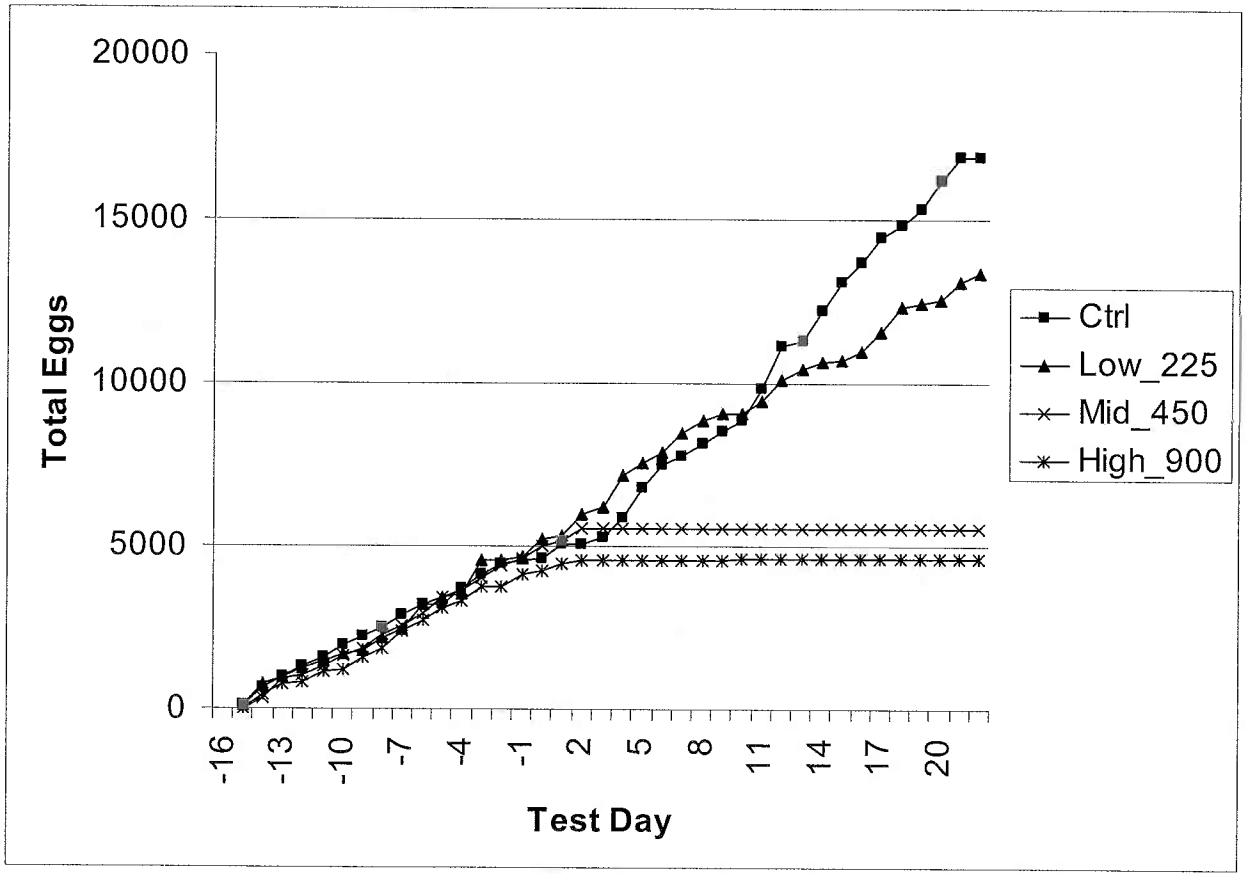

Figure 3.11 **Total Egg production Per Treatment for the 21-Day Potassium permanganate Assay**

(negative test days are prior to the exposure: Day 0 marks the initiation of the 21-day exposure to potassium permanganate)

The mean number of spawns for each treatment level ranged from 0.50 to 13 (Table 3.12). The number of spawns for the control treatment ranged from 9 to 16 for the 21-day exposure period, which equates to a range of a spawn every 1.3 to 2.3 days. Therefore the control fish met the acceptability criteria of a spawn every 3 to 4 days. The fish in the low treatment spawned 11 to 14 times during the 21-day testing period, a spawn every to 1.5 to 1.9 days. The middle and high level treatment fish only spawned 2 to 3 times during the 21-day exposure, respectively. The middle and high treatments both displayed statistically significant reductions in the mean cumulative egg values (Kruskal-Wallis, $H = 11.34$, $p = 0.010$, df = 3).

Table 3.12 Summary Statistics for Number of Spawns Data for the 21-Day Potassium permanganate Assay

Level	N	Mean Number of Spawns	SD	CV
CTRL	4	13	3.30	25.38
Low	4	12	1.41	11.75
Middle	4	0.50	0.58	116.00
High	4	0.75	0.96	128.00

The mean number of cumulative number of eggs produced for each treatment level ranged from 45 to 2,958 (Table 3.13). During the 21-day exposure phase, total egg counts in the control ranged from 2,072 to 3,907 (Figure 3.12). Total egg production in the low treatment was slightly lower than the control with a mean value of 2,007 eggs and replicate values ranging from 936 to 3,669, but the output was not significantly different from the control (Mann-Whitney U, p = 0.194). The middle and high treatment groups were adversely affected by the presence of potassium permanganate, which shut down the reproductive activity within these two treatment levels during the 21-day assay. The middle and high treatments both displayed statistically significant reductions in the mean cumulative egg values (Kruskal-Wallis, H = 11.85, p = 0.008, df = 3).

Table 3.13 Summary Statistics for Cumulative Number of Egg Data for the 21-Day Potassium permanganate Assay

Level	N	Mean Cumulative Number of Eggs	SD	CV
CTRL	4	2,958	857	28.97
Low	4	2,007	1,195	59.54
Middle	4	90	173	192.22
High	4	45	68	151.11

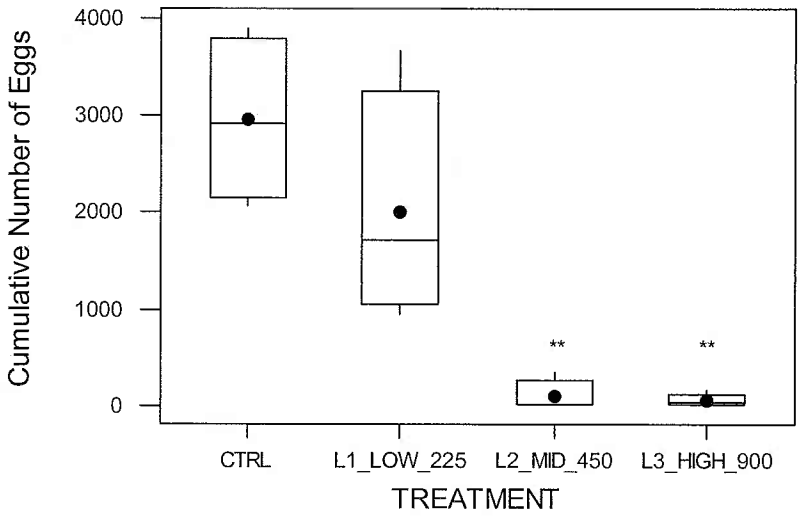

Figure 3.12 Box Plot of Cumulative Egg Numbers Data by Treatment for the Potassium permanganate Assay

(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 double asterisks represent values that are significantly ($p<0.05$) different from the control)

Fecundity per Female Reproductive Day:

During the exposure period, only the control treatment fish achieved the maximum number of female reproductive days. The replicate number of eggs per female reproductive day ranged from 24.7 to 46.5 in the control (Figure 3.13) and the means for control replicates was 38.6 eggs per female reproductive day (Table 3.14). The mean number of eggs per female reproductive day for the treatments ranged from 1.1 to 25.9 eggs per female reproductive day (Table 3.14). The middle and high treatments both displayed statistically significant reductions in the mean number of eggs per female reproductive day values (Kruskal-Wallis, $H = 11.65$, $p = 0.009$, df = 3).

Table 3.14 Summary Statistics for Number of Eggs per Female Reproductive Day for the 21-Day Potassium permanganate Assay

Level	N	Mean Number of Eggs per Reproductive Day	SD	CV
CTRL	4	38.6	14.2	36.79
Low	4	25.9	14.9	57.53
Middle	4	1.1	2.1	190.91
High	4	1.2	2.0	154.68

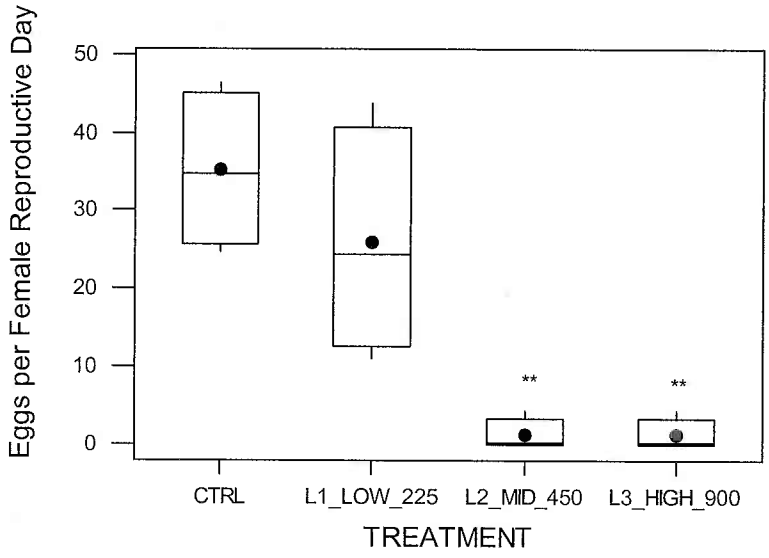

Figure 3.13 **Box Plot of Eggs per Female Reproductive Day Data by Treatment for the Potassium permanganate Assay**

(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 double asterisks represent values that are significantly (p<0.05) different from the control)

During the 21-day assay exposure, each spawn was estimated with a semi-quantitative estimate (10, 25, 50, 100, 150, 200, 250, 300, and >300 egg categories) as well as an actual count of the eggs. Most spawns that were fewer than five eggs were assessed with an actual count. Only one was estimated with the semi-quantitative criteria. Table 3.15 summarizes the number of spawns within each category, the range of actual number of eggs (direct assessment), and the number of spawns that fell within 20 percent of the estimated values. The best estimates were for spawns within the 150, 200, 250, and >300 egg categories.

Table 3.15 Summary Statistics for Estimated versus Actual Egg Count for the 21-Day Potassium permanganate Assay

Estimate Category	N	Range of Actual Eggs	No. of Spawns within 20% of estimate
10	7	4 to 28	2
25	6	21 to 43	2
50	11	48 to 85	7
100	14	65 to 180	8
150	9	109 to 197	6
200	14	173 to 320	13
250	14	195 to 306	12
300	8	321 to 425	4
>300	16	254 to 821	3 <300

3.7 Fertilization Success

During each spawning event the number of non-viable (Table 3.16) and viable eggs were recorded and the percent fertilization success was calculated (% fertile = (number of viable eggs/total number of eggs)*100). The mean percent fertility for the control replicates was 89% (Table 3.17) with replicate values ranging from 76 to 95% (Figure 3.14). Except for control replicate C, which had a percent fertilization rate of 76%, the percent fertilization would have exceeded 90% for the control. There were no statistically significant differences ($p < 0.05$) in mean percent fertilization among treatments (Kruskal-Wallis, $H = 0.61$, $p = 0.894$, df = 3).

Table 3.16 Summary Statistics for Cumulative Number of Non-Viable Eggs for the 21-Day Potassium permanganate Assay

Level	N	Mean Number Of Non-viable Eggs	SD	CV
CTRL	4	233	99.66	42.77
Low	4	83.3	46.53	55.86
Middle	4	5.5	4.95	90.00
High	4	25	18.38	73.52

Table 3.17 Summary Statistics for Percent Fertility Data for the 21-Day Potassium permanganate Assay

Level	N	Mean Percent Fertilization	SD	CV
CTRL	4	89	8.50	9.55
Low	4	91	5.12	5.63
Middle	4	79	26.16	33.11
High	4	46	65.05	141.41

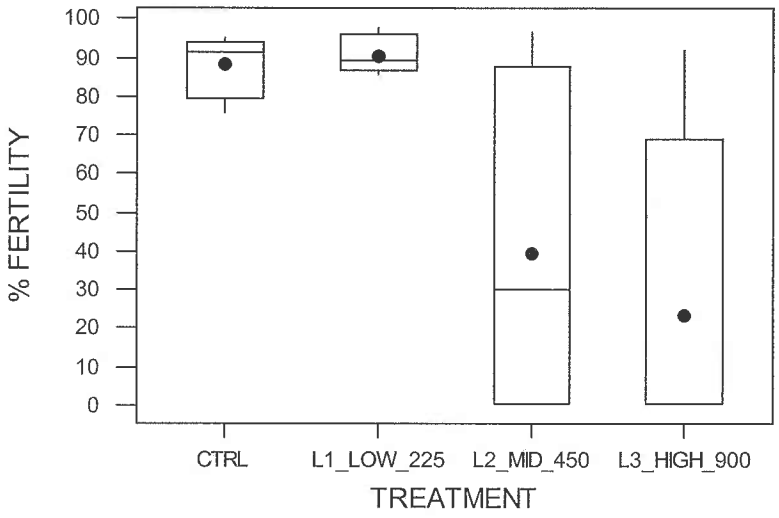

Figure 3.14 **Box Plot of Percent Fertility Data by Treatment for the Potassium permanganate Assay**

(box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, and the circle is the mean value)

3.8 Vitellogenin

The measured plasma vitellogenin (Vtg) concentrations excluding a suspected outlier value of 2,046 mg/mL ranged from 16.8 to 171.8 mg/mL in the individual control female fish (Figure 3.15). The replicate mean plasma Vtg concentrations for the control with and without the 2,046 mg/mL value ranged from 23.7 to 552.9 mg/mL and 23.7 to 137.2 mg/mL, respectively. The mean female plasma Vtg concentrations inclusive of the 2,046 mg/mL control value ranged from 9.5 to 189.7 mg/mL (Table 3.18). The mean female plasma Vtg concentrations without the inclusion of the 2,046 mg/mL control value ranged from 9.5 to 74.6 mg/mL (Table 3.19). With the 2,046 mg/mL control value there were no statistically significant reductions in the replicate mean plasma Vtg concentrations (Kruskal-Wallis, $H = 6.38$, $p = 0.095$, Mann-Whitney U, $p = 0.0518$ control versus middle treatment, Mann-Whitney U, $p = 0.488$ control versus high treatment). Without the 2,046 mg/mL control value, there was still no statistically significant reductions in the replicate mean plasma Vtg concentrations (Kruskal-Wallis, $H = 6.58$, $p = 0.087$, Mann-Whitney U, $p = 0.0518$ control versus middle treatment, Mann-Whitney U, $p = 0.488$ control versus high treatment).

There were only four male fish that had quantifiable vitellogenin concentrations (concentrations > 0.00070 mg/mL). One male in the control had a plasma Vtg concentration of 0.0014 mg/mL. Two males in the middle treatment had measured plasma Vtg concentrations of 0.0013 and 0.0082 mg/mL. One male in the high treatment had a measured plasma Vtg concentration of 0.0012 mg/mL.

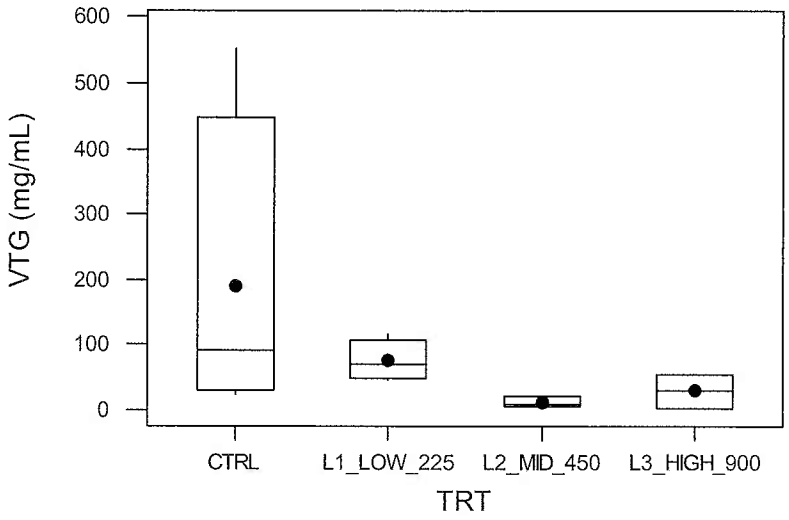

Figure 3.15 **Box Plot of Female Vitellogenin Plasma Concentrations (analysis of replicate means including 2,046 mg/mL control value) by Treatment for the Potassium permanganate Assay**

(box represents the interquartile range, whiskers represent the data range of the replicate means, the horizontal line is the median value, the circle is the mean value, and the possible outliers have not been removed from the mean and median calculations)

Table 3.18 **Summary Statistics for Female Vitellogenin Plasma Concentrations (With Suspected Outliers, 2,046 mg/mL control value) for the 21-Day Potassium permanganate Assay**

Level	N	Mean Female Vtg Concentration [a] (mg/mL)	SD	CV
CTRL	4	189.7	247.09	130.25
Low	4	74.6	31.23	41.86
Middle	4	9.5	7.47	78.63
High	4	27.3	35.64	130.55

[a] The mean value is inclusive of 2,046 mg/mL control value utilized to generate a replicate mean concentration of 552.9 for control replicate C.

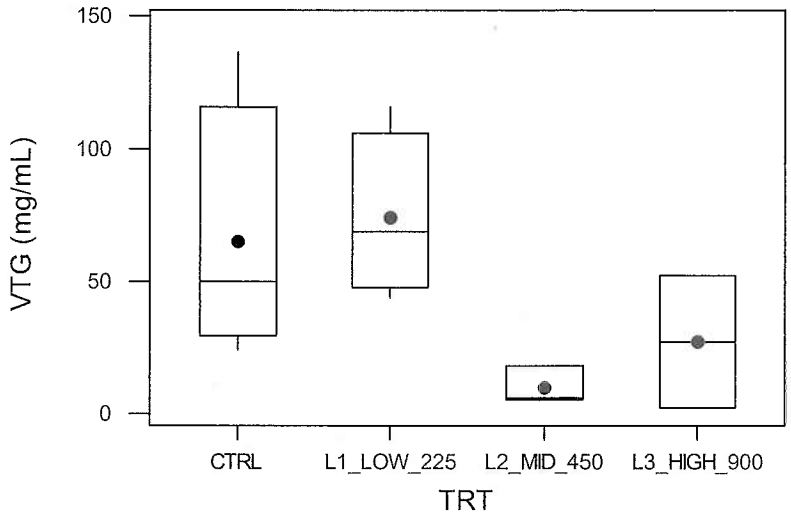

Figure 3.16 **Box Plot of Female Vitellogenin Plasma Concentrations (analysis of replicate means excluding the 2,046 mg/mL control value) by Treatment for the Potassium permanganate Assay**

(box represents the interquartile range, whiskers represent the data range of the replicate means, the horizontal line is the median value, and the circle is the mean value)

Table 3.19 **Summary Statistics for Female Vitellogenin Plasma Concentrations (Excluding the 2,046 mg/mL control value) for the 21-Day Potassium permanganate Assay**

Level	N	Mean Female Vtg Concentration (mg/mL)	SD	CV
CTRL	4	65.3	49.70	76.11
Low	4	74.6	31.23	41.86
Middle	4	9.5	7.47	78.63
High	4	27.3	35.64	130.55

An overall summary of the variability of the biological endpoints is presented in Table 3.20.

Table 3.20 Coefficient of Variance (%CV) Comparison for the Biological Endpoints determined During the 21-Day Potassium permanganate Assay

Endpoint	% CV (control)	% CV (Low)	% CV (Middle)	% CV (High)
Survival	0	8.85	22.00	82.26
Female Length	3.42	4.72	5.01	4.95
Male Length	4.59	3.62	4.22	2.81
Female Weight	11.81	10.60	25.23	17.77
Male Weight	13.54	14.64	23.22	7.08
Female GSI	19.45	30.84	78.42	52.17
Male GSI	29.20	33.12	51.64	42.73
Male Fat pad Score	50.00	31.79	54.44	0
Male Fat pad Index	67.09	58.97	62.35	63.31
Tubercle Score (mapping)	49.92	41.49	72.69	46.67
Number of Spawns	25.38	11.75	116.00	128.00
Cumulative Number of Eggs	28.97	59.54	192.22	151.11
Number of Eggs/Female Reproductive Day	36.79	57.53	190.91	154.68
Number of Non-viable eggs	42.77	55.86	90.00	73.52
Percent Fertility	9.55	5.63	33.11	141.41
Female Vitellogenin (w/o outlier values 2,046 mg/mL)	76.11	41.86	78.63	130.55
Male vitellogenin	NA	NA	NA	NA

NA = Not Applicable

3.9 Water Quality

The general water quality remained within acceptable levels throughout the exposure period. The ranges of water quality parameters are presented in Table 3.21. The periodic average temperature for the test solutions from the continuous data logger recordings was 25.3 ± 0.1°C with a minimum and maximum temperature recording of 24.9 and 25.7°C, respectively.

Table 3.21 Summary Statistics for Water Quality Data for the 21-Day Potassium permanganate Assay

Level	Temperature (°C)	Dissolved O_2 (mg/L)	pH	Alkalinity (mg $CaCO_3$/L)	Hardness (mg $CaCO_3$/L)
CTRL	25.0 to 25.3	4.6[a] to 8.0	7.8 to 8.1	156 to 170	140 to 154
Low	25.0 to 25.3	4.8[b] to 8.0	7.9 to 8.1	---	---
Middle	25.0 to 25.4	5.4 to 8.0	7.9 to 8.2	---	---
High	24.8 to 25.1	5.5 to 8.0	7.9 to 8.2	156 to 168	142 to 154

[a] This value recorded on day 7. Adjustments were made to the diluter cycle rate and the DO value from the next day was 6.28 mg/L in this replicate test chamber. The next lowest value was 5.25 mg/L.

[b] This value recorded on day 1. Adjustments were made to the diluter cycle rate and the DO value from the next day was 5.96 mg/L in this replicate test chamber.

4.0 CONCLUSIONS

Exposure to potassium permanganate under the test conditions described above was acutely and chronically toxic to the test organisms. Overall fish survival and reproductive output (i.e., number of spawns, cumulative number of eggs and eggs per reproductive day) were the parameters that were most severely affected by the potassium permanganate exposure. Statistically significant reductions in survival had occurred the middle (450 µg/L) and high (900 µg/L) treatment concentrations as compared to the control survival. The estimated LC_{50} value was 662 µg/L (95% confidence limits: 510 to 1,014 µg/L). Exposure to potassium permanganate shut down the reproduction within the middle and high treatment levels. In the middle treatment, the only spawning activity (i.e., one spawn in replicates B and C) occurred during the first 24-hours of exposure. This same trend occurred in the D replicate of the high treatment level with only one spawn within the first 24-hours of exposure. Although the females in the high treatment A replicate did actually spawn on days 7 (4 eggs) and 9 (34 eggs) of the exposure, but they were not fertile. Although not as dramatic, the mean female GSI values of all of the treatment groups all displayed statistically significant reductions in GSI values as compared to the control value.

Length and weight values from the surviving fish showed the least effect of the exposure with a statistically significant reduction only in the female body weights of the surviving fish exposed to the middle treatment only. Also these parameters displayed the least variability over the exposure with %CV values of ≤5.01 and ≤25.23, respectively, which can be attributed to the fact that the exposure duration was relatively short and adult fish were being tested.

Secondary male characteristics, i.e., fat pad scores, fat pad index (i.e., FPI), and tubercle scoring, were reduced with increasing potassium permanganate concentrations, but these were not statistically significant reductions. These characteristics also displayed some of the highest %CV values in the control animals 49.94% for the tubercle scoring to 67.09% for the control FPI values.

Female plasma vitellogenin concentrations displayed a downward trend with mean values of 65.3 (excluding 2,046 mg/mL value), 74.6, 9.5, and 27.3 mg/mL in the control, low, middle, and high treatments, respectively, but there were no statistically significant effects at $p < 0.050$. Even without the inclusion of a plasma Vtg concentration of 2,046 mg/mL from one control female, this parameter displayed a high variability (i.e., 76.11% CV) between control females.

In a comparison of the semi-quantitative egg estimate versus the actual counts, the egg estimates were most accurate (i.e., number of spawns in which the estimate was within 20% of the actual count) for the 200 and 250 egg estimate categories with 85 to 92% accuracy. With the number of females that could spawn within a replicate chamber, it was common that the >350 egg category did not closely represent the actual egg values. Many of which were much greater than 350 eggs.

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(4) American Public Health Association. 1998. Methods 2320 and 2340 In: *Standard Methods for the Examination for Water and Wastewater.* 20th ed. Washington, D.C. 2-27 to 2-29, 2-36 to 2-39.

APPENDIX A. CERTIFICATE OF ANALYSIS

SIGMA-ALDRICH

3050 Spruce Street
Saint Louis, Missouri 63105 USA
Telephone (800) 521-8956 • (314) 771-5765
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Certificate of Analysis

BATTELLE NORTHWEST
11352255MEC
MARINE SCIENCES LAB
1529 W SEQUIM BAY RD
SEQUIM WA 98382

PO NBR: CC/Smith

PRODUCT NUMBER: 223468-500G LOT NUMBER: 00310LC

PRODUCT NAME: POTASSIUM PERMANGANATE, 99+%, A.C.S. REAGENT

FORMULA: KMNO4 FORMULA WEIGHT: 158.04

APPEARANCE	DARK BLACK BEADS
TITRATION	99.5% (WITH KMNO4) *
ICP ASSAY	CONFIRMS POTASSIUM AND MANGANESE COMPONENTS
INSOLUBLE MATTER	0.005% *
CHLORIDE AND CHLORATE	0.002% *
SULFATE	0.008% *
	* SUPPLIER DATA
	MEETS REQUIREMENTS OF ACS 9TH ED
QUALITY CONTROL ACCEPTANCE DATE	SEPTEMBER, 2004

ALDRICH CHEMICAL COMPANY
RONNIE MARTIN
MAY 2, 2005

We are Committed to the success of our Customers, Employees and Shareholders through leadership in Life Science, High Technology and Service.

APPENDIX B. DILUTION WATER CHARACTERIZATION

Chemical Characteristics of ABC Blended Water Used by ABC Laboratories' Chemical Services Group

December 2004 ABC Blended Water Screen

Chlorinated Hydrocarbons (µg/L)	2004	Historical Range 98-03	Elements (mg/L)	2004	Historical Range 98-03
DDE	<0.040	<0.040	Arsenic	<0.010	<0.050
DDD	<0.040	<0.040	Boron	0.30	0.255-0.308
DDT	<0.040	<0.040	Cadmium	<0.0050	<0.0050
Dieldrin	<0.040	<0.040	Chromium	<0.010	<0.010
α-BHC	<0.040	<0.040	Copper	<0.010	<0.010
β-BHC	<0.040	<0.040	Iron	<0.10	<0.10
γ-BHC	<0.040	<0.040	Lead	<0.0050	<0.0065
Δ-BHC	<0.040	<0.040	Mercury	<0.00050	<0.00060
Heptachlor epoxide	<0.040	<0.040	Nickel	<0.020	<0.020
Endrin	<0.040	<0.040	Selenium	<0.050	<0.0059
Methoxychlor	<0.040	<0.095	Silver	<0.0050	<0.0050
Toxaphene	<0.10	<3.8	Zinc	<0.020	<0.020-0.034
Chlordane	<0.10	<0.48	Calcium	28	28-33
Endosulfan I	<0.040	<0.040	Magnesium	15	14-16
Endosulfan II	<0.040	<0.040	Potassium	4.0	3.8-4.1
Endosulfan sulfate	<0.040	<0.040	Sodium	16	15-16
Aroclor 1016	<0.10	<0.20			
Aroclor 1221	<0.10	<0.20	Organophosphate (µg/L)		
Aroclor 1248	<0.10	<0.20	Diazinon	<1.0	<1.0
Aroclor 1232	<0.10	<0.20	Parathion	<1.0	<1.0
Aroclor 1242	<0.10	<0.20	Malathion	<1.0	<1.0
Aroclor 1254	<0.10	<0.20	Ethion	<1.0	<1.0
Aroclor 1260	<0.10	<0.20	Disulfoton	<1.0	<1.0
Aldrin	<0.040	<0.040	Azinphos ethyl	<1.0	<1.0
Endrin aldehyde	<0.040	<0.040	Demeton, Total	<1.0	<1.0
Endrin Ketone	<0.040	<0.040			
Heptachlor	<0.040	<0.040	Miscellaneous (mg/L)		
2,4,5-TP (silvex)	<2.0	<50	Nitrite N	<0.030	<0.050
2,4-D	<10	<250	Nitrate N	0.16	<0.12-0.18
			Total Phosphorus as P	<0.050	<0.050-0.98

Note: Data supporting these values are on file at ABC Laboratories. Less than (<) values indicate recovery was below the limit of detection.

BATTELLE PACIFIC NORTHWEST DIVISION,
BATTELLE MEMORIAL INSTITUTE
STUDY NUMBER WA 5-11
EPL PROJECT NUMBER 237-026

POTASSIUM PERMANGANATE: PHASE 1B VALIDATION – FISH SCREENING ASSAY FOR ENDOCRINE ACTIVE SUBSTANCE WITH THE FATHEAD MINNOW (PIMEPHALES PROMELAS)

DRAFT PATHOLOGY SUMMARY

INTRODUCTION

The objective of this study was to determine the effects, if any, of potassium permanganate administered via water bath on gonadal tissue of adult fathead minnows (FHM, *Pimephales promelas*).

The experimental design is presented in the following table:

Table 1. Experimental Design for Potassium Permanganate Study

Exposure Group	Potassium Permanganate	Male Replicates				Female Replicates			
		A	B	C	D	A	B	C	D
C	0 µg/L (Control)	2	2	2	3*	4	4	4	3
1	225 µg/L	2	2	2	2	4	4	3	4**
2	450 µg/L	2	2	1	1*	3	3	4**	2
3	900 µg/L	2	0	1	1	3	1	1	0

*One of these animals was submitted as a female but was determined to be a male upon histological examination.
**No gonadal tissue was present for one of these animals.

METHODS

The tissues were submitted by ABC Laboratories, Inc. Unless otherwise indicated, histopathological procedures were performed according to the draft form of the "OECD Guidance Document for Performing Gonadal Histopathology in Small Fish." Briefly, following routine processing the left and right gonads were embedded horizontal to their long axis to allow for longitudinal sectioning. During microtomy, the first section from each block was acquired at the point at which approximately half of the gonad had been cut away and the size of the section was maximized. The second and third sections were then obtained at 50 micron intervals. Sections were stained with hematoxylin and eosin, and mounted with glass coverslips. Labels included the EPL Project No. (237-026), the group/replicate designation (e.g., CA F), the study ID (WA 5-11), and the Animal No. (e.g., CA1F).

The pathologist evaluated the slides by brightfield microscopy for changes that included, but were not limited to, the types of findings that are listed in the aforementioned guidance document. As per that document, severity grading of findings was performed according to the following scale: NR = not remarkable, grade 1 = minimal, grade 2 = mild, grade 3 = moderate, grade 4 = severe. Ovarian oocyte atresia was graded according to the following scale: Grade 1 = 3 to 5 atretic oocytes per ovary; Grade 2 = 6 to 9 atretic oocytes per ovary; Grade 3 = greater than 9 atretic oocytes per ovary, but less than the vast majority; and Grade 4 = the vast majority of oocytes were atretic. The pathologist recorded findings on a spreadsheet. This original spreadsheet as contained within the guidance document was modified slightly by the study pathologist to include the addition of a column in order to accommodate the animal numbers of the female fathead minnows. The data collection spreadsheet is incorporated into this report. Results were simultaneously recorded into EPL's Pathology Data Reporting System, and tabulated in the accompanying Histopathology Incidence Tables (HIT) and summarized in the Summary Incidence Tables (SIT).

RESULTS

Males

Based on incidence and/or severity data, findings that were substantially different in the testes of potassium permanganate-exposed males as compared to control males included: increased spermatogonia (minimal to moderate) in the 450 and 900 μg/L groups (Figures 3-6); increased testicular degeneration (minimal to mild) in the 450 and 900 μg/L groups (Figures 4, 6, and 8); and increased testicular stage scores in the 450 and 900 μg/L groups (Figures 5-8). These three changes often, but not always, occurred concurrently within the same gonad.

The morphological appearances of these findings were essentially as described in the "OECD Guidance Document for Performing Gonadal Histopathology in Small Fish."

Two animals that were submitted as females were determined to be males upon histological examination: Animal Nos. CD2F and 2D1F.

The incidence and severity of selected histopathologic results for male FHM are presented in the following table:

Table 2. Combined Incidence and Severity of Selected Histopathologic Findings in Male Fathead Minnnows

Exposure Group	C					1					2					3				
Potassium Permanganate Dose (µg/L)	0					225					450					900				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	2	2	2	3	9	2	2	2	2	8	2	2	1	1	6	2	0	1	1	4
Increased Cells, Spermatogonia	0	1	0	0	1	0	1	0	0	1	1	1	1	1	4	1	0	1	0	2
Minimal	-	1	-	-	1	-	1	-	-	1	-	-	1	-	1	-	-	1	-	1
Mild	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1	1	-	-	-	1
Moderate	-	-	-	-	-	-	-	-	-	-	-	1	-	1	2	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testicular Degeneration, Increased	1	1	1	2	5	1	1	1	1	4	2	2	1	1	6	2	0	1	1	4
Minimal	-	1	1	2	4	1	-	1	-	2	1	1	1	1	4	-	-	1	1	2
Mild	1	-	-	-	1	-	1	-	1	2	1	1	-	-	2	2	-	-	-	2
Moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testicular Stage																				
Stage 1	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stage 2	1	1	2	3	7	2	1	1	2	6	-	-	-	1	1	-	-	-	-	-
Stage 3	-	1	-	-	1	-	1	1	-	2	2	2	1	-	5	2	-	1	1	4
Average	2.0					2.3					2.8					3.0				

*T = total

Females

Based on incidence and/or severity data, findings that were substantially different in potassium permanganate-exposed females as compared to control females included: increased oocyte atresia (minimal to severe) in the 225, 450, and 900 µg/L groups (Figures 10-11); and decreased ovarian stage scores in the 450 and 900 µg/L groups (Figure 11).

The morphological appearances of these findings were essentially as described in the "OECD Guidance Document for Performing Gonadal Histopathology in Small Fish."

The incidence and severity of selected histopathologic results for female fathead minnows are presented in the following table:

Table 3. Combined Incidence and Severity of Selected Histopathologic Findings in Female Fathead Minnnows

Exposure Group	C					1					2					3				
Potassium Permanganate Dose (µg/L)	0					225					450					900				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	4	4	4	3	15	4	4	3	3	14	3	3	3	2	11	3	1	1	0	5
Oocyte Atresia, Increased	0	0	0	0	0	1	2	1	0	5	2	2	2	1	7	1	0	1	0	2
Minimal	-	-	-	-	-	1	-	-	-	1	-	1	1	1	3	-	-	-	-	-
Mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1
Moderate	-	-	-	-	-	-	3	1	-	4	2	1	1	-	4	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1
Ovarian Stage																				
Stage 0	-	-	-	-	-	-	-	-	-	-	2	-	1	-	3	-	-	-	-	-
Stage 1	-	-	-	-	-	-	-	-	-	-	-	2	2	1	5	-	1	1	-	2
Stage 2	1	1	1	2	5	2	3	1	1	7	1	1	-	1	3	1	-	-	-	1
Stage 3	3	3	2	1	9	1	1	2	2	6	-	-	-	-	-	1	-	-	-	1
Stage 4	-	-	1	-	1	1	-	-	-	1	-	-	-	-	-	1	-	-	-	1
Average	2.7					2.6					1.0					2.2				

*T = total

DISCUSSION

Potassium permanganate, an oxidizing agent that reacts with organic matter, is used as an external parasiticide and bactericide in fish. Studies that document the histopathological effects of potassium permanganate toxicity in fish are scarce. In one such report involving channel catfish *Ictalurus punctatus* (Darwish et al., 2002), the sole affected tissue was gill; however, gill, liver, and trunk kidney were the only tissues examined. A brief literature review did not yield any publications in which the potential gonadal effects of potassium permanganate were evaluated.

In the present study, exposure-related results in both male and female FHM were similar in that the gonads of both sexes were characterized by increased degeneration and loss of germinal cells (testicular degeneration in males and oocyte atresia in females). This loss of cells was accompanied by an overall decrease in histologically evident germinal tissue and by increased germ cell immaturity (these changes were represented by higher average testicular stage scores with increased spermatogonia in males, and lower ovarian stage scores in females). In males it is possible that the increased spermatogonia may have been a compensatory response for the loss of germinal tissue. Although there was a decrease in the average ovarian stage scores in females, which signifies a more immature ovarian cell population, an analogous increase in oocyte progenitor cells (oogonia) was not observed.

The above findings in the testes and ovaries appear to coincide generally with an increased incidence of mortality in the higher potassium permanganate dose groups of both males and females. As such, this situation seems to suggest that these types of non-specific degenerative changes may have occurred via "non-endocrine" mechanisms related to potassium permanganate toxicity. Examples of non-endocrine mechanisms for such effects could include oxidative damage to the gonads and/or stress-induced changes associated with toxicity in other organ systems (e.g., the gills). Notably, two of the current study

results, increased spermatogonia in males and decreased ovarian stage scores in females, were also observed as "potential" effects (i.e., not quite statistically significant) of potassium permanganate in a previous study: SSL No. 13784.6109, EPL Project No. 237-023. Although there was no evidence of mortality associated with potassium permanganate in that previous study, it is possible that those gonad changes were effects of toxicity that was consistently sublethal. Not surprisingly, gonad histology did not provide any clues regarding the cause(s) of mortality in the present study.

Although the incidences of certain findings (increased spermatogonia and increased testicular degeneration in males, and oocyte atresia in females) were increased in the 900 μg/L dose groups as compared to controls, these increases occurred to degrees that probably approach, but do not quite reach, statistical significance; however, the incidence and severity data trends suggests that this lack of significance is most likely a function of the small number of animals in the 900 μg/L male and female groups rather than a true lack of exposure-related effects at this dose level.

Other findings in this study either occurred in comparable numbers of control and potassium permanganate-exposed fish (background lesions), or as low-frequency (incidental) findings, and are therefore not considered to be exposure-related.

CONCLUSION AND SUMMARY

Histopathological findings in male FHM that were attributable to potassium permanganate exposure included increased spermatogonia in the testes, increased testicular degeneration, and increased testicular stage scores in the 450 and 900 μg/L groups as compared to controls.

Histopathological findings in female FHM that were attributable to potassium permanganate exposure included increased oocyte atresia in the ovaries of the 225, 450, and 900 μg/L groups as compared to controls, and substantially decreased ovarian stage scores in the 450 and 900 μg/L groups as compared to controls.

Other findings in this study either occurred in comparable numbers of control and potassium permanganate-exposed fish (background lesions), or as low-frequency (incidental) findings, and are therefore not considered to be exposure-related.

JEFFREY C. WOLF, DVM, Diplomate, ACVP Date
Veterinary Pathologist

JCW/cb

REFERENCES

Darwish AM, Griffin BR, Straus DL, Mitchell AJ (2002) Histological and hematological evaluation of potassium permanganate toxicity in channel catfish. *Journal of Aquatic Animal Health*, 14: 134-144.

OECD Draft Guidance Document for Performing Gonadal Histopathology in Small Fish: Histology and Histopathology Guidelines for Phase 1B of the OECD Fish Screening Assay for EDC's. (2004).

1/24/06 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Potassium Permanganate: Phase 1B Validation – Fish Screening Assay for Endocrine Active Substance with the Fathead Minnow (Pimephales promelas)

Client Study: WA 5-11 EPL Project Coordinator: Dr. Jeffrey C. Wolf

EPL Project Number: 237-026 EPL Pathologist: Dr. Jeffrey C. Wolf

The following aspects of this study were inspected by the Quality Assurance Unit of Experimental Pathology Laboratories, Inc. Dates inspections were performed and findings reported to the EPL Project Coordinator and Management are indicated below.

	Dates	
Area Inspected	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management XXX

Date of last quarterly facility inspection 7/05

EPL Quality Assurance Unit Date

SUMMARY INCIDENCE TABLES

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP CA	GROUP CB	GROUP CC	GROUP CD	GROUP 1A	GROUP 1B
TESTIS (NO. EXAMINED)	(2)	(2)	(2)	(3)	(2)	(2)
Granulomatous Inflammation	1			1	1	1
Histiocytic Cells,						
Intraluminal						
Increased Cell, Spermatogonia		1				1
Mineralization	1	2	2	2	2	
Mineralization, Collecting						
Duct	1	1	2	2	2	
Stage 1	1					
Stage 2	1	1	2	3	2	1
Stage 3		1				1
Testicular Degeneration,						
Increased	1	1	1	2	1	1

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 1C	GROUP 1D	GROUP 2A	GROUP 2B	GROUP 2C	GROUP 2D
TESTIS (NO. EXAMINED)	(2)	(2)	(2)	(2)	(1)	(1)
Granulomatous Inflammation			1			
Histiocytic Cells,						
Intraluminal			2		1	
Increased Cell, Spermatogonia			1	1	1	1
Mineralization	2	1	1	2		
Mineralization, Collecting						
Duct	2	1	2	2		
Stage 1						
Stage 2	1	2				1
Stage 3	1		2	2	1	
Testicular Degeneration,						
Increased	1	1	2	2	1	1

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 3A	GROUP 3C	GROUP 3D			
TESTIS (NO. EXAMINED)	(2)	(1)	(1)			
Granulomatous Inflammation						
Histiocytic Cells,						
Intraluminal		1				
Increased Cell, Spermatogonia	1	1				
Mineralization	1	1				
Mineralization, Collecting						
Duct	1	1				
Stage 1						
Stage 2						
Stage 3	2	1	1			
Testicular Degeneration,						
Increased	2	1	1			

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP CA	GROUP CB	GROUP CC	GROUP CD	GROUP 1A	GROUP 1B
OVARY (NO. EXAMINED)	(4)	(4)	(4)	(3)	(4)	(4)
Asynchronous Development, Gonad				1		
Granulomatous Inflammation						
Macrophage Aggregates, Increased						
Oocyte Atresia, Increased					1	3
Stage 0						
Stage 1						
Stage 2	1	1	1	2	2	3
Stage 3	3	3	2	1	1	1
Stage 4			1		1	

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 1C	GROUP 1D	GROUP 2A	GROUP 2B	GROUP 2C	GROUP 2D
OVARY (NO. EXAMINED)	(3)	(3)	(3)	(3)	(3)	(2)
Asynchronous Development,						
Gonad		1				
Granulomatous Inflammation		2				
Macrophage Aggregates,						
Increased			1		1	1
Oocyte Atresia, Increased	1		2	2	2	1
Stage 0			2		1	
Stage 1				2	2	1
Stage 2	1	1	1	1		1
Stage 3	2	2				
Stage 4						

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 3A	GROUP 3B	GROUP 3C			
OVARY (NO. EXAMINED)	(3)	(1)	(1)			
Asynchronous Development, Gonad						
Granulomatous Inflammation						
Macrophage Aggregates, Increased		1				
Oocyte Atresia, Increased	1		1			
Stage 0						
Stage 1		1	1			
Stage 2	1					
Stage 3	1					
Stage 4	1					

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP CA			GROUP CB			GROUP CC			GROUP CD			GROUP 1A			GROUP 1B		
ANIMAL	CA1M	CA2M		CB1M	CB2M		CC1M	CC2M		CD1M	CD2F	CD2M	1A1M	1A2M		1B1M	1B2M	
TESTIS																		
Granulomatous Inflammation	2											2			2			
Histiocytic Cells, Intraluminal																		
Increased Cell, Spermatogonia				1													1	
Mineralization	1			1	2		1	2		2		1	2	2				
Mineralization, Collecting Duct	1				2		1	1		2		1	2	2				
Stage 1	P																	
Stage 2		P			P		P	P		P	P	P	P	P		P		
Stage 3				P													P	
Testicular Degeneration, Increased	2			1				1			1	1		1			2	

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 1C		GROUP 1D		GROUP 2A		GROUP 2B		GROUP 2C	GROUP 2D	GROUP 3A	
ANIMAL	1C1M	1C2M	1D1M	1D2M	2A1M	2A2M	2B1M	2B2M	2C1M	2D1F	3A1M	3A2M
TESTIS												
Granulomatous Inflammation					2							
Histiocytic Cells, Intraluminal					1	1			1			
Increased Cell, Spermatogonia						2		3	1	3		2
Mineralization	1	1		1		1	1	1				1
Mineralization, Collecting Duct	2	1		2	1	2	2	3				2
Stage 1												
Stage 2	P		P	P						P		
Stage 3		P			P	P	P	P	P		P	P
Testicular Degeneration, Increased		1	2		1	2	1	2	1	1	2	2

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 3C		GROUP 3D																			
ANIMAL	3C1M		3D1M																			
TESTIS																						
Granulomatous Inflammation																						
Histiocytic Cells,																						
Intraluminal	1																					
Increased Cell, Spermatogonia	1																					
Mineralization	1																					
Mineralization, Collecting																						
Duct	1																					
Stage 1																						
Stage 2																						
Stage 3	P		P																			
Testicular Degeneration,																						
Increased	1		1																			

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP CA					GROUP CB					GROUP CC					GROUP CD				
ANIMAL	CA1F	CA2F	CA3F	CA4F		CB1F	CB2F	CB3F	CB4F		CC1F	CC2F	CC3F	CC4F		CD1F	CD3F	CD4F		
OVARY																				
Asynchronous Development, Gonad																1				
Granulomatous Inflammation																				
Macrophage Aggregates, Increased																				
Oocyte Atresia, Increased																				
Stage 0																				
Stage 1																				
Stage 2				P		P					P					P		P		
Stage 3	P	P	P				P	P	P			P	P				P			
Stage 4														P						

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 1A				GROUP 1B				GROUP 1C			GROUP 1D			
ANIMAL	1A1F	1A2F	1A3F	1A4F	1B1F	1B2F	1B3F	1B4F	1C1F	1C2F	1C3F	1D1F	1D2F	1D3F	1D4F
OVARY															N
Asynchronous Development, Gonad														1	
Granulomatous Inflammation												1	1		
Macrophage Aggregates, Increased															
Oocyte Atresia, Increased				1	3		3	3			3				
Stage 0															
Stage 1															
Stage 2		P	P		P	P		P			P			P	
Stage 3				P			P		P	P		P	P		
Stage 4	P														

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 2A			GROUP 2B			GROUP 2C				GROUP 2D		GROUP 3A		
ANIMAL	2A1F	2A2F	2A3F	2B1F	2B2F	2B3F	2C1F	2C2F	2C3F	2C4F	2D2F	2D3F	3A1F	3A2F	3A3F
OVARY									N						
Asynchronous Development, Gonad															
Granulomatous Inflammation															
Macrophage Aggregates, Increased	1						1				1				
Oocyte Atresia, Increased		3	3	1		3	3			1		1	4		
Stage 0	P	P					P								
Stage 1				P		P		P		P	P				
Stage 2			P		P							P		P	
Stage 3															P
Stage 4													P		

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 3B		GROUP 3C																			
ANIMAL	3B1F		3C1F																			
OVARY																						
Asynchronous Development, Gonad																						
Granulomatous Inflammation																						
Macrophage Aggregates, Increased	1																					
Oocyte Atresia, Increased			2																			
Stage 0																						
Stage 1	P		P																			
Stage 2																						
Stage 3																						
Stage 4																						

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

APPENDIX A

GONAD HISTOPATHOLOGY RESULTS WORKSHEET

Animal	Sex	Group	Days	Fate	Examed	Tumors	Lcode	Lesion	Tcode	Tissue	WOD	TumorType	COD	TisGrade	LesGrade	Records
CA1M	M	0	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	2	5
CA1M	M	0	.	.	1	0	1002	Granulomatous Inflammation	1560	TESTIS	.	.	2	.	2	5
CA1M	M	0	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	5
CA1M	M	0	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	1	5
CA1M	M	0	.	.	1	0	1005	Stage 1	1560	TESTIS	.	.	2	.	P	5
CA2M	M	0	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	1
CB1M	M	0	.	.	1	0	1007	Increased Cell, Spermatogonia	1560	TESTIS	.	.	2	.	1	4
CB1M	M	0	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	4
CB1M	M	0	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	4
CB1M	M	0	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	.	P	4
CB2M	M	0	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	2	3
CB2M	M	0	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	2	3
CB2M	M	0	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	3
CC1M	M	0	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	3
CC1M	M	0	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	1	3
CC1M	M	0	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	3
CC2M	M	0	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	4
CC2M	M	0	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	2	4
CC2M	M	0	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	1	4
CC2M	M	0	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	4
CD1M	M	0	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	2	3
CD1M	M	0	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	2	3
CD1M	M	0	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	3
CD2F	M	0	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	2
CD2F	M	0	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	2
CD2M	M	0	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	5
CD2M	M	0	.	.	1	0	1002	Granulomatous Inflammation	1560	TESTIS	.	.	2	.	2	5
CD2M	M	0	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	5
CD2M	M	0	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	1	5
CD2M	M	0	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	5
1A1M	M	1	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	2	3
1A1M	M	1	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	2	3
1A1M	M	1	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	3
1A2M	M	1	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	5
1A2M	M	1	.	.	1	0	1002	Granulomatous Inflammation	1560	TESTIS	.	.	2	.	2	5
1A2M	M	1	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	2	5
1A2M	M	1	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	2	5
1A2M	M	1	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	5
1B1M	M	2	.	.	1	0	1002	Granulomatous Inflammation	1560	TESTIS	.	.	2	.	2	2
1B1M	M	2	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	.	P	2
1B2M	M	2	.	.	1	0	1007	Increased Cell, Spermatogonia	1560	TESTIS	.	.	2	.	1	3

Animal	Sex	Group	Days	Fate	Examed	Tumors	Lcode	Lesion	Tcode	Tissue	WOD	TumorType	COD	TisGrade	LesGrade	Records
1B2M	M	2	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	2	3
1B2M	M	2	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	3
1C1M	M	3	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	3
1C1M	M	3	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	2	3
1C1M	M	3	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	P	.	3
1C2M	M	3	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	4
1C2M	M	3	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	4
1C2M	M	3	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	1	4
1C2M	M	3	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	4
1D1M	M	4	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	2	2
1D1M	M	4	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	P	.	2
1D2M	M	4	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	3
1D2M	M	4	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	2	3
1D2M	M	4	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	P	.	3
2A1M	M	7	.	.	1	0	1009	Histiocytic Cells, Intraluminal	1560	TESTIS	.	.	2	.	1	5
2A1M	M	7	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	5
2A1M	M	7	.	.	1	0	1002	Granulomatous Inflammation	1560	TESTIS	.	.	2	.	2	5
2A1M	M	7	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	1	5
2A1M	M	7	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	5
2A2M	M	7	.	.	1	0	1009	Histiocytic Cells, Intraluminal	1560	TESTIS	.	.	2	.	1	6
2A2M	M	7	.	.	1	0	1007	Increased Cell, Spermatogonia	1560	TESTIS	.	.	2	.	2	6
2A2M	M	7	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	2	6
2A2M	M	7	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	6
2A2M	M	7	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	2	6
2A2M	M	7	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	6
2B1M	M	5	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	4
2B1M	M	5	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	4
2B1M	M	5	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	2	4
2B1M	M	5	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	4
2B2M	M	5	.	.	1	0	1007	Increased Cell, Spermatogonia	1560	TESTIS	.	.	2	.	3	5
2B2M	M	5	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	2	5
2B2M	M	5	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	5
2B2M	M	5	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	3	5
2B2M	M	5	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	5
2C1M	M	6	.	.	1	0	1009	Histiocytic Cells, Intraluminal	1560	TESTIS	.	.	2	.	1	4
2C1M	M	6	.	.	1	0	1007	Increased Cell, Spermatogonia	1560	TESTIS	.	.	2	.	1	4
2C1M	M	6	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	4
2C1M	M	6	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	4
2D1F	M	8	.	.	1	0	1007	Increased Cell, Spermatogonia	1560	TESTIS	.	.	2	.	3	3
2D1F	M	8	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	3
2D1F	M	8	.	.	1	0	1006	Stage 2	1560	TESTIS	.	.	2	P	.	3

Animal	Sex	Group	Days	Fate	Examed	Tumors	Lcode	Lesion	Tcode	Tissue	WOD	TumorType	COD	TisGrade	LesGrade	Records
3A1M	M	10	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	2	2
3A1M	M	10	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	2
3A2M	M	10	.	.	1	0	1007	Increased Cell, Spermatogonia	1560	TESTIS	.	.	2	.	2	5
3A2M	M	10	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	2	5
3A2M	M	10	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	5
3A2M	M	10	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	2	5
3A2M	M	10	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	5
3C1M	M	11	.	.	1	0	1009	Histiocytic Cells, Intraluminal	1560	TESTIS	.	.	2	.	1	6
3C1M	M	11	.	.	1	0	1007	Increased Cell, Spermatogonia	1560	TESTIS	.	.	2	.	1	6
3C1M	M	11	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	6
3C1M	M	11	.	.	1	0	1003	Mineralization	1560	TESTIS	.	.	2	.	1	6
3C1M	M	11	.	.	1	0	1004	Mineralization, Collecting Duct	1560	TESTIS	.	.	2	.	1	6
3C1M	M	11	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	6
3D1M	M	9	.	.	1	0	1001	Testicular Degeneration, Increased	1560	TESTIS	.	.	2	.	1	2
3D1M	M	9	.	.	1	0	1008	Stage 3	1560	TESTIS	.	.	2	P	.	2
CA1F	F	0	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	1
CA2F	F	0	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	1
CA3F	F	0	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	1
CA4F	F	0	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	P	.	1
CB1F	F	0	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	P	.	1
CB2F	F	0	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	1
CB3F	F	0	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	1
CB4F	F	0	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	1
CC1F	F	0	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	P	.	1
CC2F	F	0	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	1
CC3F	F	0	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	1
CC4F	F	0	.	.	1	0	1010	Stage 4	1550	OVARY	.	.	2	P	.	1
CD1F	F	0	.	.	1	0	1011	Asynchronous Development, Gonad	1550	OVARY	.	.	2	.	1	2
CD1F	F	0	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	P	.	2
CD3F	F	0	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	1
CD4F	F	0	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	P	.	1
1A1F	F	1	.	.	1	0	1010	Stage 4	1550	OVARY	.	.	2	P	.	1
1A2F	F	1	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	P	.	1
1A3F	F	1	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	P	.	1
1A4F	F	1	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	1	2
1A4F	F	1	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	2
1B1F	F	2	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	3	2
1B1F	F	2	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	P	.	2
1B2F	F	2	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	P	.	1
1B3F	F	2	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	3	2
1B3F	F	2	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	P	.	2

Animal	Sex	Group	Days	Fate	Examed	Tumors	Lcode	Lesion	Tcode	Tissue	WOD	TumorType	COD	TisGrade	LesGrade	Records
1B4F	F	2	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	3	2
1B4F	F	2	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	.	P	2
1C1F	F	3	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	.	P	1
1C2F	F	3	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	.	P	1
1C3F	F	3	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	3	2
1C3F	F	3	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	.	P	2
1D1F	F	4	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	.	P	2
1D1F	F	4	.	.	1	0	1002	Granulomatous Inflammation	1550	OVARY	.	.	2	.	1	2
1D2F	F	4	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	.	P	2
1D2F	F	4	.	.	1	0	1002	Granulomatous Inflammation	1550	OVARY	.	.	2	.	1	2
1D3F	F	4	.	.	1	0	1011	Asynchronous Development, Gonad	1550	OVARY	.	.	2	.	1	2
1D3F	F	4	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	.	P	2
1D4F	F	4	.	.	1	0	.	.	1550	OVARY	.	.	.	3	.	1
2A1F	F	7	.	.	1	0	1014	Macrophage Aggregates, Increased	1550	OVARY	.	.	2	.	1	2
2A1F	F	7	.	.	1	0	1013	Stage 0	1550	OVARY	.	.	2	.	P	2
2A2F	F	7	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	3	2
2A2F	F	7	.	.	1	0	1013	Stage 0	1550	OVARY	.	.	2	.	P	2
2A3F	F	7	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	3	2
2A3F	F	7	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	.	P	2
2B1F	F	5	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	1	2
2B1F	F	5	.	.	1	0	1005	Stage 1	1550	OVARY	.	.	2	.	P	2
2B2F	F	5	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	.	P	1
2B3F	F	5	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	3	2
2B3F	F	5	.	.	1	0	1005	Stage 1	1550	OVARY	.	.	2	.	P	2
2C1F	F	6	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	3	3
2C1F	F	6	.	.	1	0	1014	Macrophage Aggregates, Increased	1550	OVARY	.	.	2	.	1	3
2C1F	F	6	.	.	1	0	1013	Stage 0	1550	OVARY	.	.	2	.	P	3
2C2F	F	6	.	.	1	0	1005	Stage 1	1550	OVARY	.	.	2	.	P	1
2C3F	F	6	.	.	1	0	.	.	1550	OVARY	.	.	.	3	.	1
2C4F	F	6	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	1	2
2C4F	F	6	.	.	1	0	1005	Stage 1	1550	OVARY	.	.	2	.	P	2
2D2F	F	8	.	.	1	0	1014	Macrophage Aggregates, Increased	1550	OVARY	.	.	2	.	1	2
2D2F	F	8	.	.	1	0	1005	Stage 1	1550	OVARY	.	.	2	.	P	2
2D3F	F	8	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	1	2
2D3F	F	8	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	.	P	2
3A1F	F	10	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	4	2
3A1F	F	10	.	.	1	0	1010	Stage 4	1550	OVARY	.	.	2	.	P	2
3A2F	F	10	.	.	1	0	1006	Stage 2	1550	OVARY	.	.	2	.	P	1
3A3F	F	10	.	.	1	0	1008	Stage 3	1550	OVARY	.	.	2	.	P	1
3B1F	F	11	.	.	1	0	1014	Macrophage Aggregates, Increased	1550	OVARY	.	.	2	.	1	2
3B1F	F	11	.	.	1	0	1005	Stage 1	1550	OVARY	.	.	2	.	P	2

Animal	Sex	Group	Days	Fate	Examed	Tumors	Lcode	Lesion	Tcode	Tissue	WOD	TumorType	COD	TisGrade	LesGrade	Records
3C1F	F	9	.	.	1	0	1012	Oocyte Atresia, Increased	1550	OVARY	.	.	2	.	2	2
3C1F	F	9	.	.	1	0	1005	Stage 1	1550	OVARY	.	.	2	P	.	2

APPENDIX B
FIGURES AND LEGENDS

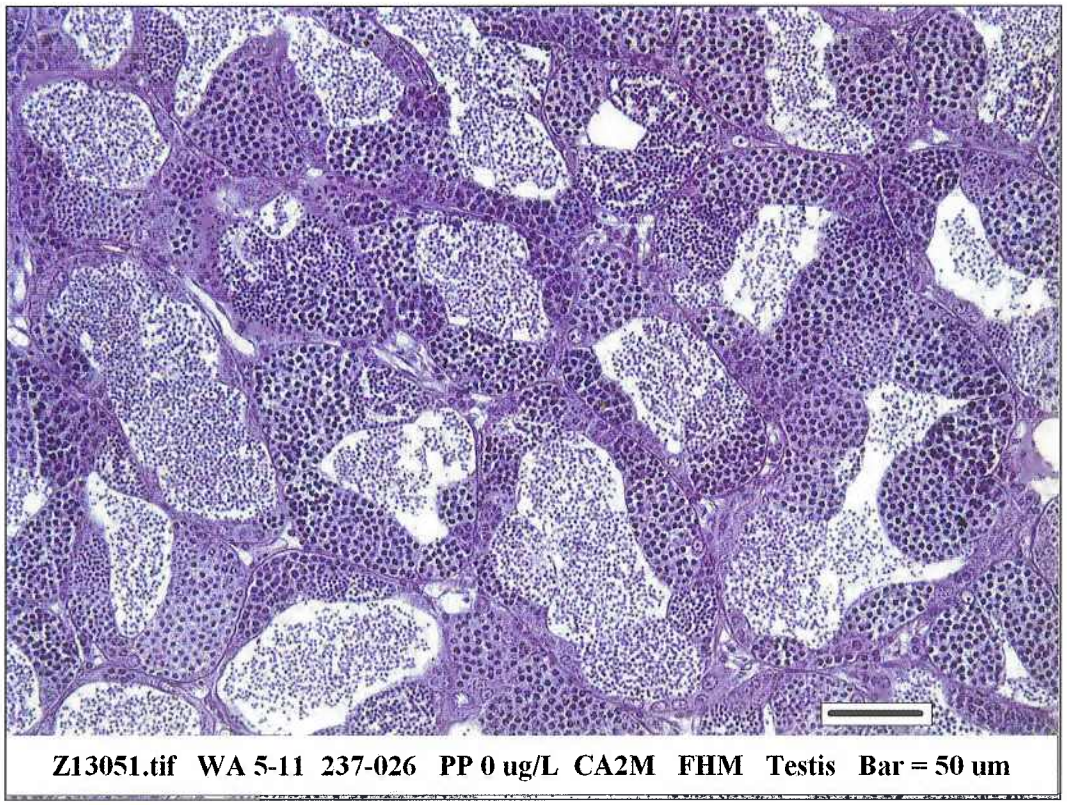

Figure 1 (Z13051). Testis from a control group male. This is a typical Stage 2 testis characterized by a moderately thick germinal epithelium as compared to the size of the tubule lumina. H&E.

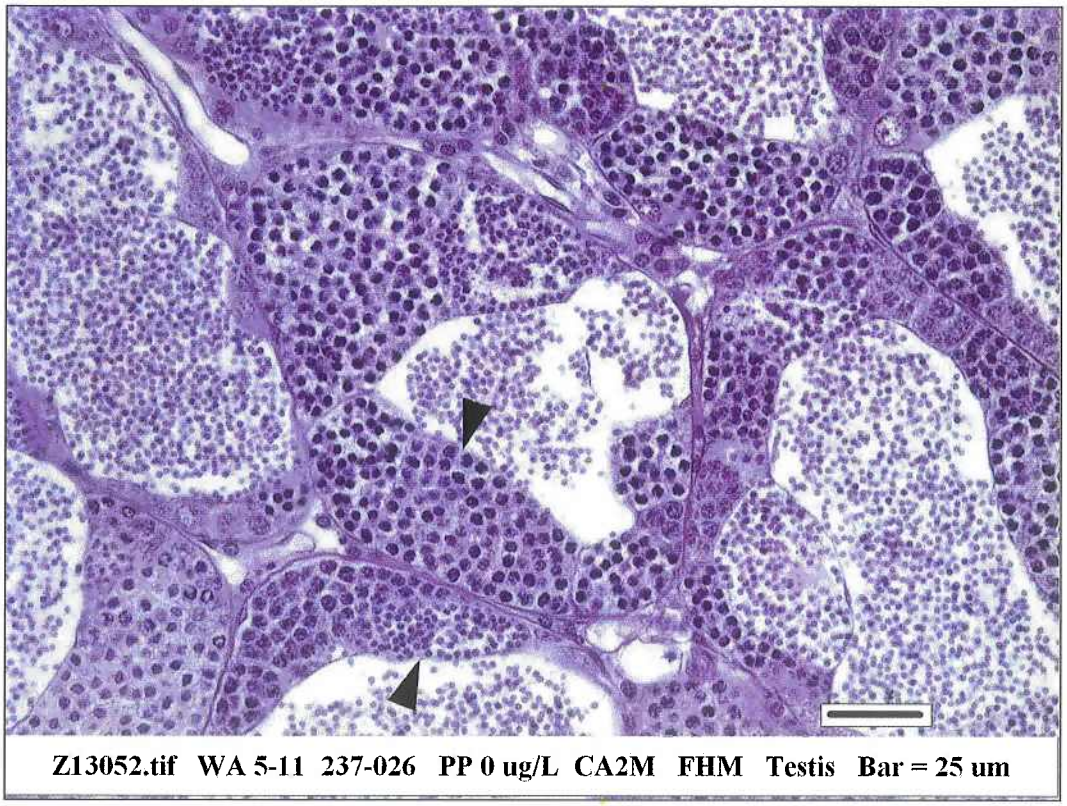

Figure 2 (Z13052). Higher magnification of Fig.1. Arrowheads indicate an area of germinal epithelium which overall is primarily comprised of spermatogenic cells including spermatogonia, spermatocytes, and spermatids. H&E.

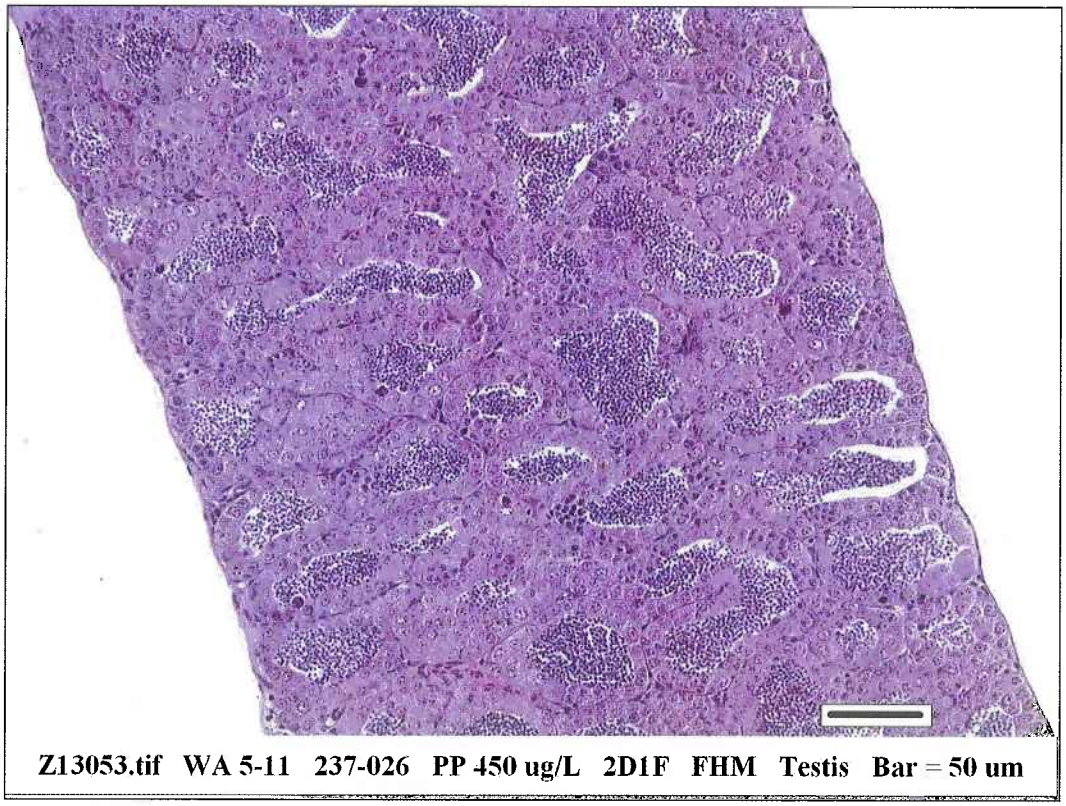

Figure 3 (Z13053). Stage 2 testis from a 450 µg/L group male. This fish was initially submitted as a female. The germinal epithelium is primarily comprised of spermatogonia (increased spermatogonia). H&E.

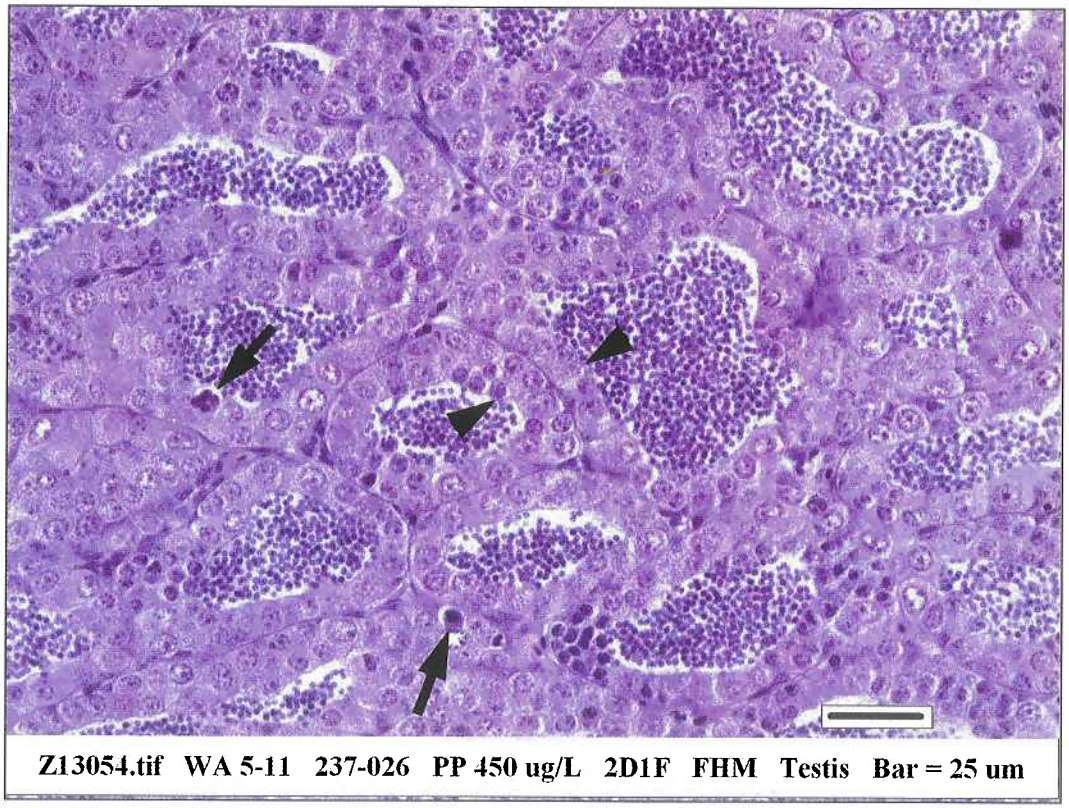

Figure 4 (Z13054). Higher magnification of Fig. 3. Arrowheads indicate the width of the germinal epithelium. Degenerating spermatogenic cells (testicular degeneration) are indicated by the arrows. H&E.

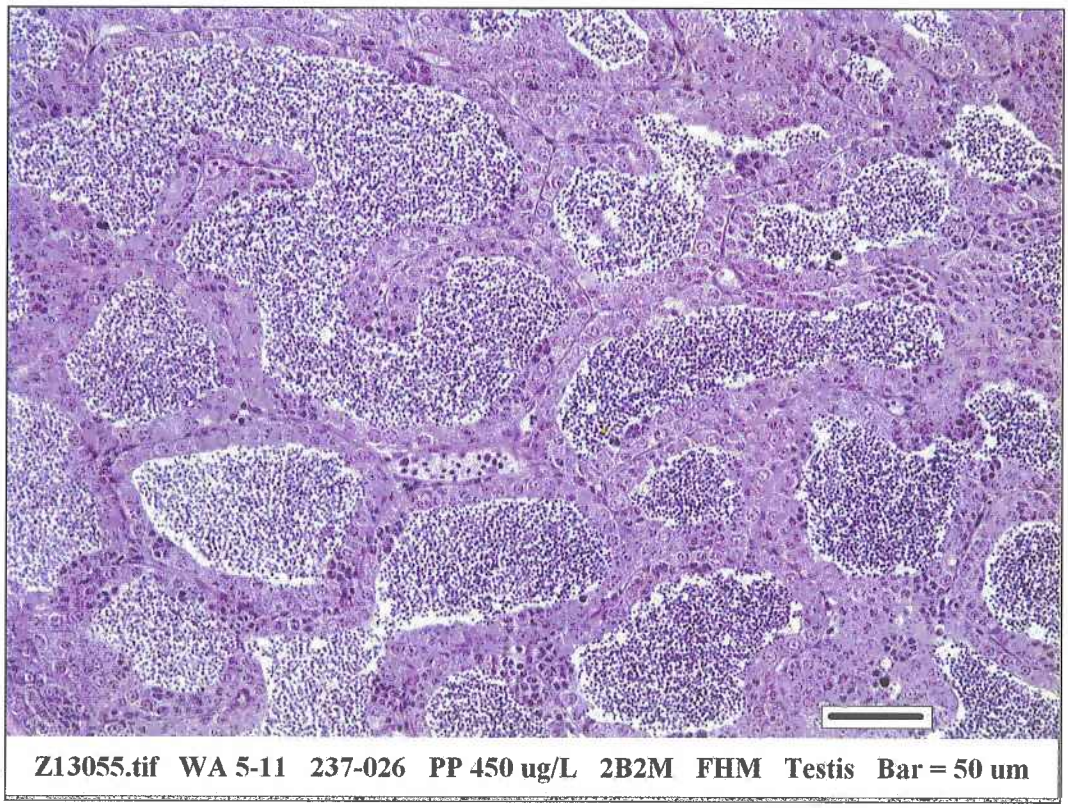

Figure 5 (Z13055). Stage 3 testis from a 450 µg/L group male. As compared to the testes in the previous figures, the germinal epithelium is thinner relative to the tubule lumina. Spermatogonia are proportionally increased. H&E.

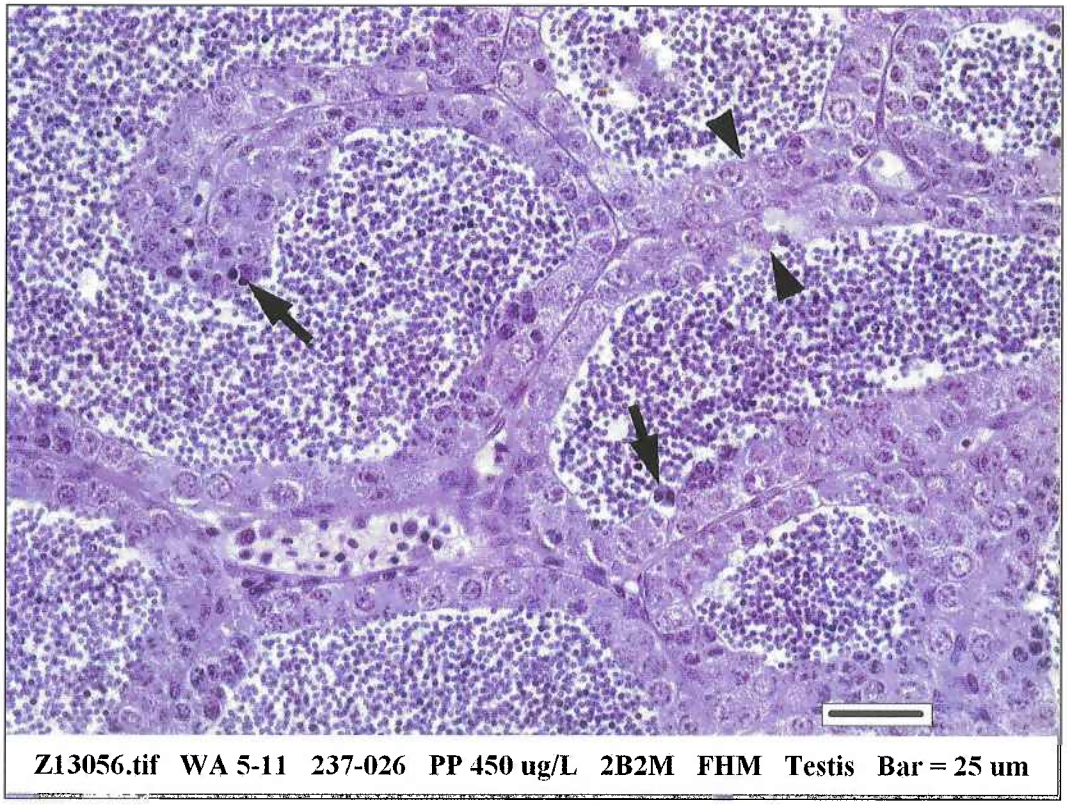

Figure 6 (Z13056). Higher magnification of Fig. 5. Arrowheads indicate the germinal epithelium, and arrows indicate degenerating spermatogenic cells (testicular degeneration) that are present singly or in clusters. H&E.

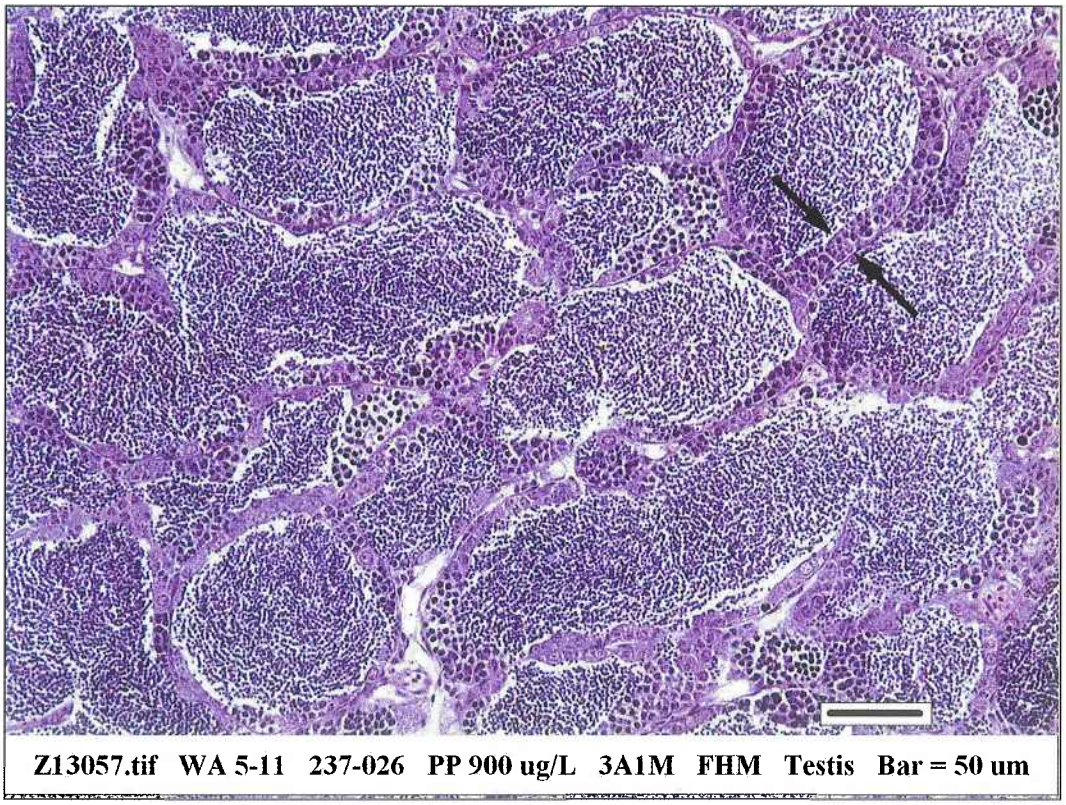

Figure 7 (Z13057). Stage 3 testis from a 900 µg/L group male. This particular testis did not have increased spermatogonia. The germinal epithelium is indicated by the arrows. H&E.

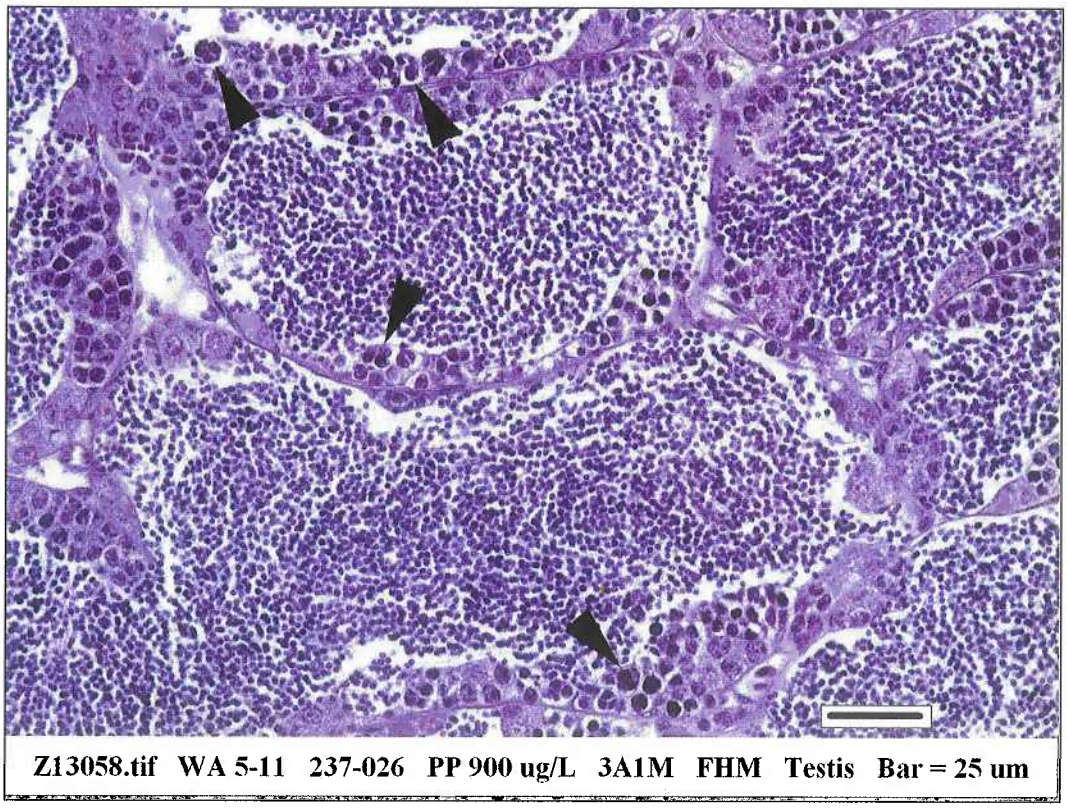

Figure 8 (Z13058). Higher magnification of Fig. 7. The arrowheads indicate numerous clusters of degenerating spermatogenic cells (testicular degeneration). H&E.

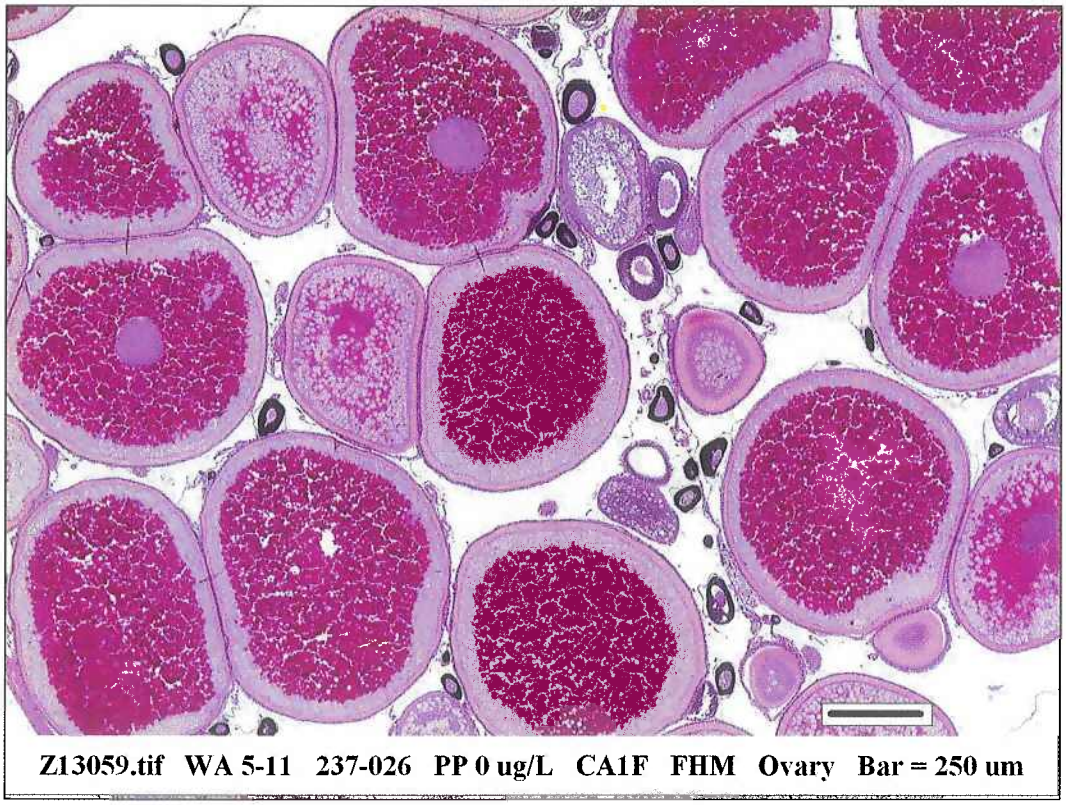

Figure 9 (Z13059). Stage 3 ovary from a control group female. Mid to late vitellogenic oocytes predominate in this field, although cortical alveolar and perinucleolar oocytes are also evident. H&E.

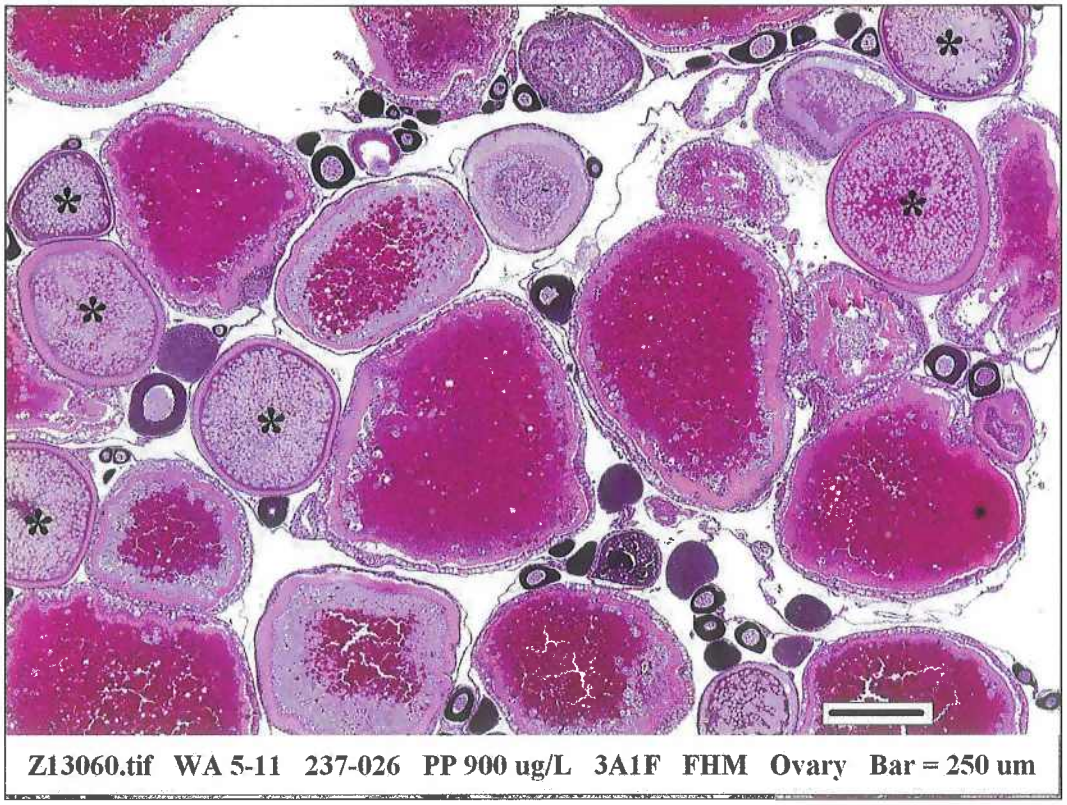

Figure 10 (Z13060). Stage 4 ovary from 900 µg/L female. This ovary is characterized by severe oocyte atresia. Asterisks indicate the relatively few non-atretic oocytes. H&E.

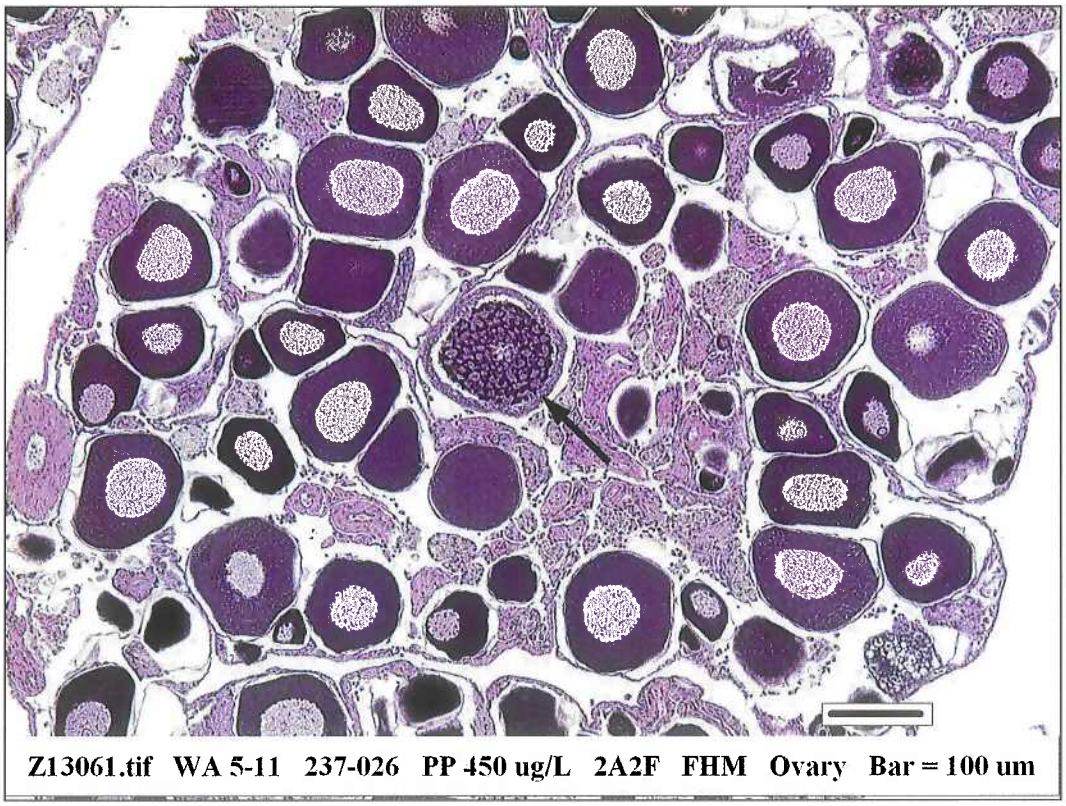

Figure 11 (Z13061). Stage 0 ovary from 450 µg/L female. Only perinucleolar ooocytes are evident in this image. The arrow indicates an atretic oocyte. H&E.

Appendix B – Springborn Smithers Report

Study Title

Fathead Minnow (*Pimephales promelas*) Fish Screening Assay OECD Phase 1 B Follow-Up

Springborn Smithers Authors

Arthur E. Putt
Mark A. Cafarella

Study Completion Date

Revised Draft Version
28 March 2006

Submitted To

Battelle Marine Sciences Laboratory
1529 West Sequim Bay Road
Sequim, Washington 98382

Performing Laboratory

Springborn Smithers Laboratories
790 Main Street
Wareham, Massachusetts 02571-1037

Laboratory Project ID

Springborn Smithers Study No.	13784.6109 13784.6110 13784.6112
Battelle Project No.	43495
EPA Contract No.	68-W-01-023

QUALITY ASSURANCE STATEMENT

The study conduct, raw data, and report for **"Fathead Minnow (*Pimephales promelas*) Fish Screening Assay OECD Phase 1 B Follow-Up"** were inspected by the Quality Assurance Unit (QAU) at Springborn Smithers Laboratories to determine adherence with the Quality Assurance Project Plan (QAPP) and laboratory standard operating procedures. Dates of study inspections, inspection types, and dates reported to the Work Assignment Leader and to Management are listed below.

Inspection Date	Inspection Type	Reported to Work Assignment Leader/ Management
	13784.6109 (flutamide)	
7/14/05	In-life (observations of egg count)	7/14/05
8/12/05	Data, chemistry data	8/18/05
10/21 & 10/20/05	Data, biology	11/4/05
12/14/05	Data, survival statistics	12/14/05
12/7, 12/8 & 12/12/05	Draft report	12/14/05
12/22/05	Data, vitellogenin	12/23/05
1/5/06	Data, statistics	1/5/06
1/6/06	Data, statistics	1/6/06
1/6/06	Revised draft report	1/6/06
3/27/06	Revised draft report	3/28/06
	13784.6110 (potassium permanganate)	
8/29/05	Data, chemistry	8/30/05
9/6/05	Data, analytical methods verification	9/9/05
9/29/05	In-life (biological observations, assessment of sex characteristics)	9/29/05
11/4/05	Data, biology	11/28/05
11/4 & 11/11/05	Data, biology	11/28/05
11/30/05	Vitellogenin analysis	11/30/05
12/7, 12/8 & 12/12/05	Draft report	12/14/05
12/14/05	Data, survival statistics	12/14/05
1/5/06	Data, statistics	1/5/06
1/6/06	Data, statistics	1/6/06
1/6/06	Revised draft report	1/6/06
3/27/06	Revised draft report	3/28/06
	13784.6112 (ketoconazole)	
10/3/05	In-life (biological observations)	10/3/05
11/14/05	Data, biology	11/28/05
11/14/05	Data, biology	11/28/05

11/14 & 11/15/05	Data, biology	11/28/05
11/16/05	Data, analytical methods verification	11/18/05
11/16/05	Data, chemistry	11/18/05
11/16/05	Data, chemistry	11/18/05
11/16/05	Data, chemistry	11/18/05
12/14/05	Data, survival statistics	12/14/05
12/7, 12/8 & 12/12/05	Draft report	12/14/05
12/22/05	Data, vitellogenin	12/23/05
1/5/06	Data, statistics	1/5/06
1/6/06	Data, statistics	1/6/06
1/6/06	Revised draft report	1/6/06
3/27/06	Revised draft report	3/28/06

SPRINGBORN SMITHERS LABORATORIES

Jean Ellen D'Alessandro
Manager, Quality Assurance

Date

KEY STUDY PERSONNEL

The following Springborn Smithers personnel were responsible for the conduct of the work and reporting of the study results.

Arthur E. Putt	Principal Investigator
Mark A. Cafarella	Principal Investigator
Duncan O. York	Biologist I
Marjorie E. Dix	Senior Research Chemist
Michael A. Orsini, Ph.D.	Senior Research Chemist
Joaquim B. Livramento	Chemist II
Catherine Lafontaine	Assistant Chemist
Robyne Zuendel	Assistant Chemist
David A. Bassett	Manager, Chemistry
Andrea M. Soares	Technical Report Writer
Ronald C. Biever	Vice President, Operations

TABLE OF CONTENTS

SUMMARY

Fathead Minnow (*Pimephales promelas*) Fish Screening Assay OECD Phase 1 B Follow-Up

SPONSOR: Battelle Marine Sciences Laboratory

SPRINGBORN SMITHERS STUDY NUMBERS:
13784.6109
13784.6110
13784.6112

BATTELLE PROJECT NUMBER: 43495

EPA CONTRACT NUMBER: 68-W-01-023

TEST SUBSTANCES:

Flutamide, Lot No. 121K1083, CAS No. 13311-84-7, reported to have a purity of 100% was received from Battelle Marine Sciences Laboratory on 9 May 2005.

Potassium permanganate, Lot No. 00310LC, CAS No. 7722-64-7, reported to have a purity of 99.5% was received from Battelle Marine Sciences Laboratory on 9 May 2005.

Ketoconazole, Lot No. QL0352, CAS No. 65277-42-1, reported to have a purity of 99.73% was received from Battelle Marine Sciences Laboratory on 23 August 2005.

DEFINITIVE TEST DATES:

flutamide	24 June to 15 July 2005
potassium permanganate	4 to 25 August 2005
ketoconazole	21 September to 12 October 2005

TEST ORGANISM:

Pimephales promelas
Age at test initiation
- flutamide: 18 weeks old
- potassium permanganate: 20 weeks old
- ketoconazole: 29 weeks old

Source: Springborn Smithers culture

DILUTION WATER:

Laboratory well water
pH: 7.2 to 7.8
Specific conductance: 130 to 230 µmhos/cm
Total hardness as $CaCO_3$: 40 to 54 mg/L
Total alkalinity as $CaCO_3$: 25 to 36 mg/L

TEST CONDITIONS:

Test duration:	21 days
Temperature range:	24 to 26 °C
Dissolved oxygen range:	4.1 to 8.5 mg/L
pH range:	6.8 to 7.8
Light intensity range:	60 to 90 footcandles (630 to 965 lux)
Photoperiod:	illumination of 16 hours light: 8 hours dark

NOMINAL TEST CONCENTRATIONS:

100, 500, and 1000 µg a.i./L (flutamide)

225, 450, and 900 µg a.i./L (potassium permanganate)

25, 100, and 400 µg a.i./L (ketoconazole)

ENDPOINTS:

- Survival
- Fecundity
- Fertilization success
- Appearance of adults (secondary sex characteristics)
- Body weight
- Body length
- Male Fatpad Index (expressed as FPI)
- Vitellogenin (VTG) concentration
- Gonad size (expressed as GSI)

RESULTS:

Flutamide

The percent fertile embryos and the fatpad index (FPI) were affected at the 1000 µg a.i./L treatment level. There were no adverse effects observed for the remaining endpoints statistically analyzed (survival, tubercle score, fecundity, length, weight, GSI, VTG) for flutamide. A summary of the endpoint results for this study is presented in the following table:

Endpoint	NOEC (µg a.i./L)	LOEC (µg a.i./L)	Statistical Test
Survival	1000	>1000	Williams'
Tubercle score	1000	>1000	Williams'
Female length	1000	>1000	Williams'
Female weight	1000	>1000	Williams'
Female GSI	1000	>1000	Williams'
Male length	1000	>1000	Williams'
Male weight	1000	>1000	Williams'
Male GSI	1000	>1000	Williams'
# of eggs estimated	1000	>1000	Steel's
# of eggs counted	1000	>1000	Steel's
# of spawns	1000	>1000	Williams'
# of infertile eggs	1000	>1000	Steel's
Percent fertile embryos	500	1000	Steel's
Vitellogenin – male	1000	>1000	Williams'
Vitellogenin - female	1000	>1000	Williams'
FPI	500	1000	Williams'

Potassium permanganate

Survival was adversely affected at the 900 µg a.i./L treatment level. None of the remaining endpoints statistically analyzed (tubercle score, fecundity, length, weight, GSI, FPI, VTG) were adversely affected at a nominal concentration of 900 µg a.i./L for potassium permanganate. Because survival was affected at the highest treatment level, it was not possible to test at higher concentrations to evaluate potential endocrine disruptor effects. A summary of the endpoint results during this study is presented in the following table:

Endpoint	NOEC (µg a.i./L)	LOEC (µg a.i./L)	Statistical Test
Survival	450	900	Williams'
Tubercle score	900	>900	Williams'
Female length	900	>900	Williams'
Female weight	900	>900	Williams'
Female GSI	900	>900	Williams'
Male length	900	>900	Williams'
Male weight	900	>900	Williams'
Male GSI	900	>900	Williams'
# of eggs estimated	900	>900	Williams'
# of eggs counted	900	>900	Williams'
# of spawns	900	>900	Williams'
# of infertile eggs	900	>900	Williams'
Percent fertile embryos	900	>900	Williams'
Vitellogenin – male	900	>900	Williams'
Vitellogenin – female	900	>900	Williams'
FPI	900	>900	Williams'

Ketoconazole

Vitellogenin was affected at the 400 µg a.i./L treatment level for males only. None of the remaining endpoints statistically analyzed (survival, tubercle score, fecundity, length, weight, GSI, FPI) were adversely affected at a nominal concentration of 400 µg a.i./L for ketoconazole. A summary of the endpoint results during this study is presented in the following table:

Endpoint	NOEC (µg a.i./L)	LOEC (µg a.i./L)	Statistical Test
Survival	400	>400	Williams'
Tubercle score	400	>400	Williams'
Female length	400	>400	Williams'
Female weight	400	>400	Williams'
Female GSI	400	>400	Steel's
Male length	400	>400	Williams'
Male weight	400	>400	Williams'
Male GSI	400	>400	Williams'
# of eggs estimated	400	>400	Williams'
# of eggs counted	400	>400	Williams'
# of spawns	400	>400	Williams'
# of infertile eggs	400	>400	Steel's
Percent fertile embryos	400	>400	Wilcoxon's
Vitellogenin – male	100	400	Kruskal-Wallis'
Vitellogenin - female	400	>400	Williams'
FPI	400	>400	Kruskal-Wallis/Steel's

1.0 INTRODUCTION

The U.S. Environmental Protection Agency (EPA) is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is a short-term screening assay designed to detect substances that interact with the estrogen and androgen systems of fish. It is thought that the inclusion of the fish screening assay in Tier 1 is important because estrogenic and androgenic controls on reproduction and development in fish may differ significantly enough from that of higher vertebrates such that mammalian screening methods may not identify potential endocrine disrupting chemicals (EDCs) in this important class of animals. As an example, dihydrotestosterone is a potent androgen in mammals, but 11-ketotestosterone is generally the more prevalent androgen in fish.

U.S. EPA (2001) has described a short-term test with the fathead minnow *(Pimephales promelas)* 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 (Ankley et al., 2001) 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 is 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 and sex steroids (17ß-estradiol, testosterone, and 11-ketotestosterone).

The Organization for Economic Cooperation and Development (OECD) initiated a fish screening assay validation activity and has completed its Phase 1A and Phase 1B trials. Phase 1A evaluated a non-spawning version of a 21-day exposure assay with fathead minnow, medaka, and zebra fish. The results of the Phase 1A led to the Phase 1B trials where spawning was included in the method. The results of the Phase 1B trial raised questions regarding the spawning conditions utilized for the fathead minnow.

Previous work assignments under this contract (WA 2-18 and WA 2-29) were initiated to evaluate a short-term reproduction assay with fathead minnow and compare the EPA (2001) method to two other related assays to contribute to the optimization of the assay for use as a screen in the EDSP. An overview of the methodology and relevant test conditions is provided in Appendix A, the WA 5-11 Fish Protocol (located in Appendix 1).

The purpose of the work conducted under this assignment was to demonstrate the Fish Screening assay test method, based on the OECD Phase 1B study for short-term reproduction assay with the fathead minnow as described in EPA (2001). This follow-up study incorporated an increased number of replicates and used a semi-quantitative and quantitative egg-counting method.

The experimental phases of the study were conducted from 24 June to 15 July 2005 (flutamide), 4 to 25 August 2005 (potassium permanganate), and from 21 September to 12 October 2005 (ketoconazole) at Springborn Smithers Laboratories (SSL), located in Wareham, Massachusetts. The original raw data, protocol, and the original final report produced during this study are stored at Springborn Smithers Laboratories at the above location. Experimental Pathology Laboratories, Inc. (EPL), Sterling, Virginia, performed the histopathology work.

2.0 MATERIALS AND METHODS

2.1 QAPP/Protocol

This study was conducted according to the procedures outlined in the Quality Assurance Project Plan (QAPP) for Work Assignment 5-11 (Appendix 1). The study protocol describes a short-term test with the fathead minnow (*Pimephales promelas*) that considers reproductive fitness as an integrated measure of toxicant exposure. It also enables measurement of a suite of histological and biochemical endpoints that reflect effects associated with [anti-] estrogens and androgens. The test described in the guideline is an extension of existing standard practice for conducting a short-term reproduction test with fathead minnow (Ankley et al., 2001).

2.2 Test Substances

Samples of the test substances were received from the EDSP Chemical Repository, and housed at Battelle's Pacific Northwest Division, Sequim, Washington. Upon receipt at Springborn Smithers, the test substances were stored at room temperature in the original container in a dark ventilated cabinet. All test concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.). The following information was provided:

Test Substance	SSL Numbers	Lot or Batch Number	CAS No.	Date Received	Purity	Recertification Date
Flutamide	112-52	121K1083	13311-84-7	9 May 2005	100%	1 April 2006
Potassium permanganate	112-51	00310LC	7722-64-7	9 May 2005	99.5%	May 2010
Ketoconazole	114-20	QL0352	65277-42-1	23 August 2005	99.73%	30 September 2005

2.3 Test Organism

The fathead minnows (*Pimephales promelas)* used during this study (SSL Lot Nos. 05A36, 05A59 and 05A78) were obtained from a laboratory supply of reproductively mature animals (18 to 29 weeks old), in spawning condition. Prior to testing, the fish were held in a 39 x 20 x 25 cm (L x W x H) glass aquarium under a photoperiod of 16 hours light and 8 hours darkness. The culture water was drawn from a 100-meter deep bedrock well into an epoxy-coated concrete reservoir where it was aerated and supplemented with well water supplied by the Town of Wareham, Massachusetts. The water which flowed into this holding tank was characterized as having total hardness and total alkalinity ranges as calcium carbonate ($CaCO_3$) of 36 to 56 mg/L and 24 to 35 mg/L, respectively, a pH range of 7.2 to 7.8, and a specific conductance range of 130 to 220 micromhos per centimeter (µmhos/cm). Fish used during the definitive exposure were maintained under these conditions for a minimum of 14 days prior to testing. The

temperature in the holding tanks ranged from 22 to 26 °C during this period. Fish did not receive treatment for disease in the two-week acclimation period preceding the test, or during the exposure period.

Prior to test initiation and throughout the exposure period, the fish were fed, a measured amount twice daily with brine shrimp. Biannual analyses of representative samples of the food were conducted by GeoLabs, Inc., Braintree, Massachusetts in accordance with Springborn Smithers' SOP 7.92 and using U.S. EPA standard methods-to ensure the absence of potential toxicants, including pesticides, PCBs, and selected toxic metals, at concentrations that may be harmful to fish. Based on these analyses, the food sources were considered to be of acceptable quality since all analyte concentrations were below levels of concern based on ASTM, 1985; Suter and Tsao, 1996; Jones et al., 1997; and Smith, 1995. No mortality was observed in the test fish population during the 48-hour period prior to testing. A representative sample of the male (N = 9) and female (N = 13) fish from the test population selected for the flutamide exposure had a mean wet weight of 3.3 g (range 2.4 to 4.0 g) and 1.1 g (range 0.90 to 1.4 g), respectively. A representative sample of the male (N = 15) and female (N = 15) fish from the test population selected for the potassium permanganate exposure had a mean wet weight of 3.5 g (range 3.1 to 4.4 g) and 1.5 g (range 0.83 to 2.0 g), respectively. A representative sample of the male (N = 15) and female (N = 15) fish from the test population selected for the ketoconazole exposure had a mean wet weight of 3.1 g (range 2.5 to 3.8 g) and 1.4 g (1.0 to 1.9 g), respectively.

2.4 Dilution Water

The dilution water used during this study was well water which was pumped into an epoxy-coated concrete reservoir where it was supplemented on demand with untreated Town of Wareham well water and aerated. During these studies, characterization of the well water established total hardness and alkalinity ranges as $CaCO_3$ of 40 to 54 mg/L and 25 to 36 mg/L, respectively, a pH range of 7.2 to 7.8, and a specific conductance range of 130 to 230 µmhos/cm. Biannual analyses were conducted on representative samples of the dilution water source for the presence of pesticides, PCBs, and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds were detected in any of the water samples analyzed at concentrations that are considered toxic according to ASTM (2002) standard practice. In addition, samples of

the dilution water source were analyzed monthly for total organic carbon (TOC) concentration. These analyses established TOC concentrations ranging from 0.45 mg/L to 1.1 mg/L for the months of June to October 2005.

2.5 Test Conditions

The exposure system consisted of a 2000-mL intermittent-flow proportional diluter (Mount and Brungs, 1967) and a two-tiered water bath, consisting of an upper and a lower level water bath (one positioned over the other). Each water bath contained a set of eight exposure aquaria. The exposure system was designed to provide three concentrations of the test substance and a dilution water control consisting of four replicates.

The aquaria were impartially positioned in a water bath containing circulating water designed to maintain the test solution temperatures at 25 ± 1 °C. Gentle, oil-free aeration was used during all tests to maintain total dissolved oxygen concentrations ≥60% of saturation. Illumination was provided by Sylvania Oktron® fluorescent bulbs centrally located above the test aquaria. Sixteen hours of light at 60 to 90 footcandles (630 to 965 lux) at the exposure solution surface was provided each day. Light intensity was measured with a VWR Model 41406661 light meter.

2.6 Test Substance Concentrations

Test substance concentrations for this study were selected as indicated in the table below. Range-finding for potassium permanganate is described in Section 3.0.

Test Substance	Nominal Concentrations	Selection Process
flutamide	100, 500, and 1000 µg a.i./L	selected by Study Sponsor
potassium permanganate	225, 450, and 900 µg a.i./L	based on range-finding results and consultation with the Study Sponsor
ketoconazole	25, 100, and 400 µg a.i./L	selected by Study Sponsor

2.7 Stock Preparation

2.7.1 Flutamide

The flutamide stock solution was prepared prior to test initiation by using a chemical coating procedure to apply the test substance to the diluter apparatus. Approximately 3.5 g of flutamide (3.5 g active ingredient) was first dissolved in 40 mL of acetone (CAS No. 67-64-1). The flutamide solution was then added to a carboy, which had been placed on a rolling mill set at low speed. A constant flow of nitrogen was then applied to evaporate the acetone from the solution. Rolling was discontinued when the acetone had evaporated and nitrogen flow was continued for an additional 60 minutes. The coated carboy was then placed on a stir plate with a slight vortex after bringing to volume with dilution water. Dilution water was then metered through the carboy at a rate of 12.6 mL/minute to allow the flutamide concentration to come to equilibrium. The resulting solution was observed to be yellow in color with floating undissolved test substance present and was analytically confirmed to have a concentration of 20 µg a.i./L. Following the overnight equilibration period, the solution was engaged on the diluter system.

2.7.2 Potassium Permanganate

An 18 mg a.i./mL primary stock solution was prepared by placing approximately 0.4523 g of potassium permanganate (0.4500 g as active ingredient) in a volumetric flask and bringing it to a volume of 25 mL with reagent grade water. The primary stock solution was observed to be dark purple in color with no visible undissolved test substance.

2.7.3 Ketoconazole

The ketoconazole stock solution was prepared by placing approximately 2.0047 g of ketoconazole (1.9993 g as active ingredient) in 50 mL of acetone (CAS No. 67-64-1). The resulting solution was observed to be milky white in color with undissolved test substance present. The solution was poured into a 1 x 12 inch stainless steel column packed with glass wool, which was then capped. To draw off the solvent, the other end of the column was attached to a vacuum pump with silicone tubing. The tubing was clamped off and the pump turned on. Once the pump was running, the tubing was slowly opened until air flow was started, but no

solution was being drawn through. The pump end of the column was elevated. After 40 minutes, the column was cold and condensation was observed on the outside. The vacuum was increased and pumping continued for two hours at which time all condensation had disappeared and the column was at ambient temperature. The column was then connected to an FMI piston pump and dilution water was then pumped through at a flow of 100 mL/min. The flow was recirculated within a 55-gallon Teflon®-line drum to allow equilibration over a period of four days prior to use on the diluter system. Following the equilibration period, the stock solution was analytically confirmed to have a concentration of 3.2 µg a.i./mL.

2.8 Exposure System

2.8.1 Flutamide

Prior to test initiation, an FMI pump was calibrated to deliver 12.6 mL/minute of the 20 µg a.i./L stock solution into the chemical mixing chamber of the diluter. The mixing chamber of the diluter was positioned over a magnetic stir plate. The continuous stirring aided the homogenization of the test substance with the dilution water. The solution contained in the mixing chamber constituted the highest nominal test concentration (1000 µg a.i./L) and was subsequently diluted to provide the remaining nominal exposure concentrations (500 and 100 µg a.i./L).

2.8.2 Potassium Permanganate

Prior to test initiation, a Harvard syringe pump in conjunction with a 25-mL Hamilton gas-tight syringe was calibrated to deliver 0.175 mL/cycle of the 18 mg a.i./mL stock solution to the diluter's mixing chamber, which also received 3.5 L of dilution water per cycle. The mixing chamber of the diluter was positioned over a magnetic stir plate. The continuous stirring aided the homogenization of the test substance with the dilution water. The solution contained in the mixing chamber constituted the highest nominal test concentration (900 µg a.i./L) and was subsequently diluted to provide the remaining nominal exposure concentrations (450 and 225 µg a.i./L).

2.8.3 Ketoconazole

Prior to test initiation, an FMI pump was calibrated to deliver 328 mL/cycle of the 3.2 µg a.i./mL stock solution to the diluter's mixing chamber, which also received 2.625 L of dilution water per cycle. The mixing chamber of the diluter was positioned over a magnetic stir plate. The continuous stirring aided the homogenization of the test substance with the dilution water. The solution contained in the mixing chamber constituted the highest nominal test concentration (400 µg a.i./L) and was subsequently diluted to provide the remaining nominal exposure concentrations (100 and 25 µg a.i./L).

Calibration of each individual diluter system was confirmed prior to test initiation by measuring delivery volumes of the chemically-dosed solutions and the dilution water. The function of the diluter (e.g., dilution water flow rate, stock solution consumption) was monitored daily and a visual check was performed twice daily. In addition, analysis of exposure solutions for flutamide, potassium permanganate, and ketoconazole concentration was also used to verify proper operation of the diluter system. Analysis of exposure solutions for flutamide, potassium permanganate, and ketoconazole was performed on test days 0, 7, 14, and 21. For all tests, the exposure systems were operating properly for at least two days prior to study initiation to allow equilibration of the test substance in the diluter apparatus and exposure aquaria. Test aquaria were labeled to identify the nominal test substance concentration and designated replicate.

The exposure system and exposure aquaria were constructed of glass, silicone sealant, and nylon. Each 18-L test aquarium measured 40 x 20 x 20 cm with a 12.5-cm high side drain that maintained a constant exposure solution volume of approximately 10 L.

Flow-splitting chambers were used between the diluter cells and the four replicate test vessels to promote mixing of the test substance solution and diluent water, and to equally split the test solution between the test vessels. Delivery rates of the test substance to each of the test vessels were equal to one volume replacement every four hours.

2.9 Test Initiation

2.9.1 Selection and Weighing of Test Fish

The range in individual weights of fish at the start of the study was kept within ± 22% of the arithmetic mean weight. A subsample of fish was weighed before the test in order to determine the mean weight.

2.9.2 Pre-Exposure Phase

Four females and two males were randomly assigned to each pre-exposure vessel 14 days prior to test initiation. Four to eight additional exposure chambers were set up during the pre-exposure phase to account for a lack of spawning in some chambers and/or mortality during this phase. The pre-exposure phase was conducted under test conditions identical to those used during the chemical exposure. The animals were fed frozen brine shrimp (*Artemia nauplii*) twice daily during this phase. Each group was monitored daily for active spawning and semi-quantitative fecundity data was collected. During this phase, suitability for testing was established when regular spawning occurred in each test chamber every 3 to 4 days. The top 16 performing spawning groups were selected for the chemical exposures.

2.9.3 Chemical Exposure

Once successful spawning was established during the pre-exposure phase, the chemical exposures were initiated and maintained for a period of 21 days. Each replicate tank contained four female and two male fish. During the exposure period, the appearance of the fish, behavior, and fecundity were assessed daily. At test termination, fish were anesthetized by transfer to a buffered solution of MS-222, measured for total length, wet weighed, and blood samples were removed for vitellogenin (VTG) analysis. The gonads were also removed and weighed for gonadosomatic index (GSI) determination and histological analyses. Where applicable, dorsal fatpads were removed and weighed for fatpad index (FPI).

2.10 Test Monitoring

2.10.1 Water Quality Measurements

At test initiation and weekly thereafter, total hardness and total alkalinity were measured and recorded in alternating replicate vessels (A, B, C, and D) of the high treatment (1000 µg flutamide/L, 900 µg potassium permanganate/L, or 400 µg ketoconazole/L) and the dilution water control. Dissolved oxygen, pH, and temperature were recorded in each concentration and control vessel on a daily basis. Test solution temperature was continuously monitored during the exposure period in the upper water bath using a Fisher minimum-maximum thermometer and in the lower water bath using a VWR minimum-maximum thermometer. Dissolved oxygen concentrations and daily temperature were determined using a Yellow Springs Instrument (YSI) Model No. 550A dissolved oxygen meter/thermometer probe. The pH was measured using a Hanna Model HI9210N pH meter. Total hardness concentrations presented in this report were measured by the EDTA titrimetric method and total alkalinity concentrations were determined by potentiometric titration to an endpoint of pH 4.5 (APHA et al., 1995).

2.10.2 Analytical Methods

Samples from one replicate of each concentration and the control were analyzed prior to the initiation of the fish screening exposure. Results of the pretest analyses were used to judge whether correct quantities of test substances were being delivered and maintained in the exposure aquaria to initiate the fish screening study. During the fish screening study, water samples were removed from each replicate of each treatment level and the control at day 0 (test initiation), 7, 14, and 21 (test termination).

Water samples were removed from a point approximately midway between the surface, bottom, and sides of each test vessel and analyzed immediately. In addition, three aqueous quality control (QC) samples were prepared and analyzed with the each set of study samples. These QC samples were prepared in dilution water at concentrations of the test substances similar to the treatment levels tested. Results of the analyses of the QC samples were used to judge the precision and quality control maintained during the analysis of exposure solution samples.

2.10.2.1 Flutamide Method Verification

A method verification was previously conducted (18 August 2004) to quantify the amount of flutamide present in freshwater (laboratory well water at Springborn Smithers Laboratories). Recovery samples (control, 100 and 1000 µg a.i./L) were analyzed by high performance liquid chromatography with ultraviolet detection (HPLC/UV). The verification of the analytical method for flutamide averaged a recovery of 99.6% ± 1.015%. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70.0 to 120%. The following instrumental conditions were utilized during the flutamide method verification and the flutamide analysis during the study:

Instrument:	Hewlett-Packard isocratic pump Series 1100 equipped with a Hewlett-Packard Series 1100 autosampler, a Hewlett-Packard Series 1100 ultraviolet detector, and a Hewlett-Packard ChemStation Version A.06.03 for data acquisition
Column:	Alltima C-18, 4.6 m x 150 mm, 3-µm thickness
Mobile Phase:	65:35 acetonitrile:purified reagent water
Run Time:	8.0 minutes
Flow Rate:	1.00 mL/minute
Injection Volume:	30 µL
Wavelength:	306 nm
Retention Time:	approximately 6.6 minutes

2.10.2.2 Potassium Permanganate Analytical Methods Verification

A verification of the analytical method was conducted (29 July 2005) to quantify the amount of potassium permanganate present in freshwater (laboratory well water at Springborn Smithers Laboratories). Recovery samples (control, 0.200, 0.500, and 1.00 mg a.i./L) were analyzed by reading the absorbance on a UV-Vis spectrophotometer. The potassium permanganate analytical method verification recovery averaged 108% ± 4.88%. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70.0 to 120%. The following instrumental conditions were utilized during the potassium permanganate analytical method verification and the potassium permanganate analysis during the fish screening study:

Instrument:	Hewlett Packard Model 8453 Spectrophotometer, Series 61103A, scanning single–beam, microprocessor-controlled
Balance:	Mettler AB 204
Cuvette:	1 cm quartz UV
Wavelength Range:	400 to 600 nm
Integration Time:	0.5 seconds
Interval:	1 nm
Wavelengths Used:	525 nm, 546 nm

2.10.2.3 Ketoconazole Analytical Methods Verification

A verification of the analytical method was conducted (16 September 2005) to quantify the amount of ketoconazole present in freshwater (laboratory well water at Springborn Smithers Laboratories). Recovery samples (control, 0.0250, 0.100, and 0.400 mg a.i./L) were analyzed by high performance liquid chromatography with fluorescent detection (HPLC/FLD). The ketoconazole analytical method verification recovery averaged 114% ± 5.09%. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70.0 to 120%. The following instrumental conditions were utilized during the ketoconazole analytical method verification and the ketoconazole analysis during the fish screening study:

Instrument:	Hewlett-Packard quaternary solvent pump Series 1100 equipped with a Hewlett-Packard Series 1100 autosampler, a Hewlett-Packard Series 1100 fluorescent detector, vacuum degasser, and a Hewlett-Packard ChemStation Version A.06.03 for data acquisition
Column:	Phenomenex Synergi, 4.6 x 150 mm
Mobile Phase:	80:20 methanol:purified reagent water
Run Time:	15.00 minutes
Flow Rate:	1.0 mL/minute
Injection Volume:	500 µL
Fluorescent Detector:	Excitation: 245 nm Emission: 370 nm
Retention Time:	approximately 8.9 minutes

2.11 Test Termination

2.11.1 Gonad Size and Histology

At the conclusion of the exposure (day 21), all surviving fish were euthanized with an overdose of MS-222 (tricaine methanesulfonate) and separated by sex. Individual total lengths and wet weights were determined. Lengths were measured to the nearest 0.01 mm and wet weights to 0.10 mg. The values presented in Appendix 2 have been rounded. Blood (30 to 80 µL) was collected from the caudal artery/vein of each fish with a heparinized microhematocrit capillary tube. Plasma was separated from the blood via centrifugation (approximately 3 minutes at 15,000 *g*) and stored with protease inhibitors at -75 °C to -85 °C until analyzed for vitellogenin.

Gonads were fixed *in situ* using Davidson's fixative to prevent autolysis and cellular deterioration, then dissected from euthanized fish by making an incision from the vent forward through the pelvic girdle to the opercula. For each fish, the gonads were identified by size, shape, and color, and then separated from the other internal organs using stainless steel forceps and scissors. Gonads were transferred to tared vials with caps and weighed to the nearest 0.10 mg for the purpose of calculating gonadosomatic index (GSI). Gonads (right and left) were placed into pre-labeled plastic tissue cassettes which were then placed into an individual container of Davidson's fixative (volume of fixative equal to at least 10 times the approximated volume of the tissues). After remaining in Davidson's fixative overnight, the tissues were transferred to 10% neutral buffered formalin. The labeled histological cassettes were then shipped to Experimental Pathology Laboratories, Inc. (EPL), Sterling, Virginia for histopathological analysis.

2.11.2 Vitellogenin (VTG) Analysis

Plasma samples were analyzed for vitellogenin using an enzyme-linked immunosorbent assay (ELISA). The vitellogenin ELISA system kits were manufactured by EnBioTec Laboratories Co., Ltd. (Japan) and were distributed by Amersham Biosciences UK Limited. For the ELISA, polyclonal fathead minnow (*Pimephales promelas*) VTG antibody and purified VTG protein, also from the fathead minnow, were utilized.

2.12 Observations and Measurements

2.12.1 Endpoints

A number of endpoints were evaluated over the course of exposure. These endpoints included survival, behavior of adults, fecundity, fertilization success, appearance of adults (secondary sex characteristics), body weight, VTG concentration, gonad size, fatpad index, and gonad morphology.

2.12.2 Performance Criteria

Mean survival of ≥90% survival of the control fish was required over the duration of the exposure and the control fish in each replicate were required to successfully spawn. In general, typical spawning occurs every three to four days, producing approximately 15 eggs per female per day per test chamber. It is widely understood that fathead minnow fecundity is highly variable. Therefore, no specific performance criteria for this endpoint have been established.

2.12.3 Statistical Analyses

All statistical analyses were conducted at the 95% level of certainty except in the case of Shapiro-Wilks' and Bartlett's Tests, in which the 99% level of certainty was applied. The 99% level of certainty is preferred for qualifying tests. The following procedures were used:

1. Shapiro-Wilks Test for normality (Weber et al., 1989) was conducted and compared the observed sample distribution with a normal distribution. The assumption that observations are normally distributed must be validated before subsequent analyses, following parametric procedures, can be performed. If the data is not normally distributed, then a non-parametric procedure is used for subsequent analyses.

2. As a check on the assumption of homogeneity of variance, implicit in parametric statistics, data for each endpoint were analyzed using Bartlett's Test (Horning and Weber, 1985).

3. For each endpoint, the performance of organisms exposed to each treatment level of the test substance was compared with the performance of the control using Williams' Test (Williams, 1971, 1972) or Steel's Test (Steel, 1959). Nonparametric procedures, Wilcoxon's Rank Sum or Kruskal-Wallis' Test, were also used for comparison.

TOXSTAT® version 3.5 (West, Inc. and Gulley, 1996) was used to perform the statistical computations. Histopathology results were evaluated qualitatively.

3.0 RESULTS

3.1 Preliminary Range-Finding Test with Potassium Permanganate

Prior to test initiation, a 96-hour range-finding exposure was conducted exposing fathead minnow to nominal concentrations of 0.63, 1.3, 2.5, 5.0, and 10 mg a.i./L and a dilution water control under flow-through conditions. Two replicate aquaria, containing two males and four females each, were established for each concentration. Following 24 hours of exposure, 100% mortality was observed among fish exposed to the 10 mg a.i./L treatment level. Following 48 hours of exposure, 100% mortality was observed among fish exposed to the 5.0 mg a.i./L treatment level. At test termination (96 hours), 8% and 42% mortality was observed among fish exposed to the 1.3 and 2.5 mg a.i./L treatment levels, respectively. One surviving fish exposed to the 1.3 mg a.i./L treatment level was observed to be at the surface of the test solution which is usually indicative of a stressed organism. One surviving fish exposed to the 2.5 mg a.i./L treatment level was observed to be lethargic at test termination. No mortality or adverse effects were observed among fish exposed to the remaining treatment level tested (0.63 mg a.i./L) or the control. Based on these results and consultation with the Study Sponsor, nominal potassium permanganate concentrations of 225, 450, and 900 µg a.i./L were selected for the definitive study.

3.2 Evaluation of Test Conditions

A summary of the water quality parameters measured during the 21-day exposure is presented in Table 1. The dissolved oxygen concentration was > 90% of saturation at test initiation and was

maintained at ≥50% of saturation throughout the remainder of the test. Any treatment levels that fell to < 60% of saturation were only at that level for < 24 hours and were corrected by adding gentle, oil-free aeration to all test aquaria in the diluter system. The reduction of dissolved oxygen levels in each test occurred in controls and treatment levels and were generally consistent. The acceptable performance of the control organisms at termination of the tests demonstrated that the lower dissolved oxygen levels did not adversely impact the performance of the fish in these tests. Continuous temperature monitoring demonstrated that the temperature ranged from 24 to 26 °C throughout the exposure periods.

3.3 In-life Analytical Results

3.3.1 Flutamide

The diluter system which prepared and delivered the test solutions to the exposure aquaria functioned properly during the pretest period and throughout the study. Throughout the study period, all exposure solutions were observed to be clear and colorless. No undissolved test substance was observed in the diluter system. Analysis of the exposure solutions during the pre-exposure period established that the concentrations of flutamide in the exposure solutions were generally consistent and that the delivery apparatus maintained the expected concentration gradient of the test substance.

The results of the analysis of exposure solutions and QC samples for flutamide concentration during the fish screening test are presented in Table 2. The limit of quantitation (LOQ) for flutamide was 100 µg a.i./L. The limit of detection (LOD) for the analytical method during this study was 40 µg a.i./L. Analyses were performed weekly throughout the exposure. Measured flutamide concentrations in the exposure solutions were consistent between replicates of the same treatment level and consistent between sampling intervals. The mean measured concentrations ranged from 68 to 74% of the nominal levels. Based on mean measured concentrations of flutamide, the treatment levels tested (and measured) were defined as 74, 340, and 690 µg a.i./L. The lowest treatment level mean measured concentration (74 µg a.i./L) is an extrapolated value since the LOQ of the analytical method was 100 µg a.i./L.

Table 2 provides the results of the analysis of the aqueous QC samples, which were consistent with the predetermined recovery range (i.e., set at 70.0 to 120%) and ranged from 95.4% to 102% (n = 12) of the nominal fortified concentrations. Based on these results, it was determined that the appropriate quality control was maintained during the analyses of the flutamide exposure solution samples.

3.3.2 Potassium Permanganate

The diluter system which prepared and delivered the test solutions to the exposure aquaria functioned properly during the pretest period and throughout the study. Throughout the study period, all exposure solutions were observed to be light purple in color, with increasing color intensity as concentration increased. No undissolved test substance was observed in the diluter system. Analysis of the exposure solutions during the pre-exposure period established that the concentrations of potassium permanganate in the exposure solutions were consistent with our expectations for this compound and that the delivery apparatus maintained the expected concentration gradient of the test substance.

The results of the analysis of exposure solutions and QC samples for potassium permanganate concentration during the fish screening test are presented in Table 3. The LOQ for potassium permanganate was 200 µg a.i./L. The LOD for the analytical method during this study was 81 µg a.i./L. Analyses were performed weekly throughout the exposure. Measured potassium permanganate concentrations in the exposure solutions decreased over the exposure period. Mean measured concentrations on day 0 ranged from 84 to 98% of nominal concentrations compared to 39 to 63% of the nominal concentrations on day 21. The decrease in concentrations over time is most likely associated with potassium permanganate oxidizing, the large and increasing biomass in the exposure vessels following the introduction of the fish, fish food, and biological growth on the exposure system surfaces. The total mean measured concentrations ranged from 58 to 76% of the nominal levels. Based on mean measured concentrations of potassium permanganate, the treatment levels tested (and measured) were defined as 150, 260, and 680 µg a.i./L. The lowest treatment level mean measured concentration (150 µg a.i./L) is an extrapolated value since the LOQ of the analytical method was 200 µg a.i./L.

Table 3 provides the results of the analysis of the aqueous QC samples which were generally consistent with the predetermined recovery range (i.e., set at 70.0 to 120%) and ranged from 89.9% to 110% (n = 11) of the nominal fortified concentrations. Based on these results, it was determined that the appropriate quality control was maintained during the analyses of the potassium permanganate exposure solution samples. One of the QC samples resulted in a recovery outside the predetermined recovery range at 133%. QC samples can be out of range because of a number of factors, some of which are spiking errors, handling errors and instrument errors. Because at least two of the three QC samples were observed to be consistent with the predetermined recovery range at each sampling interval, the analytical results were deemed acceptable.

3.3.3 Ketoconazole

The diluter system which prepared and delivered the test solutions to the exposure aquaria functioned properly during the pretest period and throughout the study. Throughout the study period, all exposure solutions were observed to be clear and colorless. No undissolved test substance was observed in the diluter system. Analysis of the exposure solutions during the pre-exposure period established that the concentrations of ketoconazole in the exposure solutions were generally consistent and that the delivery apparatus maintained the expected concentration gradient of the test substance.

The results of the analysis of exposure solutions and QC samples for ketoconazole concentration during the fish screening test are presented in Table 4. The LOQ for ketoconazole was 25 µg a.i./L. The LOD for the analytical method during this study was 10 µg a.i./L. Analyses were performed weekly throughout the exposure. Measured ketoconazole concentrations in the exposure solutions were generally consistent between sampling intervals with somewhat lower recoveries on day 7. Mean measured concentrations which ranged from 68 to 79% of the nominal levels. Based on mean measured concentrations of ketoconazole, the treatment levels tested (and measured) were defined as 18, 68, and 320 µg a.i./L. The lowest treatment level mean measured concentration (18 µg a.i./L) is an extrapolated value since the LOQ of the analytical method was 25 µg a.i./L.

Table 4 provides the results of the analysis of the aqueous QC samples which were generally consistent with the predetermined recovery range (i.e., set at 70.0 to 120%) and ranged from 87.1% to 117% (n = 11) of the nominal fortified concentrations. Based on these results, it was determined that the appropriate quality control was maintained during the analyses of the ketoconazole exposure solution samples. One of the QC samples resulted in a recovery outside the predetermined recovery range at 130%. QC samples can be out of range because of a number of factors, some of which are spiking errors, handling errors and instrument errors. Because at least two of the three QC samples were observed to be consistent with the predetermined recovery range at each sampling interval, the analytical results were deemed acceptable.

3.4 Biological Observations

3.4.1 Survival

Percent survival following 21 days of exposure to flutamide, potassium permanganate, and ketoconazole is presented in Table 5. Following 21 days of exposure, percent survival in individual replicates among fish exposed to flutamide and ketoconazole ranged from 83 to 100%. Mean percent survival in individual replicates among fish exposed to potassium permanganate following 21 days of exposure ranged from 67 to 100%. Mean percent survival in all control groups exceeded 90%.

3.4.2 Spawning Evaluation

3.4.2.1 Fecundity

A summary of the spawning endpoints collected during this study is presented in Table 6. The mean number of eggs counted was 800, 782, 543, and 603 in the control, and flutamide concentrations of 100, 500, and 1000 µg a.i./L, respectively, as illustrated in Figure 1.

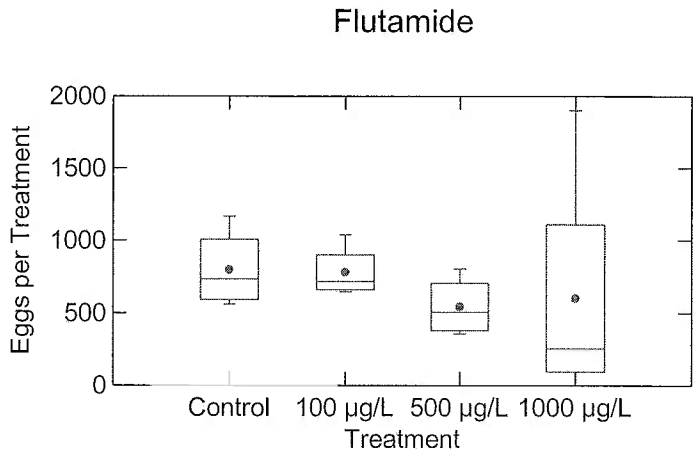

Figure 1. The mean number of eggs counted for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
----	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

The mean estimated total number of eggs produced was 625, 684, 496, and 406 in the control and flutamide concentrations of 100, 500, and 1000 µg a.i./L, respectively, as illustrated in Figure 2.

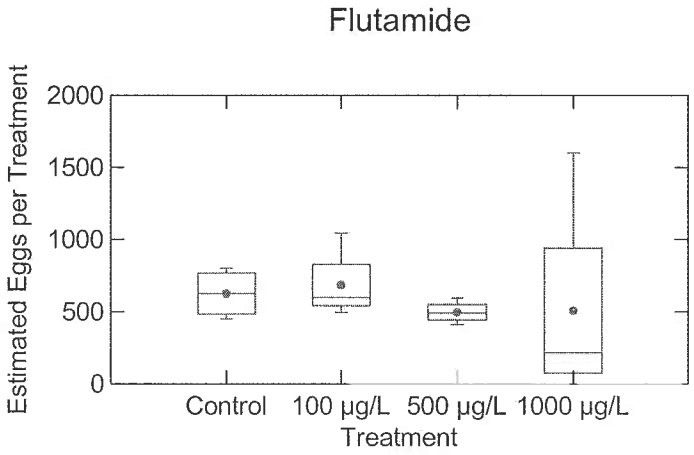

Figure 2. The mean number of eggs estimated for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

The mean number of eggs produced was 452, 291, 552, and 803 in the control, and potassium permanganate concentrations of 225, 450, and 900 µg a.i./L, respectively, as illustrated in Figure 3.

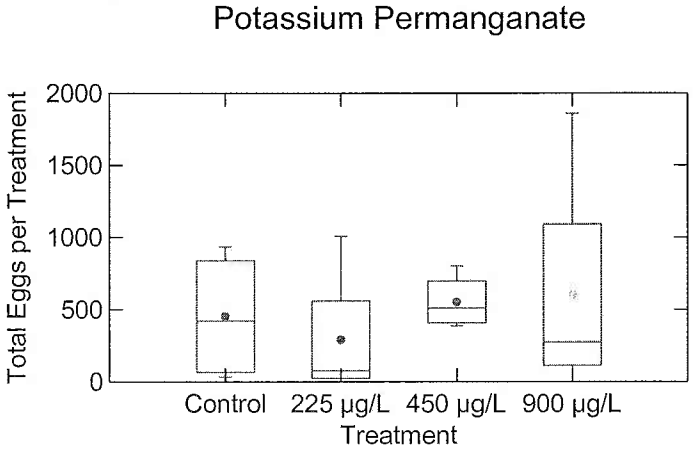

Figure 3. The mean number of eggs counted for fathead minnow exposed to potassium permanganate.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

The mean estimated total number of eggs produced was 455, 279, 601, and 409 in the control and potassium permanganate concentrations of 225, 450, and 900 µg a.i./L, respectively as illustrated in Figure 4.

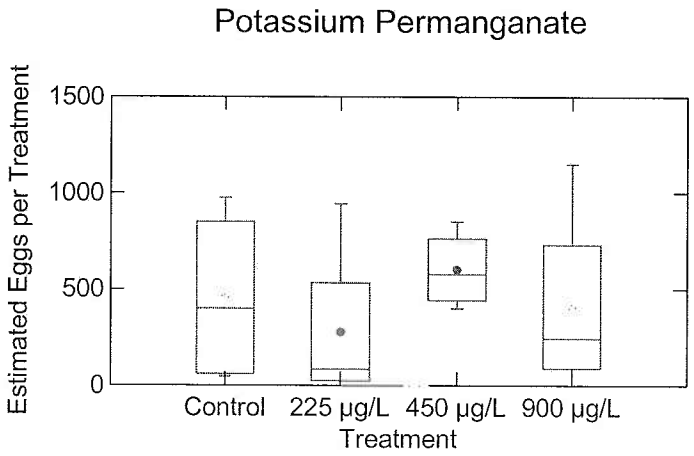

Figure 4. **The mean number of eggs estimated for fathead minnow exposed to potassium permanganate.**

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
—	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

The mean total number of eggs produced was 289, 192, 263, and 248 in the control, and ketoconazole concentrations of 25, 100, and 400 µg a.i./L, respectively, as illustrated in Figure 5.

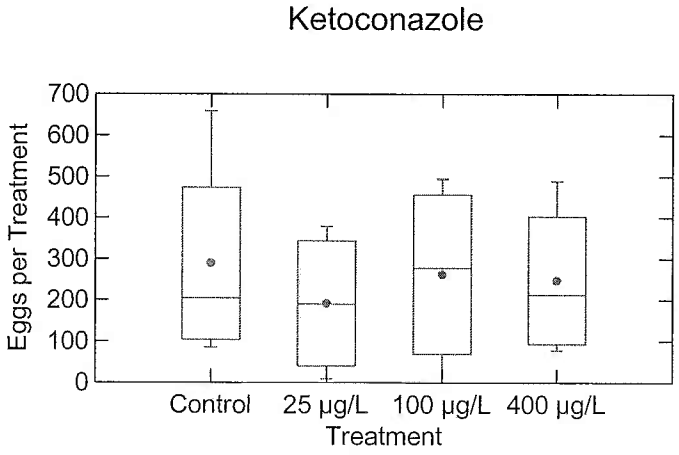

Figure 5. The mean number of eggs counted for fathead minnow exposed to ketoconazole.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

The mean estimated total number of eggs produced was 275, 193, 206, and 250 in the control and ketoconazole concentrations of 25, 100 and 400 µg a.i./L, respectively, as illustrated in Figure 6.

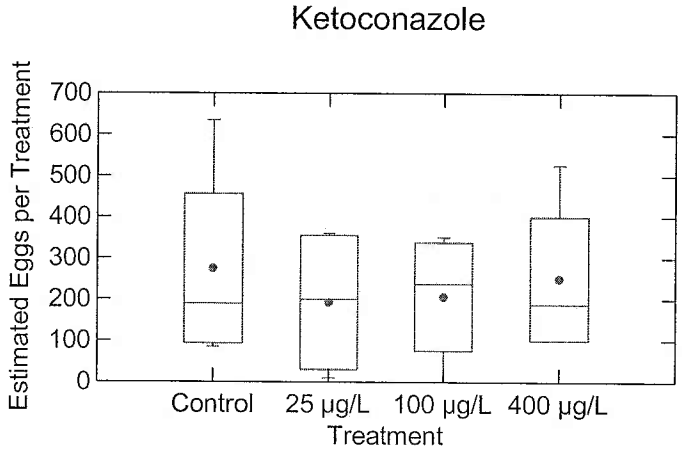

Figure 6. **The mean number of eggs estimated for fathead minnow exposed to ketoconazole.**

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•		mean
	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

In general, the estimated number of eggs was lower than the actual number of eggs but the overall trend was maintained. Seven out of the 12 estimates were within 10% of the actual number of eggs and eight of the 12 estimates were within 20% of the actual number of eggs.

There were no significant reductions in egg totals either through the actual numbers or the estimates. Figure 46 through Figure 48 show the cumulative number of eggs produced beginning in the pre-spawning exposure through the end of the exposure. The flutamide exposure suggests that there is a dose-related trend in the cumulative number of eggs produced. However, due to the natural variability in fecundity of the fathead minnow and possibly time, the difference was not significant. The potassium permanganate and ketoconazole exposures did not exhibit any dose-related trends.

3.4.2.2 Spawns

The mean number of spawns per control treatment ranged from 5 to 7.25 spawns. The mean number of spawns was not affected by any of the three exposures, as illustrated in Figure 7 through Figure 9.

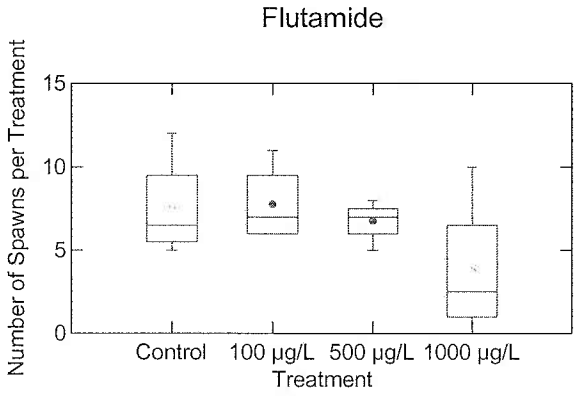

Figure 7. The number of spawns per treatment for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

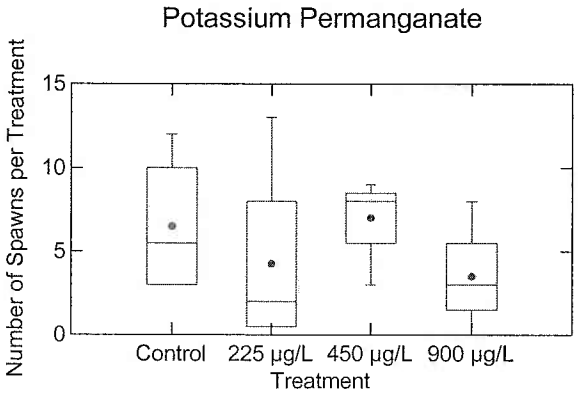

Figure 8. **The number of spawns per treatment for fathead minnow exposed to potassium permanganate.**

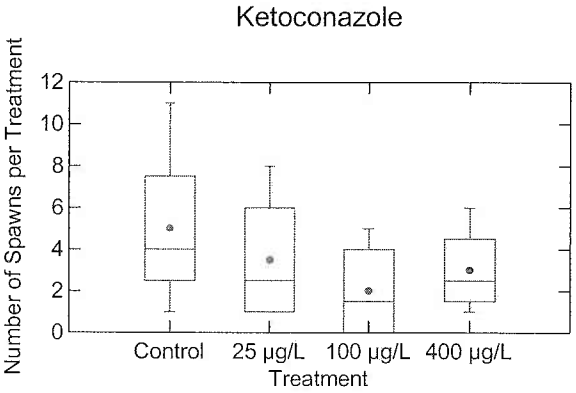

Figure 9. **The number of spawns per treatment for fathead minnow exposed to ketoconazole.**

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

3.4.2.3 Mean Number of Infertile Eggs

The mean number of infertile eggs per control treatment ranged from 1.5 to 6.75. The mean number of infertile eggs was not affected by any of the three exposures as illustrated in Figure 10 through Figure 12, below.

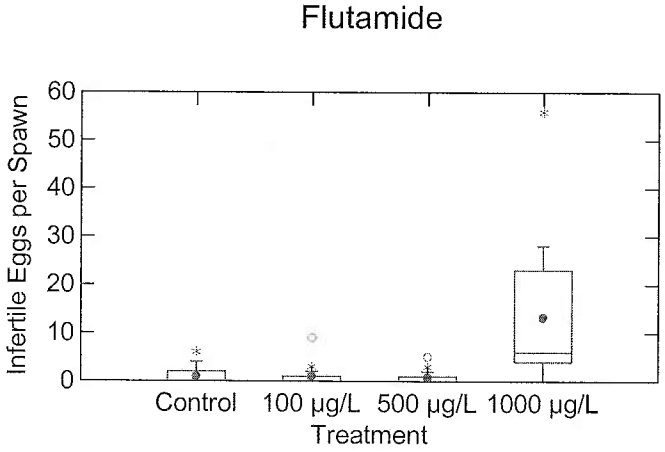

Figure 10. **The mean number of infertile eggs per spawn for fathead minnow exposed to flutamide.**

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
⊕	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

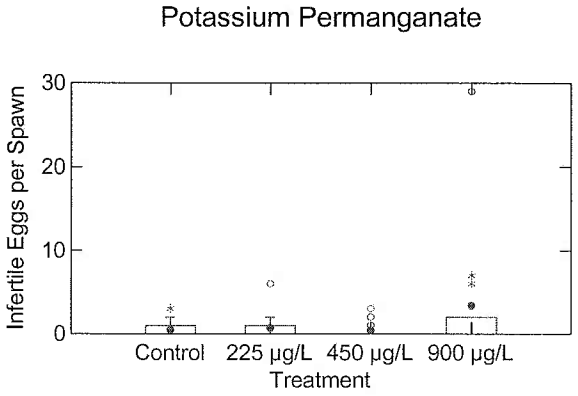

Figure 11. **The mean number of infertile eggs per spawn for fathead minnow exposed to potassium permanganate.**

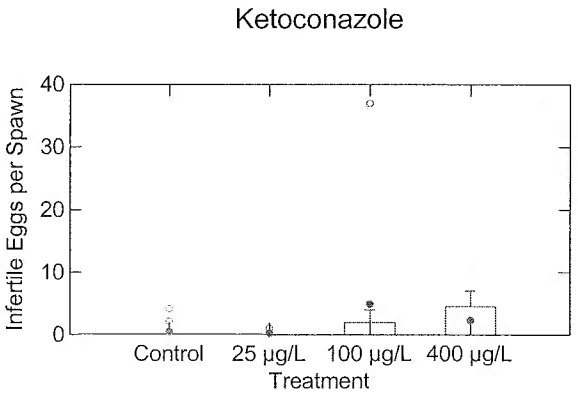

Figure 12. **The mean number of infertile eggs per spawn for fathead minnow exposed to ketoconazole.**

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
----	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

3.4.2.4 Percent Fertility

Mean percent fertility per control treatment ranged from 99 to 100%. Mean percent fertility in all treatments exceeded 90% and ranged between 91 and 100%. The mean percent fertility was not affected by either the potassium permanganate or the ketoconazole exposures. Mean percent fertility was significantly reduced in the highest treatment level of the flutamide exposure.

Mean percent fertilization was 99, 99, 99, and 91% in the control, and flutamide concentrations of 100, 500, and 1000 µg a.i./L, respectively, and is illustrated in Figure 13. A statistically significant difference was detected in the 1000 µg a.i./L treatment level compared to the control, based on Steel's Test.

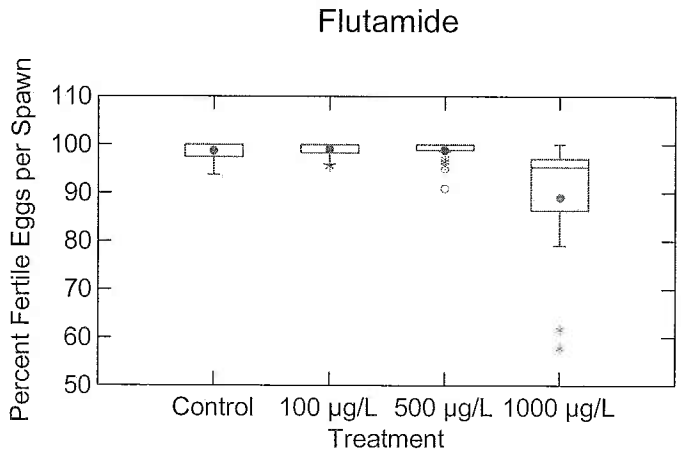

Figure 13. Percent fertile eggs per spawn for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

Mean percent fertilization was 99, 100, 100, and 99% in the control, and potassium permanganate concentrations of 225, 450, and 900 µg a.i./L, respectively, and is illustrated in Figure 14. No statistically significant differences were detected compared to the control, based on Williams' Test.

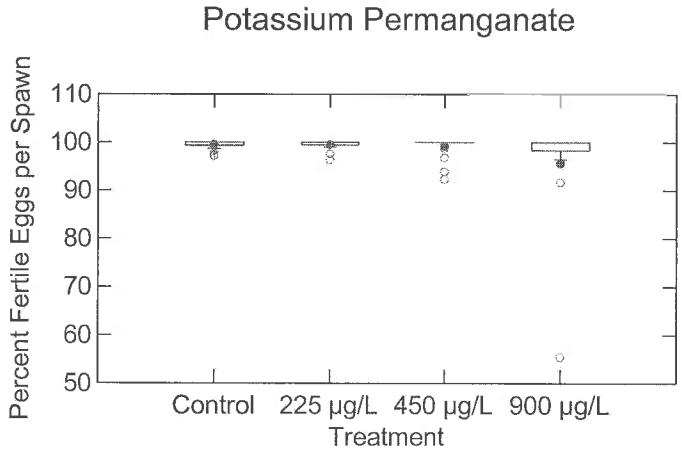

Figure 14. **Percent fertile eggs per spawn for fathead minnow exposed to potassium permanganate.**

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

Mean percent fertilization was 100, 97, 91, and 98% in the control, and ketoconazole concentrations of 25, 100, and 400 µg a.i./L, respectively, and is illustrated in Figure 15. No statistically significant differences were detected compared to the control, based on Wilcoxon's Rank Sum Test.

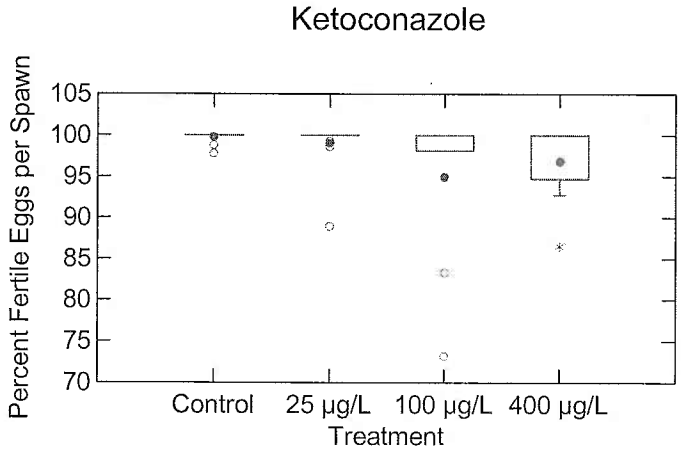

Figure 15. Percent fertile eggs per spawn for fathead minnow exposed to ketoconazole.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

3.4.3 Male Termination Endpoints

Raw data spreadsheets for male and female termination endpoints are presented in Appendix 2.

3.4.3.1 Fatpad Index

Fatpads were evaluated using the scoring system outlined in Appendix C-2 of Protocol WA 5-11 – Fish Screening Assay OECD Phase 1B Follow-Up (p. 141).

1. No fatpad visible
2. Small fatpad present
3. Fatpad is clearly visible and just above the body surface
4. Fatpad is prominent and clearly above the body surface but not overhanging
5. Fatpad is very prominent and overhanging the body surface

Since the fatpad score is a qualitative endpoint and thus subjective, only one person per test was responsible for scoring the fatpad development for all male fish at test termination. If an elevated pad was not present, the fish was scored a 1 and no attempt was made to remove the skin area in the head and dorsal pad regions of the fish. A fish would have to score at least a 2 with at least a small elevated pad present before a fatpad was dissected. Thus, if a fish was scored 1, a fatpad was not dissected and the weight of the fatpad and resulting fatpad index were recorded as 0.

The mean fatpad index per control treatment ranged from 0.38 to 0.52 (Table 7). The mean fatpad indices for fathead minnow exposed to flutamide, potassium permanganate, and ketoconazole are presented in the figures below.

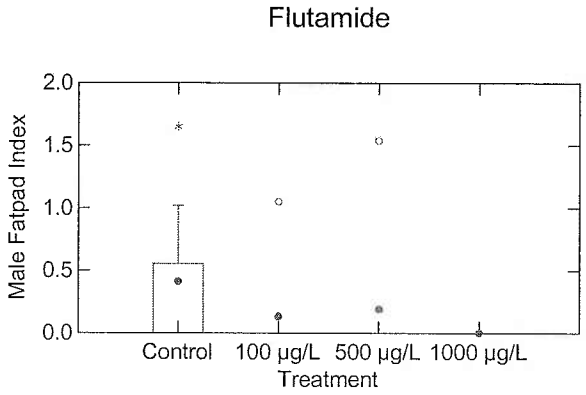

Figure 16. Male fatpad index for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

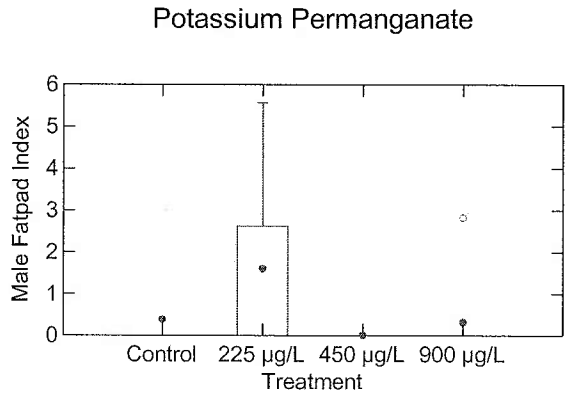

Figure 17. Male fatpad index for fathead minnow exposed to potassium permanganate.

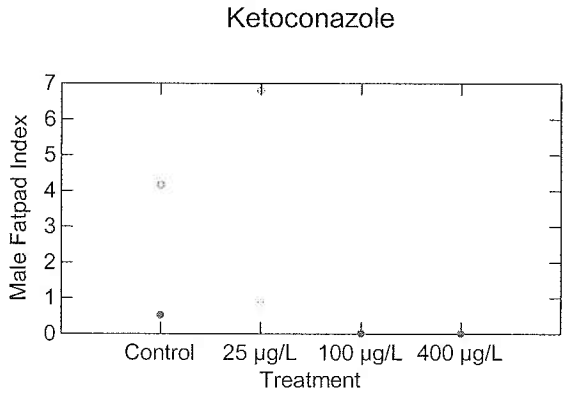

Figure 18. Male fatpad index for fathead minnow exposed to ketoconazole.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
*	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

There were more male fish with fatpad scores greater than 1 in the control and lowest treatment level in all three exposures compared to the number of male fish with fatpad scores greater than 1 in the two highest treatment level in all three exposures. The mean fatpad index was not affected by either the potassium permanganate or the ketoconazole exposures. However, the fatpad index from the highest treatment level in the flutamide exposure was significantly less than the control fatpad index based on Williams' Test.

3.4.3.2 Tubercle Score

The mean tubercle score per control treatment ranged from 25.1 to 32.4 (Table 7). Mean tubercle score was not affected by any of the three exposures as illustrated in the following figures.

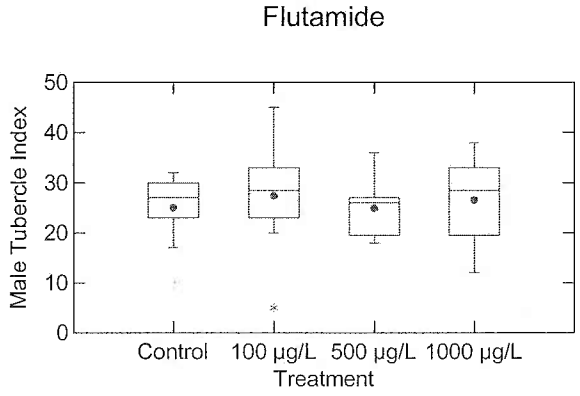

Figure 19. Male tubercle index for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

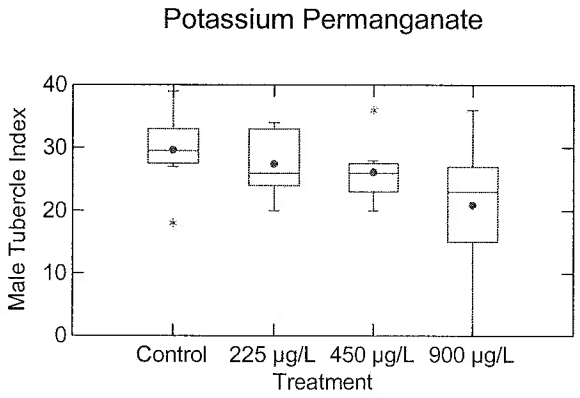

Figure 20. Male tubercle index for fathead minnow exposed to potassium permanganate.

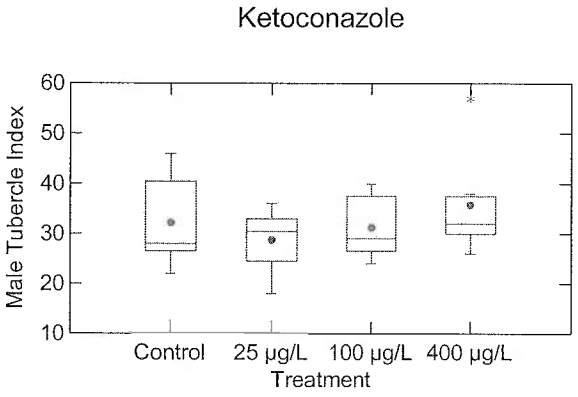

Figure 21. Male tubercle index for fathead minnow exposed to ketoconazole.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
--	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

3.4.3.3 Male GSI

The mean male GSI score per control treatment ranged from 1.01 to 1.29 (Table 7). Mean male GSI was not affected by any of the three exposures based on Williams' Test as illustrated in the following figures. For ketoconazole, there was a dose-related trend (through not statistically significant) of increased male GSI.

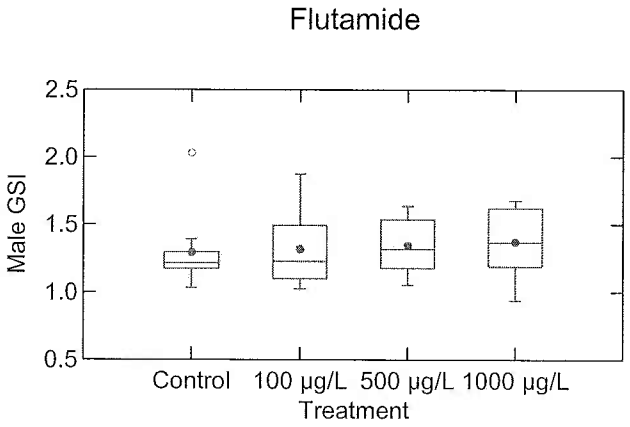

Figure 22. Male GSI for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

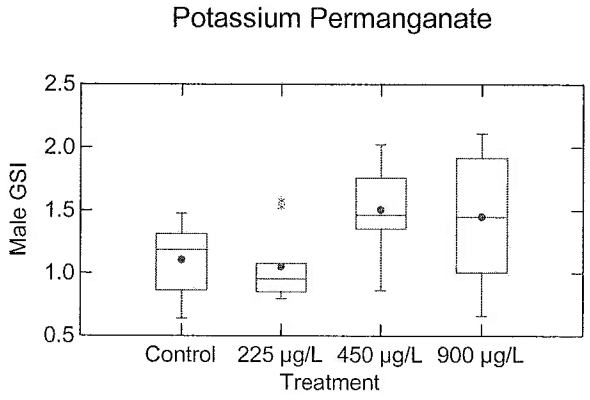

Figure 23. Male GSI for fathead minnow exposed to potassium permanganate.

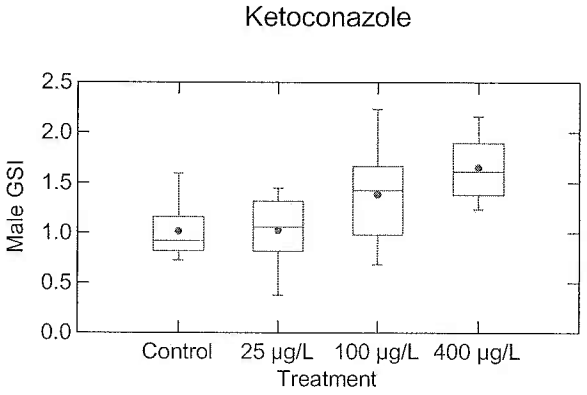

Figure 24. Male GSI for fathead minnow exposed to ketoconazole.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

3.4.3.4 Male Length

The mean male length per control treatment ranged from 61.4 to 66.4 mm (Table 7). Mean male length for fathead minnow exposed to flutamide, potassium permanganate and ketoconazole are presented in Figure 25, Figure 26, and Figure 27, respectively. Mean male length was not affected by any of the three exposures based on Williams' Test.

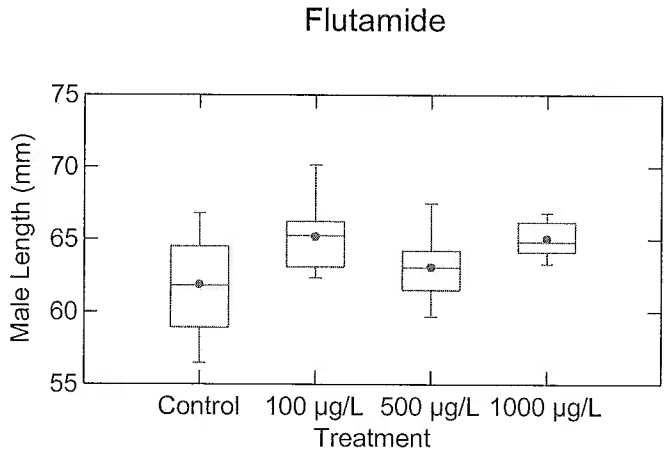

Figure 25. Male length for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

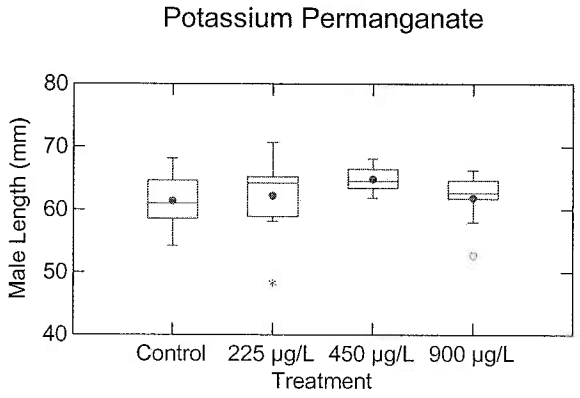

Figure 26. Male length for fathead minnow exposed to potassium permanganate.

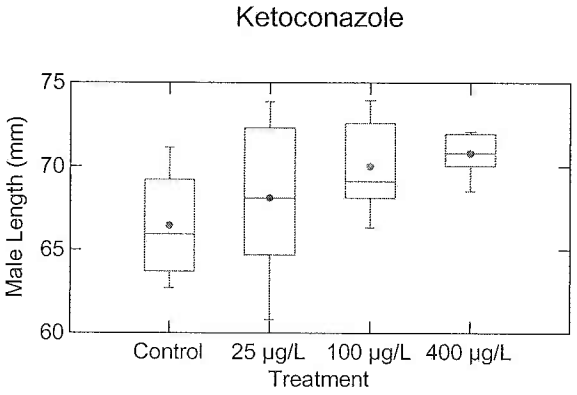

Figure 27. Male length for fathead minnow exposed to ketoconazole.

Figure Legend:

☐	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

3.4.3.5 Male Weight

The mean male weight per control treatment ranged from 2.99 to 3.49 g (Table 7). Mean male weight for fathead minnow exposed to flutamide, potassium permanganate, and ketoconazole are presented in Figure 28, Figure 29, and Figure 30, respectively. Mean male weight was not affected by any of the three exposures. The male length, weight, and GSI of the control fish demonstrated that these fish were of similar size and spawning condition.

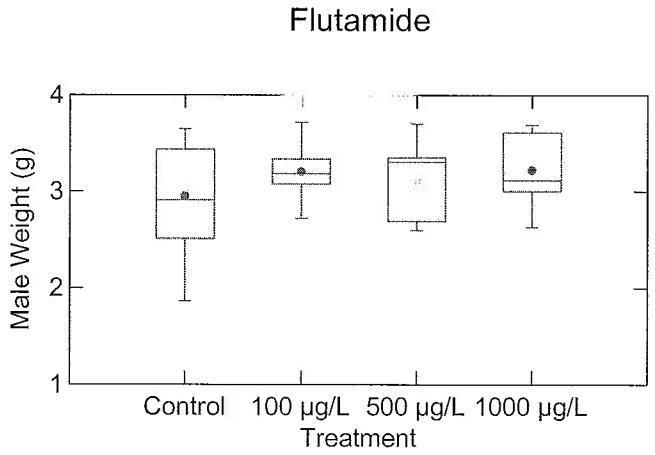

Figure 28. Male weight for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

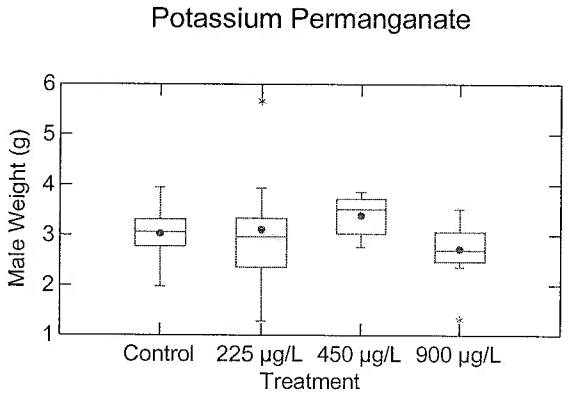

Figure 29. Male weight for fathead minnow exposed to potassium permanganate.

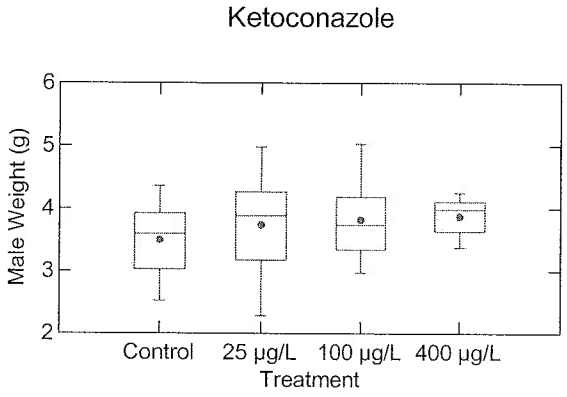

Figure 30. Male weight for fathead minnow exposed to ketoconazole.

Figure Legend:

	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

3.4.3.6 Male Vitellogenin

The mean male vitellogenin concentration per control treatment ranged from 0.0040 to 0.013 mg/mL (Table 9 through Table 11). One male fish from the ketoconazole exposure had a plasma vitellogenin concentration of 8.7 mg/mL. This measurement was considered an outlier, based on review of the data and dilutions used, and was excluded from further analysis. Male vitellogenin concentration for fathead minnow exposed to flutamide, potassium permanganate, and ketoconazole are presented in the figures below.

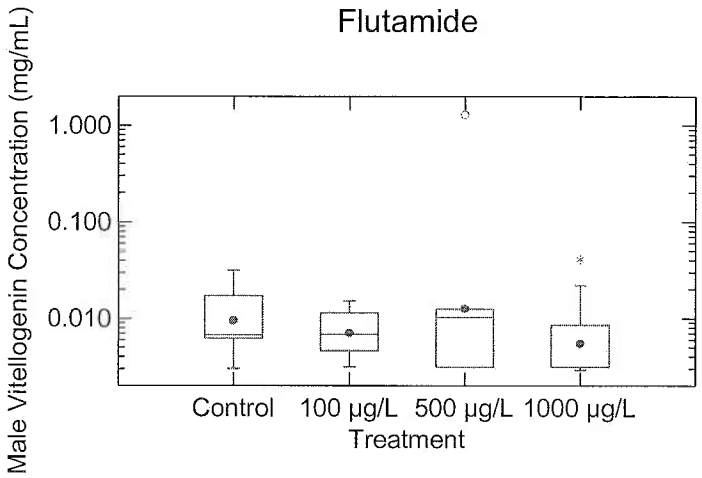

Figure 31. Male vitellogenin concentration for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

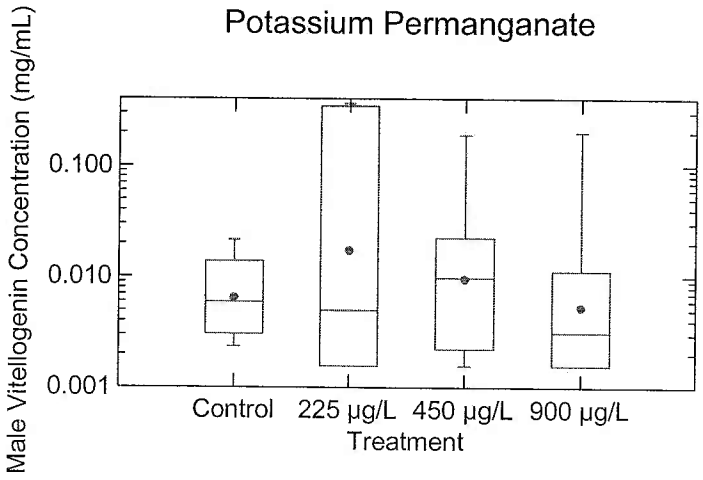

Figure 32. **Male vitellogenin concentration for fathead minnow exposed to potassium permanganate.**

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

Mean male vitellogenin concentration was not affected by either the potassium permanganate or the flutamide exposures. Vitellogenin samples from the A and B replicates from the control and 225 µg a.i./L potassium permanganate exposure were lost due to faulty ELISA kits. Based on the results from the other two replicates in each treatment level, this did not affect the interpretation of the results.

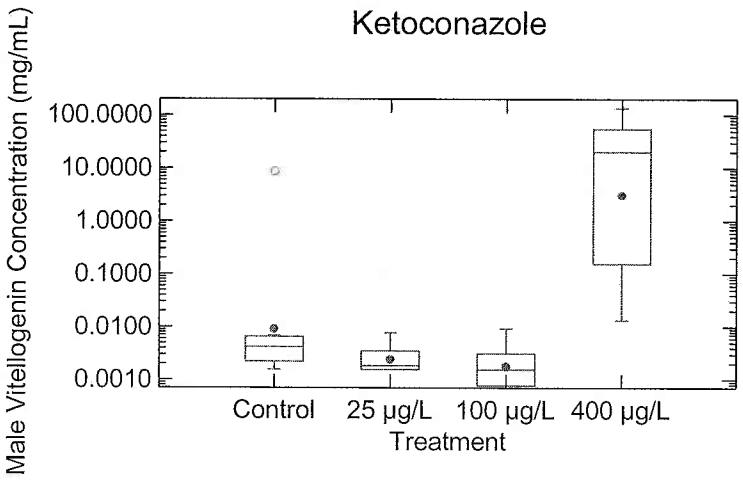

Figure 33. Male vitellogenin concentration for fathead minnow exposed to ketoconazole.

Figure Legend:

☐	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

Vitellogenin induction was observed in at least four of the seven male fish exposed to 400 µg a.i./L of ketoconazole, and the mean vitellogenin concentration for this treatment level was significantly greater than the control vitellogenin concentration.

3.4.4 Female Termination Endpoints

Secondary sex characteristics were visually evaluated for females at test termination. A urogenital opening was observed in all female fish. Therefore, empirically, no effects were apparent.

3.4.4.1 Female GSI

The mean female GSI score per control treatment ranged from 8.59 to 11.8 (Table 8). Mean female GSI was not affected by any of the three exposures as illustrated in the following figures.

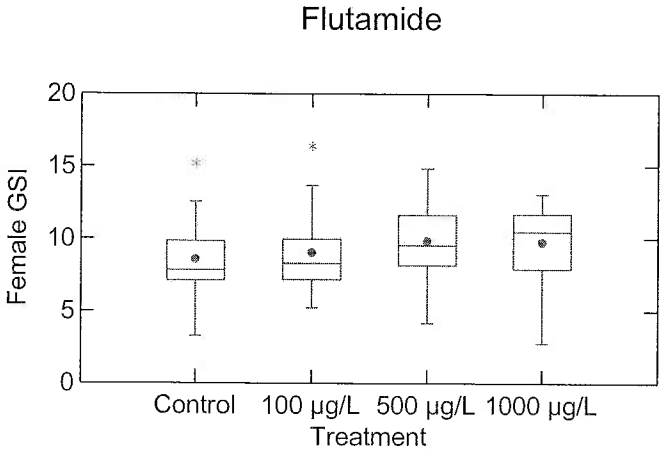

Figure 34. Female GSI for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
●	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

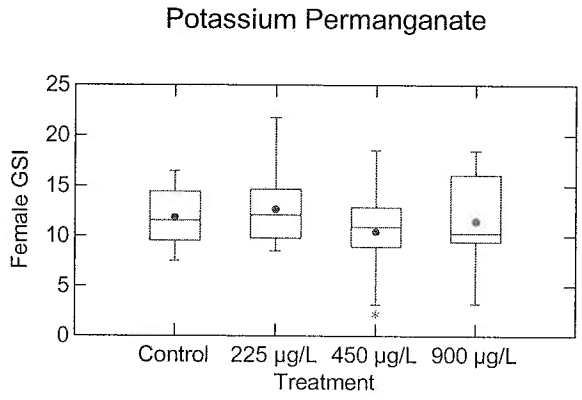

Figure 35. Female GSI for fathead minnow exposed to potassium permanganate.

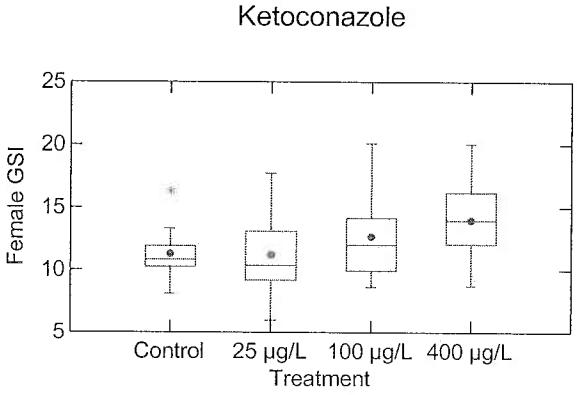

Figure 36. Female GSI for fathead minnow exposed to ketoconazole.

Figure Legend:

▭		50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

3.4.4.2 Female Length

The mean female length per control treatment ranged from 48.7 to 52.7 mm (Table 8). Mean female length was not affected by any of the three exposures. Mean female length for fathead minnow exposed to flutamide, potassium permanganate, and ketoconazole is presented in Figure 37, Figure 38, and Figure 39, respectively.

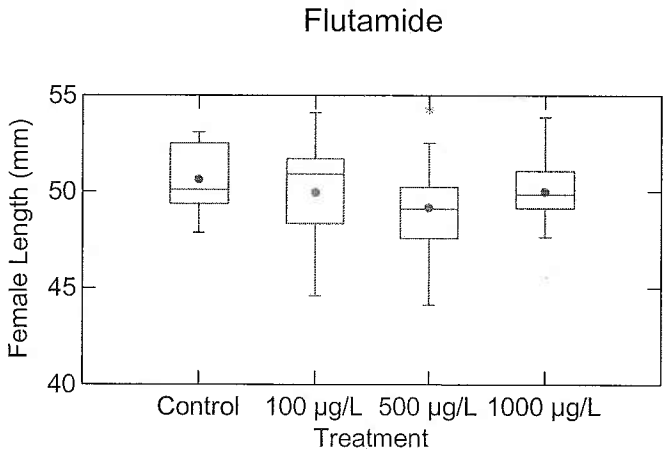

Figure 37. Female length for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

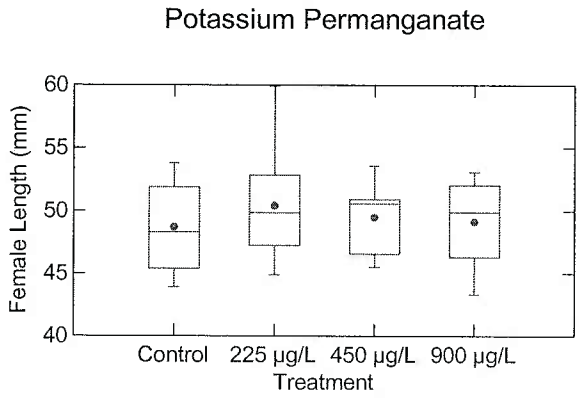

Figure 38. Female length for fathead minnow exposed to potassium permanganate.

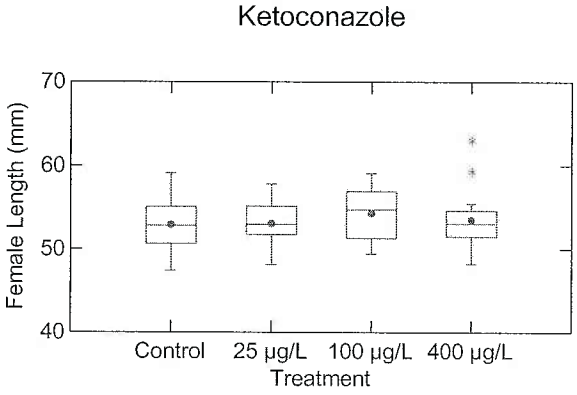

Figure 39. Female length for fathead minnow exposed to ketoconazole.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•		mean
----	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

3.4.4.3 Female Weight

The mean female weight per control treatment ranged from 1.17 to 1.45 g (Table 8). Mean female weight was not affected by any of the three exposures. Mean female weight for fathead minnow exposed to flutamide, potassium permanganate, and ketoconazole is presented in Figure 40, Figure 41, and Figure 42, respectively.

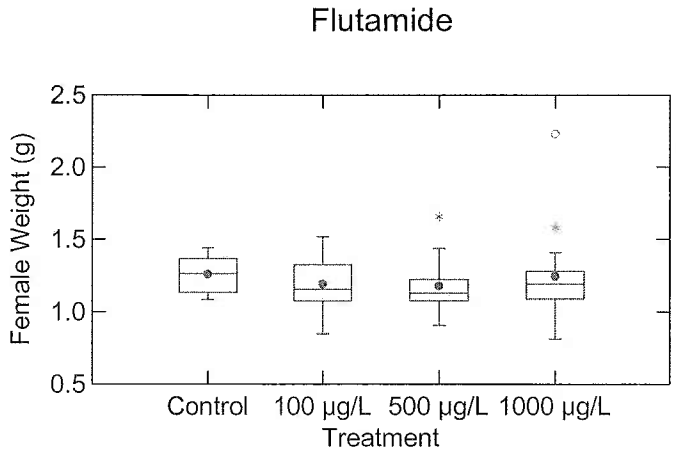

Figure 40. Female weight for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

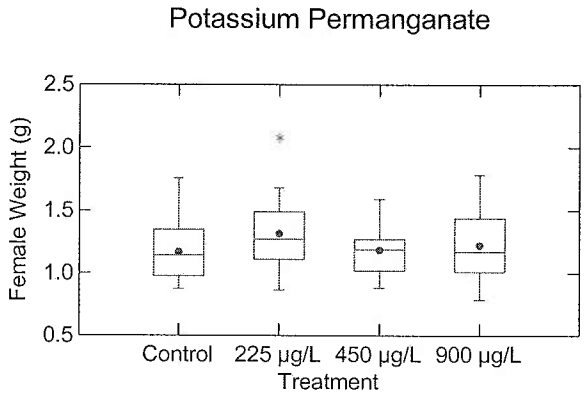

Figure 41. Female weight for fathead minnow exposed to potassium permanganate.

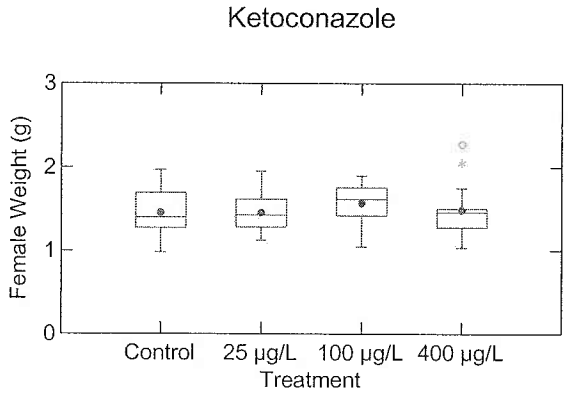

Figure 42. Female weight for fathead minnow exposed to ketoconazole.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

The female length, weight, and GSI of the control fish demonstrated that these fish were of similar size and spawning condition.

3.4.4.4 Female Vitellogenin

The mean female vitellogenin concentration per control treatment ranged from 15 to 53 mg/mL (Table 9 through Table 11). Mean female vitellogenin concentrations during the three exposures are presented in the following figures.

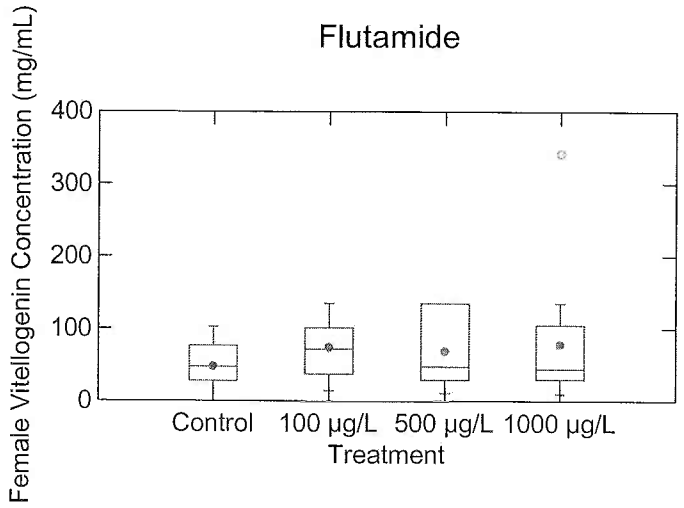

Figure 43. Female vitellogenin concentration for fathead minnow exposed to flutamide.

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

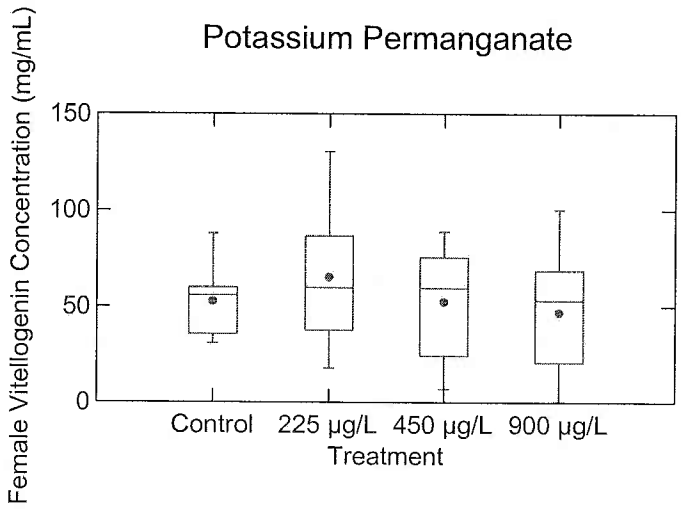

Figure 44. **Female vitellogenin concentration for fathead minnow exposed to potassium permanganate.**

Figure Legend:

▭	=	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

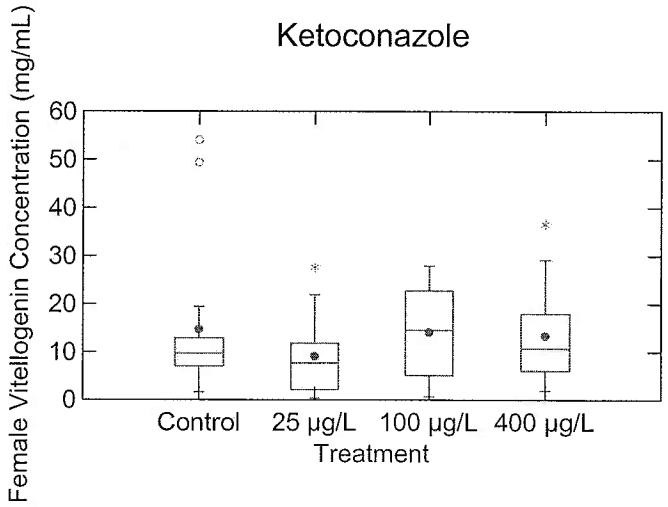

Figure 45. Female vitellogenin concentration for fathead minnow exposed to ketoconazole.

Figure Legend:

▭	–	50% of the values
I (bars)	=	1.5 times the upper of lower 50% of the values
•	=	mean
___	=	median
o	=	> 1.5 times, <3 times the upper or lower 50% of the values
*	=	> 3 times the upper or lower 50% of the values

Potassium permanganate, flutamide, and ketoconazole exposures did not affect female vitellogenin concentration. Vitellogenin samples from the A and B replicates from the control and 225 µg a.i./L potassium permanganate exposure were lost due to faulty ELISA kits. Based on the results from the other two replicates in each treatment level, this did not affect the interpretation of the results. Vitellogenin concentration in the female fish in the ketoconazole exposure (including control fish) were three to eight times lower than the potassium permanganate and flutamide exposures.

3.4.5 Histopathology

Histopathology reports for this study are presented in Appendix 3. There were no histopathological findings that were clearly attributable to flutamide exposure. The incidence of increased interstitial cells was slightly higher in the males from 1000 µg a.i./L group as compared to controls; however, this difference is unlikely to be statistically significant. Ovarian developmental stage average scores were generally higher in the two highest dose groups (500 and 1000 µg a.i./L) as compared to controls, but this difference is unlikely to be statistically significant.

There were no histopathological findings that were clearly attributable to potassium permanganate exposure. Potential effects include an increase in the proportion of spermatogonia in the testis in the 900 µg a.i./L group males and decreased ovarian stage scores in the 450 and 900 µg a.i./L group females.

The sole histopathological finding that was attributable to ketoconazole exposure was a the presence of increased interstitial (Leydig) cells in the testes of the 25, 100, and 400 µg a.i./L group males as compared to controls. This finding appeared to be somewhat dose-responsive in terms of incidence and severity, although the incidence did not reach 100% in any of the ketoconazole-exposed male groups.

4.0 CONCLUSIONS

The screening test was sensitive in identifying potential endocrine disrupting effects in both the flutamide and the ketoconazole exposures, where effects were expected. The screening test was also non-sensitive in identifying false positive effects in the potassium permanganate exposure, where endocrine-related effects were not expected.

In the potassium permanganate exposure, with the exception of fish survival, no significant adverse effects were observed on any of the endpoints evaluated in this screening test. Survival was significantly reduced at the highest treatment concentration.

Multiple endpoints were sensitive in detecting effects, suggesting that a successful screening study will need multiple endpoints to identify and corroborate endocrine-related effects. Percent fertile embryos, and fatpad index were significantly reduced in the highest treatment level in the flutamide exposure. Additionally, three other observations, though not significant, corroborate the effects seen in percent fertile embryos and fatpad index. The incidence of increased interstitial cells was slightly higher in the males from 1000 µg a.i./L group as compared to controls in the flutamide exposure. The flutamide exposure suggests that there is a dose-related trend in the cumulative number of eggs produced at the two highest treatment levels. Ovarian developmental stage average scores were generally higher in the two highest dose groups (500 and 1000 µg a.i./L) as compared to controls.

Two significant findings were attributable to ketoconazole exposure. There was an increased presence of interstitial (Leydig) cells in the testes of the 25, 100, and 400 µg a.i./L group males as compared to controls. This finding appeared to be somewhat dose-responsive in terms of incidence and severity. Vitellogenin production was induced in the male fish exposed to the 400 µg a.i./L treatment. There was also a dose-related trend (though not statistically significant) of increased male GSI.

A total of 16 endpoints were statistically evaluated in the three exposures. Table 12 compares the coefficient of variance (CV) for each control group and their average CV. Nine of the 16 endpoints have mean CV less than 20%. However, five of these endpoints (male and female length and weight and survival) may be not be sensitive to endocrine disruption. However, these endpoints are useful in demonstrating that the fish were of similar size, which is important when working to minimize the variability of spawning and fecundity endpoints.

The seven remaining endpoints have average CVs greater than 50%. The fecundity endpoints (number of eggs and number of spawns) are typically variable in fathead minnows. However, they can be sensitive endpoints. Buikema (1992) found that the reproductive endpoints in fish full life cycle studies were the most variable. Eggs per female and spawns per female were the most variable endpoints with CVs of 106% and 80%, respectively.

Blood plasma vitellogenin concentration is also rather variable, but the three- to four-order of magnitude differences between control males and females and between affected and non-affected males may make the high CVs less of a concern. The ELISA kits used for analysis of plasma vitellogenin presented were problematic. The initial lot of kits supplied by the manufacturer was defective, therefore, the kits did not develop properly and samples were lost. In addition, due to the small size of some female fish, the blood plasma volumes collected were low. Due to these low volumes archived samples were not available for reanalysis.

The number of infertile eggs is a highly variable endpoint, but integrating this observation into percent fertile embryos results in potentially the most sensitive apical endpoint of the screening assay. However, the biological relevance of this endpoint could be challenged. For example, in the flutamide exposure the percent of fertile embryos in the highest treatment level was 91%. A 91% reduction in fertilization is unlikely to have a biologically relevant impact on a fathead minnow population.

Male fatpad index may have been less variable if the skin region of the fatpads in male fish that scored a 1 had been dissected and weighed. Even with considerable variability, the male fatpad index was significantly reduced in the highest treatment level of the flutamide exposure. In all three chemical exposures, there were more male fish with fatpad scores greater than 1 in the control and lowest treatment level than in the two highest treatment levels.

The histopathological examination of the gonads, though qualitative, was important in identifying potential effects in the ketoconazole exposure. The histopathological examination of the gonads corroborated other apical and biochemical effects observed in the flutamide exposure.

Endpoint CV for Each Treatment

Endpoint	Flutamide (µg a.i./L)				Potassium Permanganate (µg a.i./L)				Ketoconazole (µg a.i./L)				Average
	Control	100	500	1000	Control	225	450	900	Control	25	100	400	
Percent Fertile Eggs	0.61%	0.51%	0.51%	8.13%	0.46%	0.63%	0.43%	1.02%	0.46%	5.49%	16.66%	1.81%	3.06%
Female Length	1.98%	4.19%	2.24%	1.22%	5.75%	5.99%	3.03%	6.26%	2.85%	0.83%	3.67%	4.69%	3.56%
Male Length	4.03%	2.91%	3.65%	1.69%	7.82%	7.01%	2.93%	4.29%	3.61%	5.73%	1.86%	1.17%	3.89%
Survival	8.88%	0.00%	8.88%	0.00%	0.00%	8.88%	0.00%	18.11%	0.00%	0.00%	8.88%	0.00%	4.47%
Female Weight	6.88%	7.82%	8.38%	11.29%	15.38%	19.53%	9.32%	22.03%	5.03%	2.90%	10.13%	13.61%	11.02%
Female GSI	11.64%	25.84%	9.87%	20.77%	13.56%	15.87%	13.46%	48.29%	10.53%	12.50%	17.60%	13.67%	17.80%
Male Weight	13.04%	5.30%	12.14%	7.76%	18.21%	27.19%	12.68%	18.03%	10.60%	16.58%	8.40%	3.61%	12.80%
Male GSI	19.38%	17.42%	16.42%	13.14%	4.95%	17.31%	12.00%	12.50%	20.79%	18.63%	11.59%	15.48%	14.97%
Tubercle score	7.57%	27.74%	16.87%	12.08%	19.26%	12.36%	16.86%	28.07%	25.62%	8.68%	7.99%	33.85%	18.08%
Plasma Vitellogenin - Female	49.58%	26.16%	70.31%	70.71%	35.49%	63.02%	55.99%	68.45%	84.18%	62.09%	32.28%	40.65%	54.91%
# of Spawns	42.76%	30.97%	19.26%	114.67%	67.69%	141.18%	38.57%	94.29%	84.00%	94.29%	97.78%	73.33%	74.90%
# of Eggs - Estimated	27.04%	35.96%	15.52%	181.53%	102.86%	160.22%	33.61%	124.69%	93.09%	97.41%	79.61%	80.40%	86.00%
# of Eggs - Actual	34.38%	22.12%	37.94%	145.44%	100.44%	164.95%	34.24%	141.20%	90.66%	93.75%	88.59%	77.82%	85.96%
Plasma Vitellogenin - Male	70.00%	16.66%	191.54%	88.88%	94.79%	193.88%	195.89%	246.81%	56.30%	69.89%	94.34%	97.50%	118.04%
# of Infertile Eggs	62.22%	74.67%	48.00%	153.94%	120.00%	172.97%	73.91%	145.86%	200.00%	66.67%	160.00%	124.80%	116.92%
Male Fatpad Index	80.95%	200.00%	205.26%	NA[a]	200.00%	47.85%	NA	303.23%	200.00%	200.00%	NA	NA	179.66%

[a] NA = Not applicable since no data to calculate CV.

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Table 1. Water quality measurements during the 21-day exposure of fathead minnow (*Pimephales promelas*) to flutamide, potassium permanganate and ketoconazole.

Nominal Concentration	Ranges		
	pH	Dissolved Oxygen[a] (mg/L)	Temperature (°C)
flutamide			
Control	7.0 – 7.8	4.7 – 8.5	24 – 26
100 µg a.i./L	7.0 – 7.8	4.4 – 8.5	24 – 26
500 µg a.i./L	7.0 – 7.8	4.5 – 8.5	24 – 26
1000 µg a.i./L	7.1 – 7.8	4.8 – 8.4	24 – 26
potassium permanganate			
Control	6.8 – 7.5	5.1 – 7.8	24 – 26
225 µg a.i./L	7.0 – 7.5	4.4 – 7.6	24 – 26
450 µg a.i./L	7.1 – 7.5	5.4 – 7.8	24 – 26
900 µg a.i./L	7.1 – 7.6	6.5 – 7.9	24 – 26
ketoconazole			
Control	7.0 – 7.8	5.3 – 7.9	24 – 26
25 µg a.i./L	7.1 – 7.8	5.0 – 7.8	25 – 26
100 µg a.i./L	7.1 – 7.8	5.3 – 7.8	25 – 26
400 µg a.i./L	7.1 – 7.8	4.1 – 7.6	25 – 26

[a] Percent saturation of 60% = 4.9 mg/L at 25 °C. Any treatment levels that fell to < 60% were only at that level for < 24 hours and were corrected by gentle, oil-free aeration.
4.7 mg/L at control flutamide = 56% saturation, occurred on test day 16
4.8 mg/L at 500 and 1000 µg a.i./L flutamide = 58% saturation, occurred on test day 16
4.4 mg/L at 100 µg a.i./L flutamide = 52% saturation, occurred on test day 16
4.5 mg/L at 500 µg a.i./L flutamide = 55% saturation, occurred on test day 16
4.4 mg/L at 225 µg a.i./L potassium permanganate = 53% saturation, occurred on test day 14
4.1 mg/L at 400 mg a.i./L ketoconazole = 50% saturation, occurred on test day 19
4.5 mg/L at 400 mg a.i./L ketoconazole = 55% saturation, occurred on test day 20

Table 2. Concentrations of flutamide measured in the exposure solutions during the fish screening assay with fathead minnow (*Pimephales promelas*).

Nominal Concentration (µg a.i./L)	Measured Concentration (µg a.i./L) Day 0	Day 7	Day 14	Day 21	Mean (SD)[a]	Mean Percent of Nominal (%)
Control	< 44 < 44 < 44 < 44	< 41 < 41 < 41 < 41	< 40 < 40 < 40 < 40	< 42 < 42 < 42 < 42	NA[b] (NA)	NA
100	74 71 79 79	70 66 77 76	69 66 73 74	76 74 82 80	74 (5.0)	74
500	350 350 340 340	340 340 330 340	320 330 320 320	350 360 350 350	340 (13)	68
1000	690 700 710 710	680 680 680 690	660 650 650 660	720 710 710 710	690 (22)	69
QC[c] #1 100	99.0 (99.0)[d]	96.3 (96.3)	95.4 (95.4)	99.7 (99.7)		
QC #2 500	503 (101)	509 (102)	504 (101)	505 (101)		
QC #3 1000	989 (98.9)	1020 (102)	981 (98.1)	996 (99.6)		

[a] Mean measured values are presented with the standard deviations in parentheses and were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table.
[b] NA = Not Applicable.
[c] QC = Quality Control sample.
[d] Percent recovery is presented in parentheses.

LOQ = 100 µg a.i./L
LOD = 40 µg a.i./L

Table 3. Concentrations of potassium permanganate measured in the exposure solutions during the fish screening assay with fathead minnow (*Pimephales promelas*).

Nominal Concentration (µg a.i./L)	Measured Concentration (µg a.i./L) Day 0	Day 7	Day 14	Day 21	Mean (SD)[a]	Mean Percent of Nominal (%)
Control	< 81 < 81 < 81 < 81	< 150 < 150 < 150 < 150	< 130 < 130 < 130 < 130	< 110 < 110 < 110 < 110	NA[b] (NA)	NA
225	190 170 200 190	< 150 < 150 160 < 150	< 130 < 130 140 < 130	130 < 110 < 110 < 110	150 (28)	76
450	410 430 410 400	270 320 180 330	160 180 180 240	160 220 130 190	260 (110)	58
900	910 850 900 880	760 690 710 690	560 520 610 550	570 540 550 630	680 (140)	76
QC[c] #1 200	206 (103)[d]	267 (133)[e]	220 (110)	180 (89.9)		
QC #2 500	481 (96.2)	475 (95.0)	525 (105)	523 (105)		
QC #3 1000	1030 (103)	978 (97.8)	1010 (101)	998 (99.8)		

[a] Mean measured values are presented with the standard deviations in parentheses and were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table.
[b] NA = Not Applicable.
[c] QC = Quality Control sample.
[d] Percent recovery is presented in parentheses.
[e] Result for this QC sample is outside of the acceptable range (i.e., 70.0 to 120%, Section 2.10.2.2).

LOQ = 200 µg a.i./L
LOD = 81 µg a.i./L

Table 4. Concentrations of ketoconazole measured in the exposure solutions during the fish screening assay with fathead minnow (*Pimephales promelas*).

Nominal Concentration (µg a.i./L)	Measured Concentration (µg a.i./L) Day 0	Day 7	Day 14	Day 21	Mean (SD)[a]	Mean Percent of Nominal (%)
Control	< 15 < 15 < 15 < 15	< 10 < 10 < 10 < 10	< 10 < 10 < 10 < 10	< 10 < 10 < 10 < 10	NA[b] (NA)	NA
25	19 18 20 19	11 14 13 16	17 13 14 23	22 21 24 23	18 (4.2)	72
100	62 70 67 66	34 53 54 53	68 79 69 76	78 96 87 81	68 (15)	68
400	300 310 300 290	270 250 260 250	390 360 370 380	380 320 290 320	320 (46)	79
QC[c] #1 25.0	25.5 (102)[d]	21.8 (87.1)	28.0 (112)	28.4 (114)		
QC #2 100	99.5 (99.5)	97.0 (97.0)	117 (117)	112 (112)		
QC #3 400	433 (108)	443 (111)	519 (130)[e]	422 (105)		

[a] Mean measured values are presented with the standard deviations in parentheses and were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table.
[b] NA = Not Applicable.
[c] QC = Quality Control sample.
[d] Percent recovery is presented in parentheses.
[e] Result for this QC sample is outside of the acceptable range (i.e., 70.0 to 120%, Section 2.10.2.3).

LOQ = 25 µg a.i./L
LOD = 10 µg a.i./L

Table 5. Survival at test termination of the 21-day exposure of fathead minnow (*Pimephales promelas*) to flutamide, potassium permanganate, and ketoconazole.

Nominal Concentration	Replicate	Cumulative Number Dead Fish (Day 21)	Percent Survival
flutamide			
Control	A	0	100
	B	0	100
	C	0	100
	D	1	83
100 µg a.i./L	A	0	100
	B	0	100
	C	0	100
	D	0	100
500 µg a.i./L	A	0	100
	B	0	100
	C	0	100
	D	1	83
1000 µg a.i./L	A	0	100
	B	0	100
	C	0	100
	D	0	100
potassium permanganate			
Control	A	0	100
	B	0	100
	C	0	100
	D	0	100
225 µg a.i./L	A	1	83
	B	0	100
	C	0	100
	D	0	100
450 µg a.i./L	A	0	100
	B	0	100
	C	0	100
	D	0	100
900 µg a.i./L[a]	A	2	67
	B	0	100
	C	1	83
	D	0	100
ketoconazole			
Control	A	0	100
	B	0	100
	C	0	100
	D	0	100
25 µg a.i./L	A	0	100
	B	0	100
	C	0	100
	D	0	100
100 µg a.i./L	A	0	100
	B	1	83
	C	0	100
	D	0	100
400 µg a.i./L	A	0	100
	B	0	100
	C	0	100
	D	0	100

[a] Significantly reduced compared to the control, based on Williams' Test.

Table 6. Summary of spawning endpoints collected during the 21-day exposure of fathead minnow (*Pimephales promelas*) to flutamide, potassium permanganate, and ketoconazole.

Nominal Concentration	Mean Number of Eggs Estimated	Std Dev	Mean Number of Eggs Counted	Std Dev	Mean Number of Spawns	Std Dev	Mean Number of Infertile Eggs	Std Dev	Mean Percent Fertile Eggs	Std Dev
flutamide										
Control	625	169	800	275	7.25	3.1	6.75	4.2	99.05	0.60
100 µg a.i./L	684	246	782	173	8.00	2.2	7.5	5.6	99.10	0.51
500 µg a.i./L	496	77	543	206	6.75	1.3	5.0	2.4	99.00	0.50
1000 µg a.i./L	406	737	603	877	3.75	4.3	49.5	76.2	91.25[a]	7.42
potassium permanganate										
Control	455	468	452	454	6.5	4.4	3	3.6	99.43	0.46
225 µg a.i./L	279	447	291	480	4.25	6.0	3.7	6.4	99.64	0.63
450 µg a.i./L	601	202	552	189	7	2.7	2.3	1.7	99.53	0.43
900 µg a.i./L	409	510	602	850	3.5	3.3	15.7	22.9	98.55	1.01
ketoconazole										
Control	275	256	289	262	5	4.2	1.5	3.0	99.77	0.46
25 µg a.i./L	193	188	192	180	3.5	3.3	0.75	0.5	96.80	5.31
100 µg a.i./L	206	164	263	233	2.25	2.2	11.0	17.6	90.59	15.09
400 µg a.i./L	250	201	248	193	3	2.2	6.25	7.8	98.47	1.78

[a] Significantly reduced compared to the control, based on Steel's Test.

Table 7. **Male termination endpoint summary during the 21-day exposure of fathead minnow (*Pimephales promelas*) to flutamide, potassium permanganate, and ketoconazole.**

Nominal Concentration	Mean FPI	Std Dev	Mean Tubercle Score	Std Dev	Mean Length (mm)	Std Dev	Mean Weight (g)	Std Dev	Mean GSI	Std Dev
flutamide										
Control	0.42	0.34	25.1	1.9	62.1	2.5	2.99	0.39	1.29	0.25
100 µg a.i./L	0.13	0.26	27.4	7.6	65.2	1.9	3.21	0.17	1.32	0.23
500 µg a.i./L	0.19	0.39	24.9	4.2	63.1	2.3	3.13	0.38	1.34	0.22
1000 µg a.i./L	0.00[a]	0.00	26.5	3.2	65.0	1.1	3.22	0.25	1.37	0.18
potassium permanganate										
Control	0.38	0.76	29.6	5.7	61.4	4.8	3.02	0.55	1.11	0.055
225 µg a.i./L	1.63	0.78	27.5	3.4	62.8	4.4	3.20	0.87	1.04	0.18
450 µg a.i./L	0.00	0.00	26.1	4.4	64.8	1.9	3.39	0.43	1.50	0.18
900 µg a.i./L	0.31	0.94	22.8	6.4	62.9	2.7	2.94	0.53	1.44	0.18
ketoconazole										
Control	0.52	1.04	32.4	8.3	66.4	2.4	3.49	0.37	1.01	0.21
25 µg a.i./L	0.85	1.70	28.8	2.5	68.1	3.9	3.74	0.62	1.02	0.19
100 µg a.i./L	0.00	0.00	31.3	2.5	70.0	1.3	3.81	0.32	1.38	0.16
400 µg a.i./L	0.00	0.00	38.4	13	70.9	0.83	3.88	0.14	1.68	0.26

[a] Significantly reduced compared to the control, based on Williams' Test.

Table 8. Female termination endpoint summary during the 21-day exposure of fathead minnow (*Pimephales promelas*) to flutamide, potassium permanganate, and ketoconazole.

Nominal Concentration	Mean Length (mm)	Std Dev	Mean Weight (g)	Std Dev	Mean GSI	Std Dev
flutamide						
Control	50.6	1.0	1.25	0.086	8.59	1.0
100 µg a.i./L	50.1	2.1	1.19	0.093	8.90	2.3
500 µg a.i./L	49.1	1.1	1.17	0.098	9.93	0.98
1000 µg a.i./L	50.0	0.61	1.24	0.14	9.15	1.9
potassium permanganate						
Control	48.7	2.8	1.17	0.18	11.8	1.6
225 µg a.i./L	50.1	3.0	1.28	0.25	12.6	2.0
450 µg a.i./L	49.5	1.5	1.18	0.11	10.4	1.4
900 µg a.i./L	49.5	3.1	1.18	0.26	9.94	4.8
ketoconazole						
Control	52.7	1.5	1.45	0.073	11.4	1.2
25 µg a.i./L	53.1	0.44	1.45	0.042	11.2	1.4
100 µg a.i./L	54.5	2.0	1.58	0.16	12.5	2.2
400 µg a.i./L	53.3	2.5	1.47	0.20	13.9	1.9

Table 9. Results of vitellogenin analysis during the 21-day exposure of fathead minnow (*Pimephales promelas*) to flutamide.

Nominal Concentration	Replicate	Mean Conc. (mg/mL)	SD	Treatment Mean (mg/mL)	SD	% CV
Males						
Control	A[a]	0.0063	NA[b]			
	B	0.0228	0.0079			
	C	0.0158	0.0137			
	D	0.0043	0.0019	0.0123	0.0086	70.00
100 µg a.i./L	A	0.0069	0.0009			
	B	0.0091	0.0041			
	C	0.0094	0.0083			
	D	0.0070	0.0054	0.0081	0.0013	16.66
500 µg a.i./L	A	0.6527	0.9054			
	B	0.0125	0.0000			
	C	0.0058	0.0038			
	D	0.0031	0.0000	0.1685	0.3228	191.54
1000 µg a.i./L	A	0.0126	0.0134			
	B	0.0221	0.0269			
	C	0.0030	0.0001			
	D	0.0032	0.0001	0.0102	0.0091	88.88
Females						
Control	A	45.41	37.94			
	B	51.66	23.48			
	C	32.87	21.49			
	D	60.98	51.05	47.73	23.66	49.58
100 µg a.i./L	A	87.64	35.36			
	B	90.05	52.63			
	C	61.88	46.45			
	D	51.58	33.37	72.79	19.04	26.16
500 µg a.i./L	A	108.99	43.22			
	B	95.45	45.94			
	C	28.41	8.47			
	D	22.18	15.90	63.76	44.83	70.31
1000 µg a.i./L	A	58.16	19.55			
	B	60.25	56.07			
	C	160.91	130.16			
	D	36.15	24.45	78.87	55.77	70.71

[a] No standard deviation could be calculated since this replicate contained only one fish.
[b] NA = Not Applicable.

Table 10. Results of vitellogenin analysis during the 21-day exposure of fathead minnow (*Pimephales promelas*) to potassium permanganate.

Nominal Concentration	Replicate	Mean Conc. (mg/mL)	SD	Treatment Mean (mg/mL)	SD	% CV
Males						
Control	A	--[a]	--			
	B	--[a]	--			
	C	0.0064	0.0034			
	D	0.0120	0.0136	0.0092	0.0087	94.79
225 µg a.i./L	A	--[a]	--			
	B	--[a]	--			
	C	0.1731	0.2426			
	D	0.0033	0.0024	0.0882	0.1710	193.88
450 µg a.i./L	A	0.0139	0.0152			
	B	0.0986	0.1294			
	C	0.0016	0.0000			
	D	0.0169	0.0050	0.0328	0.0642	195.89
900 µg a.i./L	A	0.0031	NA[b]			
	B	0.0031	0.0000			
	C	0.0563	0.0958			
	D	0.0016	0.0000	0.0264	0.0652	246.81
Females						
Control	A	--[a]	--			
	B	--[a]	--			
	C	65.33	15.38			
	D	40.03	12.34	52.68	18.70	35.49
225 µg a.i./L	A	--[a]	--			
	B	--[a]	--			
	C	72.98	32.83			
	D	59.49	50.61	65.27	41.13	63.02
450 µg a.i./L	A	46.98	39.10			
	B	47.30	31.17			
	C	67.62	12.92			
	D	51.73	33.30	52.46	29.37	55.99
900 µg a.i./L	A	66.42	5.28			
	B	51.75	41.08			
	C	0.28	NA[b]			
	D	30.87	14.36	46.83	32.06	68.45

[a] Sample lost due to faulty ELISA kit.

[b] NA = Not Applicable. Standard deviation could not be calculated since this replicate contained only one fish.

Table 11. Results of vitellogenin analysis during the 21-day exposure of fathead minnow (*Pimephales promelas*) to ketoconazole.

Nominal Concentration	Replicate	Mean Conc. (mg/mL)	SD	Treatment Mean (mg/mL)	SD	% CV
Males						
Control	A	0.0031	0.0000			
	B	0.0066	0.0005			
	C[a]	0.0016	6.1303			
	D	0.0036	0.0029	0.0037	0.0021	56.30
25 µg a.i./L	A	0.0023	0.0010			
	B	0.0016	0.0000			
	C	0.0019	0.0004			
	D	0.0059	0.0027	0.0029	0.0020	69.89
100 µg a.i./L	A	0.0008	0.0000			
	B	0.0008	0.0000			
	C	0.0031	0.0000			
	D	0.0062	0.0043	0.0027	0.0026	94.34
400 µg a.i./L	A	29.3375	40.3956			
	B	25.8834	36.5856			
	C	77.7443	80.9717			
	D	0.0315	NA[b]	33.2492[c]	32.4180	97.50
Females						
Control	A	31.31	23.75			
	B	5.04	3.94			
	C	7.40	4.98			
	D	12.79	6.03	14.13	11.90	84.18
25 µg a.i./L	A	9.82	3.31			
	B	9.91	8.35			
	C	13.68	11.59			
	D	1.06	0.67	8.62	5.35	62.09
100 µg a.i./L	A	8.65	8.52			
	B	19.87	5.48			
	C	15.82	11.50			
	D	13.61	7.71	14.48	4.68	32.28
400 µg a.i./L	A	7.83	5.34			
	B	8.78	5.59			
	C	16.17	10.90			
	D	18.09	14.34	12.71	5.17	40.65

[a] One male fish had a vitellogenin concentration of 8.7 mg/mL, which was excluded from the treatment mean as an outlier.

[b] Standard deviation is not applicable - data is from one replicate.

[c] Statistically different compared to controls using Kruskal-Wallis Test.

Table 12. Coefficient of variance (% CV) comparison for the 16 endpoints determined during the 21-day exposure of fathead minnow (*Pimephales promelas*) to flutamide, potassium permanganate, and ketoconazole.

Endpoint	Endpoint CV for Each Treatment												Average
	Flutamide (µg a.i./L)				Potassium Permanganate (µg a.i./L)				Ketoconazole (µg a.i./L)				
	Control	100	500	1000	Control	225	450	900	Control	25	100	400	
Percent Fertile Eggs	0.61%	0.51%	0.51%	8.13%	0.46%	0.63%	0.43%	1.02%	0.46%	5.49%	16.66%	1.81%	3.06%
Female Length	1.98%	4.19%	2.24%	1.22%	5.75%	5.99%	3.03%	6.26%	2.85%	0.83%	3.67%	4.69%	3.56%
Male Length	4.03%	2.91%	3.65%	1.69%	7.82%	7.01%	2.93%	4.29%	3.61%	5.73%	1.86%	1.17%	3.89%
Survival	8.88%	0.00%	8.88%	0.00%	0.00%	8.88%	0.00%	18.11%	0.00%	0.00%	8.88%	0.00%	4.47%
Female Weight	6.88%	7.82%	8.38%	11.29%	15.38%	19.53%	9.32%	22.03%	5.03%	2.90%	10.13%	13.61%	11.02%
Female GSI	11.64%	25.84%	9.87%	20.77%	13.56%	15.87%	13.46%	48.29%	10.53%	12.50%	17.60%	13.67%	17.80%
Male Weight	13.04%	5.30%	12.14%	7.76%	18.21%	27.19%	12.68%	18.03%	10.60%	16.58%	8.40%	3.61%	12.80%
Male GSI	19.38%	17.42%	16.42%	13.14%	4.95%	17.31%	12.00%	12.50%	20.79%	18.63%	11.59%	15.48%	14.97%
Tubercle score	7.57%	27.74%	16.87%	12.08%	19.26%	12.36%	16.86%	28.07%	25.62%	8.68%	7.99%	33.85%	18.08%
Plasma Vitellogenin - Female	49.58%	26.16%	70.31%	70.71%	35.49%	63.02%	55.99%	68.45%	84.18%	62.09%	32.28%	40.65%	54.91%
# of Spawns	42.76%	30.97%	19.26%	114.67%	67.69%	141.18%	38.57%	94.29%	84.00%	94.29%	97.78%	73.33%	74.90%
# of Eggs - Estimated	27.04%	35.96%	15.52%	181.53%	102.86%	160.22%	33.61%	124.69%	93.09%	97.41%	79.61%	80.40%	86.00%
# of Eggs - Actual	34.38%	22.12%	37.94%	145.44%	100.44%	164.95%	34.24%	141.20%	90.66%	93.75%	88.59%	77.82%	85.96%
Plasma Vitellogenin - Male	70.00%	16.66%	191.54%	88.88%	94.79%	193.88%	195.89%	246.81%	56.30%	69.89%	94.34%	97.50%	118.04%
# of Infertile Eggs	62.22%	74.67%	48.00%	153.94%	120.00%	172.97%	73.91%	145.86%	200.00%	66.67%	160.00%	124.80%	116.92%
Male Fatpad Index	80.95%	200.00%	205.26%	NA[a]	200.00%	47.85%	NA	303.23%	200.00%	200.00%	NA	NA	179.66%

[a] NA = Not applicable since no data to calculate CV.

Figure 46. Cumulative number of eggs per day during the pre-exposure period and 21-day exposure of fathead minnow (*Pimephales promelas*) to flutamide.

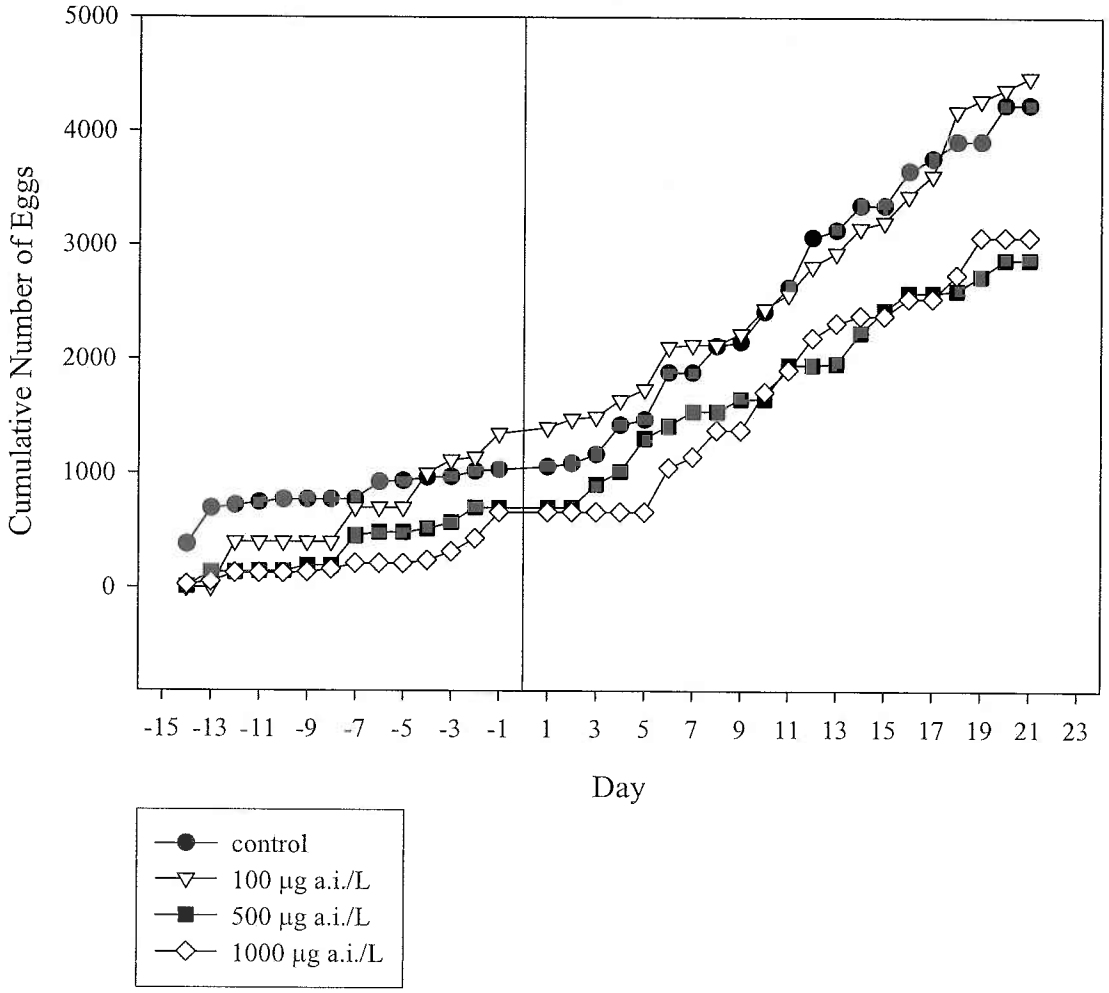


Figure 47. Cumulative number of eggs per day during the pre-exposure period and 21-day exposure of fathead minnow (*Pimephales promelas*) to potassium permanganate.

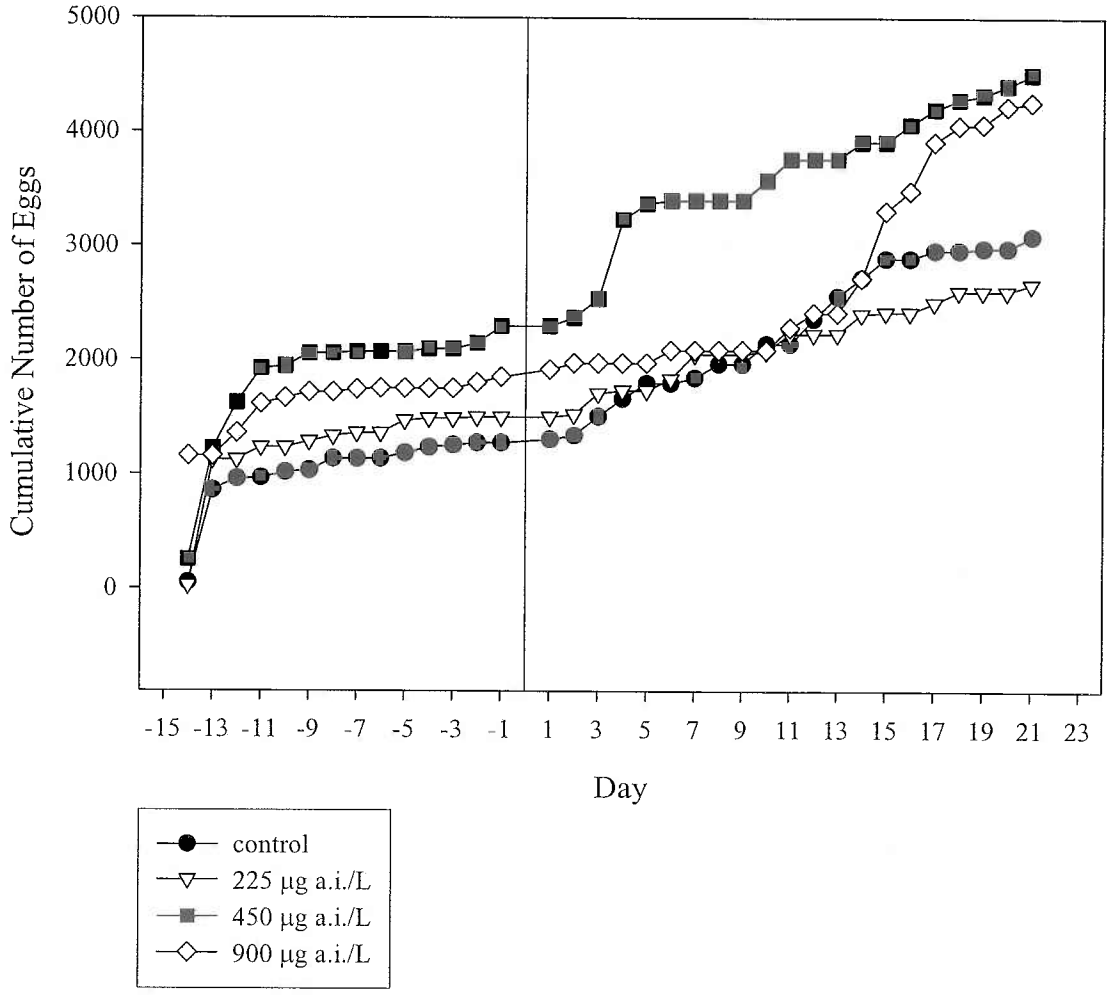
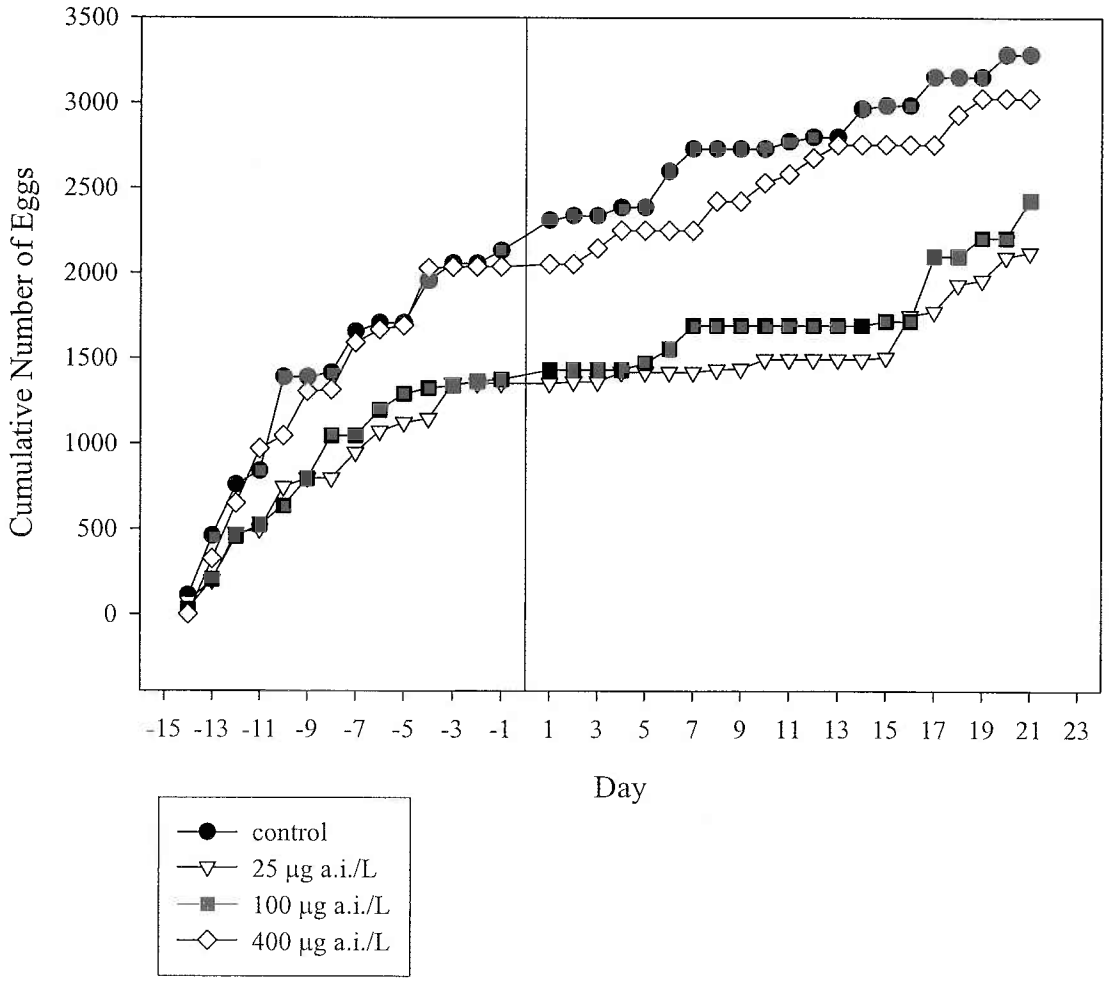


Figure 48. Cumulative number of eggs per day during the pre-exposure period and 21-day exposure of fathead minnow (*Pimephales promelas*) to ketoconazole.



APPENDIX 1 - QAPP

1.0 TITLE AND APPROVAL

QUALITY ASSURANCE PROJECT PLAN

FOR WORK ASSIGNMENT 5-11
FISH SCREENING ASSAY OECD PHASE 1B FOLLOW-UP

for

EPA CONTRACT NUMBER 68-W-01-023
Project Number: Battelle Pacific Northwest Division Project 43495
Work Assignment Leader: Michael Blanton
Institution: Battelle's Pacific Northwest Division

SIGNATURE PAGE
Quality Assurance Project Plan for WA 5-11

Fish Screening Assay OECD Phase 1B Follow-Up
EPA Contract Number 68-W-01-023
Concurrences and Approvals

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2.0 TABLE OF CONTENTS

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4.0 PROJECT/TASK ORGANIZATION

4.1 PROJECT MANAGEMENT

The U.S. Environmental Protection Agency is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is a short-term screening assay designed to detect substances that interact with the estrogen and androgen systems of fish. It is thought that the inclusion of the fish screening assay in Tier 1 is important because estrogenic and androgenic controls on reproduction and development in fish may differ significantly enough from those of higher vertebrates such that mammalian screening methods may not identify potential EDCs in this important class of animals. As an example, dihydrotestosterone is a potent androgen in mammals, but 11-ketotestosterone is generally the more prevalent androgen in fish.

U.S. EPA (2001) has described a short-term test with the fathead minnow *(Pimephales promelas)* 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 (Ankley et al. 2001) is initiated with mature male and female fish. During a 21-day chemical exposure, survival, behavior, and secondary sexual characteristics are observed, and fecundity is 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 and sex steroids (17β-estradiol, testosterone, and 11-ketotestosterone)..

The Organization for Economic Cooperation and Development (OECD) initiated a fish screening assay validation activity and has completed its Phase 1A and Phase 1B trials. Phase 1A evaluated a non-spawning version of a 21-day exposure assay with fathead minnow, medaka, and zebrafish. The results of the Phase 1A led to the Phase 1B trials, where spawning was included in the method. The results of the Phase 1B trial raised questions regarding the spawning conditions utilized for the fathead minnow.

Previous work assignments under this contract (WA 2-18, and WA 2-29) were initiated to evaluate a short-term reproduction assay with fathead minnow and compare the EPA (2001) method to two other related assays to contribute to the optimization of the assay for use as a screen in the EDSP. The laboratories that participated in the OECD Phase 1B work were ABC Laboratories, Springborn Smithers Laboratories, and Wildlife International, Ltd. Springborn Smithers performed the Phase 1B study with flutamide and 4-tert-pentylphenol (4-PP) using fathead minnows, ABC used 4-PP and prochloraz with fathead minnows, and Wildlife International, Ltd. used 4-PP and flutamide with medaka. Experimental Pathology Laboratories (EPL) performed the histopathology work under Phase 1B for the OECD effort.

4.2 GOALS

The purpose of work to be conducted under this work assignment is to perform a follow-on study to the OECD Phase 1B study based on features in the short-term reproduction assay with the fathead minnow as described by the U.S. EPA (2001). Specifically, this study will incorporate an increased number of replicates and uses a semi-quantitative and quantitative egg-counting method. The studies will include two different laboratories (Springborn Smithers and Wildlife International Ltd.), each running flutamide and potassium permanganate. Ketoconazole and trenbolone will be run by Battelle.

The purpose of WA 5-11 is to demonstrate the Fish Screening assay test method, based on the proposed test guidelines.

The assays will be initiated with mature ("first time spawners") spawning adults. Active spawning for all replicate groups will be established during a "pre-exposure" period of 14 days. The pre-exposure observation period will be used to monitor reproductive performance (semi-quantitative only) as described for the chemical exposure period.

The pre-exposure observations will occur in the same system/tanks as will be utilized for the chemical test. An overview of the tests and relevant test substance concentrations are provided in Table 1. The assays will be initiated with mature male and female fish.

Table 1. Test Chemicals and Exposure Concentrations

Test Chemical	Testing Lab	Exposure Concentration (µg/L)		
		Low	Med	High
Flutamide	Springborn Smithers Wildlife International Ltd.	100	500	1000
Potassium permanganate	Springborn Smithers* Wildlife International Ltd	TBD	TBD	TBD
Trenbolone	Battelle	0.1	0.5	1
Ketoconazole	Battelle	25	100	400

*Laboratory conducting concentration range-finding study for this compound
TBD = to be determined.

During a 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics will be observed, while fecundity and fertilization success will be monitored daily. At termination of the assay, measurements will be made of a number of endpoints reflective of the status of the reproductive endocrine system, including the GSI, gonadal histology, and plasma concentrations of vitellogenin.

There are four administrative and two research-related tasks required under WA 5-11. None of the tasks will be performed under Good Laboratory Practice (GLP) requirements (40 CFR 160). The tasks specified in the work assignment are shown in Table 2. This QAPP was prepared in Task 4, and it covers work in Tasks 5 and 6.

Table 2. Tasks as Identified in WA 5-11 and this QAPP

WA 5-11 Task No.	GLP	Task Description
1	No	Prepare a Work Plan
2	No	Consultation with Fish Experts
3	No	Prepare a Study Plan/Protocol
4	No	Prepare a Quality Assurance Project Plan
5	No	Conduct Studies with flutamide, potassium permanganate, ketoconazole and trenbolone
6	No	Data Analysis and Final Report

4.3 ORGANIZATION

A summary of the work assignment organization is shown in Figure 1. The overall work assignment will be managed by **Mr. Michael Blanton**, the Work Assignment Leader (WAL). Mr. Blanton is responsible for preparing the technical work assignment, assigning appropriate staff to complete specified tasks within this work assignment, and monitoring the progress of both technical and fiscal milestones, as outlined in the technical work plan. Mr. Blanton will report progress on the work assignment to **Dr. David Houchens**, the EDSP Program Manager at Battelle, through a series of planned conference calls and through written monthly reports.

General scientific direction and supervision of the work performed under this work assignment is also provided by Mr. Blanton, Principal Investigator (PI), who has 11 years of project management experience. Mr. Blanton is responsible for following the technical work assignment, preparing the study protocol, assigning appropriate staff to complete specified tasks within this work assignment, maintaining project records, preparing the final project reports and data summaries, and reporting the progress of technical milestones as outlined in the technical work plan. Mr. Blanton also prepares and defends the Battelle Pacific Northwest Division Institutional Animal Care and Use Committee (IACUC) study protocol (a document separate and distinct from the study protocol) and ensures that the study is being conducted according to the appropriate study protocol and animal care and use guidelines.

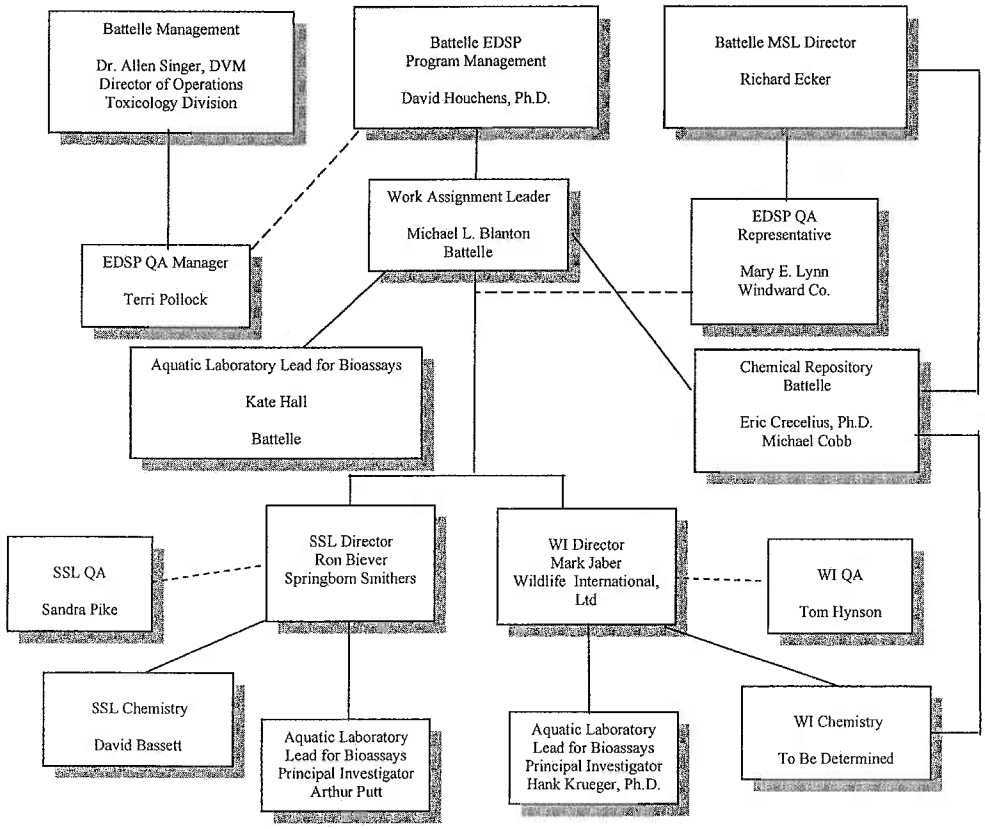

Figure 1. WA 5-11 Project Organization Overview

Together, this team of individuals is responsible for implementing the necessary tasks required in the study protocol and ensuring that the data are collected and handled appropriately. All of these tasks are clearly defined in the study protocol, and include:

- Animal ordering and chain of custody
- Animal husbandry, to include housing, feed, and water monitoring
- Monitoring environmental conditions
- Limitation of stress and discomfort
- Daily observations and water quality monitoring
- Clinical observations
- Necropsy and histology
- Collection of study endpoint data
- Retention of specimens and records.

The QA Unit is established as an objective, independent monitor of the work performed at Battelle. Thus, the QA Unit Representative, **Ms. Mary Lynn**, operationally informs **Mr. Richard Ecker**, who has overall responsibility for operations at Battelle Pacific Northwest Division at Sequim, WA, of QA Program status and implementation. Ms. Lynn is designated to audit all practices and data for their compliance with this QAPP for Tasks 5 and 6 at Battelle. The specific responsibilities for QA representatives from Battelle, Wildlife International and Springborn Smithers include but are not limited to:

1. Interact with the PI to ensure that WA personnel understand quality assurance (QA) and quality control (QC) procedures.

2. Conduct technical systems audits (TSAs) and audits of data quality (ADQs) to evaluate the implementation of the program WAs with respect to the EDSP Quality Management Plan (QMP) (Battelle 2003), the WA Quality Assurance Project Plan (QAPP), the study protocol, and applicable program and facility Standard Operating Procedures (SOPs). Prepare and distribute reports of the audits as described in Section 20.0.

3. Consult with the PI and, as necessary, the EDSP Battelle QA Manager and Program Manager on actions required to correct deficiencies noted during the conduct of the WA.

4. Ensure, during the conduct of TSAs, that all staff participating on the EDSP WA are adequately trained.

5. Maintain complete facility and study-specific QA records related to the program.

6. Submit copies of resolved audits to the EDSP Battelle QA Manager.

7. Submit a QA statement to the EDSP QA Manager and Program Manager with each written deliverable that describes the audit and review activities completed.

8. Maintain effective communication with the EDSP QA Manager.

Dr. Eric Crecelius is the Manager for the Environmental Chemistry group at the Battelle Marine Sciences Laboratory (MSL). He is responsible for ensuring that appropriate and comparable technical procedures are used for sample analysis, ensuring that performance evaluation samples, standard reference materials, and certified standards are a routine part of the laboratory quality control program, and for providing technical expertise to the analytical laboratories. Dr. Crecelius reports to the MSL Director. The Chemical Repository functions under a separate work assignment that is subject to GLP guidelines; **Mr. Michael Cobb** is the Study Director for the Chemical Repository. Mr. Cobb directs a team of professional chemists and laboratory technicians within the EDSP Chemical Repository who will prepare, store, and deliver the chemical test solutions to be used by all the participating laboratories on the program for the various tasks upon receiving a chemical request. He will have ultimate responsibility for the quality of all chemical test samples used on the program and for their timely delivery to the participating laboratories. Mr. Cobb is also responsible for ensuring that analyses are scheduled and completed by their due dates, that holding times are met, and that reporting schedules are not compromised. He will also ensure that corrective actions are assigned and completed. He reports to Dr. Crecelius the status of laboratory analyses and potential problems to the MSL Laboratory Director, Mr. Ecker, the Work Assignment Leader, Mr. Blanton and the EDSP Program and QA Managers.

As EDSP Program Manager, Dr. Houchens will have ultimate responsibility for quality, timeliness, and budget adherence for all activities on the contract. He will also serve as the principal interface with the EPA's Project Officer on all contract-level administrative and technical issues. Because of the high level of subcontracting and purchases required by the program, such as test laboratory subcontracts and purchases of chemical supplies, Dr. Houchens will be assisted by an administrative deputy manager, **Mr. James Easley**. Mr. Easley will manage the procurement of all subcontracts, consultants, and purchased materials and services, and will facilitate schedule and cost control. He has played a similar role on ten other large, multi-year, level-of-effort task-order contracts for EPA. Thus, he will be able to assure that all purchases are compliant with government regulations and that EPA is provided timely, accurate accounting of these substantial costs in our monthly progress reports.

Ms. Terri Pollock, the EDSP QA Manager at Battelle, will direct a team of QA specialists who will monitor the technical activities and provide oversight to all associated QA functions. Ms. Pollock and Ms. Lynn coordinate their respective QA efforts. Ms. Pollock will be responsible for reporting her findings and any quality concerns to Dr. Houchens. Ms. Pollock reports to **Dr. Allen Singer**, Director of Operations for Battelle's Toxicology Division. This

reporting relationship assures that the QA function is independent of the technical activities on the program.

Dr. Hank Krueger will serve as study director for Wildlife International Ltd. **Mr. Tom Hynson,** who has more than 30 years of experience in quality assurance, will be the Wildlife International Ltd. QA officer. Both Dr. Krueger and Mr. Hynson report to **Mr. Mark Jaber,** who will provide management oversight for Wildlife International Ltd.'s involvement on this work assignment. Likewise, **Mr. Arthur Putt** will serve as PI for Springborn Smithers. **Ms. Sandra Pike** will be the Springborn Smithers QA officer. **Mr. David Bassett** will be Springborn Smithers' chemistry leader. Mr. Putt, Ms. Pike, and Mr. Bassett report to **Mr. Ron Biever,** who will provide management oversight for Springborn Smithers' involvement on this work assignment. Pathology support will be provided to Battelle, Wildlife International Ltd., and Springborn Smithers by Experimental Pathology Laboratory (EPL).

5.0 PROBLEM DEFINITION/BACKGROUND

5.1 PROBLEM DEFINITION

The Food Quality Protection Act of 1996 requires the EPA to develop and implement a screening program using valid tests for determining the potential in humans for estrogenic effects from pesticides. EPA proposed a two-tiered screening program in a Federal Register notice in 1998 (63 FR 71542-71568, Dec. 28, 1998). One of the assays being considered for inclusion in the screening program is a Fish Screening Assay

This test will be based on a combination of OECD Phase 1B outcomes and fish screening methods developed by EPA (U.S. EPA 2001, Ankley et al., 2001).

5.2 BACKGROUND

The purpose of work to be conducted under this work assignment is to perform a follow-on study to the OECD Phase 1B study based on features in the short-term reproduction assay with the fathead minnow as described by the U.S. EPA (2001). Specifically, this will incorporate an increased number of replicates and will use a semi-quantitative and quantitative egg-counting method. The studies will include two different laboratories (Springborn Smithers and Wildlife International Ltd.), each running flutamide and potassium permanganate. Ketoconazole and trenbolone will be run by Battelle.

6.0 PROJECT TASK DESCRIPTION

6.1 PROJECT OVERVIEW

The primary experimental objective is to demonstrate the fish screening assay based on the proposed test guidelines. The endpoints will include survival, behavior, and secondary sexual characteristics, while fecundity and fertilization success will be monitored daily. At termination of the assay, measurements will be made of a number of endpoints reflective of the status of the reproductive endocrine system, including the GSI, gonadal histology, and plasma concentrations of vitellogenin.

6.2 TASK 5 AND TASK 6

Laboratory. Battelle, in addition to Springborn Smithers and Wildlife International Ltd., will participate in Tasks 5 and 6. All testing labs have prior experience with reliable performance of conducting work with similar fish screening assay protocols.

Assay. The assay to be conducted is the Fish Screening Assay modified from U.S. EPA (2001).

Chemicals. Battelle will run the assay with ketoconazole and trenbolone. Both Wildlife International Ltd. and Springborn Smithers will run studies with flutamide and potassium permanganate.

Data Interpretation Procedure. Working with EPA, Battelle will make certain that predictions about the potency of the test substances chosen are documented and expected results articulated.

Measurement Endpoints. Table 3 shows the quality objectives and measurement endpoints.

Table 3. Quality Objectives and Criteria for Measurement Data

Parameter	Units	Expected Results
Survival: Daily assessment of survival will be made to provide a basis for expression and interpretation of reproductive output.	Not Applicable	90% or greater survival in controls. Mortality is expected to be low based on previous studies at these exposure rates.
Behavior of Adults: Abnormal behavior (relative to controls), during the daily observations will be noted.	Not Applicable	Expected observations may include: Hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and feeding abstinence. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males. Qualitative anecdotal observations.
Fecundity: Egg production will be determined daily, but only during the morning. Both quantitative and semi-quantitative measurements will be performed.	Fecundity will be expressed either on the basis of average number of eggs laid by surviving females per reproductive (test) day per replicate or as cumulative eggs laid over the test.	It is expected that one spawn typically will be composed of 10 to 250 eggs. If no embryos are present, the substrate will be left in the tank; new substrates will be added to replace any that are removed.

Parameter	Units	Expected Results
Fertilization Success: If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate is easily achieved.	Number embryos/number of eggs x 100	Fertilized eggs will be apparent within a few hours of fertilization. Infertile eggs will be opaque or clear with a white dot where the yolk has precipitated. Control fertilization should be ≥95%.
Appearance of Adults: The external appearance of the adults will be assessed as part of the daily observations, and any unusual changes will be noted. These observations are especially important for assessing endocrine active agents that are (anti)-androgenic. Fatpad index and weight will be collected	Grams	External features of particular importance include body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad (fatpad), number of nuptial tubercles in males; ovipositor size in females).
Body Weights Samples	Grams	Normal/increased/decreased relative weights to control animals.
Blood Samples: will be collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule and analyzed for VTG	Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 µL.	Plasma will be separated from the blood sample via centrifugation (approx. 3 minutes at 15,000 x g) and stored with protease inhibitors at -75°C to -85°C until analysis.
Vitellogenin (VTG) Concentration	pg/mL	The measurement of VTG in plasma samples will be performed using an enzyme-linked immunosorbent assay (ELISA). For the ELISA, fathead minnow (FHM) *(Pimephales promelas)* Amersham VTG kits with monoclonal antibodies, VTG antibody, and purified VTG protein, also from the FHM, will be utilized.
Gonad Size: After sampling the blood, the fixed gonads will be removed and weighed (to the nearest 0.1 mg) to determine the GSI (GSI=100 x gonad wt/body wt).	Not Applicable	Typical GSI values for reproductively active fathead minnows range from 8 to 13% for females and from 1 to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes.
Gonad Morphology: Routine histological procedures will be used to assess the condition of testes and ovaries from the fish. Gonads will be fixed in Davidson's fixative. EPL will perform histology procedures and will follow the protocol from the OECD Phase 1B Study.	Not Applicable	Evaluation of the testis will be based on the amount of germinal epithelium present and the degree of spermatogenic activity. The ovary will be evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Not Applicable. No unit can be defined for this parameter.

6.3 DELIVERABLES

Tasks 5 and 6: Fish Screening Assay Phase 1B Follow-Up Protocol and Reporting

Task 5: Monthly technical reports will be provided to the EPA Work Assignment Manager (WAM) by Battelle to detail progress, interim results, and any problems/deviations encountered. These reports are in addition to the monthly progress reports required under the terms of the overall contract and will summarize the technical aspects of the progress and interim results. Assuming an initiation date of May 16, 2005, every attempt will be made to complete the in-life testing phase of the WA by the end of July, 2005.

Task 6: A draft final report will be submitted to EPA by November 18, 2005. Battelle will notify the EPA WAM in writing when 70 percent of the technical hours and/or 70 percent of the funds approved for this work assignment have been expended. In this notification, Battelle will

state whether the work assignment can be completed with the remaining technical hours and funds.

7.0 QUALITY OBJECTIVES AND CRITERIA

7.1 QUALITY CONTROL OVERVIEW

Quality objectives for the measures to be performed for this study are expressed in terms of accuracy, precision, completeness, representativeness, and sensitivity. Analytical accuracy and precision are monitored through the analysis of QC samples. Table 3 (see Section 6.2) lists specific quality objectives, criteria, and endpoints for the Fish Screening Assay.

Accuracy is defined as the degree of agreement between an observed value and an accepted reference value. Accuracy can be affected by a combination of random error (precision) and systematic error (bias), both of which are due to sampling and analytical operations. In this study, accuracy of body weight measures will be controlled by the use of balances whose calibration is verified with every use, and that are on an external calibration schedule. Analytical chemistry objectives for accuracy will be implemented when an analytical method is selected and will follow the accuracy requirements for initial calibration verification (ICV) and continuing calibration verification (CCV) samples, and percent recovery of standard and certified reference materials, blank and matrix spike samples, laboratory control samples, and surrogate and internal standards, as specified in the method. Chemistry accuracy will be expressed as percent recovery from the known concentration.

Precision is defined as the degree of variability inherent in a set of observations or measurements of the same property, obtained under similar conditions. Precision is usually expressed in either relative or absolute terms. In this study, relative percent difference (RPD) and relative standard deviation (RSD) will be used. Because much of the data to be collected under this work assignment are observational, accuracy will be addressed primarily by defining observation and count measures to be made, preparing data collection forms specifying the data to be collected, and training personnel prior to data collection so that staff consistently record these types of data between data recorders. Analytical chemistry objectives for precision will be implemented when an analytical method is selected and will follow the precision requirements for replicates and blank spike and matrix spike duplicate pairs specified in the method. Precision will be expressed as the relative percent difference between two measures or the relative standard deviation among 3 or more values.

Completeness is the amount of data collected, as compared to the amount needed to ensure that the uncertainty or error is within acceptable limits. The goal for data completeness is 100%. However, the project will not be compromised if 90% or greater of the samples collected are analyzed with acceptable quality.

Representativeness is the degree to which data accurately and precisely represent a characteristic of a population. This is a qualitative assessment and is addressed primarily in the study design and through the selection of test subjects.

Method sensitivity is the capability of a test method to discriminate between measurement responses representing different levels (e.g., concentrations) of the four test chemicals of interest. Sensitivity is addressed primarily through the selection of appropriate test method endpoints, equipment, and instrumentation.

8.0 SPECIAL TRAINING/CERTIFICATION

The in-life portion of this WA is not required to be conducted under Good Laboratory Practices (GLP). However, personnel conducting the laboratory work for the Tasks listed in this document are trained to perform the assay and have done so for previous work assignments. Additionally, GLP training has been provided to personnel.

9.0 DOCUMENTS AND RECORDS

9.1 QUALITY ASSURANCE PROJECT PLAN

This QAPP will be distributed to project participants, initially, and whenever revised. Previous versions either will be marked as "obsolete" when newer versions are distributed, or will be collected and destroyed so that there is no confusion regarding the version in effect. The right-justified document control header example shown here

Version 1
Month Year
Page 1 of 1

is used to ensure that revision numbers and dates are obvious to document users. The QAPP will be reviewed annually if the project extends beyond one year, and a determination made to either modify the document based on new or modified project requirements, or leave as is. Controlled copies of the QAPP are maintained, tracked, and managed by the QA Unit through the use of a master distribution list.

9.2 DATA PACKAGE CONTENTS

The data package for Task 5 will contain the raw data generated as a result of, or in support of each of the three studies (two chemicals by each of three participating laboratories). These records include, but are not limited to, test substance receipt, storage and distribution; test, stock and dilution preparation; sample preparation/ extraction; instrument printouts; spreadsheets and outputs from graph programs, as applicable; all observational data.

Additional task-specific documentation such as the protocol, protocol changes (amendments/deviations), SOP deviations, QAPP deviations, and a listing of equipment used will also be retained in the data package. Any deviations from the protocol, study plan, QAPP, or SOPs will be documented and acknowledged by the Principal Investigator, Work Assignment Leader, EDSP Program Manager, and EDSP Quality Assurance Manager.

Documentation that is applicable to multiple studies, such as training records for personnel involved in each Task, equipment calibration and maintenance records, and storage temperature records may be retained either in the data package for the specific Task or as facility-related records.

9.3 DATA REPORTING

The test results will be evaluated using the specifications and statistical analyses as specified in the study plan and protocol. Springborn Smithers and Wildlife International Ltd. will provide completed reports to Battelle. Battelle will integrate results into one report to be submitted to EPA. No additional statistical evaluation (power analysis) will be done on data provided by sub-contractors in the final report.

9.3.1 Draft and Final Reports

Data will be reported on the schedule indicated in Section 6.3. Interim reports will be prepared as required by the study plan and protocol. At the conclusion of Task 5 a final report will be prepared. The final report (Task 6) will contain, but not be limited to:

- A description of each test, control and reference substance, including information on their CAS numbers, sources, lot numbers and purities, as provided by Battelle.
- A description of the preparation of test, control and reference substance stocks and dilutions, if applicable.
- A description of sample preparation.
- A listing of sample results including any calculations, statistical analysis, graphs, as required by the protocol and study plan.
- The protocol including any amendments or deviations.
- A quality assurance statement listing any audits performed and the date(s) audits were reported.
- Reports from subcontractors:
  - Springborn Smithers
  - Wildlife International Ltd.
  - Experimental Pathology Laboratory (EPL).

9.3.2 Quality Assurance Reports

Each Task will be audited by the Quality Assurance Unit (QAU) at each facility as indicated in the QMP. Reports from audits conducted by the QAU will be issued as described in

the QMP and/or as specified in each facility's SOPs and maintained as confidential files by the QAU.

9.3.3 Status Reports

Progress, results, and other information will be provided by monthly reports and conference calls with the WAM and the Battelle Team.

9.4 RETENTION OF SPECIMENS AND RECORDS

All specimens and records generated at Battelle and that remain the responsibility of Battelle will be retained in the project data files for the length of time stipulated in the contract. The files will remain with the PI until the study is complete. The project files will include a file index. At the conclusion of WA 5-11, the project files generated at MSL will be placed in the archived files for Battelle in Richland, Washington, as per the Records Inventory and Disposal Schedule (RIDS). Records are maintained according to a policy of limited access. The Battelle archivist is responsible for archiving and retrieving materials. An archive inventory will be maintained and storage capability will provide for the expedient retrieval of materials. Specimens and samples will be disposed of only after project management staff have determined that they no longer afford evaluation.

The raw data and specimens generated at Springborn Smithers, Wildlife International Ltd., and EPL will be stored at each laboratory's archives. No records will be disposed of without the authorization of Battelle.

10.0 SAMPLING PROCESS DESIGN

A study protocol to meet the requirements of the project (Section 6.0) will be developed and will define the experimental design. The study protocol will minimally contain the objective; name of all tests, control and reference substances; test system to be used; number and type of samples to be analyzed; detailed description of the assay to be used; and proposed statistical analyses.

11.0 SAMPLING METHODS

Sampling methods are outlined in the study protocol.

12.0 SAMPLE HANDLING AND CUSTODY

Specific test chemical sample handling will be detailed in the protocol and facility SOPs, and will be documented in the study file at each facility. Custody of samples and test chemicals is addressed in facility SOPs. The EDSP Chemical Repository will provide test chemicals and

accompanying Chain of Custody documentation. Chain of Custody documentation will be signed and dated upon receipt and the forms maintained in the project files. Use and disposition of test chemicals and samples will be documented per facility SOPs.

13.0 ANALYTICAL METHODS

The studies will be performed as specified in the study protocols. A chemistry study protocol will be prepared for this WA that covers each test substance and describes the methods to be used to assay the samples.

14.0 QUALITY CONTROL

Concurrent negative controls are included in each study. The negative control provides assurance that the solvent (if used) does not interact with the test system.

15.0 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

Each participating laboratory is responsible to provide the equipment necessary to perform the assay and is responsible for testing, inspecting, and maintaining the required equipment. Each participating laboratory has been pre-qualified to participate in this project. During the prequalification process it was determined that the laboratory has approved SOPs detailing how equipment is tested, calibrated, inspected, and maintained as appropriate. Additionally, each laboratory maintains records to demonstrate that calibration, testing, inspections, and routine and nonroutine maintenance have been performed as specified in the SOPs.

16.0 INSTRUMENT/EQUIPMENT CALIBRATION AND FREQUENCY

Each piece of equipment used to obtain a measurement or used in the generation of data (e.g., balances, pipettes, diluter system, high-performance liquid chromatographs, gas chromatographs, and thermometers) will be calibrated or tested on the schedule specified in the SOP for that piece of equipment (see section 15.0 for additional information). In general, equipment will be calibrated on a schedule as recommended by the manufacturer or more frequently.

Equipment will be calibrated using NIST traceable or other certified standard(s). Calibration will be conducted either by qualified facility personnel or equipment service representatives. The procedure for calibration is specified in the facility SOPs, equipment manual, or service representative procedures. Documentation of any calibration conducted will be retained. In general, this documentation will consist of the unique identification of the piece

of equipment being calibrated, the date of calibration, who conducted the calibration, the expected result, the actual result and if the calibration was within the acceptable limit. If the calibration is outside the acceptable limit, the corrective action will be documented.

17.0 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

Upon receipt, purchased items will be inspected for conformance to quality requirements and again prior to use. All products will be used prior to their expiration dates, when applicable.

18.0 NONDIRECT MEASUREMENTS

No collection of any samples or sample data for this study will be obtained from nondirect measures.

19.0 DATA MANAGEMENT

19.1 DATA MANAGEMENT OVERVIEW

The data for this study will be collected on preprinted data collection forms that are appropriate for the information being collected and will either have one animal per data sheet or multiple animals per data sheet when the measurements are being conducted on multiple fish per tank.

The data form will include, as appropriate, the following items: study code, tank number, and treatment (Rx) code. The dates for data collection will be hand printed on the forms as needed prior to, or on the day of, collection of the data. All data forms will be initialed and dated by the person collecting the data, and all forms will receive documented technical review and signature approval. Corrections to data entries will be made by drawing a single line through the error, recording the correct entry, initials, date, and error code that explains the reason for the correction. The data will then be entered into an Excel spreadsheet.

The original raw data collected on the data forms will be stored with other project files until there is a signed final report. Electronic and paper files will be stored at each facility location unless the sponsor requests that the data be transferred to an archive location other than the one at Battelle (or Springborn Smithers, Wildlife International Ltd., EPL).

19.2 DATA TRANSFER

All raw data will be presented as an Excel workbook. Information will be sent from Springborn Smithers, Wildlife International Ltd., and EPL to Battelle Sequim in electronic format.

Information will then be sent to the Data Coordination Center at Battelle Columbus in electronic format, as specified in:

EDSP.D-003-01	Transmission of Information to the EDSP Data Coordination Center (DCC)
EDSP.D-013-01	Information to be Delivered for QA Documentation to the EDSP Data Coordination Center (DCC).

In addition, data files (e.g., SAS data sets), statistical analysis programs (e.g., SAS programs), and all study documents will be sent to the EDSP Data Coordination Center in electronic format.

20.0 ASSESSMENTS AND RESPONSE ACTIONS

The Quality Assurance Unit (QAU) at each facility will perform assessments on WA 5-11, Tasks 5 and 6 activities and operations affecting data quality. Additionally, the QAU at each facility will review the raw data and report resulting from this Task. Assessments of critical phase activities and operations will be identified, scheduled, and reported as specified in each facility's SOPs. The assessments for this WA include Technical Systems Audits (TSAs) and Audits of Data Quality (ADQs). Performance Evaluations do not apply to this QAPP.

20.1 TECHNICAL SYSTEMS AUDITS

A Technical Systems Audit (TSA) is a process by which the quality of work performed under a WA is assessed through evaluating a WA activity's conformance with the study protocol(s), applicable facility or program SOPs, the WA QAPP, and the EDSP QMP, as applicable. The acceptance criteria are that WA activities and operations must meet the requirements of these planning documents or be explained and evaluated in a deviation report by the Principal Investigator.

A minimum of one TSA will be conducted during each study in Task 5. Based on the activities specified in the protocols and procedures specified in each facility's SOPs, critical phase(s) to be audited during each Task will be determined by the respective facility QAU. Critical phases targeted for TSAs may include, but are not limited to:

- Analysis of exposure solutions
- Biological observations at critical time points during the in-life phase of the study
- Chemical diluter calibration.

The procedure followed by the QAU representative is outlined in the SOPs for each facility. The procedure at Battelle is specified in the QAU SOPs and EDSP QMP (QMP,

Battelle Northwest, 2003) and is briefly outlined here. EDSP QA team members observe the procedure, data recording, and any equipment maintenance and calibration procedures and/or documentation, noting whether or not the activities adhered to the WA study protocols and QAPP, applicable SOPs, and the EDSP QMP. During the TSA, EDSP QA team members record observations to be used later in preparing the audit report. Any findings will be communicated to the technical personnel at the completion of the WA activity unless an error could compromise the WA (e.g., lost or broken samples). EDSP QA team members will immediately notify the Principal Investigator in person or by telephone and/or e-mail of any adverse findings that could impact the conduct of the WA. Findings will be detailed in the audit report (see below).

20.2 AUDITS OF DATA QUALITY

An ADQ is a process by which the accuracy of data calculations and reporting is assessed to ensure that the reported results are of high quality and accurately reflect the raw data and that the executive summary and final report accurately describe the materials used in the WA. The acceptance criteria for the ADQ are that data collection, analysis, and reporting must meet the requirements of the applicable facility and program SOPs, the WA study protocols and QAPP, and the EDSP QMP, as applicable, or be explained and evaluated in a deviation report.

The QAU at each facility will be responsible to conduct ADQ(s) on each Task to assure that the data meet the facility and program requirements and that the report accurately reflects the data. Each facility QAU will conduct ADQ(s) as specified in their SOPs.

Personnel will submit all data and records for review at the time of the ADQ. An EDSP QA team member or delegate will review the data package for completeness and, if incomplete, request that the additional records needed for review be submitted. EDSP QA team members review a minimum of 10% of the raw data, the tabulated data, and WA records of performance and methods to ensure compliance with the planning documents mentioned previously. At least 10% of the raw data will be compared to the tabulated individual data, and a minimum 10% of the summary data tables are checked. EDSP QA team members will also check all tabulated data designated as statistically significant. EDSP QA team members will review any data summary using the audited data and corrected tables to ensure that the reported results are of high quality and accurately reflect the raw data, and that the summary accurately describes the materials used in the WA. Findings will be reported as described below.

20.3 AUDIT REPORT FORMAT

The QAU at each facility reports audits conducted during each Task to the Principal Investigator and management at their respective facility. The procedure for reporting audits is described in the SOPs at each facility.

At Battelle, the procedure is as follows. The audit report consists of a cover page for WA information and additional page(s) with the audit findings. All pages have header information containing the WA study protocol number, audit date(s), and auditor. The audit report date is the

date on which the EDSP QA Unit signs the audit report and sends it to the Principal Investigator and EDSP management.

The cover page contains the WA number; Principal Investigator; audit type; audit date(s); EDSP QA Unit; distribution list; the dated signature of the auditor; the date that the WAL received the audit report; and the dated signatures of the Principal Investigator; and management. The distribution list may include additional names for individuals who have findings pertaining to their area of responsibility. The official audit report is the hard copy containing corrective actions and dated signatures of the EDSP QA Unit, Principal Investigator, and management. Subsequent page(s) contain the audit finding(s), any recommended remedial actions, and space for the Principal Investigator to respond to the findings and document remedial actions taken or to be taken.

20.4 RESPONSE ACTIONS

The manner by which audit reports will be responded to at each contracted facility are specified in their respective SOPs.

At Battelle, the Principal Investigator shall be requested to respond to a TSA report within 10 working days of receipt of the report. There is no deadline for the PI's response to an ADQ report except for the time constraint deriving from the submission date of the final WA report. The Principal Investigator forwards the audit report to management for review. Management adds comments as necessary, signs and dates the report, and returns it to the EDSP QA Team Member. The EDSP QA Team Member assesses the responses and verifies the corrective actions. If a disagreement between the Principal Investigator and EDSP QA Team Member arises over a finding, these individuals will attempt to resolve the issue. If necessary, the EDSP Quality Assurance Manager may be consulted and/or the issue brought to the attention of management for resolution. The final resolution will be documented in the QA files.

During an assessment, if the auditor determines that adverse health effects could result or WA objectives of acceptable quality cannot be achieved, the auditor will follow the Stop Work Procedure specified in the EDSP QMP (Section 3.3).

20.5 INDEPENDENT ASSESSMENTS

The EDSP Battelle QAM or designee may conduct one or more independent TSA(s) and ADQ(s) during the conduct of this work assignment. Typically, one independent audit will be conducted during the work assignment. If major deficiencies are uncovered, additional independent audits may be scheduled. The conduct and reporting of the audits will be consistent with the procedures described in the EDSP QMP (Section 3.3).

21.0 REPORTS TO MANAGEMENT

The manner by which audit reports are distributed to the Principal Investigator and management are defined in each facility's SOPs.

At Battelle, each audit report prepared is distributed to the Principal Investigator, Work Assignment Leader, and management of the applicable facility (i.e., Principal Investigator's manager). The QA Unit will send copies of resolved audit reports containing findings, responses, and corrective actions taken to the Battelle's EDSP QA Manager as they are completed. The EDSP QA Manager routinely prepares and distributes quarterly reports to EDSP program management.

22.0 DATA REVIEW, VERIFICATION, AND VALIDATION

The data produced under this work assignment will be reviewed by the technical personnel to determine data validity based on pre-established acceptance criteria. The QAU at each facility will review the data and verify reporting accuracy (see section 20.0 for discussion of Audits of Data Quality). The Work Assignment Leader will review the data from the participating laboratories and communicate with the laboratories as needed.

23.0 VERIFICATION AND VALIDATION METHODS

Based on the pre-established acceptance criteria defined in this document (Table 3 in Section 6) and the study protocol, the technical personnel will review the data produced to determine data validity and that data meet project objectives. Data will also be reviewed to determine if any samples were compromised during the analysis. Any sample data determined to be invalid will be documented and reported as such and, generally, will not be used in the data analysis. The review for data validity will be conducted prior to the ADQ (verification) conducted by the QAU.

The QAU verification review (i.e., ADQ) is conducted to assure that data were collected in accordance with the planning documents (e.g., study protocol, QAPP, facility and EDSP SOPs) and that the reported results accurately reflect the raw data. The ADQ(s) conducted by the QAU will be conducted and reported as specified in approved facility QA SOPs and/or the QMP and as described previously in section 20.0.

24.0 RECONCILIATION AND USER REQUIREMENTS

The data requirements and proposed methods for data analysis for this Task have been specified in previous sections and will be included in the study protocol. Results will be

communicated to EDSP program management and EPA as indicated in the Study Plan for this Task.

25.0 REFERENCES

Ankley GT, Jensen KJ, Kahl MD, Korte JJ, and Makynen EA (2001). Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*)." Environ Toxicol Chem 20: 1276-1290.

Battelle Northwest, 2003. Endocrine Disruptor Screening Program, Quality Management Plan, Version 2, Battelle, Columbus, Ohio. 12 May 2003.

U.S. EPA Mid-Continent Ecology Division (2001). A Short-term Method for Assessing the Reproductive Toxicity of Endocrine Disrupting Chemicals Using the Fathead Minnow (*Pimephales promelas*). EPA/600/R-01/067.

Battelle
The Business of Innovation

EFFECTIVE DATE: 05-01-05

TEST PROTOCOL

PROTOCOL TITLE: FISH SCREENING ASSAY OECD PHASE 1 B FOLLOW-UP

TO BE COMPLETED BY THE STUDY SPONSOR:

Study Sponsor: U.S. Environmental Protection Agency

Address: EPA East Building, 1201 Constitution Avenue, N.W. Room 4106, Mail Code 7201M, Washington, D.C. 20004 Phone:

Sponsor Protocol/Project No.:

Test Substance Name(s): Ketoconazole CAS #: 65277-42-1

Purity: 99.73% Batch or Lot #: QL0352

Analytical Standard Same as above

Purity: Same as above Batch or Lot #: Same as above

Additional Comments and Modifications:

Sponsor Approval: Date:

TO BE COMPLETED BY TESTING LABORATORY BEFORE EXPERIMENT INITIATION:

Testing Facility: BATTELLE PACIFIC NORTHWEST DIVISION

Principal Investigator: Mark A. Cafarella Study No.: 13784.6112

Test Concentration: * 400, 100, 25 ug ai/L

Proposed Experimental Dates: (Start) 9/21/05 (Termination) 10/12/05

Mark A. Cafarella
Principal Investigator Signature

10/13/05
Study Initiation Date

* To be provided by protocol amendment, if applicable.

Battelle
The Business of Innovation

EFFECTIVE DATE: 05-01-05

TEST PROTOCOL

PROTOCOL TITLE: FISH SCREENING ASSAY OECD PHASE 1 B FOLLOW-UP

TO BE COMPLETED BY THE STUDY SPONSOR:
Study Sponsor: U.S. Environmental Protection Agency
Address: EPA East Building, 1201 Constitution Avenue, N.W.
Room 4106, Mail Code 7201M, Washington, DC. **Phone:**
Sponsor Protocol/Project No.:
Test Substance Name(s): see below **CAS #:** see below
Purity: see below **Batch or Lot #:** see below
Analytical Standard Not applicable
Purity: Not applicable **Batch or Lot #:** Not applicable
Additional Comments and Modifications:
① Flutamide Lot# 00310LC, purity 99.5%, CAS# 7722-64-7
② Potassium permanganate, Lot# 121K1083, purity 100%, CAS# 13311-84-7
Sponsor Approval: **Date:**

TO BE COMPLETED BY TESTING LABORATORY BEFORE EXPERIMENT INITIATION:

Testing Facility: BATTELLE PACIFIC NORTHWEST DIVISION

Principal Investigator: Arthur E. Puff Study No.: ① 13784.6109 ② 13784.6110

Test Concentration: *

Proposed Experimental Dates: (Start) 6/1/05 (Termination) 8/31/05

Principal Investigator Signature 5/19/05 **Study Initiation Date**

* To be provided by protocol amendment, if applicable.

1.0 INTRODUCTION

The U.S. Environmental Protection Agency (EPA) is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is a short-term screening assay designed to detect substances that interact with the estrogen and androgen systems of fish. It is thought that the inclusion of the fish screening assay in Tier 1 is important because estrogenic and androgenic controls on reproduction and development in fish may differ significantly enough from that of higher vertebrates such that mammalian screening methods may not identify potential endocrine disrupting chemicals (EDCs) in this important class of animals. As an example, dihydrotestosterone is a potent androgen in mammals, but 11-ketotestosterone is generally the more prevalent androgen in fish.

EPA (2001) has described a short-term test with the fathead minnow *(Pimephales promelas)* 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 (Ankley et al. 2001) is initiated with mature male and female fish. During a 21-day chemical exposure, survival, behavior, and secondary sexual characteristics are observed, and fecundity is 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.

1.1 OECD Assay

The Organization for Economic Cooperation and Development (OECD) initiated a fish screening assay validation activity and has completed its Phase 1A and Phase 1B trials. Phase 1A evaluated a non-spawning version of a 21-day exposure assay with fathead minnow, medaka, and zebrafish. The results of the Phase 1A led to the Phase 1B trials where spawning was included in the method. The results of the Phase 1B trial raised questions regarding the spawning conditions utilized for the fathead minnow.

Previous work assignments under this contract (WA 2-18 and WA 2-29) were initiated to evaluate a short-term reproduction assay with fathead minnow and compare the EPA (2001) method to two other related assays to contribute to the optimization of the assay for use as a screen in the EDSP. The laboratories that participated in the OECD Phase 1B work were ABC Laboratories, Springborn Smithers Laboratories, and Wildlife International, Ltd. Springborn Smithers performed the Phase 1B study with flutamide and 4-tert-pentylphenol (4-PP) using fathead minnows, ABC used 4-PP and prochloraz with fathead minnows, and Wildlife International used 4-PP and flutamide with medaka. Experimental Pathology Laboratories (EPL) performed the histopathology work under Phase 1B for the OECD effort.

The purpose of work to be conducted under this work assignment is to perform a follow-on study to the OECD Phase 1B study based on features in the short-term reproduction assay with the fathead minnow as described in EPA (2001). Specifically, this study will incorporate an increased number of replicates and will use a semi-quantitative and quantitative egg-counting

method. The studies will include two different labs (Springborn Smithers and Wildlife International), each running flutamide and potassium permanganate. Ketoconazole and trenbolone will be run by Battelle.

This protocol describes an *in vivo* screening assay for identifying endocrine active chemicals in sexually dimorphic fish. It has been adapted from OECD Phase 1B protocol with specific changes to various measurement endpoints. Battelle will conduct its studies using this protocol. Springborn Smithers and Wildlife International will adapt this protocol and will use their own standard operating procedures related to their systems and organizational structures.

1.2 Principle of the Assay

The experimental protocol for a short-term reproduction assay is based upon the protocol developed by Ankley et al. (2001) using the fathead minnow (*Pimephales promelas*). This assay will measure the reproductive performance of groups of fathead minnows as the primary indicator for endocrine disruption. Additional measurements of morphology, histopathology, and biochemical endpoints will be performed to aid identification of the specific toxicological mode of action of the test chemical.

The assays will be initiated with mature male and female fish. During a 21-day chemical exposure, survival, behavior, and secondary sexual characteristics will be observed while fecundity and fertilization success will be monitored daily. At termination of the assay, measurements will be made of a number of endpoints reflective of the status of the reproductive endocrine system, including the GSI, gonadal histology, and plasma concentrations of vitellogenin.

The assays will be initiated with mature ("first time spawners") spawning adults. Active spawning for all replicate groups will be established during a "pre-exposure" period of 14 days. The pre-exposure observation period will be used to monitor reproductive performance (semi-quantitative only) as described for the chemical exposure period.

The pre-exposure observations will occur in the same system/tanks as will be utilized for the chemical test. An overview of the tests and relevant test conditions are provided in Table 1.

Table 1. Experimental Design for the Assay Method

Parameter	Assay Protocol
Test species:	Reproductively active fathead minnows (20 ±2 weeks old)
Fish husbandry conditions:	Temp: 25EC ± 1EC; D.O. >5.0 mg/L; Light: Austro sunrise/sunset system; at time of testing the lighting will be approximately 16 h light : 8 h dark with 540 to 1080 lux; Feed: frozen brine shrimp or trout chow twice daily.
Pre-exposure evaluation	Duration: 14 days; Data Collected: fecundity semi-quantitative (daily)
Dilution water	Clean, surface, well or reconstituted water
Test material	Chemicals listed in Table 2
Test chamber size	18 L (40 x20 x 20 cm)
Test volume:	10 L
# Exchanges/day	6 tank volume exchanges
Flow rate:	2.5 L / hr
# Concentration / chemical	3; identified in Table 2
# Replicates:	4
Weight of each fish	NS
# Fish/vessel	4 females and 2 males
Total # fish/concentration	16 females and 8 males
Feeding regime	Frozen brine shrimp, twice a day
# Controls	1; Dilution water control
# Fish/control	4 adult females and 2 adults males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)
Photo period:	Austro sunrise/sunset system; at time of testing the lighting will be approximately 16 h light : 8 h dark
Temperature:	25EC ± 1EC
Light intensity	540 - 1080 lux
Aeration:	None unless D.O. <4.9 mg/L
pH	NS
Biological endpoints:	Adult survival, behavior, secondary sexual characteristics, GSI, gonadal histology, VTG, fecundity and fertility
Test validity criteria:	D.O. = 60% saturation; Mean temp. 25EC ± 2EC; 90% survival in the controls and successful egg production in controls. Typical spawning occurs every 3 to 4 days in controls, or approximately 15 eggs/female/day/test chamber.

NS = Not specified in procedure.

2.0 DESCRIPTION OF THE METHOD

2.1 Selection of Test Organisms

The exposure phase will be started with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals, in spawning conditions. As a general guidance for phase 1B, based on the technical judgment of experienced laboratory personnel, fish will be reproductively mature (namely, with clear secondary sexual characteristics visible) and actively spawning. For general guidance only (and not to be considered in isolation from observing the actual reproductive status of a given batch of fish), fathead minnows typically reach reproductive status at approximately 20 (± 2) weeks of age, assuming they have been cultured at 25 ± 2°C

Test fish will be selected from a laboratory population, preferably from a single stock, which has been acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test (note, this acclimation period is not an *in situ* pre-exposure period). Fish will be fed twice per day throughout the holding period and during the exposure phase. However, fish will not be fed within 12 hours of necropsy.

Following a 48-hour settling-in period, mortalities will be recorded and the following criteria applied:

- mortalities of greater than 10% of population in seven days: reject the entire batch;
- mortalities of between 5% and 10% of population: acclimation for seven additional days; if more than 5% mortality during second seven days, reject the entire batch;
- mortalities of less than 5% of population in seven days: accept the batch.

Fish will not receive treatment for disease in the two week acclimation period preceding the test, or during the exposure period. Fish for studies to be conducted at Battelle facilities will be purchased from the Osage Fish company and will be accumulated and cultured at our facility for approximately 30 days prior to first use. Springborn Smithers' in-house fish culture will be utilized for their studies and in addition will supply fish to Wildlife International for testing at their facility.

2.2 Water

It is well established that the fathead minnow can reproduce successfully over a wide range of water quality. Therefore, no specific water type is required for this test. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully should be acceptable. The animals will be tested using a flow-through water renewal system that maintains adequate water quality (temperature, dissolved oxygen, low ammonia, etc.), and ensures a consistent exposure to the parent chemical.

2.3 Assay System

Five-gallon glass exposure vessels are used for the test system. As recommended by Ankley et al. (2001), dimensions of the test chambers must be such that the animals can interact in a fashion conducive to successful spawning. The test chamber contains 10 L of test solution,

which is renewed once every 4 hours. This particular animal loading/water renewal rate is within recommended guidelines and in studies conducted according to this method, has maintained acceptable water quality while not utilizing an excessive amount of test material.

A complete randomized design will be used for the reproductive assay. This design is intended to randomize out the effects associated with the local environment (i.e., light and water) and possible trends associated with the diluter during testing. All fish will be transferred from pre-validation tank and then randomly assigned a treatment. Thus, when one evaluates the difference between treatment means, the variability associated with experimental environment, experimental containers, and organisms being treated is removed and only the effect of the treatment remains.

2.4 Dosage Formulations

Dosage formulations will be requested and received from the Chemical Repository (CR) (for work conducted at Battelle). The formulations will be logged in following the procedures for sample receipt, distribution and storage, Sample Log-In Procedure (MSL-A-001-04) and Sample Chain of Custody (MSL-A-002-03).

The dosage formulations will be stored under appropriate conditions defined by the CR until transfer to the Toxicology Laboratory. A copy of the Chain of Custody form will accompany all formulations. The sub-sampling method will be chosen to facilitate collection of a representative sample of the dosage formulations received from the CR. Purity and stability testing will not be required for trenbolone and flutamide due to previous work conducted with these chemicals in past work assignments. The CR will provide neat material to Springborn Smithers and Wildlife International for potassium permanganate and flutamide and each facility will then be responsible for dose formulation.

2.5 Preparation and Testing of Chemical Exposure Water

The test chemicals possess some varied physicochemical properties that will likely require different approaches for preparation of the chemical exposure water. When possible, direct addition to water will be utilized if the test chemical has sufficiently high water solubility. This approach appears to be suitable for ketoconazole, trenbolone, and potassium permanganate. For test chemicals with reduced water solubility such as flutamide, the use of a saturator column will be required to prepare the concentrated stock solution. All aqueous stock solutions will be encased in black tarpaulin during agitation and subsequent storage to prevent photo-oxidation. The proposed exposure concentrations are provided in Table 2.

2.6 Analytical Determinations

After preparation of the stock solution, determinations of the concentration will be made using the methods described below. The concentrations of the test chemical in the exposure chambers will be measured prior to adding fish to verify that target concentrations are reached. Additionally, water samples will be removed weekly and analyzed for the test chemicals in each test chamber (High, Med, Low, and Control, in each of four replicates).

All test chemicals will be directly measured using various analytical methods summarized below for each test chemical:

Ketoconazole : This substance was studied in EDSP WA 2-14, a male and female pubertal study in rats. This study utilized corn oil as the carrier. The CR expects to employ high-performance liquid chromatography (HPLC) for the quantitation of ketoconazole in water for WA 5-11.

Trenbolone: Samples will be analyzed by gas chromatography-mass spectrometry (GC-MS) and HPLC methods depending on the treatment level to be analyzed. The CR has documented analytical methods for trenbolone employed in an earlier EDSP study (WA 2-18).

Potassium Permanganate: The CR will not be conducting stability or purity on this compound. Methods to be utilized by subcontractors will be developed based upon the outcome of the dose range-finding tests (see below).

Flutamide: This substance was studied by the CR for WA 2-18. Sampling was done by taking 0.025 mL of the sample and placing it into a 1 mL HP autosampler vial with 0.975 mL of 60:40 acetonitrile:water, this sample was then mixed with a pipetter and transferred to a Gilson autosampler vial. The vial was then agitated and analyzed on a HPLC-ultraviolet/visible (UV/VIS) system.

96 Hour Range-finding: A range-finding test for potassium permanganate will be necessary. Guidance on test chemical concentrations for trenbolone, ketoconazole, and flutamide has been obtained based upon previous research with these test substances.

The highest target test concentration for the range-finder will be based upon toxicity data for other fish species. If such information is lacking, the highest concentration will be near the solubility limit of the chemical in water. Test concentration will then be decreased by a factor of 10 for each successively lower exposure (six exposure concentrations). Range-finding tests will be performed with fish of similar age and size that will be utilized in the 21-day test. The 96-hour range finder exposure will utilize five test concentrations plus a control (six total), two replicates for each treatment of four females and two males per exposure tank (72 fish total). The number of mortalities that occur will be used to develop a dose response curve. Based upon the results, the highest concentration that does not result in increased mortality or signs of overt morbidity compared to controls will serve as the highest exposure concentration in the 21-day test.

Table 2. Test Chemicals and Exposure Concentrations

Test Chemical	Testing Lab	Exposure Concentration (µg/L)		
		Low	Med	High
Flutamide	Springborn Smithers Wildlife International	100	500	1000
Potassium permanganate	Springborn Smithers* Wildlife International	TBD	TBD	TBD
Trenbolone	Battelle	0.1	0.5	1
Ketoconazole	Battelle	25	100	400

*Laboratory conducting concentration range-finding study for this compound
TBD = to be determined.

2.7 Biochemical Determinations

Vitellogenin (VTG): Enzyme-linked immunosorbent assay (ELISA) tests will be conducted using commercially available test kits from Amersham Bioscience. The methods used for the bioanalytical measurements of VTG will follow manufacturer's specifications.

2.8 21-day Assay Initiation and Conduct

Selection and weighing of test fish

It is important to minimize variation in weight of the fish at the beginning of the assay. For the whole batch of fish used in the test, the range in individual weights at the start of the test will be kept, if possible, within ± 20% of the arithmetic mean weight. A subsample of fish will be weighed before the test in order to estimate the mean weight.

Length of exposure

The test duration is 21 days.

Feeding

The fish will be fed twice per day with brine shrimp or trout chow at a sufficient rate to maintain body condition. Food will be withheld from the fish for 12 hours prior to the day of sampling to aid in histology processing of small fish. Uneaten food and fecal material will be removed from the test vessels at least twice weekly, e.g., by carefully cleaning the bottom of each tank using suction.

Pre-exposure

The pre-exposure phase will last 14 days. The assay will use fish that are approximately 20 ±2 weeks old, previously maintained in communal culture tanks. Four females and two males will be randomly assigned to the replicate exposure chambers at each treatment concentration. Additional exposure chambers may be set up for pre-exposure to account for a lack of spawning in some chambers and/or mortality during the pre-exposure spawning. Any specimens whose

gender cannot be identified will be excluded from the assay. It has been reported that, at 5 to 6 months of age, males are larger and darker and exhibit nuptial tubercles, while females possess an ovipositor. The pre-exposure phase of the assay will be conducted under conditions (temperature, photo period, feeding, etc.) identical to those used during the chemical exposure. The animals will be fed frozen *Artemia* twice daily. Semi-quantitative fecundity data will be collected daily. For each assay, successful pre-exposure (suitability for testing) is established when regular spawning occurs in each test chamber every 3 to 4 days.

Chemical Exposure

After successful spawning is verified during pre-exposure as per the requirements of the assay, the chemical exposure will be initiated and continued for 21 days. The assays will be conducted at three chemical concentrations (identified in Table 2), as well as a diluent water control, with four experimental units (replicates) per treatment. Each replicate tank will contain four female and two male fish. The test chemical will be delivered to the exposure chamber using a proportional diluter (concentrated aqueous stock solutions will be prepared without using carrier solvents). The exposure will be conducted for 21 days, during which time the appearance of the fish, behavior, and fecundity will be assessed daily. At termination of the exposure, blood samples will be removed from adults and analyzed for VTG. The gonads will also be removed for GSI determination and later histological analyses.

Frequency of Analytical Determinations and Measurements

Prior to initiation of the exposure period, proper function of the chemical delivery system will be ensured. Just as well, all analytical methods needed will be established, including sufficient knowledge on the substances' stability in the test system. During the test, the concentrations of the test substance will be determined at regular intervals, as follows: the flow rates of diluent and toxicant stock solution will be checked at intervals, at least twice per week, and will not vary by more than 15% throughout the test. Actual test chemical concentrations will be measured in all vessels at the start of the test and at weekly intervals thereafter.

- Results will be based on measured concentrations and will be included in reporting.
- Samples may need to be filtered (e.g., using a 0.45 μm pore size) or centrifuged. If needed, then centrifugation is the recommended procedure. However, if the test material does not adsorb to filters, filtration may also be acceptable.
- During the test, dissolved oxygen, temperature, and pH will be measured in all test vessels at least once per week. Total hardness and alkalinity will be measured in the controls and one vessel at the highest concentration at least once per week. Temperature should preferably be monitored continuously in at least one test vessel.

3.0 OBSERVATIONS AND MEASUREMENTS

3.1 Endpoints

A number of endpoints will be assessed over the course of, and/or at conclusion of the assays. A description of these endpoints (Table 3 below) and their utility, particularly in the context of the assay as an EDC screen, follows:

Survival: Daily assessment of survival will be made to provide a basis for expression and interpretation of reproductive output, that is, number of eggs/female/day. Fish will be examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) noted. Any mortality will be recorded and the dead fish removed as soon as possible. Dead fish will not be replaced in either the control or treatment vessels.

Body Weights: Relative to control animals.

Behavior of Adults: Abnormal behavior (relative to controls), such as hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and feeding abstinence, will be noted during the daily observations. Alterations in behavior, particularly loss of territorial aggressiveness by males, also will be noted.

Fecundity: Egg production will be determined daily. Because fathead minnows spawn within a few hours after the lights are turned on, they will not be disturbed (except for feeding) until late morning. This will allow time for spawning and fertilization to be completed and for eggs to water-harden. The spawning substrates will be removed from the tanks to enumerate any eggs that are present. Based on the published report (and our past experience), it is expected that one spawn typically will be composed of 50 to 250 eggs. If no embryos are present, the substrate is left in the tank; new substrates should be added to replace any that are removed. Fecundity will be expressed on the basis of surviving females per reproductive (test) day per replicate or as cumulative eggs laid over the test. Semi-quantitative fecundity measurements will also be measured by visual estimation using a matrix, such as 0, <50, 50 to 200, >200 eggs.

Fertilization Success: After the spawning substrate has been removed from the tank, the embryos will be carefully rolled off with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100) will be easily achieved. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated; viable embryos remain clear for 36 to 48 hours until reaching the eyed stage.

Appearance and observation of secondary sex characteristics: Secondary sexual characteristics are under endocrine control; therefore observations of physical appearance of the fish should be made over the course of the test, and at conclusion of the study (Appendix C). Experience to date with fathead minnows suggests that some endocrine active chemicals may initially induce changes in the following external characteristics: body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in

male fathead minnow, ovipositor size in females). Notably, chemicals with certain modes of action may cause abnormal occurrence of secondary sex characteristic in animals of the opposite sex; for example, androgen receptor agonists, such as methyltestosterone and dihydrotestosterone, can cause female fathead minnows to develop pronounced nuptial tubercles (Ankley et al. 2001; Smith 1974; and Panter et al., in press). It also has been reported that estrogen receptor agonists can decrease nuptial tubercle numbers and size of the dorsal nape pad in adult males (Miles-Richardson et al., 1999; Holbech et al., 2001). Such gross morphological observations may provide useful qualitative and quantitative information to contribute to potential future fish testing requirements.

Because some aspects of appearance (primarily color) can change quickly with handling, it is important that qualitative observations be made prior to removal of animals from the test system. Other endpoints, such as the number and size of nuptial tubercles in fathead minnow, can be quantified directly. Methods for the evaluation of secondary sex characteristics in fathead minnow are provided in Appendix C-1. In addition, the weight of the fatpad of all fish will be determined following procedures outlined in Appendix C-2.

Humane killing of fish: At the conclusion of the exposure, the fish will be anesthetized by transfer to an oxygenated solution of MS-222 (appropriately buffered dependent on the pH/ hardness of the water) and weighed.

Blood Sampling: Blood will be collected from the caudal artery/vein (Appendix A) with a heparinized microhematocrit capillary tubule. Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 µL. Plasma is separated from the blood via centrifugation (approximately 3 minutes at 15,000 x g) and stored with protease inhibitors at -75°C to -85°C until analyzed for VTG.

Gonad Size and Histology: The first step of gonad histological analysis is necropsy and rapid gonad fixation in Davidson's fixative to prevent autolysis and cellular deterioration. Immediately after humane killing of an individual fish, gonads will be removed (Appendix B). Length and weight measurements and collection of fresh tissues, e.g., blood, will also be performed. The eight criteria for histopathology are the following:

- **Males:** Increased proportion of spermatogonia, presence of testis-ova, increased testicular degeneration, and interstitial (Leydig) cell hyperplasia/hypertrophy

- **Females:** Increased oocyte atresia, perifollicular cell hyperplasia/hypertrophy, decreased vitellogenesis, gonadal staging.

The steps in the process are as follows:

1. After sampling the blood, the "fixed" gonads will be removed and weighed (fixed weight to the nearest 0.1 mg) to determine the GSI (GSI=100 x gonad wt/body wt). Typical GSI values for reproductively active fathead minnows range from 8 to 13% for females and from 1 to 2% for males.

2. Using a syringe, approximately 0.5 mL of Davidson's fixative will be gently applied to the gonads *in situ*. Approximately 90 seconds following the application of fixative, the liquid fixative within the abdomen will be removed with a gauze sponge, and the gonads will be excised in a manner similar to the abdominal viscera:

   a. Using the microdissection scissors, the spermatic ducts or oviducts will be severed proximal to the genital pore.

   b. Microdissection forceps will then be applied to the spermatic ducts/oviducts. Using gentle traction, the gonads will be dissected out of the abdominal cavity in a caudal to cranial direction, severing the mesorchial/mesovarial attachments as needed using the microdissection scissors. The left and right gonads may be excised individually or they may be excised simultaneously and subsequently divided at their caudal attachment.

3. The gonads (right and left) will be placed into a pre-labeled plastic tissue cassette which will then be placed into an individual container of Davidson's fixative accompanied by the abdominal viscera. The volume of fixative in the container will be at least 10 times the approximated volume of the tissues. The fixative container will be gently agitated for 5 seconds to dislodge air bubbles from the cassette.

4. Using the carcass, the secondary sex characteristics will be assessed (e.g., dorsal nape pad, nuptial tubercles; see Appendix C-1 and C-2). After gonads are fixed in place and then excised, material will be placed in labeled histological cassettes to then be sent to EPL for histological analysis. Routine histological procedures will be used to assess the condition of testes and ovaries from the fish using procedures previously used in the Phase 1B exercise (Histopathology Guidelines for Phase 1B of the OECD Fish Screening Assay for EDCs May 20, 2004). After remaining in Davidson's fixative overnight, the tissues will be transferred to 10% neutral buffered formalin the next day. Appendix D details the post mortem and histotechnical procedures that will be used.

Vitellogenin (VTG): The measurement of VTG in plasma samples will be performed using an enzyme-linked immunoabsorbant assay (ELISA). For the ELISA, polyclonal fathead minnow (*Pimephales promelas*) VTG antibody and purified VTG protein, also from the fathead minnow, will be utilized.

Table 3 provides a summary of the measurement endpoints previously discussed. In addition, Appendix E provides an example product guide.

3.2 Statistical Methods

Descriptive statistics, including the mean, standard deviation, minimum, maximum, and quartiles, will be used to characterize each endpoint measured in the tests. Statistical significance for each endpoint and chemical will be 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

will be considered classifications of fixed effects (i.e., control, low dose, mid-dose, and high dose). Box plots will be used to visually characterize the effect of each treatment.

Appropriate data transformations will be 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 will be used when the common data transformation is not successful in controlling heterogeneity (Daniel 1978).

Analysis may be conducted both with and without suspected outliers (Chapman et al. 1996). Potential outliers may be 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 cannot be made for the divergence of data, then both analyses will be presented, assuming that the results differ. If there are no changes to the results, then the analysis including the outliers will be presented. If differences occur, then the implications of removing the outliers will be carefully documented. If an explanation can be made for the existence of outliers, the analysis excluding outliers may be sufficient.

3.3 Performance Criteria

- Water quality characteristics will remain within the limits of tolerance described in Table 1 (water temperature did not differ by more than ± 1 EC between test vessels at any one time during the exposure period and was maintained within a range of 2 EC within the temperature ranges specified for the test species).

- There will be greater than or equal to 90% survival of control animals over the duration of the chemical exposure, and the control fish in each replicate will spawn, at a minimum, every 3 to 4 days. Typically, there will be approximately 15 eggs/female/day/test chamber.

- There will be greater than or equal to 95% fertility of eggs from the control animals.

3.4 Data Reporting

Test report: The test report will include the following information:

Test substance: physical nature and relevant physical-chemical properties, chemical identification data including purity and analytical method for quantification of the test substance where appropriate, source, CAS number, lot number.

Test species: at a minimum scientific name, supplier and any pretreatment.

Test conditions: test procedure used (test type, loading rate, stocking density, etc.); method of preparation of stock solutions and flow-rate; nominal test concentrations; means of the measured values and standard deviations in test vessels and method by which these were attained and

evidence that the measurements refer to the concentrations of test substance in true solution; dilution water characteristics (including pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels, total organic carbon, suspended solids and any other measurements made); water quality within test vessels: pH, hardness, temperature, and dissolved oxygen concentration; detailed information on feeding [e.g., type of food(s), source, amount given and frequency and analyses for relevant contaminants if necessary, e.g., PCBs, PAHs, and organochlorine pesticides]; source and treatment of dilution water; average and ranges of water chemistry parameters; photoperiod; light intensity; chamber size; numbers of male and female fish per replicate; number and composition of spawning substrate; lot number of feed; number of daily water volume exchanges.

Results: evidence that controls met the validity criterion for survival, and data on mortalities occurring in any of the test concentrations; statistical analytical techniques used, statistics based on fish, treatment of data and justification of techniques used; tabulated data on biological observations of gross morphology (including secondary sex characteristics) and vitellogenin; detailed report on gonadal histology; results of the statistical analysis preferably in tabular and graphical form; incidence of any unusual reactions by the fish and any visible effects produced by the test substance; average, standard deviation, and range for each test endpoint.

Table 3. Measurement Endpoints and Associated Criteria

Parameter	Units	Expected Results
Survival: Daily assessment of survival will be made to provide a basis for expression and interpretation of reproductive output.	Not Applicable	90% or greater survival in controls. Mortality expected to be low based on previous studies at these exposure rates.
Behavior of Adults: Abnormal behavior (relative to controls), during the daily observations will be noted.	Not Applicable	Expected observations: Hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and feeding abstinence. Alterations in behavior, particularly loss of territorial aggressiveness by males. Qualitative anecdotal observations.
Fecundity: Egg production will be determined daily by both quantitative and semi-quantitative measurements, but only during the morning.	Fecundity will be expressed either on the basis of average number of eggs laid by surviving females per reproductive (test) day per replicate or as cumulative eggs laid over the test.	One spawn typically composed of 10 to 250 eggs. If no embryos present, substrate will be left in the tank; new substrates will be added to replace any that are removed.
Fertilization Success: If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate is easily achieved.	Number embryos/number of eggs x 100	Fertilized eggs apparent within a few hours of fertilization. Infertile eggs opaque or clear with a white dot where the yolk has precipitated. Control fertilization ≥ 95%.
Appearance of Adults: The external appearance of the adults will be assessed as part of the daily observations, and any unusual changes will be noted. These observations are especially important for assessing endocrine active agents that are (anti)-androgenic. Fatpad index and weight measurement will be collected.	Grams	External features of particular importance: body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad or fatpad), number of nuptial tubercles in males.
Body Weight Samples	Grams	Normal/increased/decreased relative weights to control animals.
Vitellogenin (VTG) Concentration Blood Samples: will be collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule and analyzed for VTG.	Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 µL. pg/mL	Plasma separated from the blood sample via centrifugation (approx. 3 minutes at 15,000 x g) and stored with protease inhibitors at -75°C to -85°C until analysis. Measurement of VTG in plasma samples performed using an enzyme-linked immunosorbent assay (ELISA). ELISA to use monoclonal fathead minnow (FHM) *(Pimephales promelas)* VTG antibody and purified VTG protein, also from the FHM.
Gonad Size: After sampling the blood, the fixed gonads will be removed and weighed (to the nearest 0.1 mg) to determine the GSI (GSI=100 x gonad wt/body wt).	Not Applicable	Typical GSI values for reproductively active fathead minnows: 8 to 13% (females) and 1 to 2% (males). Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes.

Parameter	Units	Expected Results
Gonad Morphology: Routine histological procedures will be used to assess the condition of testes and ovaries from the fish. Gonads will be fixed in Davidson's fixative. After remaining in Davidson's fixative overnight, the tissues will be transferred to 10% neutral buffered formalin the next day. EPL will perform histology procedures and will follow the protocol from the OECD Phase 1B Study.	Not Applicable	Evaluation of testis based on amount of germinal epithelium present and degree of spermatogenic activity. Ovary evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Not Applicable: No unit can be defined for this parameter.

4.0 CONTINGENCIES

The three problems most likely to be encountered are related to insufficient numbers of spawning tanks that are successfully "pre-validated," unplanned mortality in control or exposure tanks, and low or high measured concentrations relative to the nominal level of the test chemicals. These problems will be dealt with in the following manner:

- For each chemical, extra spawning tanks (up to 8 additional tanks) will be pre-validated to ensure an adequate supply of spawning fathead minnows.
- If there is excessive unscheduled mortality in any tank, Battelle will discuss the course of action with EPA.
- Prior to initiation of the chemical exposure, each diluter will be tested for up to 1 week for its ability to maintain the desired concentration. If, during the exposures the measured concentration becomes unacceptably low or high, adjustments will be made to the diluter to correct the problem.

5.0 LITERATURE

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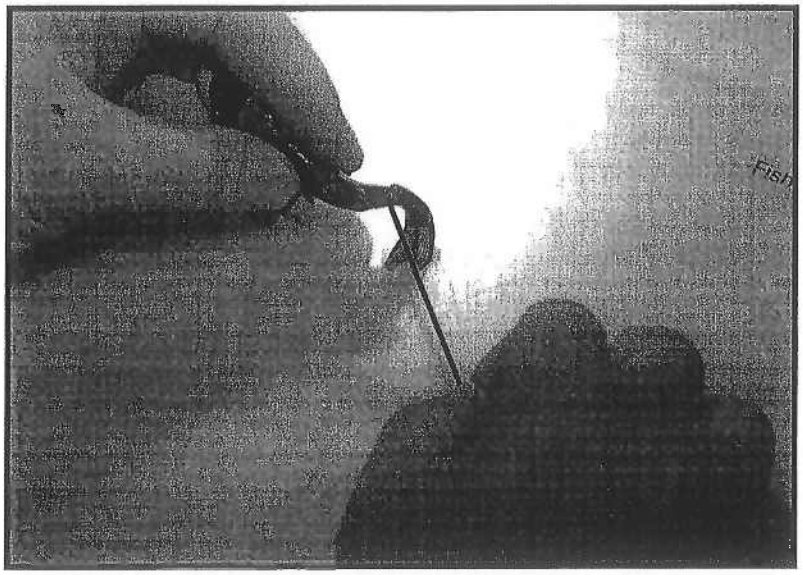
APPENDIX A

SAMPLE COLLECTION PROCEDURES FOR VITELLOGENIN ANALYSIS

Procedure 1A: Fathead Minnow, Blood Collection from the Caudal Vein/Artery



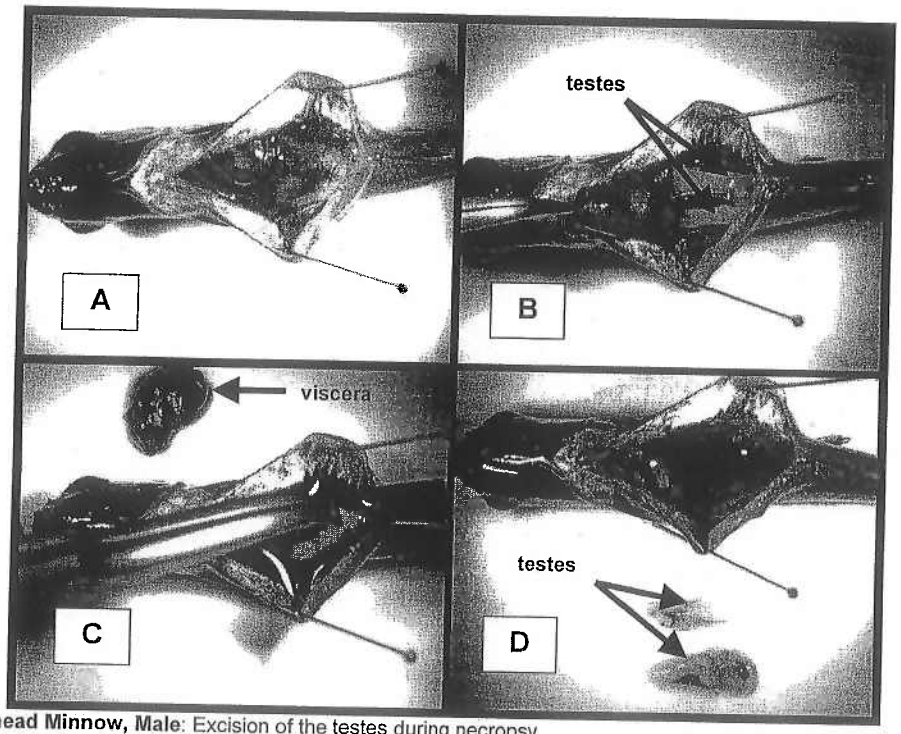
After anaesthetization, the caudal peduncle is partially severed with a scalpel blade and blood is collected from the caudal vein/artery with a heparinized microhematocrit capillary tube. After the blood has been collected, the plasma is quickly isolated by centrifugation for 3 min at 15,000 *g*. If desired, percent hematocrit can be determined following centrifugation. The plasma portion is then removed from the microhematocrit tube and stored in a centrifuge tube with 0.13 units of aprotinin (a protease inhibitor) at -75°C to -85°C until determination of vitellogenin can be made. Depending on the size of the fathead minnow (which is sex-dependent), collectable plasma volumes generally range from 20 to 60 microliters per fish (Jensen *et al.* 2001).

Procedure 1B: Fathead Minnow, Blood Collection from Heart

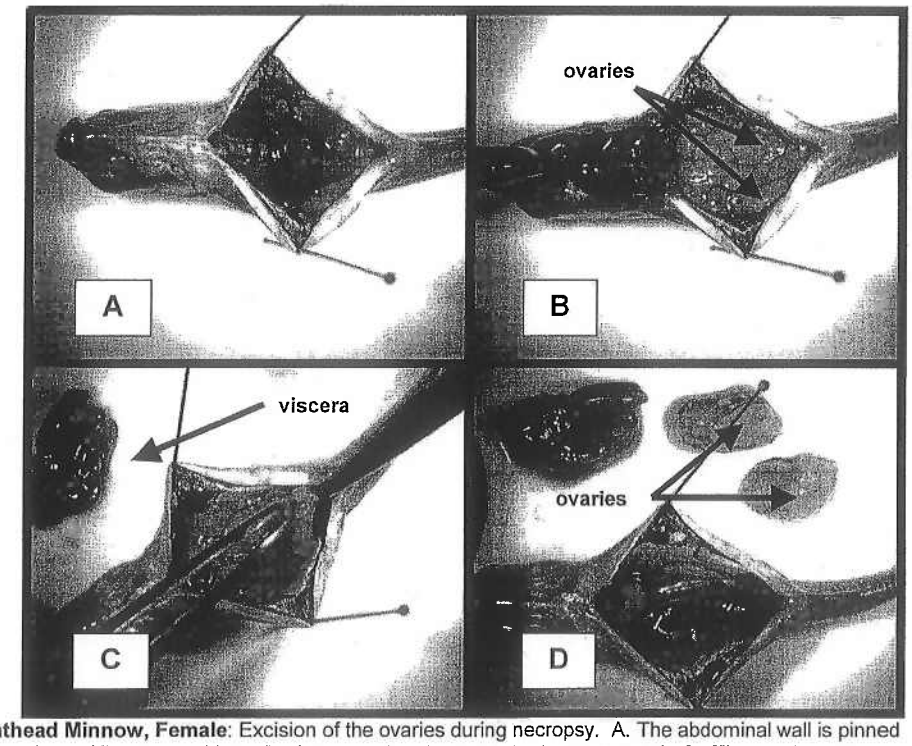
Alternatively, blood may also be collected by cardiac puncture using a heparinized syringe (1000 units of heparin per ml). The blood is transferred into Eppendorf tubes (held on ice) and then centrifuged (5 min, 7,000 g, room temperature). The plasma should be transferred into clean Eppendorf tubes (in aliquots if the volume of plasma makes this feasible) and promptly frozen at -75°C to -85°C, until analyzed (Panter et al., 1998).

APPENDIX B

Removal of gonads from Fathead minnows



Fathead Minnow, Male: Excision of the testes during necropsy.
A. The abdominal wall is pinned laterally. B. The terminal intestine is severed and retracted prior to removal. C. The testes are grasped near the spermatic ducts. D. Removal of the testes is complete.



Fathead Minnow, Female: Excision of the ovaries during necropsy. A. The abdominal wall is pinned laterally. B. The terminal intestine is severed and retracted prior to removal. C. The ovaries are grasped near the oviducts. D. Removal of the ovaries is complete.

APPENDIX C-1

Assessment of Secondary Sex Characteristics (Nuptial Tubercles) in EDC Tests with Fathead Minnows

Michael Kahl and Gerald Ankley

Overview

Potentially important characteristics of physical appearance in adult fathead minnows in endocrine disrupter testing include body color (i.e., light/dark), coloration patterns (i.e., presence or absence of vertical bands), body shape (i.e., shape of head and pectoral region, distension of abdomen), and specialized secondary sex characteristics (i.e., number and size of nuptial tubercles, size of dorsal pad and ovipositor).

Nuptial tubercles are located on the head (dorsal pad) of reproductively-active male fathead minnows, and are usually arranged in a bilaterally-symmetric pattern (Jensen et al. 2001). Control females and juvenile males and females exhibit no tubercle development (Jensen et al. 2001). There can be up to eight individual tubercles around the eyes and between the nares of the males. The greatest numbers and largest tubercles are located in two parallel lines immediately below the nares and above the mouth. In many fish there are groups of tubercles below the lower jaw; those closest to the mouth generally occur as a single pair, while the more ventral set can be comprised of up to four tubercles. The actual numbers of tubercles is seldom more than 30 (range, 18-28; Jensen et al. 2001). The predominant tubercles (in terms of numbers) are present as a single, relatively round structure, with the height approximately equivalent to the radius. Most reproductively-active males also have, at least some, tubercles which are enlarged and pronounced such that they are indistinguishable as individual structures.

Some types of endocrine-disrupting chemicals (EDCs) can cause the abnormal occurrence of certain secondary sex characteristics in the opposite sex; for example, androgen receptor agonists, such as 17α-methyltestosterone or 17β-trenbolone, can cause female fathead minnows to develop nuptial tubercles (Smith 1974; Ankley *et al.* 2001; 2003), while estrogen receptor agonists may decrease number or size of nuptial tubercles in males (Miles-Richardson et al. 1999; Harries et al. 2000).

This protocol describes characterization of nuptial tubercles in fathead minnows based on procedures used at the U.S. Environmental Protection Agency lab in Duluth, MN. Specific products and/or equipment can be substituted with comparable materials available in participating labs.

Protocol

Anesthetic

MS-222 is used as an anesthetic for fish sampling/assessment. Sodium bicarbonate is used as a buffering agent for the sedative.

Reagents:

MS-222 - Fenquel™ (Tricaine Methanesulfonate, Argent Chemical Laboratories, Redmond, WA 98052, USA).

Sodium bicarbonate - $NaHCO_3$, (J.T. Baker Inc., Phillipsburg, NJ 08865, USA).

Procedure:

1. Collect 1 L of control test water at nominal test temperature (e.g., 25°C) in a beaker
   -allocate 100 mg of MS-222 to weigh pan
   -allocate 200 mg of sodium bicarbonate to weigh pan
   1. Add weighed chemicals to control water and stir (ca., 1 minute)
   2. Transfer dissolved chemical solution to stainless steel bowl for easy fish handling
   3. Solution will accommodate 20 to 30 organisms (added individually); fresh solution will need to be prepared for additional animals

Sampling Methods

Procedure:

1. Using a 12.5cmX10cm (125mm) fine mesh nylon net, carefully net organism from culture or test chamber.
   - If handling toxicant-exposed fish, start with control fish and work up with increasing EDC concentrations.
2. Place organisms in MS-222 solution.
   - Activity level may be momentarily high with rapid swimming or darting. Activity will decrease but gill ventilation rate may become elevated or rapid.
3. Within about 1 minute fish will start to show loss of equilibrium.
   - Spiral or erratic swimming.
   - Loss of movement, listlessness.
   - Gentle probing with the net will cause little physical response. Organisms are still actively ventilating.
4. Remove fish from anesthetic with a net. Wipe excess moisture from net and fish into an absorbent towel. Gently place fish on petri dish - lack of movement occurs.
   - Fish should not be actively moving; muscle tissue should still be rigid without loss of character. Continued emersion into MS-222 may be required. If potency of MS-222 is not adequate, additional chemical ($\leq$ 10 mg) may be added to strengthen effectiveness.
5. Viewing is best accomplished using an illuminated magnifying glass or 3X illuminated dissection scope. View fish dorsally and anterior forward (head toward viewer).
   a. Place fish in small petri dish (e.g., 100 mm in diameter), anterior forward, ventral side down. Focus viewfinder to allow identification of tubercles. Gently and slowly roll fish from side to side to identify tubercle areas. Count and score tubercles.
   b. Repeat the observation on the ventral head surface by placing the fish dorsal anterior forward in the petri dish.
   c. Observations should be completed within 2 min for each fish.
   d. Return fish to control water to revive, if desired.

6. If fish are handled in a gentle manner within a reasonable amount of time during tubercle assessment recovery will occur within a few minutes without lasting adverse effects. To avoid mortality during and after this procedure be alert to the following details.
   - Keep fish moist during procedure.
   - Limit the amount of time used to score tubercles.
   - When placing fish into clean water gently move the fish back and forth, aiding water movement across the gill membranes.
7. If tubercles are assessed at test conclusion, animal may be subjected to additional sampling at this time (e.g., removal of blood for vitellogenin measurements; dissection of gonads).

Tubercle Counting and Rating

Six specific areas have been identified for assessment of tubercle presence and development in adult fathead minnows. A template was developed to map the location and quantity of tubercles present (attachment 1). The number of tubercles is recorded and their size can be quantitatively ranked as: 1-present, 2-enlarged and 3-pronounced for each organism (Fig. 1).

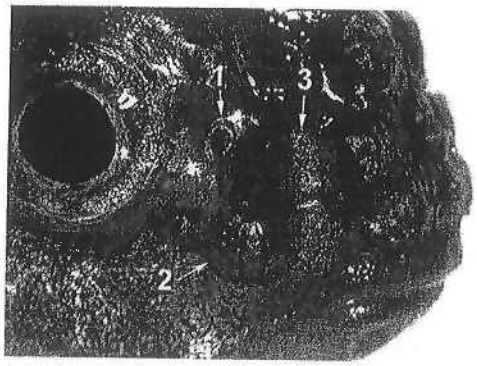


Figure1. The actual number of tubercles in some fish may be greater than the template boxes (Attachment 1) for a particular rating area. If this happens, additional rating numbers may be marked within, to the right or to the left of the box. The template therefore does not have to display symmetry. An additional technique for mapping tubercles which are paired or joined vertically along the horizontal plane of the mouth could be done by double-marking two tubercle rating points in a single box.

Rating 1-present, is identified as any tubercle having a single point whose height is nearly equivalent to its radius (diameter). Rating 2- enlarged; identified by tissue resembling an asterisk in appearance; usually has a large radial base with grooves or furrows emerging from the center. Tubercle height is often more jagged but can be somewhat rounded at times. Rating 3- pronounced; usually quite large and rounded with less definition in structure. At times these tubercles will run together forming a single mass along an individual or combination of areas (B, C and D, described below). Coloration and design are similar to rating 2 but at times are fairly indiscriminate. Using this rating system generally will result in overall tubercle scores of <50 in a normal control male possessing a tubercle count of 18 to 20 (Jensen et al. 2001).

Mapping regions:

A - Tubercles located around eye. Mapped dorsal to ventral around anterior rim of eye. Commonly multiple in mature control males, not present in control females, generally paired (one near each eye) or single in females exposed to androgens.

B - Tubercles located between nares, (sensory canal pores). Normally in pairs for control males at more elevated levels (2- enlarged or 3- pronounced) of development. Not present in control females with some occurrence and development in females exposed to androgens.

C - Tubercles located immediately anterior to nares, parallel to mouth. Generally enlarged or pronounced in mature control males. Present or enlarged in less developed males or androgen-treated females.

D - Tubercles located parallel along mouth line. Generally rated developed in control males. Absent in control females but present in androgen-exposed females.

E - Tubercles located on lower jaw, close to mouth, usually small and commonly in pairs. Varying in control or treated males, and treated females.

F - Tubercles located ventral to E. Commonly small and paired. Present in control males and androgen-exposed females.

ID_____

Tubercle Template

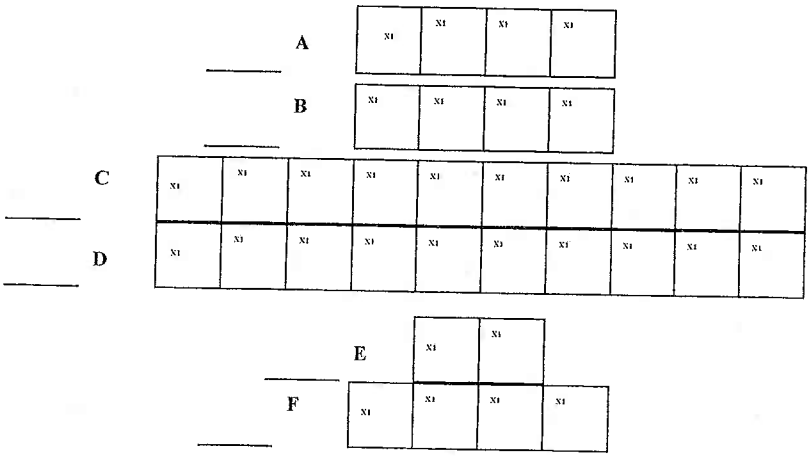
1-present

2-enlarged

3-pronounced

Numerical Rating **Date**

Total Score_____



APPENDIX C-2

Procedure for Determining Fatpad Weight

Fatpad Score

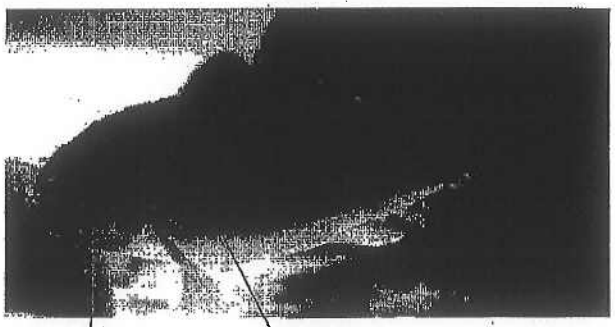
For evaluating the fatpads, use a very similar system as to that used in tubercle assessment:

1	No fatpad visible
2	Small fatpad evident
3	Fatpad is clearly visible and is just above body surface
4	Fatpad is prominent, and is clearly above the body surface, but not 'overhanging'
5	Fatpad is very prominent and is starting to 'overhang' the body surface

These evaluations are rather sufficient to identify chemical effects during exposure and should be accompanied by fatpad removal at the end of the experiment for a more accurate assessment of the fatpad.

Fatpad Index

The fatpad index is expressed as a percentage of the body weight, i.e. Fatpad index = fatpad weight/total wet body weight



Fatpad Index (FPI) – score around the edge of the fatpad using a scalpel and then starting from the dorsal fin and working towards the head, gently peel the fatpad away from the dorsal musculature. Be careful not to remove the muscle with the fatpad, as this will affect the overall weight. The fatpad consists of two regions, the head pad and the dorsal pad. Once you reach the head pad, stop, and then starting from the head and working back to the dorsal pad carefully slice of the head pad to the point where it is attached to the dorsal pad. Carefully sever any points at which the fatpad is still attached to the body of the fish. Weigh the fatpad.
Fatpad index (FPI) = (fatpad weight (mg)/total wet body weight (mg))*100

APPENDIX D

I. POST-MORTEM AND HISTOTECHNICAL PROCEDURES

The purpose of this section is to outline all of the post-mortem steps and procedures that occur prior to the evaluation of histologic sections on glass slides, to include euthanasia, necropsy, tissue fixation.

Post-mortem procedures:

1. Substrate obtained for vitellogenin analysis.
   a. FHM: blood sample from the caudal vein/artery or heart
2. Tissue specimen for gonad histopathology. For each species, a technique was selected that would most optimally: 1) preserve the cellular structure of the gonads; 2) maximize the amount of gonad tissue available for analysis; 3) sample the gonads in a representative and consistent fashion; and 4) allow the pathologist to examine at least three step sections of both gonads on a single glass slide.
   a. FHM: gonads excised from fish.

Davidson's fixative was selected as the recommended fixative. Compared to other common fixatives such as 10% neutral buffered formalin or Bouin's fixative, the advantages of Davidson's fixative are: 1) the morphologic appearance of gonad sections is generally considered to be comparable to sections fixed in Bouin's fixative and superior to sections fixed in formalin; 2) compared to Bouin's fixative, which contains picric acid, Davidson's fixative is generally considered to be less noxious, less hazardous, and more easily disposed of; 3) there is anecdotal information which suggests that Bouin's fixative may be difficult to obtain in the near future; 4) specimens fixed in Bouin's fixative require multiple rinses prior to transfer to alcohol or formalin. Please see photographic comparison of specimens fixed in Davidson's versus Bouin's fixatives (Histology Figures 1 provided below). Please be aware that different recipes and products that are designated as "Davidson's fixative" may actually be modifications of the original formula if a modified Davidson's fixative is used, this should be noted by the participating laboratory.
Dividson's recipe is provided below:

Davidson's Fixative

Formaldehyde (37-40%)	200 ml
Glycerol	100 ml
Glacial acetic acid	100 ml
Absolute alcohol	300 ml
Distilled water	300 ml

Modified Davidson's Fixative

Formaldehyde (37-40%)	220 ml
Glacial acetic acid	115 ml
95% Ethyl alcohol	330 ml
Distilled water	335 ml

HISTOLOGY FIGURES 1

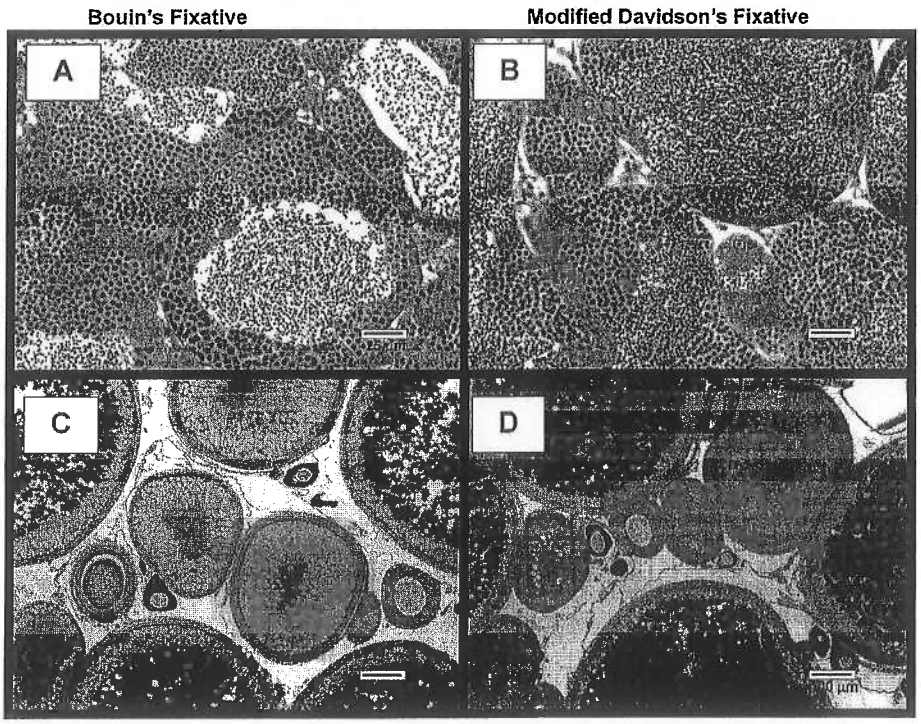


Fig. 1. Fathead Minnows, Testis (A&B) and Ovary (C&D): Gonads fixed in Bouin's fixative (A&C) and modified Davidson's fixative (B&D). Color contrast was slightly superior in testes fixed with Davidson's fixative and was clearly superior in ovaries fixed with Bouin's fixative. Either fixative is satisfactory for diagnostic purposes; however, Davidson's fixative was selected for the Phase 1B assay.

Fathead Minnow

1. Euthanasia, Necropsy, and Tissue Fixation

Objectives:

1. Provide for the humane sacrifice of fish.
2. Obtain necessary body weights and measurements.
3. Obtain specimens for vitellogenin analysis.
4. Excise gonad specimens.
5. Evaluate secondary sex characteristics.
6. Provide for adequate fixation of the gonads and the carcass.

Materials:

1. Fish transport container (Approx. ~500 ml, contains water from the experimental tank or system reservoir).
2. Small dip net.
3. Euthanasia chamber (Approx. ~500 ml vessel).
4. Euthanasia solution
5. Electronic slide caliper (minimum display: ≤ 0.1mm)
6. Electronic analytical balance (minimum display: ≤ 0.1mg) and tared vessels.
7. Stereoscopic microscope.
8. Pins and corkboard.
9. Small scissors (e.g., iris scissors).
10. Small forceps.
11. Microdissection forceps.
12. Microdissection scissors.
13. Gauze sponges.
14. Davidson's fixative
15. Plastic syringe (3ml).
16. Standard plastic tissue cassettes (one per fish).
17. Fixation containers (100 ml, one per fish).

Procedures:

1. Fish should be sacrificed within one to two minutes prior to necropsy. Therefore, unless multiple prosectors are available, multiple fish should not be sacrificed simultaneously.
2. Using the small dip net, a fish is removed from the experimental chamber and transported to the necropsy area in the transport container. For each test chamber, all male fish are sacrificed prior to the sacrifice of female fish; the sex of each fish is determined by external body characteristics (e.g., presence or absence of nuptial tubercles, dorsal pad, etc.).

1. The fish is placed into the euthanasia solution. The fish is removed from the solution when there is cessation of respiration and the fish is unresponsive to external stimuli.
2. The fish is wet weighed, measured according to protocol, and a blood sample is obtained from the caudal artery/vein or heart.
3. The fish is placed on a corkboard on the stage of a dissecting microscope. Using iris scissors and small forceps, the abdomen is opened via a carefully made incision that extends along the ventral midline from the pectoral girdle to a point just cranial to the anus.

4. The fish is placed in dorsal recumbency and the opposing flaps of body wall are pinned laterally to expose the abdominal viscera.
5. Using the small forceps and small scissors, the abdominal viscera (liver, gastrointestinal tract, spleen, pancreatic tissue, and abdominal mesentery) are carefully removed *en masse* in the following manner:
    a. The intestine is severed proximal to the anus.
    b. A forceps is applied to the terminal portion of the intestine. Using gentle traction and taking care not to disturb the gonads, the viscera are dissected out of the abdominal cavity in a caudal to cranial direction.
    c. The distal esophagus is severed just proximal to the liver.
6. Using a syringe, approximately 0.5 ml of Davidson's fixative is then gently applied to the gonads *in situ*. Approximately 90 seconds following the application of fixative, the liquid fixative within the abdomen is removed with a gauze sponge, and the gonads are excised in a manner similar to the abdominal viscera:
    a. Using the microdissection scissors, the spermatic ducts or oviducts are severed proximal to the genital pore.
    b. Microdissection forceps are then applied to the spermatic ducts/oviducts. Using gentle traction, the gonads are dissected out of the abdominal cavity in a caudal to cranial direction, severing the mesorchial/mesovarial attachments as needed using the microdissection scissors. The left and right gonads may be excised individually or they may be excised simultaneously and subsequently divided at their caudal attachment.
7. The gonads (right and left) are placed into a pre-labeled plastic tissue cassette which is then placed into an individual container of Davidson's fixative accompanied by the abdominal viscera. The volume of fixative in the container should be at least 10 times the approximated volume of the tissues. The fixative container is gently agitated for five seconds to dislodge air bubbles from the cassette.
8. Using the carcass, the secondary sex characteristics are assessed (e.g., dorsal nape pad, nuptial tubercles
9. All tissues remain in Davidson's fixative overnight, followed by transfer to individual containers of 10% neutral buffered formalin the next day. Containers with cassettes are gently agitated for 5 seconds to ensure adequate penetration of formalin into cassettes (it is not necessary to rinse with water or perform multiple changes in formalin).

APPENDIX E: EXAMPLE PRODUCT GUIDE

Example Product	Catalogue #	Supplier/Manufacturer
Clear Rite-3™	6901	Richard Allen Medical Industries 8850 M89 Box 351 Richland, MI 49083 800-522-7270 http://www.rallansci.com.
Coverglass, 24x50 premier nonstick Thinness: 0.13mm-0.17mm	00145-ACS	Surgipath Medical Industries, Inc. P. O. Box 528 Richmond, IL 60071 800-225-3035
Davidson's Fixative	S2250	Poly Scientific R&D Corp. 70 Cleveland Avenue Bay Shore, NY 11706 631-586-0400
Decalcifier: Formical-2000®	1354	Decal Chemical Corp. PO Box 916 Tallman, NY 10982-0916 800-428-5856
Eosin Y (for H&E Stain) Eosin-Y Reagent Alcohol Deionized Water Glacial Acetic Acid	7111	Richard Allen Medical Industries 8850 M89 Box 351 Richland, MI 49083 800-522-7270 http://www.rallansci.com.
Hematoxylin 2 (for H&E Stain) Hematoxylin Aluminum Sulfate Sodium Iodate Ethylene Glycol Deionized Water Glacial Acetic Acid	7231	Richard Allen Medical Industries 8850 M89 Box 351 Richland, MI 49083 800-522-7270 http://www.rallansci.com.
MS-222 Fenquel™ (Tricaine Methanesulfonate)	C-FINQ-UE	Argent Chemical Laboratories, Redmond, WA 98052, USA.
Paraplast® (CSMP) Kendall Paraplast Tissue Embedding Medium 8889 501006	SHM8889-501006	Supplier: Laboratory Supply Co. 800-888-9004 Manufacturer: Sherwood Services AG Tyco Healthcare Group L 15 Hampshire Street Mansfield, MA 02048
Permount® Mounting Media Toluene 55% BHT < 1% Polymer Alpha pinene & Betapinene 45%	SP15-500	Fisher HealthCare 800-640-0640
Slide, Single Frosted, ground edge Crystal Line Premier Brand	8105	C&A Scientific Co., Inc. 7241 Gabe Court Manassas, VA 20109 703-330-1413

APPENDIX 2 - RAW DATA

Male termination endpoints during the 21-day exposure of fathead minnow (*Pimephales promelas*) to flutamide.

Treatment	ID	Sex	Tubercle score	Length (mm)	Wet Weight (g)	Gonad weight (g)	GSI	Fatpad Weight (g)	FPI	replicate mean tubercle score	treatment mean tubercle score	treatment std dev	replicate mean length (mm)	treatment mean length (mm)	treatment std dev	replicate mean weight (g)	treatment mean weight (g)	treatment std dev	replicate mean GSI	treatment mean GSI	treatment std dev
Control	6AM1	M	26	64.5	3.37	0.0350	1.04	0	0	**26.5**	**25.1**	1.93	**62.8**	**62.1**	2.51	**3.12**	**2.99**	0.385	**1.04**	**1.29**	0.248
Control	6AM2	M	27	61.0	2.87	0.0296	1.03	0.0473	1.65												
Control	6BM1	M	17	61.8	2.91	0.0591	2.03	0	0	**23.0**			**60.0**			**2.71**			**1.62**		
Control	6BM2	M	29	58.1	2.51	0.0305	1.21	0	0												
Control	6CM1	M	32	58.9	2.44	0.0340	1.39	0.0249	1.02	**24.0**			**60.3**			**2.65**			**1.31**		
Control	6CM2	M	30	65.4	3.65	0.0456	1.25	0	0												
Control[a]	6CM3	M	10	56.6	1.87	0.0242	1.30	0	0												
Control	6DM1	M	23	63.9	3.52	0.0413	1.17	0.0167	0.474	**27.0**			**65.4**			**3.48**			**1.19**		
Control	6DM2	M	31	66.8	3.43	0.0414	1.21	0.0191	0.556												
100 µg a.i./L	5AM1	M	33	62.4	2.72	0.0279	1.02	0.0286	1.05	**26.5**	**27.4**	7.62	**64.4**	**65.2**	1.90	**3.01**	**3.21**	0.169	**1.04**	**1.32**	0.227
100 µg a.i./L	5AM2	M	20	66.5	3.29	0.0347	1.05	0	0												
100 µg a.i./L	5BM1	M	33	66.0	3.09	0.0515	1.67	0	0	**19.0**			**68.1**			**3.40**			**1.46**		
100 µg a.i./L	5BM2	M	5	70.1	3.72	0.0464	1.25	0	0												
100 µg a.i./L	5CM1	M	27	65.1	3.07	0.0352	1.15	0	0	**26.5**			**64.4**			**3.14**			**1.23**		
100 µg a.i./L	5CM2	M	26	63.6	3.21	0.0423	1.32	0	0												
100 µg a.i./L	5DM1	M	30	65.4	3.38	0.0634	1.87	0	0	**37.5**			**64.0**			**3.27**			**1.54**		
100 µg a.i./L	5DM2	M	45	62.6	3.16	0.0382	1.21	0	0												
500 µg a.i./L	3AM1	M	26	64.1	3.32	0.0372	1.12	0	0	**31.0**	**24.9**	4.17	**65.8**	**63.1**	2.27	**3.51**	**3.13**	0.375	**1.19**	**1.34**	0.215
500 µg a.i./L	3AM2	M	36	67.5	3.70	0.0466	1.26	0	0												
500 µg a.i./L	3BM1	M	18	59.7	2.69	0.0331	1.23	0.0413	1.54	**22.0**			**60.4**			**2.64**			**1.14**		
500 µg a.i./L	3BM2	M	26	61.2	2.60	0.0273	1.05	0	0												
500 µg a.i./L	3CM1	M	21	62.7	2.70	0.0410	1.52	0	0	**24.0**			**62.3**			**3.04**			**1.45**		
500 µg a.i./L	3CM2	M	27	61.8	3.38	0.0465	1.37	0	0												
500 µg a.i./L	3DM1	M	27	64.4	3.30	0.0513	1.55	0	0	**22.5**			**63.9**			**3.31**			**1.59**		
500 µg a.i./L	3DM2	M	18	63.4	3.32	0.0543	1.64	0	0												
1000 µg a.i./L	1AM1	M	29	66.8	3.69	0.0588	1.59	0	0	**30.0**	**26.5**	3.24	**66.2**	**65.0**	1.08	**3.35**	**3.22**	0.249	**1.52**	**1.37**	0.184
1000 µg a.i./L	1AM2	M	31	65.6	3.00	0.0435	1.45	0	0												
1000 µg a.i./L	1BM1	M	20	66.7	3.60	0.0393	1.09	0	0	**24.0**			**65.7**			**3.30**			**1.37**		
1000 µg a.i./L	1BM2	M	28	64.6	3.01	0.0495	1.65	0	0												
1000 µg a.i./L	1CM1	M	38	65.0	3.08	0.0396	1.29	0	0	**28.5**			**64.1**			**2.85**			**1.48**		
1000 µg a.i./L	1CM2	M	19	63.3	2.63	0.0441	1.68	0	0												
1000 µg a.i./L	1DM1	M	35	63.6	3.15	0.0405	1.29	0	0	**23.5**			**64.1**			**3.39**			**1.11**		
1000 µg a.i./L	1DM2	M	12	64.6	3.63	0.0341	0.938	0	0												

[a] This fish was added to the exposure system as a female fish based on morphological features. At test termination, this fish was dissected and identified as a male (based on presence of testes).
NOTE: Male fish had a fatpad score of 1 (no fatpad visible). Male fish were sexually active but insufficient fatpad was present to allow removal and weighing, therefore, a fatpad index (FPI) of 0 was recorded.

Male termination endpoints during the 21-day exposure of fathead minnow (*Pimephales promelas*) to potassium permanganate.

Treatment	ID	Sex	Tubercle score	Length (mm)	Wet Weight (g)	Gonad weight (g)	GSI	Fatpad Weight (g)	FPI	replicate mean tubercle score	treatment mean tubercle score	treatment std dev	replicate mean length (mm)	treatment mean length (mm)	treatment std dev	replicate mean weight (g)	treatment mean weight (g)	treatment std dev	replicate mean GSI	treatment mean GSI	treatment std dev
Control	6AM1	M	30	63.1	3.13	0.0354	1.13	0	0	**30.5**	**29.6**	5.69	**62.1**	**61.4**	4.77	**3.06**	**3.02**	0.552	**1.19**	**1.11**	0.0550
Control	6AM2	M	31	61.1	2.99	0.0371	1.24	0	0												
Control	6BM1	M	27	54.2	1.97	0.0259	1.32	0	0	**27.5**			**55.6**			**2.34**			**1.09**		
Control	6BM2	M	28	57.0	2.71	0.0237	0.875	0	0												
Control	6CM1	M	35	60.9	3.19	0.0204	0.640	0	0	**37.0**			**60.5**			**3.01**			**1.06**		
Control	6CM2	M	39	60.2	2.83	0.0418	1.48	0	0												
Control	6DM1	M	29	66.3	3.95	0.0338	0.856	0.119	3.02	**23.5**			**67.2**			**3.69**			**1.08**		
Control	6DM2	M	18	68.2	3.43	0.0449	1.31	0	0												
225 µg a.i./L	5AM1	M	29	65.2	3.94	0.0314	0.797	0.0832	2.11	**31.5**	**27.5**	3.35	**65.2**	**62.8**	4.44	**3.64**	**3.20**	0.865	**0.94**	**1.04**	0.176
225 µg a.i./L	5AM2	M	34	65.2	3.33	0.0359	1.08	0	0												
225 µg a.i./L	5BM1	M	25	62.9	2.97	0.0246	0.830	0.0776	2.62	**23.5**			**60.9**			**2.66**			**0.84**		
225 µg a.i./L	5BM2	M	22	58.9	2.35	0.0200	0.849	0	0												
225 µg a.i./L	5CM1	M	26	66.1	3.30	0.0322	0.975	0.135	4.1	**26.7**			**57.5**			**2.31**			**1.17**		
225 µg a.i./L	5CM2	M	34	58.2	2.36	0.0225	0.955	0	0												
225 µg a.i./L	5CM3	M[b]	20	48.3	1.28	0.0201	1.58	0	0												
225 µg a.i./L	5DM1	M	33	70.7	5.67	0.0493	0.870	0.316	5.57	**28.5**			**67.5**			**4.19**			**1.20**		
225 µg a.i./L	5DM2	M	24	64.2	2.71	0.0414	1.53	0	0												
450 µg a.i./L	3AM1	M	25	61.8	2.76	0.0378	1.37	0	0	**23.5**	**26.1**	4.44	**62.3**	**64.8**	1.92	**2.85**	**3.39**	0.429	**1.35**	**1.50**	0.176
450 µg a.i./L	3AM2	M	22	62.8	2.95	0.0394	1.34	0	0												
450 µg a.i./L	3BM1	M	24	64.1	3.37	0.0550	1.63	0	0	**22.0**			**64.4**			**3.24**			**1.76**		
450 µg a.i./L	3BM2	M	20	64.7	3.10	0.0584	1.89	0	0												
450 µg a.i./L	3CM1	M	27	66.1	3.80	0.0571	1.50	0	0	**27.0**			**66.4**			**3.72**			**1.46**		
450 µg a.i./L	3CM2	M	27	66.8	3.65	0.0519	1.42	0	0												
450 µg a.i./L	3DM1	M	28	64.3	3.64	0.0737	2.02	0	0	**32.0**			**66.2**			**3.75**			**1.44**		
450 µg a.i./L	3DM2	M	36	68.1	3.86	0.0332	0.861	0	0												
900 µg a.i./L	1AM1	M	27	64.6	3.46	0.0458	1.32	0.0977	2.82	**27.0**	**22.8**	6.43	**64.6**	**62.9**	2.74	**3.46**	**2.94**	0.528	**1.32**	**1.44**	0.176
900 µg a.i./L	1AM2	M	[a]	[a]	[a]	[a]	[a]														
900 µg a.i./L	1BM1	M	36	66.22	3.52	0.0509	1.45	0	0	**29.5**			**64.0**			**3.04**			**1.69**		
900 µg a.i./L	1BM2	M	23	61.76	2.57	0.0498	1.94	0	0												
900 µg a.i./L	1CM1	M	24	57.97	2.35	0.0451	1.92	0	0	**16.3**			**58.8**			**2.21**			**1.42**		
900 µg a.i./L	1CM2	M	13	62.87	2.46	0.0248	1.01	0	0												
900 µg a.i./L	1CM3	M[b]	28	61.73	2.69	0.0568	2.11	0	0												
900 µg a.i./L	1CM4	M[b]	0	52.7	1.31	0.00870	0.662	0	0												
900 µg a.i./L	1DM1	M	22	62.63	3.03	0.0567	1.87	0	0	**18.5**			**64.2**			**3.05**			**1.32**		
900 µg a.i./L	1DM2	M	15	65.81	3.06	0.0234	0.765	0	0												

[a] Male fish died prior to test termination.

[b] These three fish were added to the exposure system as female fish based on morphological features. At test termination, these three fish were dissected and were identified as males (based on presence of testes).

NOTE: Male fish had a fatpad score of 1 (no fatpad visible). Male fish were sexually active but insufficient fatpad was present to allow removal and weighing, therefore, a fatpad index (FPI) of 0 was recorded.

Male termination endpoints during the 21-day exposure of fathead minnow (*Pimephales promelas*) to ketoconazole.

Treatment	ID	Sex	Tubercle score	Length (mm)	Wet Weight (g)	Gonad weight (g)	GSI	Fatpad Weight (g)	FPI	replicate mean tubercle score	treatment mean tubercle score	treatment t std dev	replicate mean length (mm)	treatment mean length (mm)	treatment std dev	replicate mean weight (g)	treatment mean weight (g)	treatment std dev	replicate mean GSI	treatment mean GSI	treatment std dev
Control	6AM1	M	38	68.9	4.01	0.0398	0.993	0	0	**42.0**	**32.4**	8.26	**69.2**	**66.4**	2.37	**3.81**	**3.49**	0.369	**1.16**	**1.01**	0.205
Control	6AM2	M	46	69.5	3.62	0.0478	1.32	0	0												
Control	6BM1	M	27	66.2	3.83	0.0362	0.946	0	0	**27.0**			**64.5**			**3.34**			**0.87**		
Control	6BM2	M	27	62.7	2.85	0.0225	0.790	0	0												
Control	6CM1	M	43	71.1	4.36	0.0370	0.849	0	0	**36.5**			**67.6**			**3.78**			**1.22**		
Control	6CM2	M	30	64.1	3.19	0.0508	1.59	0	0												
Control	6DM1	M	26	65.6	3.57	0.0258	0.724	0.149	4.17	**24.0**			**64.5**			**3.04**			**0.81**		
Control	6DM2	M	22	63.3	2.52	0.0226	0.896	0	0												
25 µg a.i./L	5AM1	M	34	67.6	3.63	0.0526	1.45	0	0	**32.0**	**28.8**	2.53	**64.2**	**68.1**	3.89	**3.19**	**3.74**	0.622	**0.910**	**1.02**	0.194
25 µg a.i./L	5AM2	M	30	60.8	2.75	0.0103	0.375	0.187	6.8												
25 µg a.i./L	5BM1	M	28	72.2	4.36	0.0359	0.823	0	0	**29.5**			**72.3**			**4.26**			**0.92**		
25 µg a.i./L	5BM2	M	31	72.4	4.16	0.0426	1.02	0	0												
25 µg a.i./L	5CM1	M	32	68.6	4.12	0.0333	0.808	0	0	**26.5**			**65.5**			**3.20**			**0.95**		
25 µg a.i./L	5CM2	M	21	62.3	2.28	0.0248	1.09	0	0												
25 µg a.i./L	5DM1	M	36	73.9	4.98	0.0592	1.19	0	0	**27.0**			**70.5**			**4.29**			**1.31**		
25 µg a.i./L	5DM2	M	18	67.1	3.59	0.0517	1.44	0	0												
100 µg a.i./L	3AM1	M	40	71.5	4.04	0.0372	0.921	0	0	**33.0**	**31.3**	2.53	**70.5**	**70.0**	1.32	**3.93**	**3.81**	0.319	**1.20**	**1.38**	0.164
100 µg a.i./L	3AM2	M	26	69.4	3.81	0.0563	1.48	0	0												
100 µg a.i./L	3BM1	M	31	73.6	5.03	0.0342	0.680	0	0	**27.5**			**70.0**			**4.00**			**1.46**		
100 µg a.i./L	3BM2	M	24	66.3	2.97	0.0663	2.23	0	0												
100 µg a.i./L	3CM1	M	27	68.6	3.66	0.0503	1.37	0	0	**32.5**			**71.3**			**3.99**			**1.57**		
100 µg a.i./L	3CM2	M	38	74.0	4.33	0.0763	1.76	0	0												
100 µg a.i./L	3DM1	M	27	68.8	3.42	0.0537	1.57	0	0	**32.0**			**68.2**			**3.34**			**1.30**		
100 µg a.i./L	3DM2	M	37	67.6	3.26	0.0338	1.04	0	0												
400 µg a.i./L	1AM1	M	38	71.9	4.23	0.0631	1.49	0	0	**32.0**	**38.4**	12.50	**71.0**	**70.9**	0.83	**3.98**	**3.88**	0.142	**1.36**	**1.68**	0.262
400 µg a.i./L	1AM2	M	26	70.0	3.74	0.0461	1.23	0	0												
400 µg a.i./L	1BM1	M	32	72.1	4.25	0.0801	1.89	0	0	**30.5**			**70.3**			**3.89**			**1.57**		
400 µg a.i./L	1BM2	M	29	68.5	3.53	0.0446	1.26	0	0												
400 µg a.i./L	1CM1	M	33	70.0	3.38	0.0544	1.61	0	0	**35.0**			**70.4**			**3.68**			**1.89**		
400 µg a.i./L	1CM2	M	37	70.8	3.98	0.0860	2.16	0	0												
400 µg a.i./L	1DM1	M	57	72.1	3.98	0.0759	1.91	0	0	**57.0**			**72.1**			**3.98**			**1.91**		
400 µg a.i./L	1DM2	M	[a]	[a]	[a]	[a]	[a]		0												

[a] This fish was added to the exposure system as a male fish based on morphological features. At test termination, this fish was dissected and identified as a female (based on the presence of ovaries).
NOTE: Male fish had a fatpad score of 1 (no fatpad visible). Male fish were sexually active but insufficient fatpad was present to allow removal and weighing, therefore, a fatpad index (FPI) of 0 was recorded.

Female termination endpoints during the 21-day exposure of fathead minnows (*Pimephales promelas*) to flutamide.

Treatment	ID	Sex	Length (mm)	Wet Weight (g)	Gonad weight (g)	GSI	replicate mean length (mm)	treatment mean length (mm)	treatment std dev	replicate mean weight (g)	treatment mean weight (g)	treatment std dev	replicate mean GSI	treatment mean GSI	treatment std dev
Control	6AF1	F	49.7	1.31	0.102	7.80	**51.8**	50.6	1.02	**1.38**	1.25	0.0860	**8.95**	8.59	1.01
Control	6AF2	F	53.1	1.43	0.0871	6.07									
Control	6AF3	F	51.5	1.33	0.203	15.2									
Control	6AF4	F	52.7	1.44	0.0965	6.69									
Control	6BF1	F	49.2	1.14	0.117	10.3	**49.5**			**1.19**			**7.99**		
Control	6BF2	F	53.0	1.43	0.123	8.59									
Control	6BF3	F	48.0	1.10	0.0358	3.26									
Control	6BF4	F	47.9	1.08	0.107	9.83									
Control	6CF1	F	50.3	1.22	0.0946	7.78	**50.1**			**1.21**			**7.59**		
Control	6CF2	F	49.9	1.27	0.0996	7.86									
Control	6CF3	F	49.9	1.14	0.0809	7.13									
Control	6CF4	F	[b]	[b]	[b]	[b]									
Control	6DF1	F	51.7	1.37	0.172	12.6	**51.2**			**1.24**			**9.85**		
Control	6DF2	F	49.4	1.10	0.0847	7.67									
Control	6DF3	F	52.5	1.26	0.117	9.30									
Control	6DF4	F	[a]	[a]	[a]	[a]									
100 µg a.i./L	5AF1	F	49.8	1.32	0.181	13.7	**47.3**	50.1	2.09	**1.05**	1.19	0.0930	**9.58**	8.90	2.33
100 µg a.i./L	5AF2	F	46.5	0.898	0.0657	7.32									
100 µg a.i./L	5AF3	F	44.6	0.848	0.0764	9.01									
100 µg a.i./L	5AF4	F	48.5	1.15	0.0952	8.30									
100 µg a.i./L	5BF1	F	51.9	1.23	0.130	10.5	**50.1**			**1.25**			**11.9**		
100 µg a.i./L	5BF2	F	45.5	1.02	0.168	16.4									
100 µg a.i./L	5BF3	F	51.4	1.33	0.180	13.6									
100 µg a.i./L	5BF4	F	51.5	1.40	0.0986	7.03									
100 µg a.i./L	5CF1	F	49.0	1.13	0.0586	5.20	**50.7**			**1.24**			**7.24**		
100 µg a.i./L	5CF2	F	54.1	1.52	0.132	8.70									
100 µg a.i./L	5CF3	F	51.5	1.32	0.124	9.38									
100 µg a.i./L	5CF4	F	48.2	0.984	0.0559	5.68									
100 µg a.i./L	5DF1	F	50.9	1.13	0.0620	5.47	**52.4**			**1.23**			**6.87**		
100 µg a.i./L	5DF2	F	53.1	1.42	0.103	7.30									
100 µg a.i./L	5DF3	F	53.1	1.16	0.0906	7.84									
100 µg a.i./L	5DF4	F	[c]	[c]	[c]	[c]									

[a] Female fish died prior to test termination.
[b] This fish was added to the exposure system as a female fish based on morphological features. At test termination, this fish was dissected and identified as a male (based on presence of testes).
[c] During the pre-spawn phase, one of the female fish was identified as a male and removed to keep the sex ratio at 2 males:3 females.
[d] Female fish was bloated. Ovaries were ruptured and could not be removed.

NOTE: No female fish had fatpads. Therefore, no data was collected for this endpoint.

Continued. Female termination endpoints during the 21-day exposure of fathead minnows (*Pimephales promelas*) to flutamide.

Treatment	ID	Sex	Length (mm)	Wet Weight (g)	Gonad weight (g)	GSI	replicate mean length (mm)	treatment mean length (mm)	treatment std dev	replicate mean weight (g)	treatment mean weight (g)	treatment std dev	replicate mean GSI	treatment mean GSI	treatment std dev
500 µg a.i./L	3AF1	F	51.7	1.44	0.161	11.2	**50.6**	49.1	1.10	**1.31**	1.17	0.0980	**9.22**	9.93	0.980
500 µg a.i./L	3AF2	F	46.8	1.03	0.141	13.6									
500 µg a.i./L	3AF3	F	54.3	1.66	0.131	7.92									
500 µg a.i./L	3AF4	F	49.5	1.11	0.0462	4.15									
500 µg a.i./L	3BF1	F	50.0	1.20	0.0782	6.52	**48.8**			**1.13**			**9.14**		
500 µg a.i./L	3BF2	F	47.6	1.05	0.0667	6.34									
500 µg a.i./L	3BF3	F	49.1	1.12	0.159	14.2									
500 µg a.i./L	3BF4	F	48.4	1.13	0.108	9.54									
500 µg a.i./L	3CF1	F	47.6	1.14	0.128	11.2	**49.1**			**1.17**			**10.1**		
500 µg a.i./L	3CF2	F	48.8	1.08	0.0949	8.82									
500 µg a.i./L	3CF3	F	50.5	1.23	0.103	8.36									
500 µg a.i./L	3CF4	F	49.5	1.22	0.147	12.0									
500 µg a.i./L	3DF1	F	52.5	1.28	0.115	8.99	**47.9**			**1.09**			**11.3**		
500 µg a.i./L	3DF2	F	47.2	1.08	0.161	14.8									
500 µg a.i./L	3DF3	F	44.1	0.905	0.0898	9.93									
500 µg a.i./L	3DF4	F	[c]	[c]	[c]	[c]									
1000 µg a.i./L	1AF1	F	53.9	2.23	[d]	0[d]	**50.9**	50.0	0.61	**1.46**	1.24	0.144	**6.44**	9.15	1.85
1000 µg a.i./L	1AF2	F	51.2	1.58	0.207	13.1									
1000 µg a.i./L	1AF3	F	52.8	1.22	0.0336	2.76									
1000 µg a.i./L	1AF4	F	45.6	0.811	0.0804	9.91									
1000 µg a.i./L	1BF1	F	50.4	1.15	0.133	11.5	**49.8**			**1.19**			**10.4**		
1000 µg a.i./L	1BF2	F	49.5	1.13	0.0871	7.74									
1000 µg a.i./L	1BF3	F	49.7	1.30	0.136	10.5									
1000 µg a.i./L	1BF4	F	49.7	1.17	0.140	11.9									
1000 µg a.i./L	1CF1	F	51.0	1.24	0.0999	8.08	**49.8**			**1.16**			**9.54**		
1000 µg a.i./L	1CF2	F	49.1	1.08	0.0827	7.67									
1000 µg a.i./L	1CF3	F	49.2	1.07	0.124	11.7									
1000 µg a.i./L	1CF4	F	50.0	1.26	0.135	10.7									
1000 µg a.i./L	1DF1	F	50.0	1.10	0.111	10.1	**49.5**			**1.17**			**10.2**		
1000 µg a.i./L	1DF2	F	47.6	0.927	0.109	11.7									
1000 µg a.i./L	1DF3	F	51.9	1.41	0.167	11.9									
1000 µg a.i./L	1DF4	F	48.3	1.26	0.0891	7.07									

[a] Female fish died prior to test termination.
[b] This fish was added to the exposure system as a female fish based on morphological features. At test termination, this fish was dissected and identified as a male (based on presence of testes).
[c] During the pre-spawn phase, one of the female fish was identified as a male and removed to keep the sex ratio at 2 males:3 females.
[d] Female fish was bloated. Ovaries were ruptured and could not be removed.

NOTE: No female fish had fatpads. Therefore, no data was collected for this endpoint.

Female termination endpoints during the 21-day exposure of fathead minnow (*Pimephales promelas*) to potassium permanganate.

Treatment	ID	Sex	Length (mm)	Wet Weight (g)	Gonad weight (g)	GSI	replicate mean length (mm)	treatment mean length (mm)	treatment std dev	replicate mean weight (g)	treatment mean weight (g)	treatment std dev	replicate mean GSI	treatment mean GSI	treatment std dev
Control	6AF1	F	53.7	1.45	0.221	15.3	**47.7**	48.7	2.75	**1.10**	1.17	0.179	**13.8**	11.8	1.61
Control	6AF2	F	45.1	0.899	0.0698	7.77									
Control	6AF3	F	46.4	0.996	0.164	16.5									
Control	6AF4	F	45.4	1.05	0.164	15.6									
Control	6BF1	F	53.8	1.76	0.172	9.76	**51.1**			**1.38**			**12.4**		
Control	6BF2	F	52.5	1.37	0.151	11.0									
Control	6BF3	F	49.2	1.20	0.156	13.0									
Control	6BF4	F	49.0	1.20	0.189	15.7									
Control	6CF1	F	45.4	1.00	0.121	12.0	**45.3**			**0.960**			**10.9**		
Control	6CF2	F	43.9	0.873	0.0916	10.5									
Control	6CF3	F	47.5	1.09	0.148	13.6									
Control	6CF4	F	44.3	0.876	0.0658	7.51									
Control	6DF1	F	50.4	1.20	0.144	12.0	**50.7**			**1.22**			**10.2**		
Control	6DF2	F	47.6	0.954	0.0884	9.27									
Control	6DF3	F	51.2	1.33	0.110	8.30									
Control	6DF4	F	53.4	1.42	0.156	11.0									
225 µg a.i./L	5AF1	F	45.0	0.862	0.126	14.6	**48.1**	50.1	2.98	**1.11**	1.28	0.253	**13.6**	12.6	2.01
225 µg a.i./L	5AF2	F	48.9	1.19	0.187	15.7									
225 µg a.i./L	5AF3	F	50.6	1.27	0.131	10.3									
225 µg a.i./L	5AF4	F	[a]	[a]	[a]	[a]									
225 µg a.i./L	5BF1	F	56.8	1.67	0.163	9.77	**53.7**			**1.56**			**11.1**		
225 µg a.i./L	5BF2	F	50.6	1.37	0.183	13.3									
225 µg a.i./L	5BF3	F	59.9	2.08	0.224	10.8									
225 µg a.i./L	5BF4	F	47.2	1.13	0.121	10.6815									
225 µg a.i./L	5CF1	F	[b]	[b]	[b]	[b]	**47.1**			**1.03**			**10.7**		
225 µg a.i./L	5CF2	F	49.1	1.27	0.171	13.5									
225 µg a.i./L	5CF3	F	44.9	0.901	0.0830	9.22									
225 µg a.i./L	5CF4	F	47.4	0.932	0.0870	9.34									
225 µg a.i./L	5DF1	F	51.3	1.43	0.201	14.1	**51.3**			**1.43**			**14.9**		
225 µg a.i./L	5DF2	F	54.0	1.68	0.257	15.3									
225 µg a.i./L	5DF3	F	52.8	1.49	0.126	8.49									
225 µg a.i./L	5DF4	F	47.2	1.11	0.242	21.8									

[a] Female fish died prior to test termination.
[b] Three female fish were identified as males (by presence of testes) at termination of the test. These three fish were then relabeled with male ID numbers and processed as male fish.

NOTE: No female fish had fatpads. Therefore, no data was collected for this endpoint.

Continued. Female termination endpoints during the 21-day exposure of fathead minnow (*Pimephales promelas*) to potassium permanganate.

Treatment	ID	Sex	Length (mm)	Wet Weight (g)	Gonad weight (g)	GSI	replicate mean length (mm)	treatment mean length (mm)	treatment std dev	replicate mean weight (g)	treatment mean weight (g)	treatment std dev	replicate mean GSI	treatment mean GSI	treatment std dev
450 µg a.i./L	3AF1	F	47.7	1.04	0.0967	9.31	**49.7**	49.5	1.50	**1.28**	1.18	0.105	**12.4**	10.4	1.39
450 µg a.i./L	3AF2	F	53.6	1.59	0.197	12.38									
450 µg a.i./L	3AF3	F	46.5	0.998	0.0939	9.41									
450 µg a.i./L	3AF4	F	51.0	1.49	0.276	18.5									
450 µg a.i./L	3BF1	F	50.8	1.29	0.155	12.1	**50.7**			**1.24**			**10.3**		
450 µg a.i./L	3BF2	F	50.7	1.25	0.109	8.70									
450 µg a.i./L	3BF3	F	50.7	1.18	0.0708	5.98									
450 µg a.i./L	3BF4	F	50.5	.23	0.177	14.4									
450 µg a.i./L	3CF1	F	45.5	0.990	0.139	14.0	**47.3**			**1.04**			**9.31**		
450 µg a.i./L	3CF2	F	50.6	.17	0.106	9.07									
450 µg a.i./L	3CF3	F	46.7	1.11	0.133	12.0									
450 µg a.i./L	3CF4	F	46.5	0.882	0.0192	2.18									
450 µg a.i./L	3DF1	F	50.0	1.19	0.157	13.2	**50.2**			**1.18**			**9.64**		
450 µg a.i./L	3DF2	F	53.3	1.23	0.0384	3.13									
450 µg a.i./L	3DF3	F	51.7	1.40	0.137	9.77									
450 µg a.i./L	3DF4	F	45.8	0.889	0.110	12.4									
900 µg a.i./L	1AF1	F	52.7	1.41	0.150	10.6	**52.0**	49.5	3.05	**1.42**	1.18	0.256	**11.5**	9.94	4.78
900 µg a.i./L	1AF2	F	50.2	1.29	0.127	9.87									
900 µg a.i./L	1AF3	F	53.1	1.56	0.221	14.2									
900 µg a.i./L	1AF4	F	[a]	[a]	[a]	[a]									
900 µg a.i./L	1BF1	F	51.5	1.78	0.327	18.3	**50.9**			**1.39**			**10.3**		
900 µg a.i./L	1BF2	F	49.8	1.24	0.123	9.89									
900 µg a.i./L	1BF3	F	52.6	1.46	0.140	9.61									
900 µg a.i./L	1BF4	F	49.5	1.07	0.0342	3.20									
900 µg a.i./L	1CF1	F	[a]	[a]	[a]	[a]	**50.0**			**0.959**			**3.32**		
900 µg a.i./L	1CF2	F	50.0	0.959	0.0318	3.32									
900 µg a.i./L	1CF3	F	[b]	[b]	[b]	[b]									
900 µg a.i./L	1CF4	F	[b]	[b]	[b]	[b]									
900 µg a.i./L	1DF1	F	44.37	0.9232	0.1657	17.9	**45.1**			**0.965**			**14.6**		
900 µg a.i./L	1DF2	F	46.69	1.0576	0.1368	12.9									
900 µg a.i./L	1DF3	F	43.32	0.7844	0.1448	18.5									
900 µg a.i./L	1DF4	F	45.88	1.0945	0.1007	9.20									

[a] Female fish died prior to test termination.
[b] Three female fish were identified as males (by presence of testes) at termination of the test. These three fish were then relabeled with male ID numbers and processed as male fish.

NOTE: No female fish had fatpads. Therefore, no data was collected for this endpoint.

Female termination endpoints during the 21-day exposure of fathead minnow (*Pimephales promelas*) to ketoconazole.

Treatment	ID	Sex	Length (mm)	Wet Weight (g)	Gonad weight (g)	GSI	replicate mean length (mm)	treatment mean length (mm)	treatment std dev	replicate mean weight (g)	treatment mean weight (g)	treatment std dev	replicate mean GSI	treatment mean GSI	treatment std dev
Control	6AF1	F	59.1	1.95	0.259	13.3	**53.0**	52.7	1.54	**1.43**	1.45	0.0730	**11.0**	11.4	1.16
Control	6AF2	F	51.7	1.47	0.187	12.7									
Control	6AF3	F	52.8	1.35	0.135	10.1									
Control	6AF4	F	48.3	0.977	0.0790	8.09									
Control	6BF1	F	55.5	1.45	0.167	11.5	**52.9**			**1.49**			**11.0**		
Control	6BF2	F	54.7	1.97	0.208	10.5									
Control	6BF3	F	50.4	1.27	0.134	10.5									
Control	6BF4	F	50.9	1.28	0.146	11.5									
Control	6CF1	F	54.4	1.72	0.213	12.4	**50.7**			**1.35**			**13.0**		
Control	6CF2	F	50.2	1.27	0.207	16.3									
Control	6CF3	F	47.4	1.06	0.111	10.4									
Control	6CF4	F	[a]	[a]	[a]	[a]									
Control	6DF1	F	55.5	1.75	0.171	9.77	**54.4**			**1.51**			**10.4**		
Control	6DF2	F	57.8	1.66	0.189	11.4									
Control	6DF3	F	53.6	1.40	0.151	10.8									
Control	6DF4	F	50.8	1.25	0.119	9.52									
25 µg a.i./L	5AF1	F	52.8	1.34	0.163	12.1	**53.5**	53.1	0.44	**1.46**	1.45	0.0420	**12.0**	11.2	1.43
25 µg a.i./L	5AF2	F	54.9	1.54	0.142	9.22									
25 µg a.i./L	5AF3	F	52.6	1.34	0.136	10.1									
25 µg a.i./L	5AF4	F	53.5	1.62	0.270	16.6									
25 µg a.i./L	5BF1	F	56.3	1.61	0.0960	5.97	**53.2**			**1.46**			**9.95**		
25 µg a.i./L	5BF2	F	56.0	1.66	0.166	9.99									
25 µg a.i./L	5BF3	F	52.0	1.31	0.129	9.85									
25 µg a.i./L	5BF4	F	48.3	1.25	0.174	14.0									
25 µg a.i./L	5CF1	F	57.8	1.95	0.284	14.6	**53.2**			**1.50**			**12.8**		
25 µg a.i./L	5CF2	F	52.8	1.62	0.287	17.7									
25 µg a.i./L	5CF3	F	50.6	1.26	0.133	10.5									
25 µg a.i./L	5CF4	F	51.6	1.16	0.0968	8.36									
25 µg a.i./L	5DF1	F	55.3	1.61	0.194	12.0	**52.4**			**1.39**			**10.0**		
25 µg a.i./L	5DF2	F	53.1	1.46	0.167	11.5									
25 µg a.i./L	5DF3	F	53.1	1.39	0.127	9.12									
25 µg a.i./L	5DF4	F	48.2	1.12	0.0842	7.51									

[a] Female fish died prior to test termination.

NOTE: No female fish had fatpads. Therefore, no data was collected for this endpoint.

Continued. Female termination endpoints during the 21-day exposure of fathead minnow (*Pimephales promelas*) to ketoconazole.

Treatment	ID	Sex	Length (mm)	Wet Weight (g)	Gonad weight (g)	GSI	replicate mean length (mm)	treatment mean length (mm)	treatment std dev	replicate mean weight (g)	treatment mean weight (g)	treatment std dev	replicate mean GSI	treatment mean GSI	treatment std dev
100 µg a.i./L	3AF1	F	56.2	1.64	0.157	9.56	**54.7**	54.5	2.00	**1.56**	1.58	0.156	**11.3**	12.5	2.18
100 µg a.i./L	3AF2	F	56.9	1.79	0.178	9.93									
100 µg a.i./L	3AF3	F	55.6	1.64	0.23	14.3									
100 µg a.i./L	3AF4	F	50.0	1.15	0.133	11.5									
100 µg a.i./L	3BF1	F	59.0	1.89	0.163	8.62	**54.9**			**1.68**			**11.6**		
100 µg a.i./L	3BF2	F	54.3	1.71	0.243	14.1									
100 µg a.i./L	3BF3	F	51.3	1.42	0.170	12.0									
100 µg a.i./L	3BF4	F	[a]	[a]	[a]	[a]									
100 µg a.i./L	3CF1	F	53.9	1.59	0.320	20.1	**51.8**			**1.37**			**15.8**		
100 µg a.i./L	3CF2	F	53.3	1.57	0.307	19.6									
100 µg a.i./L	3CF3	F	50.5	1.28	0.159	12.4									
100 µg a.i./L	3CF4	F	49.4	1.04	0.115	11.0									
100 µg a.i./L	3DF1	F	55.1	1.54	0.185	12.0	**56.6**			**1.72**			**11.3**		
100 µg a.i./L	3DF2	F	57.7	1.87	0.248	13.3									
100 µg a.i./L	3DF3	F	56.9	1.76	0.153	8.70									
100 µg a.i./L	3DF4	F	[a]	[a]	[a]	[a]									
400 µg a.i./L	1AF1	F	52.7	1.45	0.259	17.9	**52.3**	53.3	2.53	**1.37**	1.47	0.195	**12.4**	13.9	1.85
400 µg a.i./L	1AF2	F	52.0	1.34	0.175	13.1									
400 µg a.i./L	1AF3	F	51.1	1.20	0.105	8.73									
400 µg a.i./L	1AF4	F	53.5	1.50	0.151	10.1									
400 µg a.i./L	1BF1	F	59.3	2.05	0.294	14.4	**54.3**			**1.61**			**14.2**		
400 µg a.i./L	1BF2	F	53.9	1.63	0.196	12.0									
400 µg a.i./L	1BF3	F	52.6	1.46	0.237	16.2									
400 µg a.i./L	1BF4	F	51.5	1.28	0.183	14.3									
400 µg a.i./L	1CF1	F	50.2	1.24	0.151	12.2	**50.3**			**1.25**			**12.5**		
400 µg a.i./L	1CF2	F	53.3	1.46	0.160	10.9									
400 µg a.i./L	1CF3	F	48.2	1.04	0.0974	9.40									
400 µg a.i./L	1CF4	F	49.6	1.26	0.221	17.5									
400 µg a.i./L	1DF1	F	55.4	1.75	0.321	18.3	**56.2**			**1.66**			**16.4**		
400 µg a.i./L	1DF2	F	54.9	1.50	0.302	20.1									
400 µg a.i./L	1DF3	F	54.6	1.48	0.194	13.1									
400 µg a.i./L	1DF4	F	53.0	1.32	0.184	14.0									
400 µg a.i./L	1DF5	F[b]	63.1	2.27	0.348	15.2									

[a] Female fish died prior to test termination.
[b] This fish was added to the exposure system as a male fish based on morphological features. At test termination, this fish was dissected and identified as a female (based on the presence of ovaries).

NOTE: No female fish had fatpads. Therefore, no data was collected for this endpoint.

	CONTROL Replicate A					CONTROL Replicate B					CONTROL Replicate C					CONTROL Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			0	0	0			0	0	0			250	180	2	4	97.78
2	0	0	0			0	0	0			25	26	1	0	100.00	0	0	0		
3	0	0	0			0	0	0			0	0	0			0	0	0		
4	0	0	0			0	0	0			10	11	1	0	100.00	50	39	1	0	100.00
5	0	0	0			0	0	0			0	0	0			0	0	0		
6	0	0	0			50	100	1	0	100.00	25	27	1	0	100.00	75	84	2	0	100.00
7	100	122	1	0	100.00	0	0	0			0	0	0			10	10	1	0	100.00
8	0	0	0			0	0	0			0	0	0			0	0	0		
9	0	0	0			0	0	0			0	0	0			0	0	0		
10	0	0	0			0	0	0			0	0	0			0	0	0		
11	0	0	0			25	43	1	0	100.00	0	0	0			0	0	0		
12	0	0	0			0	0	0			0	0	0			25	27	2	0	100.00
13	0	0	0			0	0	0			0	0	0			0	0	0		
14	0	0	0			200	142	1	0	100.00	0	0	0			25	23	1	0	100.00
15	0	0	0			0	0	0			25	21	1	0	100.00	0	0	0		
16	0	0	0			0	0	0			0	0	0			0	0	0		
17	0	0	0			3	3	1	0	100.00	0	0	0			100	164	1	2	98.78
18	0	0	0			0	0	0			0	0	0			0	0	0		
19	0	0	0			0	0	0			0	0	0			0	0	0		
20	0	0	0			0	0	0			0	0	0			100	132	1	0	100.00
21	0	0	0			0	0	0			0	0	0			0	0	0		
Total	**100**	**122**	**1**	**0**	**100.00**	**278**	**288**	**4**	**0**	**100.00**	**85**	**85**	**4**	**0**	**100.00**	**635**	**659**	**11**	**6**	**99.09**

	25 ug/L Replicate A					25 ug/L Replicate B					25 ug/L Replicate C					25 ug/L Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			0	0	0			0	0	0			0	0	0		
2	0	0	0			0	0	0			10	10	1		100.00	0	0	0		
3	0	0	0			0	0	0			0	0	0			0	0	0		
4	50	57	1	0	100.00	0	0	0			0	0	0			0	0	0		
5	0	0	0			0	0	0			0	0	0			0	0	0		
6	0	0	0			0	0	0			0	0	0			0	0	0		
7	0	0	0			0	0	0			0	0	0			0	0	0		
8	10	14	1	0	100.00	0	0	0			0	0	0			0	0	0		
9	10	6	1	0	100.00	0	0	0			0	0	0			0	0	0		
10	100	54	1	0	100.00	0	0	0			0	0	0			0	0	0		
11	0	0	0			0	0	0			0	0	0			0	0	0		
12	0	0	0			0	0	0			0	0	0			0	0	0		
13	0	0	0			0	0	0			0	0	0			0	0	0		
14	0	0	0			0	0	0			0	0	0			0	0	0		
15	10	12	1	0	100.00	0	0	0			0	0	0			0	0	0		
16	0	0	0	0		0	0	0			300	245	1	0	100.00	0	0	0		
17	20	24	1		100.00	0	0	0			0	0	0			0	0	0		
18	50	78	1	0	100.00	10	9	1	1	88.89	0	0	0			50	71	1	1	**98.59**
19	0	0	0			0	0	0			25	27	1	0	100.00	0	0	0		
20	100	134	1	1	99.25	0	0	0			0	0	0			0	0	0		
21	0	0	0			0	0	0			25	27	1	0	100.00	0	0	0		
Total	**350**	**379**	**8**	**1**	**99.74**	**10**	**9**	**1**	**1**	**88.89**	**360**	**309**	**4**	**0**	**100.00**	**50**	**71**	**1**	**1**	**98.59**

	100 ug/L Replicate A					100 ug/L Replicate B					100 ug/L Replicate C					100 ug/L Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			0	0	0			50	55	1	0	100.00	0	0	0		
2	0	0	0			0	0	0			0	0	0			0	0	0		
3	0	0	0			0	0	0			0	0	0			0	0	0		
4	0	0	0			0	0	0			0	0	0			0	0	0		
5	0	0	0			0	0	0			0	0	0			50	41	1	0	100.00
6	0	0	0			0	0	0			100	82	1	0	100.00	0	0	0		
7	150	138	1	37	**73.19**	0	0	0			0	0	0			0	0	0		
8	0	0	0			0	0	0			0	0	0			0	0	0		
9	0	0	0			0	0	0			0	0	0			0	0	0		
10	0	0	0			0	0	0			0	0	0			0	0	0		
11	0	0	0			0	0	0			0	0	0			0	0	0		
12	0	0	0			0	0	0			0	0	0			0	0	0		
13	0	0	0			0	0	0			0	0	0			0	0	0		
14	0	0	0			0	0	0			0	0	0			0	0	0		
15	0	0	0			0	0	0			0	0	0			25	24	1	4	83.33
16	0	0	0			0	0	0			0	0	0			0	0	0		
17	0	0	0			0	0	0			200	281	1	0	100.00	100	101	1	0	100.00
18	0	0	0			0	0	0			0	0	0			0	0	0		
19	0	0	0			0	0	0			0	0	0			50	107	1	2	98.13
20	0	0	0			0	0	0			0	0	0			0	0	0		
21	0	0	0			0	0	0			0	0	0			100	221	1	1	99.55
Total	**150**	**138**	**1**	**37**	**73.19**	**0**	**0**	**0**	**0**		**350**	**418**	**3**	**0**	**100.00**	**325**	**494**	**5**	**7**	**98.58**

	400 ug/L Replicate A					400 ug/L Replicate B					400 ug/L Replicate C					400 ug/L Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			0	0	0			0	0	0			25	16	1	0	100.00
2	0	0	0			0	0	0			0	0	0			0	0	0		
3	0	0	0			0	0	0			0	0	0			100	91	1	0	100.00
4	0	0	0			0	0	0			0	0	0			100	106	1	0	100.00
5	0	0	0			0	0	0			0	0	0			0	0	0		
6	0	0	0			0	0	0			0	0	0			0	0	0		
7	0	0	0			0	0	0			0	0	0			0	0	0		
8	0	0	0			0	0	0			0	0	0			150	172	1	6	96.51
9	0	0	0			0	0	0			0	0	0			0	0	0		
10	100	108	2	0	100.00	0	0	0			0	0	0			0	0	0		
11	0	0	0			0	0	0			0	0	0			50	52	1	3	94.23
12	0	0	0			25	42	1	2	95.24	0	0	0			100	52	1	7	86.54
13	0	0	0			0	0	0			100	77	1	0	100.00	0	0	0		
14	0	0	0			0	0	0			0	0	0			0	0	0		
15	0	0	0			0	0	0			0	0	0			0	0	0		
16	0	0	0			0	0	0			0	0	0			0	0	0		
17	0	0	0			0	0	0			0	0	0			0	0	0		
18	0	0	0			150	179	1	0	100.00	0	0	0			0	0	0		
19	0	0	0			100	96	1	7	92.71	0	0	0			0	0	0		
20	0	0	0			0	0	0			0	0	0			0	0	0		
21	0	0	0			0	0	0			0	0	0			0	0	0		
Total	**100**	**108**	**2**	**0**	**100.00**	**275**	**317**	**3**	**9**	**97.16**	**100**	**77**	**1**	**0**	**100.00**	**525**	**489**	**6**	**16**	**96.73**

	CONTROL Replicate A					CONTROL Replicate B					CONTROL Replicate C					CONTROL Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			0	0	0			20	22	1	1	95.45	0	0	0		
2	0	0	0			0	0	0			0	0	0			20	32	1	2	93.75
3	0	0	0			0	0	0			100	77	1	2	97.40	0	0	0		
4	0	0	0			200	258	1	0	100.00	0	0	0			0	0	0		
5	0	0	0			0	0	0			35	46	1	0	100.00	0	0	0		
6	0	0	0			0	0	0			150	189	1	2	98.94	225	225	1	0	100.00
7	0	0	0			0	0	0			0	0	1			0	0	0		
8	0	0	0			100	125	1	2	98.40	10	8	1	0	100.00	100	100	1	0	100.00
9	20	13	1	0	100.00	0	0	0			20	22	1	1	95.45	0	0	0		
10	50	93	1	4	95.70	0	0	0			0	0	0			150	172	1	6	96.51
11	50	102	1	1	99.02	50	111	1	0	100.00	0	0	0			0	0	0		
12	30	81	1	0	100.00	100	221	1	0	100.00	100	105	1	0	100.00	20	32	1	0	100.00
13	0	0	0			0	0	0			50	64	1	2	96.88	0	0	0		
14	100	102	1	0	100.00	100	115	1	0	100.00	0	0	0			0	0	0		
15	0	0	0			0	0	0			0	0	0			0	0	0		
16	0	0	0			150	230	1	0	100.00	50	75	1	4	94.67	0	0	0		
17	0	0	0			100	109	1	0	100.00	0	0	0			0	0	0		
18	0	0	0			0	0	0			100	151	1	0	100.00	0	0	0		
19	0	0	0			0	0	0			0	0	0			0	0	0		
20	200	232	1	0	100.00	0	0	0			100	89	1	0	100.00	0	0	0		
21	0	0	0			0	0	0			0	0	0			0	0	0		
Total	**450**	**623**	**6**	**5**	**99.20**	**800**	**1169**	**7**	**2**	**99.83**	**735**	**848**	**12**	**12**	**98.58**	**515**	**561**	**5**	**8**	**98.57**

	100 ug/L Replicate A					100 ug/L Replicate B					100 ug/L Replicate C					100 ug/L Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			50	48	1	1	97.92	0	0	0			0	0	0		
2	0	0	0			100	76	1	3	96.05	0	0	0			0	0	0		
3	0	0	0			0	0	0			20	21	1	0	100.00	0	0	0		
4	0	0	0			0	0	0			0	0	0			150	147	1	0	100.00
5	0	0	0			35	41	1	0	100.00	50	54	1	1	98.15	0	0	0		
6	250	369	1	2	99.46	0	0	0			0	0	0			0	0	0		
7	0	0	0			0	0	0			20	21	1	0	100.00	0	0	0		
8	0	0	0			0	0	0			0	0	0			0	0	0		
9	100	84	2	0	100.00	10	11	1	0	100.00	0	0	0			10	3	1	0	100.00
10	0	0	0			0	0	0			50	118	1	2	98.31	100	101	1	3	97.03
11	0	0	0			50	53	1	0	100.00	0	0	0			50	75	1	0	100.00
12	100	105	1	0	100.00	200	141	2	1	99.29	0	0	0			0	0	0		
13	20	34	1	0	100.00	50	83	1	0	100.00	0	0	0			0	0	0		
14	0	0	0			0	0	0			200	216	1	1	99.54	0	0	0		
15	25	56	1	0	100.00	0	0	0			0	0	0			0	0	0		
16	0	0	0			200	208	1	9	95.67	20	22	1	1	95.45	0	0	0		
17	0	0	0			0	0	0			25	27	1	0	100.00	100	151	1	2	98.68
18	0	0	0			250	283	1	0	100.00	200	284	1	0	100.00	0	0	0		
19	0	0	0			100	97	1	1	98.97	0	0	0			0	0	0		
20	0	0	0			0	0	0			0	0	0			100	93	1	0	100.00
21	0	0	0			0	0	0			0	0	0			100	105	1	3	97.14
Total	**495**	**648**	**6**	**2**	**99.69**	**1045**	**1041**	**11**	**15**	**98.56**	**585**	**763**	**8**	**5**	**99.34**	**610**	**675**	**7**	**8**	**98.81**

	500 ug/L Replicate A					500 ug/L Replicate B					500 ug/L Replicate C					500 ug/L Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			0	0	0			0	0	0			0	0	0		
2	0	0	0			0	0	0			0	0	0			0	0	0		
3	0	0	0			100	88	1	3	96.59	150	116	1	5	95.69	0	0	0		
4	20	21	1	0	100.00	0	0	0			0	0	0			100	90	1	0	100.00
5	165	197	1	1	99.49	125	84	1	0	100.00	10	10	1	0	100.00	0	0	0		
6	35	86	1	3	96.51	0	0	0			0	0	0			25	24	1	0	100.00
7	50	38	1	0	100.00	30	69	1	0	100.00	0	0	0			20	20	1	1	95.00
8	0	0	0			0	0	0			0	0	0			0	0	0		
9	100	103	1	3	97.09	10	6	1	0	100.00	0	0	0			0	0	0		
10	0	0	0			0	0	0			0	0	0			0	0	0		
11	0	0	0			0	0	0			0	0	0			150	298	1	0	100.00
12	0	0	0			0	0	0			0	0	0			0	0	0		
13	10	11	1	1	90.91	0	0	0			0	0	0			0	0	0		
14	100	117	1	0	100.00	150	77	1	0	100.00	100	76	1	0	100.00	0	0	0		
15	0	0	0			0	0	0			50	89	1	0	100.00	100	110	1	0	100.00
16	0	0	0			0	0	0			0	0	0			150	153	1	2	98.69
17	0	0	0			0	0	0			0	0	0			0	0	0		
18	0	0	0			50	21	1	0	100.00	0	0	0			0	0	0		
19	0	0	0			10	11	1	0	100.00	100	113	1	1	99.12	0	0	0		
20	25	35	1	0	100.00	0	0	0			0	0	0			50	110	1	0	100.00
21	0	0	0			0	0	0			0	0	0			0	0	0		
Total	**505**	**608**	**8**	**8**	**98.68**	**475**	**356**	**7**	**3**	**99.16**	**410**	**404**	**5**	**6**	**98.51**	**595**	**805**	**7**	**3**	**99.63**

	1000 ug/L Replicate A					1000 ug/L Replicate B					1000 ug/L Replicate C					1000 ug/L Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			0	0	0			0	0	0			0	0	0		
2	0	0	0			0	0	0			0	0	0			0	0	0		
3	0	0	0			0	0	0			0	0	0			0	0	0		
4	0	0	0			0	0	0			0	0	0			0	0	0		
5	0	0	0			0	0	0			0	0	0			0	0	0		
6	0	0	0			0	0	0			250	302	1	22	92.72	75	86	1	4	95.35
7	30	39	1	5	87.18	0	0	0			50	57	1	24	57.89	0	0	0		
8	0	0	0			0	0	0			250	233	1	8	96.57	0	0	0		
9	0	0	0			0	0	0			0	0	0			0	0	0		
10	0	0	0			0	0	0			250	267	1	56	79.03	50	68	1	26	61.76
11	0	0	0			0	0	0			100	193	1	28	85.49	0	0	0		
12	250	280	1	0	100.00	0	0	0			0	0	0			0	0	0		
13	0	0	0			0	0	0			50	131	1	4	96.95	0	0	0		
14	0	0	0			0	0	0			100	66	1	5	92.42	0	0	0		
15	0	0	0			0	0	0			0	0	0			0	0	0		
16	0	0	0			0	0	0			100	150	1	2	98.67	0	0	0		
17	0	0	0			0	0	0			0	0	0			0	0	0		
18	0	0	0			0	0	0			150	207	1	6	97.10	0	0	0		
19	0	0	0			0	0	0			300	298	1	7	97.65	25	35	1	1	97.14
20	0	0	0			0	0	0			0	0	0			0	0	0		
21	0	0	0			0	0	0			0	0	0			0	0	0		
Total	**280**	**319**	**2**	**5**	**98.43**	**0**	**0**	**0**	**0**	**NA**	**1600**	**1904**	**10**	**162**	**91.49**	**150**	**189**	**3**	**31**	**83.60**

	CONTROL Replicate A					CONTROL Replicate B					CONTROL Replicate C					CONTROL Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			0	0	0			25	31	1	0	100.00	0	0	0		
2	0	0	0			0	0	0			25	33	1	0	100.00	0	0	0		
3	0	0	0			200	168	1	2	98.81	0	0	0			0	0	0		
4	50	48	1	1	97.92	0	0	0			100	104	1	0	100.00	0	0	0		
5	0	0	0			0	0	0			100	116	1	0	100.00	25	21	1	0	100.00
6	0	0	0			0	0	0			0	0	0			0	0	0		
7	0	0	0			0	0	0			50	47	1	0	100.00	0	0	0		
8	5	5	1	0	100.00	25	71	1	2	97.18	50	41	2	1	97.56	10	5	1	0	100.00
9	0	0	0			0	0	0			0	0	0			0	0	0		
10	0	0	0			200	169	1		100.00	0	0	0			10	7	1	0	100.00
11	0	0	0			0	0	0			0	0	0			0	0	0		
12	0	0	0			250	179	1	3	98.32	50	37	1	0	100.00	0	0	0		
13	0	0	0			50	50	1	0	100.00	150	149	1	2	98.66	0	0	0		
14	0	0	0			00	160	1	1	99.38	0	0	0			0	0	0		
15	0	0	0			100	109	1	0	100.00	100	64	1	0	100.00	0	0	0		
16	0	0	0			0	0	0			0	0	0			0	0	0		
17	20	44	1	0	100.00	50	27	1	0	100.00	0	0	0			0	0	0		
18	0	0	0			0	0	0			0	0	0			0	0	0		
19	0	0	0			0	0	0			25	21	1	0	100.00	0	0	0		
20	0	0	0			0	0	0			0	0	0			0	0	0		
21	0	0	0			0	0	0			50	103	1	0	100.00	0	0	0		
Total	**75**	**97**	**3**	**1**	**98.97**	**975**	**933**	**8**	**8**	**99.14**	**725**	**746**	**12**	**3**	**99.60**	**45**	**33**	**3**	**0**	**100.00**

	225 ug/L Replicate A					225 ug/L Replicate B					225 ug/L Replicate C					225 ug/L Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0	0			0	0	0			0	0	0			0	0	0		
2	25	21	1	0	100.00	0	0	0			0	0	0			0	0	0		
3	50	100	1	1	99.00	0	0	0			100	88	1	0	100.00	0	0	0		
4	25	23	1	0	100.00	0	0	0			0	0	0			0	0	0		
5	0	0	0			0	0	0			0	0	0			0	0	0		
6	100	103	1	0	100.00	0	0	0			0	0	0			0	0	0		
7	200	215	1	1	99.53	0	0	0			0	0	0			0	0	0		
8	0	0	0			0	0	0			0	0	0			0	0	0		
9	0	0	0			0	0	0			0	0	0			0	0	0		
10	25	24	1	0	100.00	0	0	0			0	0	0			0	0	0		
11	150	153	2		99.35	0	0	0			0	0	0			0	0	0		
12	0	0	0			0	0	0			0	0	0			0	0	0		
13	0	0	0			0	0	0			0	0	0			0	0	0		
14	100	165	1	6	96.36	0	0	0			10	10	1	0	100.00	0	0	0		
15	20	16	1	0	100.00	0	0	0			0	0	0			0	0	0		
16	0	0	0			0	0	0			0	0	0			0	0	0		
17	100	84	1	2	97.62	0	0	0			0	0	0			0	0	0		
18	50	41	1	0	100.00	50	45	1	0	100.00	10	12	1	0	100.00	0	0	0		
19	0	0	0			0	0	0			0	0	0			0	0	0		
20	0	0	0			0	0	0			0	0	0			0	0	0		
21	100	63	1	0	100.00	0	0	0			0	0	0			0	0	0		
Total	**945**	**1008**	**13**	**11**	**98.91**	**50**	**45**	**1**	**0**	**100.00**	**120**	**110**	**3**	**0**	**100.00**	**0**	**0**	**0**	**0**	

	450 ug/L Replicate A					450 ug/L Replicate B					450 ug/L Replicate C					450 ug/L Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	0	0				0	0		0		0	0	0			0	0	0		
2	50	53	1	0	100.00	25	21	1	0	100.00	0	0	0			0	0	0		
3	25	31	1	1	96.77	0	0				100	134	1	1	99.25	0	0	0		
4	10	14	1	0	100.00	0	0				250	212	1	0	100.00	450	469	1	0	100.00
5	0	0				100	71	1	0	100.00	0	0	0			100	67	2	0	100.00
6	25	31	1		100.00	0	0				0	0	0			0	0	0		
7	0	0				0	0				0	0	0			0	0	0		
8	0	0				0	0				0	0	0			0	0	0		
9	0	0				0	0				0	0	0			0	0	0		
10	50	81	1	1	98.77	50	24	1	0	100.00	50	39	1	3	92.31	50	33	1	2	93.94
11	200	120	1	0	100.00	0	0				0	0	0			50	65	1	0	100.00
12	0	0				0	0				0	0	0			0	0	0		
13	0	0				0	0				0	0	0			0	0	0		
14	100	83	1	0	100.00	100	65	1	0	100.00	0	0	0			0	0	0		
15	0	0				0	0				0	0	0			0	0	0		
16	0	0				100	155	1	0	100.00	0	0	0			0	0	0		
17	20	16	1	0	100.00	100	120	1	0	100.00	0	0	0			0	0	0		
18	0	0				0	0				0	0	0			100	83	1	1	98.80
19	0	0				0	0				0	0	0			50	41	1	0	100.00
20	0	0				100	84	2	0	100.00	0	0	0			0	0	0		
21	0	0				100	52	1	0	100.00	0	0	0			50	44	1	0	100.00
Total	**480**	**429**	**8**	**2**	**99.53**	**675**	**592**	**9**	**0**	**100.00**	**400**	**385**	**3**	**4**	**98.96**	**850**	**802**	**8**	**3**	**99.63**

	900 ug/L Replicate A					900 ug/L Replicate B					900 ug/L Replicate C					900 ug/L Replicate D				
Test Day	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs	Number of Eggs Estimated	Number of Eggs Counted	Number of Spawns	Number of Infertile Eggs	Percent Fertile Eggs
1	25	65	1	29	55.38	0	0	0			0	0	0			0	0			
2	0	0	0			0	0	0			0	0	0			50	57	1	2	
3	0	0	0			0	0	0			0	0	0			0	0			
4	0	0	0			0	0	0			0	0	0			0	0			
5	0	0	0			0	0	0			0	0	0			0	0			
6	0	0	0			50	112	1	0	100.00	0	0	0			0	0			
7	0	0	0			0	0	0			0	0	0			0	0			
8	0	0	0			0	0	0			0	0	0			0	0			
9	0	0	0			0	0	0			0	0	0			0	0			
10	0	0	0			0	0	0			0	0	0			0	0			
11	0	0	0			250	198	1	0	100.00	0	0	0			0	0			
12	0	0	0			0	0	0			0	0	0			100	128	1	2	
13	0	0	0			0	0	0			0	0	0			0	0			
14	200	301	1	0	100.00	0	0	0			0	0	0			0	0			
15	250	549	1	6	98.91	0	0	0			0	0	0			25	41	1	0	
16	100	179	1	0	100.00	0	0	0			0	0	0			0	0			
17	250	430	1	7	98.37	0	0	0			0	0	0			0	0			
18	200	142	1	0	100.00	0	0	0			0	0	0			0	0			
19	0	0	0			10	12	1	1	91.67	0	0	0			0	0			
20	100	157	1	0	100.00	0	0	0			0	0	0			0	0			
21	25	38	1	0	100.00	0	0	0			0	0	0			0	0			
Total	**1150**	**1861**	**8**	**42**	**97.74**	**310**	**322**	**3**	**1**	**99.69**	**0**	**0**	**0**	**0**		**175**	**226**	**3**	**4**	**98.23**

APPENDIX 3 - HISTOPATHOLOGY

Flutamide

Experimental Pathology Laboratories, Inc.

BATTELLE PACIFIC NORTHWEST DIVISION,
BATTELLE MEMORIAL INSTITUTE
STUDY NUMBER WA 5-11
EPL PROJECT NUMBER 237-021

FISH SCREENING ASSAY OECD PHASE 1B FOLLOW-UP

DRAFT PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.
P.O. Box 474
Herndon, VA 20172-0474
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Submitted to:

Battelle Pacific Northwest Division, Battelle Memorial Institute
Sequim, WA 98382

December 12, 2005

FINAL REPORT

EPL® Experimental Pathology Laboratories, Inc.

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DRAFT PATHOLOGY SUMMARY

EPL® Experimental Pathology Laboratories, Inc.

BATTELLE PACIFIC NORTHWEST DIVISION,
BATTELLE MEMORIAL INSTITUTE
STUDY NUMBER WA 5-11
EPL PROJECT NUMBER 237-021

FISH SCREENING ASSAY OECD PHASE 1B FOLLOW-UP

DRAFT PATHOLOGY SUMMARY

INTRODUCTION

The objective of this study was to determine the effects, if any, of flutamide administered via water bath on gonadal tissue of adult fathead minnows (FHM, *Pimephales promelas*).

The experimental design is presented in the following table:

Table 1. Experimental Design for Flutamide Study*

Exposure Group	Flutamide	Male Replicates				Female Replicates			
		A	B	C	D	A	B	C	D
6	0 µg/L (Control)	2	2	3	2	3	4	3	3
5	100 µg/L	2	2	1	3	4	4	4	3
3	500 µg/L	2	2	2	2	4	4	4	3
1	1000 µg/L	2	2	2	2	3	4	4	4

* Reflects actual numbers of animals for which gonad tissue was examined rather than numbers from the original experimental design.

METHODS

Unless otherwise indicated, histopathological procedures were performed according to the draft form of the "OECD Guidance Document for Performing Gonadal Histopathology in Small Fish." Briefly, following routine processing the left and right gonads were embedded horizontal to their long axis to allow for longitudinal sectioning. During microtomy, the first section from each block was

acquired at the point at which approximately half of the gonad had been cut away and the size of the section was maximized. The second and third sections were then obtained at 50 micron intervals. Sections were stained with hematoxylin and eosin, and mounted with glass coverslips. Labels included the EPL Project No. (237-021), the Study ID (WA 5-11), and the Animal No. (e.g., 1CF2).

The tissue cassette labeled as Animal No. 6AF1 either arrived empty or the tissue was lost during processing; therefore, there are no results for that fish. The labels of two testis tissue cassettes, representing Animal Nos. 5CM2 and 5DM2, became smudged during tissue processing and their identities could not be reconciled. Slides created from these cassettes were subsequently labeled 5DM2-1 and 5DM2-2, respectively. One animal originally designated as female (Animal No. 6CF4) was determined to be a male following microscopic examination of the gonads.

The pathologist evaluated the slides by brightfield microscopy for changes that included, but were not limited to, the types of findings that are listed in the aforementioned guidance document. As per that document, severity grading of findings was performed according to the following scale: NR = not remarkable, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, Grade 4 = severe. Ovarian oocyte atresia was graded according to the following scale: Grade 1 = 3 to 5 atretic oocytes per ovary; Grade 2 = 6 to 9 atretic oocytes per ovary; Grade 3 = greater than 9 atretic oocytes per ovary, but less than the vast majority; and Grade 4 = the vast majority of oocytes were atretic. The pathologist recorded findings on a spreadsheet. This original spreadsheet as contained within the guidance document was modified slightly by the study pathologist to include the addition of a column in order to accommodate the animal numbers of the female fathead minnows. The data collection spreadsheet is incorporated into this report (see Appendix A). Results were simultaneously recorded into

EPL's Pathology Data Reporting System, and tabulated in the accompanying Histopathology Incidence Tables (HIT) and summarized in the Summary Incidence Tables (SIT). Representative photographs are presented in Appendix B.

RESULTS

Males

Based on incidence and/or severity data, there were no findings that were substantially different in flutamide-exposed males as compared to control males. The incidence of increased interstitial cells was slightly higher in the 1000 µg/L group as compared to controls; however, this difference is unlikely to be statistically significant.

Single, approximately 15-50 µm diameter, ovoid, parasite cysts were occasionally evident within testis tubule lumina (Fig 1). Each thin-walled cyst was separated into compartments by narrow septa, and was packed with oval, amphophilic, spore-like organisms (approximately 1-2 µm diameter), that were partially birefringent under polarized light (Fig 2). These organisms were consistent with microsporidia. The cysts were generally not spatially associated with an inflammatory response.

The incidence and severity of selected histopathologic results for male fathead minnows are presented in the following table:

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
12/12/05 DRAFT

Table 2. Combined Incidence and Severity of Selected Histopathologic Findings in Male Fathead Minnows

Exposure Group	6					5					3					1				
Flutamide Dose (µg/L)	0					100					500					1000				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	2	2	3	2	9	2	2	1	3	8	2	2	2	2	8	2	2	2	2	8
Increased Cells, Spermatogonia	0	0	1	1	2	0	1	0	0	1	1	0	0	0	1	1	0	1	0	2
minimal	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1
mild	-	-	1	-	1	-	1	-	-	1	1	-	-	-	1	-	-	-	-	-
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Increased Cells, Interstitial Cells	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	3
minimal	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	1	1	1	3
mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Microsporidia	0	0	1	1	2	1	1	0	0	2	0	0	0	0	0	0	0	0	1	1
minimal	-	-	1	1	2	1	1	-	-	2	-	-	-	-	-	-	-	-	1	1
mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testicular Stage																				
Stage 1	-	-	-	-	-	-	-	-	1	1	-	1	-	-	1	-	1	-	-	1
Stage 2	2	2	2	1	7	2	1	1	2	6	1	1	1	2	5	1	-	2	2	5
Stage 3	-	-	1	1	2	-	1	-	-	1	1	-	1	-	2	1	1	-	-	2
Average	2.2					2.0					2.1					2.1				

*T = totals

Females

Based on incidence and/or severity data, there were no findings that were substantially different in flutamide-exposed females as compared to control females. Ovarian developmental stage average scores were generally higher in the two highest dose groups (500 and 1000 µg/L) as compared to controls, but this difference is unlikely to be statistically significant.

An unusual incidental finding in a single female ovary was oogonial hyperplasia of moderate severity (Grade 3) (Fig 4, compared to normal ovary Fig 3). Although a relatively high proportion of the oogonia in this ovary were observed in various phases of meiotic and/or mitotic division, the cells did not display atypical features consistent with neoplasia (Fig 5).

As in the testes of occasional males, microsporidia were evident in the ovarian interstitium of some females (Figs 6 & 7). However, unlike the situation in males, the ovarian microsporidia were consistently associated with varying degrees of granulomatous inflammation, and the spores were present within host macrophages instead of parasitic cysts. Some areas of inflammation contained only very few parasites, and in such instances the organisms were best appreciated as small fragments of birefringent material when viewed under polarized light. Other areas of granulomatous inflammation that did not contain obvious parasites appeared to be spatially associated with fragments of atretic oocytes.

The incidence and severity of selected histopathologic results for female fathead minnows are presented in the following table:

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
12/12/05 DRAFT

Table 2. Combined Incidence and Severity of Selected Histopathologic Findings in Female Fathead Minnows

Exposure Group	6					5					3					1				
Flutamide Dose (µg/L)	0					100					500					1000				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	3	4	3	3	13	4	4	4	3	15	4	4	4	3	15	3	4	4	4	15
Oocyte Atresia, Increased	1	1	1	1	4	2	2	0	1	5	2	1	0	0	3	2	1	0	0	3
minimal	-	-	-	-	-	-	2	-	-	2	-	-	-	-	-	1	-	-	-	1
mild	1	-	-	1	2	-	-	-	-	-	-	1	-	-	1	1	-	-	-	1
moderate	-	1	-	-	1	1	-	-	1	2	2	-	-	-	2	-	-	-	-	-
severe	-	-	1	-	1	1	-	-	-	1	-	-	-	-	-	-	1	-	-	1
Microsporidia	0	0	1	0	1	2	1	0	0	3	0	0	1	0	1	1	0	1	0	2
minimal	-	-	1	-	1	2	1	-	-	3	-	-	1	-	1	1	-	1	-	2
mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Granulomatous, Inflammation	0	1	1	1	3	2	3	0	0	5	0	1	1	0	2	2	1	1	1	5
minimal	-	1	1	1	3	2	3	-	-	5	-	1	1	-	2	1	1	-	1	3
mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	2
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovarian Stage																				
Stage 1	-	1	-	-	1	-	-	-	-	-	1	-	-	-	1	1	-	-	-	1
Stage 2	2	1	1	1	5	2	2	1	2	7	-	1	1	-	2	-	1	-	-	1
Stage 3	1	2	2	2	7	2	2	3	1	8	3	3	3	3	12	2	2	4	4	12
Stage 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
Average	2.5					2.5					2.7					2.9				

*T = totals

DISCUSSION

Flutamide is a non-steroidal antiandrogen which inhibits androgen uptake and/or nuclear binding of androgen in target tissues (Wester, et al., 2003). The effects of flutamide on adult gonadal histopathology have been investigated for several fishes including zebrafish (*Danio rerio*), male guppies (*Poecilia reticulata*) and Japanese medaka (*Oryzias latipes*). Findings in flutamide-exposed male zebrafish (up to 1000 µg/L flutamide in water) included an increase in interstitial cells, nuclear hypertrophy of Sertoli cells, an increase in the proportion of spermatogonia, and a decrease in the proportion of spermatocytes (Wester, et al., 2003). There were no histopathologic findings for the ovaries of exposed adult female zebrafish in that study. Histopathologic findings attributed to flutamide exposure in male guppies (up to 100 µg/mg of feed) included a reduced number of spermatogenetic cysts and increased numbers of spermatozeugmata in ducts (Kinnberg and Toft, 2003).

Fathead minnows in the previous Phase 1B experiments, which were exposed to the same nominal doses as the present study, did not exhibit consistent responses to flutamide. Effects recorded by one laboratory included increased spermatogonia in the testes of male fathead minnows and increased oocyte atresia in the ovaries of females; neither of these were observed as effects in the current study.

CONCLUSION AND SUMMARY

There were no histopathological findings in this study that were clearly attributable to flutamide exposure. The incidence of increased interstitial cells was slightly higher in the 1000 µg/L group as compared to controls; however, this difference is unlikely to be statistically significant. Ovarian developmental stage

average scores were generally higher in the two highest dose groups (500 and 1000 µg/L) as compared to controls, but this difference is unlikely to be statistically significant.

Other findings in this study either occurred in comparable numbers of control and flutamide-exposed fish (background lesions), or as low-frequency (incidental) findings, and are therefore not considered to be exposure-related.

JEFFREY C. WOLF, DVM, Diplomate, ACVP Date
Veterinary Pathologist

JCW/cb

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Kinnberg K, Toft G (2003) Effects of estrogenic and antiandrogenic compounds on the testis structure of the adult guppy (*Poecilia reticulata*). Ecotoxicology and Environmental Safety, 54:16-24.

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Wester PW, van der Ven LTM, Brandhof EJ, Vos JH (2003) Identification of endocrine disruptive effects in the aquatic environment: a partial life cycle assay in zebrafish. RIVM Report 640920001/2003, pp.58-63.

OECD Draft Guidance Document for Performing Gonadal Histopathology in Small Fish: Histology and Histopathology Guidelines for Phase 1B of the OECD Fish Screening Assay for EDC's. (2004).

EPL® Experimental Pathology Laboratories, Inc.

12/12/05 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Fish Screening Assay OECD Phase 1B Follow-Up

Client Study: WA 5-11 EPL Project Coordinator: Dr. Jeffrey C. Wolf

EPL Project Number: 237-021 EPL Pathologist: Dr. Jeffrey C. Wolf

The following aspects of this study were inspected by the Quality Assurance Unit of Experimental Pathology Laboratories, Inc. Dates inspections were performed and findings reported to the EPL Project Coordinator and Management are indicated below.

Area Inspected	Dates	
	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management _____

Date of last quarterly facility inspection 7/05

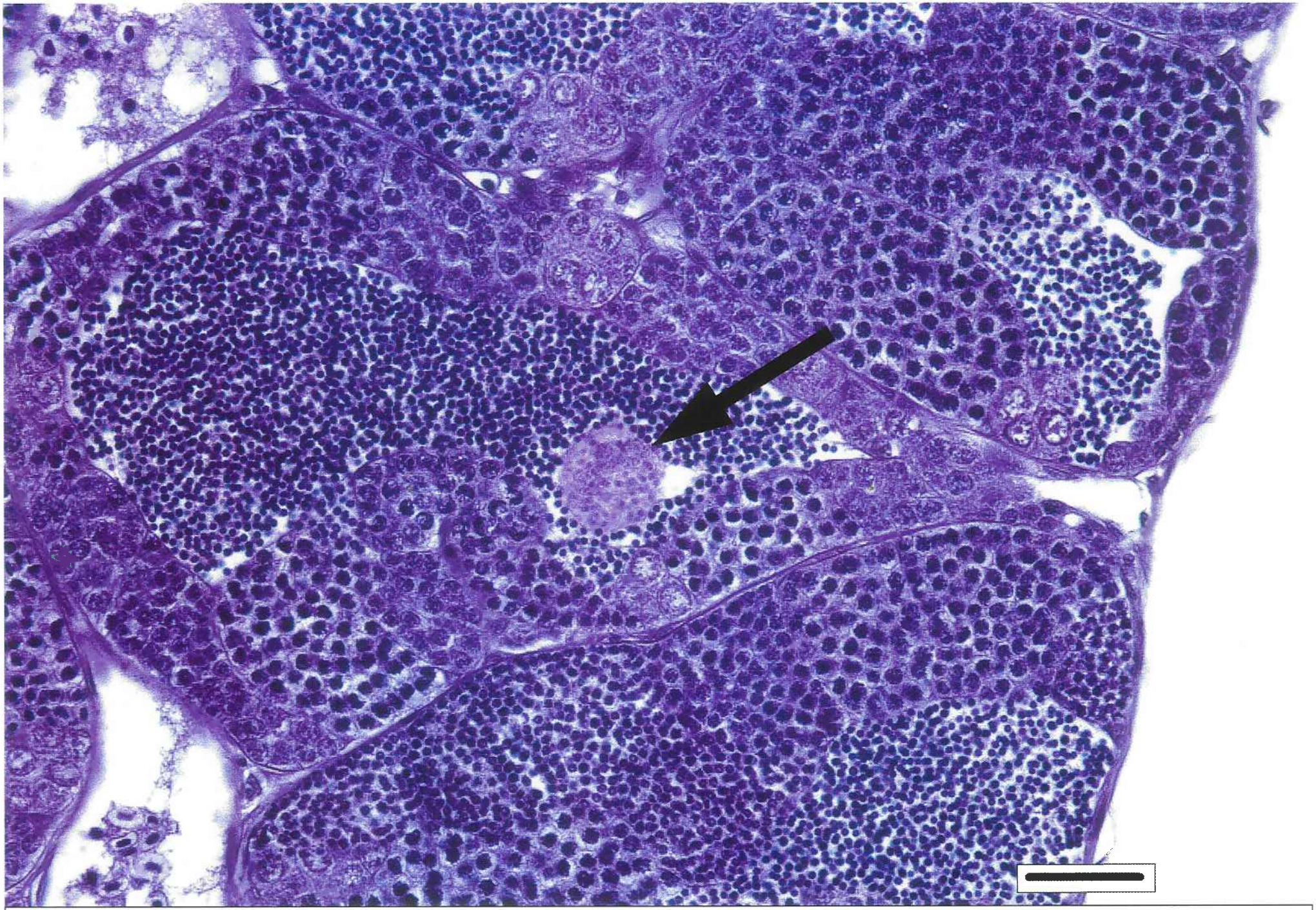
_____ _____

EPL Quality Assurance Unit Date

Form No. 6-2 (7/2/99)

EPL® Experimental Pathology Laboratories, Inc.

12/12/05 DRAFT



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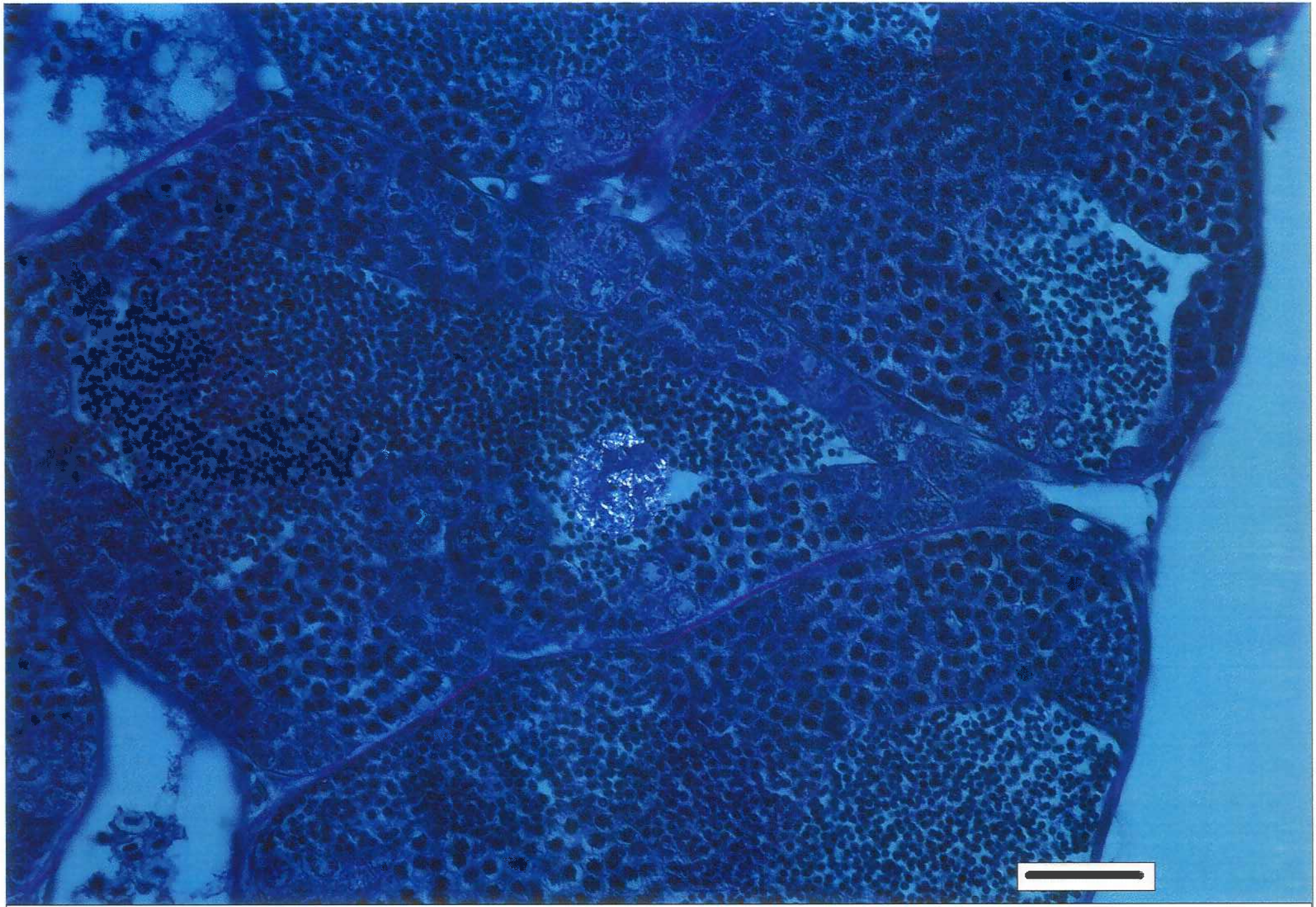

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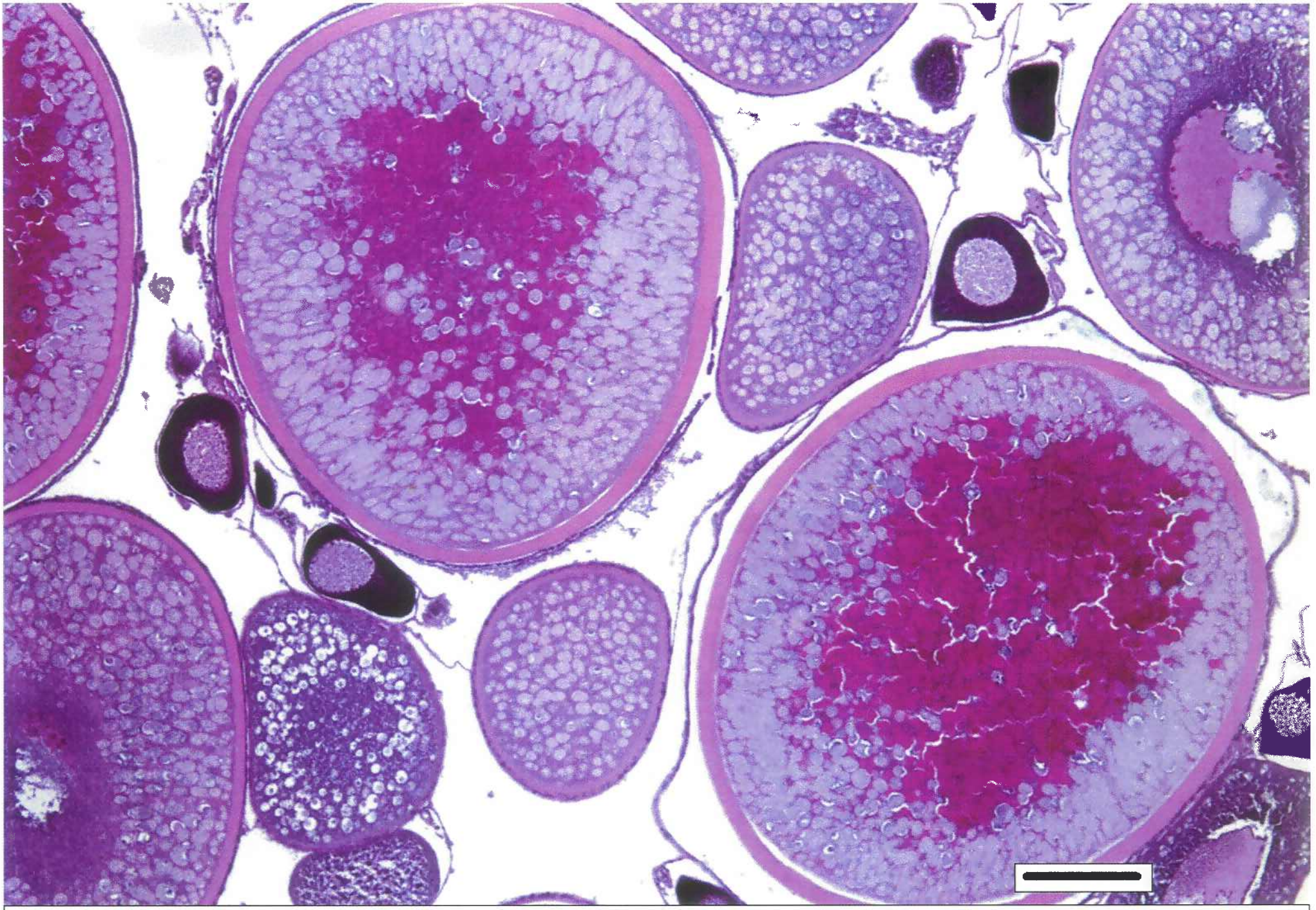

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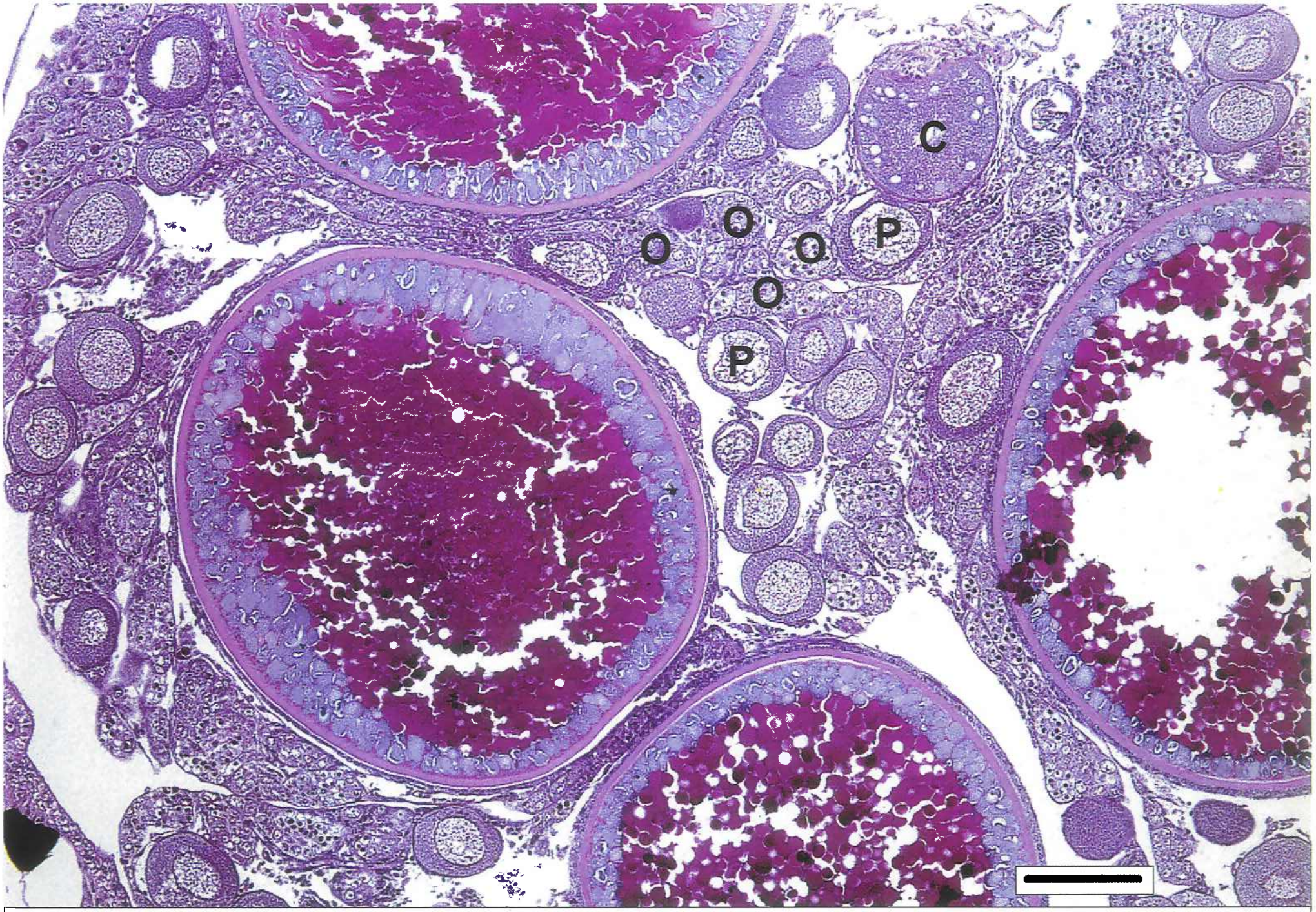

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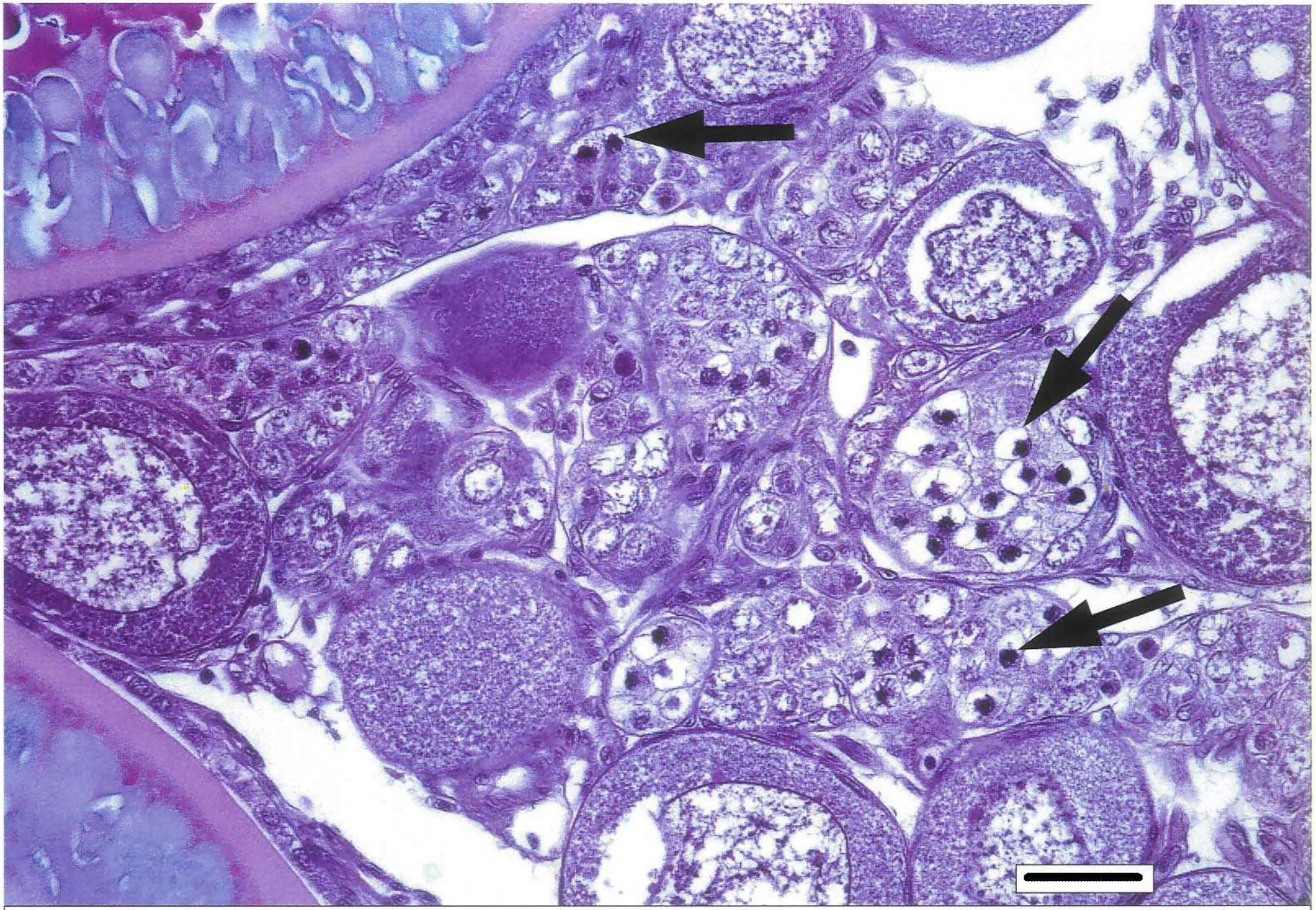

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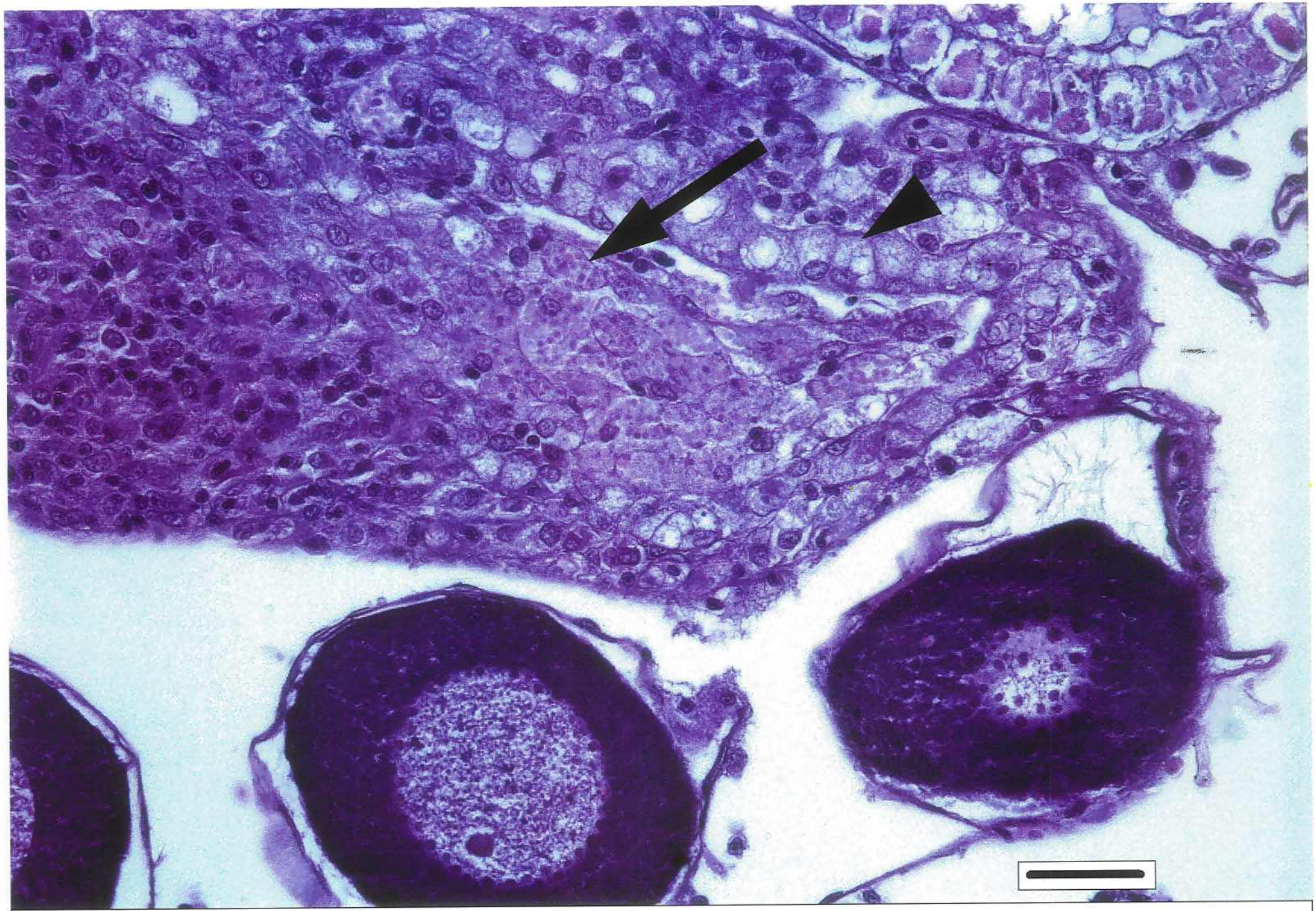

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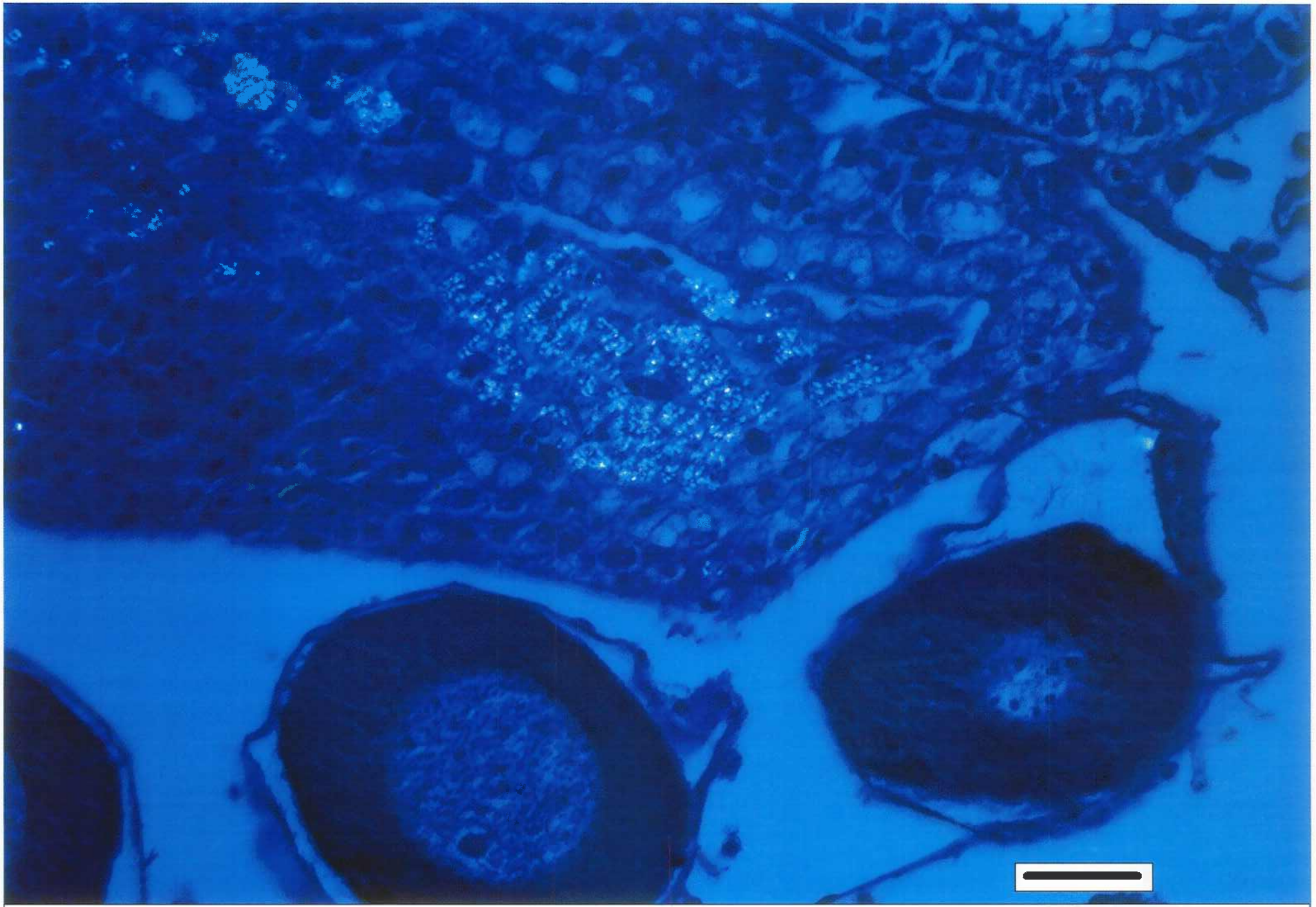

Z12635.tif WA 5-11 5AF3 FHM ovary H&E (polarized) bar = 25 microns

SUMMARY INCIDENCE TABLES

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP Cont/A	GROUP Cont/B	GROUP Cont/C	GROUP Cont/D	GROUP 100/A	GROUP 100/B
TESTIS (NO. EXAMINED)	(2)	(2)	(3)	(2)	(2)	(2)
Granulomatous Inflammation				2		1
Histiocytic Cells, Intraluminal						1
Increased Cells, Interstitial Cells						
Increased Cells, Spermatogonia			1	1		1
Interstitial Fibrosis				2		1
Microsporidia			1	1	1	1
Stage 1						
Stage 2	2	2	2	1	2	1
Stage 3			1	1		1
Testicular Degeneration, Increased						1

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 100/C	GROUP 100/D	GROUP 500/A	GROUP 500/B	GROUP 500/C	GROUP 500/D
TESTIS (NO. EXAMINED)	(1)	(3)	(2)	(2)	(2)	(2)
Granulomatous Inflammation						
Histiocytic Cells, Intraluminal						
Increased Cells, Interstitial Cells		1				
Increased Cells, Spermatogonia			1			
Interstitial Fibrosis						
Microsporidia						
Stage 1		1		1		
Stage 2	1	2	1	1	1	2
Stage 3			1		1	
Testicular Degeneration, Increased						

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 1000/A	GROUP 1000/B	GROUP 1000/C	GROUP 1000/D		
TESTIS (NO. EXAMINED)	(2)	(2)	(2)	(2)		
Granulomatous Inflammation						
Histiocytic Cells,						
Intraluminal	1		1			
Increased Cells, Interstitial						
Cells		1	1	1		
Increased Cells, Spermatogonia	1		1			
Interstitial Fibrosis						
Microsporidia				1		
Stage 1		1				
Stage 2	1		2	2		
Stage 3	1	1				
Testicular Degeneration,						
Increased	1					

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP Cont/A	GROUP Cont/B	GROUP Cont/C	GROUP Cont/D	GROUP 100/A	GROUP 100/B
OVARY (NO. EXAMINED)	(3)	(4)	(3)	(3)	(4)	(4)
Decreased Cells, Cortical Alveolar Oocytes						
Granulomatous Inflammation		1	1	1	2	3
Increased Cells, Oogonia						
Microsporidia			1		2	1
Oocyte Atresia, Increased	1	1	1	1	2	2
Stage 1		1				
Stage 2	2	1	1	1	2	2
Stage 3	1	2	2	2	2	2
Stage 4						

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 100/C	GROUP 100/D	GROUP 500/A	GROUP 500/B	GROUP 500/C	GROUP 500/D
OVARY (NO. EXAMINED)	(4)	(3)	(4)	(4)	(4)	(3)
Decreased Cells, Cortical Alveolar Oocytes	1					
Granulomatous Inflammation				1	1	
Increased Cells, Oogonia	1					
Microsporidia					1	
Oocyte Atresia, Increased		1	2	1		
Stage 1			1			
Stage 2	1	2		1	1	
Stage 3	3	1	3	3	3	3
Stage 4						

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 1000/A	GROUP 1000/B	GROUP 1000/C	GROUP 1000/D		
OVARY (NO. EXAMINED)	(3)	(4)	(4)	(4)		
Decreased Cells, Cortical Alveolar Oocytes						
Granulomatous Inflammation	2	1	1	1		
Increased Cells, Oogonia						
Microsporidia	1		1			
Oocyte Atresia, Increased	2	1				
Stage 1	1					
Stage 2		1				
Stage 3	2	2	4	4		
Stage 4		1				

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP Cont/A			GROUP Cont/B			GROUP Cont/C				GROUP Cont/D			GROUP 100/A			GROUP 100/B			
ANIMAL	6AM1	6AM2		6BM1	6BM2		6CF4	6CM1	6CM2		6DM1	6DM2		5AM1	5AM2		5BM1	5BM2		
TESTIS																				
Granulomatous Inflammation											1	1						1		
Histiocytic Cells, Intraluminal																		1		
Increased Cells, Interstitial Cells																				
Increased Cells, Spermatogonia							2				1							2		
Interstitial Fibrosis											1	1						1		
Microsporidia								1				1			1			1		
Stage 1																				
Stage 2	P	P		P	P			P	P			P		P	P		P			
Stage 3							P				P							P		
Testicular Degeneration, Increased																		1		

EPL Experimental Pathology Laboratories, Inc.

II-1

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 100/C			GROUP 100/D				GROUP 500/A			GROUP 500/B			GROUP 500/C			GROUP 500/D		
ANIMAL	5CM1	5CM2		5DM1	5DM2-1	5DM2-2		3AM1	3AM2		3BM1	3BM2		3CM1	3CM2		3DM1	3DM2	
TESTIS		N																	
Granulomatous Inflammation																			
Histiocytic Cells, Intraluminal																			
Increased Cells, Interstitial Cells				1															
Increased Cells, Spermatogonia									2										
Interstitial Fibrosis																			
Microsporidia																			
Stage 1				P								P							
Stage 2	P				P	P		P			P				P		P	P	
Stage 3									P					P					
Testicular Degeneration, Increased																			

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 1000/A		GROUP 1000/B		GROUP 1000/C		GROUP 1000/D	
ANIMAL	1AM1	1AM2	1BM1	1BM2	1CM1	1CM2	1DM1	1DM2
TESTIS								
Granulomatous Inflammation								
Histiocytic Cells, Intraluminal		1				1		
Increased Cells, Interstitial Cells			1			1		1
Increased Cells, Spermatogonia		3				1		
Interstitial Fibrosis								
Microsporidia								1
Stage 1			P					
Stage 2	P				P	P	P	P
Stage 3		P		P				
Testicular Degeneration, Increased		1						

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP Cont/A					GROUP Cont/B					GROUP Cont/C				GROUP Cont/D					
ANIMAL	6AF1	6AF2	6AF3	6AF4		6BF1	6BF2	6BF3	6BF4		6CF1	6CF2	6CF3		6DF1	6DF2	6DF3			
OVARY	N																			
Decreased Cells, Cortical Alveolar Oocytes																				
Granulomatous Inflammation						1						1			1					
Increased Cells, Oogonia																				
Microsporidia												1								
Oocyte Atresia, Increased			2					3				4					2			
Stage 1								P												
Stage 2		P		P					P				P			P				
Stage 3			P			P	P				P	P			P		P			
Stage 4																				

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11 Terminal Sacrifice Female Pimephales promelas	GROUP 100/A					GROUP 100/B					GROUP 100/C					GROUP 100/D				
ANIMAL	5AF1	5AF2	5AF3	5AF4		5BF1	5BF2	5BF3	5BF4		5CF1	5CF2	5CF3	5CF4		5DF1	5DF2	5DF3		
OVARY																				
Decreased Cells, Cortical																				
Alveolar Oocytes												2								
Granulomatous Inflammation		1	1			1		1	1											
Increased Cells, Oogonia												3								
Microsporidia		1	1			1														
Oocyte Atresia, Increased		3	4			1			1							3				
Stage 1																				
Stage 2		P		P				P	P					P			P	P		
Stage 3	P		P			P	P				P	P	P			P				
Stage 4																				

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 500/A					GROUP 500/B					GROUP 500/C					GROUP 500/D				
ANIMAL	3AF1	3AF2	3AF3	3AF4		3BF1	3BF2	3BF3	3BF4		3CF1	3CF2	3CF3	3CF4		3DF1	3DF2	3DF3		
OVARY																				
Decreased Cells, Cortical Alveolar Oocytes																				
Granulomatous Inflammation						1							1							
Increased Cells, Oogonia																				
Microsporidia													1							
Oocyte Atresia, Increased		3		3			2													
Stage 1				P																
Stage 2						P							P							
Stage 3	P	P	P				P	P	P		P	P		P		P	P	P		
Stage 4																				

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 1000/A				GROUP 1000/B				GROUP 1000/C				GROUP 1000/D			
ANIMAL	1AF2	1AF3	1AF4		1BF1	1BF2	1BF3	1BF4	1CF1	1CF2	1CF3	1CF4	1DF1	1DF2	1DF3	1DF4
OVARY																
Decreased Cells, Cortical Alveolar Oocytes																
Granulomatous Inflammation	1	2					1		2						1	
Increased Cells, Oogonia																
Microsporidia		1							1							
Oocyte Atresia, Increased	1	2					4									
Stage 1		P														
Stage 2						P										
Stage 3	P		P		P		P		P	P	P	P	P	P	P	P
Stage 4								P								

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac /death

Fish Endocrine Screening Assay Phase 1B OECD Protocol
Gonad Histopathology Results Worksheet

Fathead Minnow
Flutamide

LABORATORY'S NAME: Battelle Pacific Northwest Division, Batelle Memorial Institute

EPL Project No. 237-021
Study No. WA 5-11

VESSEL A

	Male		Female		Comments
Group CONT/A 0 µg/L	6AM1 stg2			6AF1	cassette received empty
	6AM2 stg2		6AF2 stg2		
			6AF3 stg3; F05-2		
			6AF4 stg2		
Group 10t/A 100 µg/L	5AM1 stg2		5AF1 stg3		
	5AM2 stg2; M28-1		5AF2 stg2; F05-3; F15-1; F28-1		
			5AF3 stg3; F05-4; F15-1; F28-1		
			5AF4 stg2		
Group 50t/A 500 µg/L	3AM1 stg2		3AF1 stg3		
	3AM2 stg3; M01-SPA-2		3AF2 stg3; F05-3		
			3AF3 stg3		
			3AF4 stg3; F05-3		
Group 10t/A 1000 µg/L	1AM1 stg2				
	1AM2 stg3; M01-SPA-3; M28-1; M05-1		1AF2 stg3; F15-1; R05-1		
			1AF3 stg1; F15-2; F05-2; F28-1		
			1AF4 stg3		

VESSEL B

	Male		Female		Comments
Group CONT/B 0 µg/L	6BM1 stg2		6BF1 stg3; F15-1		
	6BM2 stg2		6BF2 stg3		
			6BF3 stg1; F05-3		
			6BF4 stg2		
Group 10t/B 100 µg/L	5BM1 stg2		5BF1 stg3; F15-1; F05-1; F28-1		
	5BM2 stg3; M01-SPA-2; M05-1; M15-1; M11-1; M28-1		5BF2 stg3		
			5BF3 stg2; F15-1		
			5BF4 stg2; F05-1; F15-1		
Group 50t/B 500 µg/L	3BM1 stg2		3BF1 stg2; F15-1		
	3BM2 stg1		3BF2 stg3; F05-2		
			3BF3 stg3		
			3BF4 stg3		
Group 100t/B 1000 µg/L	1BM1 stg1; M01-SPA-1		1BF1 stg3		
	1BM2 stg3		1BF2 stg2		
			1BF3 stg3; F05-4; F15-1		
			1BF4 stg4		

VESSEL C

	Male		Female		Comments
Group CONT/C 0 µg/L	6CM1 stg2; M28-1		6CF1 stg3		
	6CM2 stg2		6CF2 stg3; F05-4; F15-1; F28-1		
			6CF3 stg2		
	6CF4 stg3; M01-SPA-2			6CF4 is a male	
Group 10t/C 100 µg/L	5CM1 stg2		5CF1 stg3		
			5CF2 stg3; F01-DOG-3; F02-DAC-2		No cassette labeled 5CM2 (two cassettes labeled 5CM2)
			5CF3 stg3		
			5CF4 stg2		
Group 50t/C 500 µg/L	3CM1 stg3		3CF1 stg3		
	3CM2 stg2		3CF2 stg3		
			3CF3 stg2; F15-1; F28-1		
			3CF4 stg3		
Group 100t/C 1000 µg/L	1CM1 stg2		1CF1 stg3; F15-2; F28-1		
	1CM2 stg2; M01-SPA-1; M16-1; M01-ISO-1		1CF2 stg3		
			1CF3 stg3		
			1CF4 stg3		

VESSEL D

	Male	Female	Comments
Group CONTID 0 µg/L	6DM1 stg3; M11-1; M15-1; M01-SPA-1	6DF1 stg3; F15-1	
	5DM3 stg3; M15-1; M15-1; M28-1	6DF2 stg2	
		6DF3 stg3; F05-2	
Group 100/D 100 µg/L	5DM1 stg3; M01-ISC-1	5DF1 stg3; F05-3	
	5DM2-1 stg2	5DF2 stg2	
	5DM2-2 stg2	5DF3 stg2	
Group 500/D 500 µg/L	3DM1 stg2	3DF1 stg3	
	3DM2 stg2	3DF2 stg3	
		3DF3 stg3	
Group 1000/D 1000 µg/L	1DM1 stg2	1DF1 stg3	
	1DM2 stg2; M01-ISC-1; M28-1	1DF2 stg3	
		1DF3 stg3; F15-1	
		1DF4 stg3	

Diagnosis Codes_Testis:	
M01	Increased cells (followed by Cell Type Code)
M02	Decreased cells (followed by Cell Type Code)
M03	Testis-ova
M04	Germ cell atrophy, male
M06	Hermaph. degeneration, increased
M06	Hermaph. degeneration, increased (comment)
M07	Asynchronous development, gonad
M08	Asynchronous development, oocyte
M09	Asynchronous development, oocyte & left gonads
M05	Proliferacaous fluid, intravascular
M06	Proliferacaous fluid, interstitial
M11	Interstitial fibrosis
M12	Sertoli cell hypertrophy
M13	Hepatocyte basophilia, increased
M14	Myo-myopathy
M15	Granulomatous inflammation
M16	Histiocytic cells, intratesticular
M17	Increased peritoneal attachments
M18	Mineralization, testes
M19	Renal tubules, mineralization
M20	Mineralization, collecting duct
M21	Sperm necrosis
M22	Hypertrophy (followed by Cell Type)
M23	Renal tubules, dilatation
M24	Kidney, granulomatous inflammation
M25	Exfoliated germ cells, increased
M26	Macrophage aggregates, increased
M27	Seminoma, spermatocytic
M28	Microsporidia

Diagnosis Codes_Ovary:	
F01	Increased cells (followed by Cell Type Code)
F02	Decreased cells (followed by Cell Type Code)
F03	Ovarian appendages
F04	Hermaphroditism, female
F05	Oocyte atresia, increased
F06	Oocyte atresia, increased, mature
F07	Asynchronous development, gonad
F08	Asynchronous development, oocyte & left gonads
F09	Proliferacaous fluid, intravascular
F10	Proliferacaous fluid, interstitial
F11	Interstitial fibrosis
F12	Post-ovulatory follicles, increased
F13	Hepatocyte basophilia, decreased
F14	Myo-myopathy
F15	Granulomatous inflammation
F16	Histiocytic cells, intratesticular
F17	Oocyte membrane folding
F18	Egg debris, ovarian
F19	Renal tubules, mineralization
F20	Mineralization, collecting duct
F21	Hypertrophy (followed by Cell Type)
F22	Hypertrophy (followed by Cell Type)
F23	Renal tubules, dilatation
F24	Post-ovulatory follicles, decreased
F25	Ovarian cyst
F26	Ovarian mineralization
F27	Oocyte malformation
F28	Microsporidia
F29	Ectopic neural tissue

Severity Codes:	
1	minimal
2	mild
3	moderate
4	severe

Cell Type Codes:	
SPA	Spermatocytes
SPC	Spermatocytes
SPT	Spermatids
SPZ	Spermatozoa
STC	Sertoli cells
ISC	Interstitial cells (Leydig cells)
PND	Primordial oocytes
CAO	Cortical alveolar oocytes
EVO	Early vitellogenic oocytes
LVO	Late vitellogenic oocytes
MSO	Mature / spawning oocytes
PFC	Perifollicular (Granulosa/theca) cells
OOG	Oogonia

Maturation Codes:		
stg1		Stage Juvenile
stg0		Stage 0
stg1		Stage 1
stg2		Stage 2
stg3		Stage 3
stg4		Stage 4
stg5		Stage 5
NR	Not remarkable	
UTS	Unable to stage	

Potassium Permanganate

EPL® Experimental Pathology Laboratories, Inc.

BATTELLE PACIFIC NORTHWEST DIVISION,
BATTELLE MEMORIAL INSTITUTE
STUDY NUMBER WA 5-11, SSL NO. 13784.6109
EPL PROJECT NUMBER 237-023

FISH SCREENING ASSAY OECD PHASE 1B FOLLOW-UP

DRAFT PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.
P.O. Box 474
Herndon, VA 20172-0474
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Submitted to:

Battelle Pacific Northwest Division, Battelle Memorial Institute
Sequim, WA 98382

November 18, 2005

EPL® Experimental Pathology Laboratories, Inc.

TABLE OF CONTENTS

DRAFT PATHOLOGY SUMMARY

EPL® Experimental Pathology Laboratories, Inc.

BATTELLE PACIFIC NORTHWEST DIVISION,
BATTELLE MEMORIAL INSTITUTE
STUDY NUMBER WA 5-11, SSL NO. 13784.6109
EPL PROJECT NUMBER 237-023

FISH SCREENING ASSAY OECD PHASE 1B FOLLOW-UP

DRAFT PATHOLOGY SUMMARY

INTRODUCTION

The objective of this study was to determine the effects, if any, of potassium permanganate administered via water bath on gonadal tissue of adult fathead minnows (FHM, *Pimephales promelas*).

The experimental design is presented in the following table:

Table 1. Experimental Design for Potassium Permanganate Study

Exposure Group	Potassium Permanganate	Male Replicates				Female Replicates			
		A	B	C	D	A	B	C	D
6	0 µg/L (Control)	2	2	2	2	4	4	4	4
5	225 µg/L	2	2	3	2	3	4	3	4
3	450 µg/L	2	2	2	2	4	4	4	4
1	900 µg/L	1	2	4	2	3	4	1	4

METHODS

Unless otherwise indicated, histopathological procedures were performed according to the draft form of the "OECD Guidance Document for Performing Gonadal Histopathology in Small Fish." Briefly, following routine processing the left and right gonads were embedded horizontal to their long axis to allow for longitudinal sectioning. During microtomy, the first section from each block was acquired at the point at which approximately half of the gonad had been cut away and the size of the section was maximized. The second and third sections were

then obtained at 50 micron intervals. Sections were stained with hematoxylin and eosin, and mounted with glass coverslips. Labels included the EPL Project No. (237-023), the group/replicate designation (e.g., PP900/D), the study ID (WA 5-11), and the Animal No. (e.g., 1AM1).

The pathologist evaluated the slides by brightfield microscopy for changes that included, but were not limited to, the types of findings that are listed in the aforementioned guidance document. As per that document, severity grading of findings was performed according to the following scale: NR = not remarkable, grade 1 = minimal, grade 2 = mild, grade 3 = moderate, grade 4 = severe. Ovarian oocyte atresia was graded according to the following scale: Grade 1 = 3 to 5 atretic oocytes per ovary; Grade 2 = 6 to 9 atretic oocytes per ovary; Grade 3 = greater than 9 atretic oocytes per ovary, but less than the vast majority; and Grade 4 = the vast majority of oocytes were atretic. The pathologist recorded findings on a spreadsheet. This original spreadsheet as contained within the guidance document was modified slightly by the study pathologist to include the addition of a column in order to accommodate the animal numbers of the female fathead minnows (which were different from what appeared on the worksheet), and corrections of some of the animal numbers to correspond with the animal numbers that were submitted by the client. The data collection spreadsheet is incorporated into this report. Results were simultaneously recorded into EPL's Pathology Data Reporting System, and tabulated in the accompanying Histopathology Incidence Tables (HIT) and summarized in the Summary Incidence Tables (SIT).

RESULTS

Males

Based on incidence and/or severity data, there were no findings that were clearly different in potassium permanganate-exposed males as compared to control males. There was a slight increase in the incidence and severity of "Increased Cells, Spermatogonia" in the 900 µg/L male group as compared to the control males. The difference in incidence alone is unlikely to be statistically significant; however, it is possible that the combination increase in incidence and severity in the 900 µg/L male group could be significant. Figures 1 through 4 illustrate the various severity grades of spermatogonial increase.

There was a very slight, non-dose-responsive, increase in the testicular stage scores of the 225, 450, and 900 µg/L male groups as compared to controls; these differences are unlikely to be statistically significant.

Single, approximately 15-50 µm diameter, ovoid, parasite cysts were occasionally evident within testis tubule lumina (Fig 5). Each thin-walled cyst was separated into compartments by narrow septa, and was packed with oval, amphophilic, spore-like organisms (approximately 1-2 µm diameter). These organisms were consistent with microsporidia. The cysts were associated with minimal, if any, inflammation in the testis. The incidence of parasitism was not associated with potassium permanganate exposure.

Animal No. 6CF4 was originally reported to be a female, but on dissection was thought to be a male. Histopathological evaluation revealed that this fish was actually (morphologically) a female.

The incidence and severity of selected histopathologic results for male FHM are presented in the following table:

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11, SSL No. 13784.6109
11/18/05 DRAFT

Table 2. Combined Incidence and Severity of Selected Histopathologic Findings in Male Fathead Minnows

Exposure Group	6					5					3					1				
Potassium Permanganate Dose (µg/L)	0					225					450					900				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	2	2	2	2	8	2	2	3	2	9	2	2	2	2	8	1	2	4	2	9
Increased Cells, Spermatogonia	0	0	0	1	1	0	0	0	1	1	0	1	0	0	1	0	0	2	1	3
minimal	-	-	-	1	1	-	-	-	1	1	-	1	-	-	1	-	-	-	-	-
mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1
Testicular Degeneration, Increased	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1
minimal	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	1	-	1
mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Microsporidia	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	0	0	0	1	1
minimal	-	1	-	-	1	-	1	-	-	1	-	-	1	-	1	-	-	-	1	1
mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testicular Stage																				
Stage 1	1	1	1	-	3	-	-	-	1	1	-	-	-	1	1	-	-	1	-	1
Stage 2	1	1	1	1	4	1	2	2	-	5	2	2	2	1	7	1	1	1	1	4
Stage 3	-	-	-	1	1	1	-	1	1	3	-	-	-	-	-	-	1	2	1	4
Average	1.8					2.2					1.9					2.3				

*T = totals

Females

Based on incidence and/or severity data, there were no findings that were clearly different in potassium permanganate-exposed females as compared to control females. One potential difference was a slight decrease in ovarian stage scores in the 450 and 900 µg/L female groups as compared to controls.

As in the testes of occasional males, microsporidia were evident in the ovarian interstitium of two females, both of which were in the 450 µg/L group (Fig. 6). However, unlike the situation in males, the ovarian microsporidia were associated with granulomatous inflammation, and the spores were present within host macrophages instead of parasitic cysts.

The incidence and severity of selected histopathologic results for female fathead minnows are presented in the following table:

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11, SSL No. 13784.6109
11/18/05 DRAFT

Table 3. Combined Incidence and Severity of Selected Histopathologic Findings in Female Fathead Minnows

Exposure Group	6					5					3					1				
Potassium Permanganate Dose (µg/L)	0					225					450					900				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	4	4	4	4	16	3	4	3	4	14	4	4	4	4	16	3	4	1	4	12
Oocyte Atresia	1	0	0	0	1	0	0	0	2	2	2	0	1	0	3	0	1	0	0	1
minimal	-	-	-	-	-	-	-	-	1	1	1	-	-	-	1	-	-	-	-	-
mild	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	1	-	-	1
moderate	1	-	-	-	1	-	-	-	-	-	1	-	1	-	2	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Microsporidia	0	0	0	0	0	0	0	0	0	0	1	0	1	0	2	-	-	0	0	-
minimal	-	-	-	-	-	-	-	-	-	-	1	-	1	-	2	-	-	-	-	-
mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Granulom. Inflammation	0	0	1	0	1	0	1	1	1	3	1	0	1	1	3	1	1	0	0	2
minimal	-	-	1	-	1	-	1	1	1	3	1	-	1	1	3	1	1	-	-	2
mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovarian Stage																				
Stage 0	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	1	1	-	2
Stage 1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-
Stage 2	-	1	3	2	6	1	2	1	1	5	2	2	2	1	7	1	2	-	-	3
Stage 3	3	2	1	2	8	1	2	2	1	6	2	1	1	2	6	2	-	-	3	5
Stage 4	1	1	-	-	2	1	-	-	2	3	-	1	-	-	1	-	1	-	1	2
Average	2.8					2.9					2.3					2.4				

*T = totals

DISCUSSION

Potassium permanganate, an oxidizing agent that reacts with organic matter, is used as an external parasiticide and bactericide in fish. Studies that document the histopathological effects of potassium permanganate toxicity in fish are scarce. In one such report involving channel catfish *Ictalurus punctatus* (Darwish et al., 2002), the sole affected tissue was gill; however, gill, liver, and trunk kidney were the only tissues examined. A brief literature review did not yield any publications in which the potential gonadal effects of potassium permanganate were evaluated.

As reported above, one possible effect of potassium permanganate in the present study was an increase in the proportion of spermatogonia in the 900 µg/L group males. This cannot be considered a clear effect with confidence due to the relatively small number of males exhibiting this finding, and the fact that even severe increases in spermatogonia can be observed occasionally in the testes of control FHM.

Another potential effect of potassium permanganate was a slight decrease in ovarian stage scores in the 450 and 900 µg/L female groups as compared to controls. This result is unlikely to be biologically significant, however, as the difference in stage scores between treated and control fish appears largely driven by the low combined incidence of Stage 0 and Stage 1 ovaries in the 450 and 900 µg/L dose groups (i.e., only three of twenty-eight fish were Stage 0 and only one of twenty-eight fish was Stage 1).

The presence of microsporidia in the testes and ovaries of some fish is not considered to be a confounding factor in this study, because the incidence and severity of the parasite infection (and the granulomatous inflammation that occasionally accompanied this infection in the ovary), were low.

Other findings in this study either occurred in comparable numbers of control and potassium permanganate-exposed fish (background lesions), or as low-frequency (incidental) findings, and are therefore not considered to be exposure-related.

CONCLUSION AND SUMMARY

There were no histopathological findings in this study that were clearly attributable to potassium permanganate exposure. Potential effects include an increase in the proportion of spermatogonia in the testis in the 900 µg/L group males and decreased ovarian stage scores in the 450 and 900 µg/L group females.

JEFFREY C. WOLF, DVM, Diplomate, ACVP Date
Veterinary Pathologist

JCW/cb

REFERENCES

Darwish AM, Griffin BR, Straus DL, Mitchell AJ. (2002) Histological and hematological evaluation of potassium permanganate toxicity in channel catfish. *Journal of Aquatic Animal Health*, 14: 134-144.

OECD Draft Guidance Document for Performing Gonadal Histopathology in Small Fish: Histology and Histopathology Guidelines for Phase 1B of the OECD Fish Screening Assay for EDC's. (2004).

EPL® Experimental Pathology Laboratories, Inc.

11/18/05 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Fish Screening Assay OECD Phase 1B Follow-Up

Client Study: WA 5-11, SSL No. 13784.6109

EPL Project Coordinator: Dr. Jeffrey C. Wolf

EPL Project Number: 237-023

EPL Pathologist: Dr. Jeffrey C. Wolf

The following aspects of this study were inspected by the Quality Assurance Unit of Experimental Pathology Laboratories, Inc. Dates inspections were performed and findings reported to the EPL Project Coordinator and Management are indicated below.

Area Inspected	Dates	
	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management _____

Date of last quarterly facility inspection 7/05

EPL Quality Assurance Unit _____ Date _____

Form No. 6-2 (7/2/99)

SUMMARY INCIDENCE TABLES

SUMMARY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Male Pimephales Promelas

	GROUP CONT/A	GROUP CONT/B	GROUP CONT/C	GROUP CONT/D		
TESTIS (NO. EXAMINED)	(2)	(2)	(2)	(2)		
Histiocytic Cells, Intraluminal				1		
Increased Cells, Interstitial Cells (Leydig Cells)			1			
Increased Cells, Spermatogonia				1		
Inflammation, Granulomatous						
Inflammation, Mixed Cells				1		
Microsporidia		1				
Mineralization			1			
Mineralization, Collecting Duct			1	1		
Stage 1	1	1	1			
Stage 2	1	1	1	1		
Stage 3				1		
Testicular Degeneration, Increased						

SUMMARY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Male Pimephales Promelas

	GROUP 225/A	GROUP 225/B	GROUP 225/C	GROUP 225/D		
TESTIS (NO. EXAMINED)	(2)	(2)	(3)	(2)		
Histiocytic Cells, Intraluminal						
Increased Cells, Interstitial Cells (Leydig Cells)		1		1		
Increased Cells, Spermatogonia				1		
Inflammation, Granulomatous						
Inflammation, Mixed Cells						
Microsporidia		1				
Mineralization			1			
Mineralization, Collecting Duct			1			
Stage 1				1		
Stage 2	1	2	2			
Stage 3	1		1	1		
Testicular Degeneration, Increased						

SUMMARY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Male Pimephales Promelas

	GROUP 450/A	GROUP 450/B	GROUP 450/C	GROUP 450/D		
TESTIS (NO. EXAMINED)	(2)	(2)	(2)	(2)		
Histiocytic Cells, Intraluminal						
Increased Cells, Interstitial Cells (Leydig Cells)						
Increased Cells, Spermatogonia		1				
Inflammation, Granulomatous	1					
Inflammation, Mixed Cells			1			
Microsporidia			1			
Mineralization			1			
Mineralization, Collecting Duct			1			
Stage 1				1		
Stage 2	2	2	2	1		
Stage 3						
Testicular Degeneration, Increased			1			

SUMMARY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Male Pimephales Promelas

	GROUP 900/A	GROUP 900/B	GROUP 900/C	GROUP 900/D		
TESTIS (NO. EXAMINED)	(1)	(2)	(4)	(2)		
Histiocytic Cells, Intraluminal						
Increased Cells, Interstitial Cells (Leydig Cells)						
Increased Cells, Spermatogonia			2	1		
Inflammation, Granulomatous						
Inflammation, Mixed Cells			1			
Microsporidia				1		
Mineralization		1	1			
Mineralization, Collecting Duct			1			
Stage 1			1			
Stage 2	1	1	1	1		
Stage 3		1	2	1		
Testicular Degeneration, Increased			1			

SUMMARY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Female Pimephales Promelas

	GROUP CONT/A	GROUP CONT/B	GROUP CONT/C	GROUP CONT/D		
OVARY (NO. EXAMINED)	(4)	(4)	(4)	(4)		
Atrophy						
Inflammation, Granulomatous			1			
Inflammation, Mixed Cells			1			
Macrophage Aggregates, Increased						
Microsporidia						
Oocyte Atresia, Increased	1					
Stage 0						
Stage 1						
Stage 2		1	3	2		
Stage 3	3	2	1	2		
Stage 4	1	1				

SUMMARY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Female Pimephales Promelas

	GROUP 225/A	GROUP 225/B	GROUP 225/C	GROUP 225/D		
OVARY (NO. EXAMINED)	(3)	(4)	(3)	(4)		
Atrophy						
Inflammation, Granulomatous		1	1	1		
Inflammation, Mixed Cells				1		
Macrophage Aggregates, Increased						
Microsporidia						
Oocyte Atresia, Increased				2		
Stage 0						
Stage 1						
Stage 2	1	2	1	1		
Stage 3	1	2	2	1		
Stage 4	1			2		

SUMMARY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Female Pimephales Promelas

	GROUP 450/A	GROUP 450/B	GROUP 450/C	GROUP 450/D		
OVARY (NO. EXAMINED)	(4)	(4)	(4)	(4)		
Atrophy			1			
Inflammation, Granulomatous	1		1	1		
Inflammation, Mixed Cells				1		
Macrophage Aggregates, Increased						
Microsporidia	1		1			
Oocyte Atresia, Increased	2		1			
Stage 0			1			
Stage 1				1		
Stage 2	2	2	2	1		
Stage 3	2	1	1	2		
Stage 4		1				

SUMMARY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Female Pimephales Promelas

	GROUP 900/A	GROUP 900/B	GROUP 900/C	GROUP 900/D		
OVARY (NO. EXAMINED)	(3)	(4)	(1)	(4)		
Atrophy		1				
Inflammation, Granulomatous	1	1				
Inflammation, Mixed Cells		1				
Macrophage Aggregates, Increased		1				
Microsporidia						
Oocyte Atresia, Increased		1				
Stage 0		1	1			
Stage 1						
Stage 2	1	2				
Stage 3	2			3		
Stage 4		1		1		

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Male Pimephales Promelas

	GROUP CONT/A			GROUP CONT/B			GROUP CONT/C			GROUP CONT/D		
ANIMAL	6AM1	6AM2		6BM1	6BM2		6CM1	6CM2		6DM1	6DM2	
TESTIS												
Histiocytic Cells, Intraluminal											1	
Increased Cells, Interstitial Cells (Leydig Cells)							2					
Increased Cells, Spermatogonia											1	
Inflammation, Granulomatous												
Inflammation, Mixed Cells											1	
Microsporidia					1							
Mineralization								1				
Mineralization, Collecting Duct								1			1	
Stage 1		P			P		P					
Stage 2	P			P				P		P		
Stage 3											P	
Testicular Degeneration, Increased												

EPL Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Male Pimephales Promelas

	GROUP 225/A			GROUP 225/B			GROUP 225/C			GROUP 225/D		
ANIMAL	5AM1	5AM2		5BM1	5BM2		5CM1	5CM2	5CM3	5DM1	5DM2	
TESTIS												
Histiocytic Cells, Intraluminal												
Increased Cells, Interstitial Cells (Leydig Cells)					1					2		
Increased Cells, Spermatogonia										1		
Inflammation, Granulomatous												
Inflammation, Mixed Cells												
Microsporidia					1							
Mineralization							1					
Mineralization, Collecting Duct							2					
Stage 1										P		
Stage 2	P			P	P		P	P				
Stage 3		P							P		P	
Testicular Degeneration, Increased												

II-2

EPL | Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Male Pimephales Promelas

	GROUP 450/A			GROUP 450/B			GROUP 450/C			GROUP 450/D		
ANIMAL	3AM1	3AM2		3BM1	3BM2		3CM1	3CM2		3DM1	3DM2	
TESTIS												
Histiocytic Cells, Intraluminal												
Increased Cells, Interstitial Cells (Leydig Cells)												
Increased Cells, Spermatogonia					1							
Inflammation, Granulomatous	1											
Inflammation, Mixed Cells							1					
Microsporidia							1					
Mineralization								3				
Mineralization, Collecting Duct								2				
Stage 1											P	
Stage 2	P	P		P	P		P	P		P		
Stage 3												
Testicular Degeneration, Increased							1					

EPL Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Male Pimephales Promelas

	GROUP 900/A		GROUP 900/B			GROUP 900/C					GROUP 900/D									
ANIMAL	1AM1		1BM1	1BM2		1CM1	1CM2	1CM3	1CM4		1DM1	1DM2								
TESTIS																				
Histiocytic Cells, Intraluminal																				
Increased Cells, Interstitial Cells (Leydig Cells)																				
Increased Cells, Spermatogonia							2		4			3								
Inflammation, Granulomatous																				
Inflammation, Mixed Cells									1											
Microsporidia												1								
Mineralization			1					1												
Mineralization, Collecting Duct								1												
Stage 1									P											
Stage 2	P		P			P					P									
Stage 3				P			P	P				P								
Testicular Degeneration, Increased							1													

II-4

EPL | Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Female Pimephales Promelas

	GROUP CONT/A					GROUP CONT/B					GROUP CONT/C					GROUP CONT/D				
ANIMAL	6AF1	6AF2	6AF3	6AF4		6BF1	6BF2	6BF3	6BF4		6CF1	6CF2	6CF3	6CF4		6DF1	6DF2	6DF3	6DF4	
OVARY																				
Atrophy																				
Inflammation, Granulomatous											1									
Inflammation, Mixed Cells											1									
Macrophage Aggregates, Increased																				
Microsporidia																				
Oocyte Atresia, Increased		3																		
Stage 0																				
Stage 1																				
Stage 2						P					P	P		P			P	P		
Stage 3	P		P	P			P	P					P			P			P	
Stage 4		P							P											

II-5

EPL Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Female Pimephales Promelas

	GROUP 225/A				GROUP 225/B				GROUP 225/C				GROUP 225/D			
ANIMAL	5AF1	5AF2	5AF3		5BF1	5BF2	5BF3	5BF4	5CF2	5CF3	5CF4		5DF1	5DF2	5DF3	5DF4
OVARY																
Atrophy																
Inflammation, Granulomatous								1			1				1	
Inflammation, Mixed Cells																1
Macrophage Aggregates, Increased																
Microsporidia																
Oocyte Atresia, Increased														1		2
Stage 0																
Stage 1																
Stage 2			P		P			P		P				P		
Stage 3	P					P	P				P					P
Stage 4		P											P		P	

EPL Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Female Pimephales Promelas

	GROUP 450/A					GROUP 450/B					GROUP 450/C					GROUP 450/D				
ANIMAL	3AF1	3AF2	3AF3	3AF4		3BF1	3BF2	3BF3	3BF4		3CF1	3CF2	3CF3	3CF4		3DF1	3DF2	3DF3	3DF4	
OVARY																				
Atrophy														3						
Inflammation, Granulomatous	1										1						1			
Inflammation, Mixed Cells																		1		
Macrophage Aggregates, Increased																				
Microsporidia	1										1									
Oocyte Atresia, Increased		1	3								3									
Stage 0														P						
Stage 1																	P			
Stage 2	P		P				P	P			P	P						P		
Stage 3		P		P		P							P			P			P	
Stage 4									P											

II-7

EPL Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11, SSL No. 13784.6109
Terminal Sacrifice
Female Pimephales Promelas

	GROUP 900/A				GROUP 900/B				GROUP 900/C		GROUP 900/D								
ANIMAL	1AF1	1AF2	1AF3		1BF1	1BF2	1BF3	1BF4		1CF2		1DF1	1DF2	1DF3	1DF4				
OVARY																			
Atrophy								1											
Inflammation, Granulomatous	1					1													
Inflammation, Mixed Cells								1											
Macrophage Aggregates, Increased							2												
Microsporidia																			
Oocyte Atresia, Increased							2												
Stage 0								P		P									
Stage 1																			
Stage 2		P				P	P												
Stage 3	P		P									P	P		P				
Stage 4					P									P					

EPL Experimental Pathology Laboratories, Inc.

Key : X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

APPENDIX A

GONAD HISTOPATHOLOGY RESULTS WORKSHEET

Fish Endocrine Screening Assay Phase 1B OECD Protocol Gonad Histopathology Results Worksheet				Fathead Minnow Pimephales promelas
LABORATORY'S NAME:	Battelle Pacific Northwest Division, Battelle Memorial Institute			EPL Project No. 237-023
				Study No. WA 5-11

VESSEL A

	Male		Female		Comments
Group CONT/A 0 µg/L	6AM1	stg2;	6AF1	stg3;	
	6AM2	stg1;	6AF2	stg4; F05-3	
			6AF3	stg3;	
			6AF4	stg3;	
Group PP225A 225 µg/L	5AM1	stg2;	5AF1	stg3	
	5AM2	stg3;	5AF2	stg4;	
			5AF3	stg2;	
Group PP450A 450 µg/L	3AM1	stg2; M15-1	3AF1	stg2; F28-1; F15-1	
	3AM2	stg3;	3AF2	stg3; F05-1	
			3AF3	stg2; F05-3;	
			3AF4	stg3;	
Group PP900A 900 µg/L	1AM1	stg2;	1AF1	stg3; F15-1	
			1AF2	stg2;	
			1AF3	stg3;	

VESSEL B

	Male		Female		Comments
Group CONT/B 0 µg/L	6BM1	stg2;	6BF1	stg2;	
	6BM2	stg1; M29-1	6BF2	stg4;	
			6BF3	stg3;	
			6BF4	stg4;	
Group PP225B 225 µg/L	5BM1	stg2;	5BF1	stg2;	
	5BM2	stg3; M28-1; M01-ISC-1	5BF2	stg3	
			5BF3	stg3;	
			5BF4	stg2; F15-1	
Group PP450B 450 µg/L	3BM1	stg2;	3BF1	stg3;	
	3BM2	stg1; M01-SPA-1	3BF2	stg2;	
			3BF3	stg2;	
			3BF4	stg4;	
Group PP900B 900 µg/L	1BM1	stg2; M18-1	1BF1	stg4;	
	1BM2	stg3;	1BF2	stg2; F15-1	
			1BF3	stg3; F05-2; F18-2	
			1BF4	stg3; F29-1; F27-1	

VESSEL C

	Male		Female		Comments
Group CONT/C 0 µg/L	6CM1	stg1; M01-ISC-2	6CF1	stg2; F29-1; F15-1	
	6CM2	stg2; M18-1; M20-1	6CF2	stg3;	
			6CF3	stg3;	
			6CF4	stg2;	
Group PP225C 225 µg/L	5CM1	stg2; M18-1; M20-2;			
	5CM2	stg2;	5CF2	stg3;	
	5CM3	stg3;	5CF3	stg2;	
			5CF4	stg3; F15-1	
Group PP450C 450 µg/L	3CM1	stg2; M28-1; M05-1; M29-1	3CF1	stg2; F05-3; F28-1; F18-1	
	3CM2	stg3; M18-1; M20-2	3CF2		
			3CF3	stg3;	
			3CF4	stg3; F27-1	
Group PP900C 900 µg/L	1CM1	stg2;			
	1CM2	stg3; M05-1; M01-SPA-1	1CF2	stg2;	
	1CM3	stg3; M18-1; M20-1			
	1CM4	stg1; M01-SPA-1; M29-1			

VESSEL D

	Male		Female		Comments
Group CONT/D 0 µg/L	6DM1	stg2;	6DF1	stg3;	
	6DM2	stg3; M29-1; M18-1; M20-1; M01-SPA-1	6DF2	stg2;	
			6DF3	stg2;	
			6DF4	stg3;	
Group PP225D 225 µg/L	5DM1	stg1; M01-ISC-2; M01-SPA-1	5DF1	stg4;	
	5DM2	stg3;	5DF2	stg2; F05-1	
			5DF3	stg4; F29-1	
			5DF4	stg4; [illegible]	
Group PP450D 450 µg/L	3DM1	stg2;	3DF1	stg3;	
	3DM2	stg3;	3DF2	stg1; F15-1	
			3DF3	stg2; F29-1;	
			3DF4	stg3;	
Group PP900D 900 µg/L	1DM1	stg2;	1DF1	stg3;	
	1DM2	stg3; M01-SPA-1; M28-1	1DF2	stg3;	
			1DF3	stg4;	
			1DF4	stg3;	

Diagnosis Codes, Testis:		Diagnosis Codes, Ovary:	
M01	Increased cells (followed by Cell Type Code)	F01	Increased cells (followed by Cell Type Code)
M02	Decreased cells (followed by Cell Type Code)	F02	Decreased cells (followed by Cell Type Code)
M03	Testis-ova	F03	Ovarian spermatogenesis
M04	Hermaphrodism, male	F04	Hermaphrodism, female
M05	Testicular degeneration, increased	F05	Oocyte atresia, increased
M06	Asynchronous development, spermatocyst	F06	Oocyte atresia, increased, mature
M07	Asynchronous development, gonad	F07	Asynchronous development, gonad
M08	Asynchronous development, right & left gonads	F08	Asynchronous development, right & left gonads
M09	Proteinaceous fluid, intravascular	F09	Proteinaceous fluid, intravascular
M10	Proteinaceous fluid, interstitial	F10	Proteinaceous fluid, interstitial
M11	Interstitial fibrosis	F11	Interstitial fibrosis
M12	Sertoli cell hypertrophy	F12	Post-ovulatory follicles, increased
M13	Hepatocyte basophilia, increased	F13	Hepatocyte basophilia, decreased
M14	Nephropathy	F14	Nephropathy
M15	Granulomatous inflammation	F15	Granulomatous inflammation
M16	Histiocytic cells, intraluminal	F16	Macrophage aggregates, increased
M17	Retained peritoneal attachments	F17	Oocyte membrane folding
M18	Mineralization	F18	Egg debris, oviduct
M19	Renal tubules, mineralization	F19	Renal tubules, mineralization
M20	Mineralization, collecting duct	F20	Skeletal muscle degeneration
M21	Sperm necrosis	F21	Decreased vitellogenesis
M22	Hypertrophy (followed by Cell Type)	F22	Hypertrophy (followed by Cell Type)
M23	Renal tubules, dilatation	F23	Renal tubules, dilatation
M24	Kidney, granulomatous inflammation	F24	Post-ovulatory follicles, decreased
M25	Exfoliated germ cells, increased	F25	Ovarian cyst
M26	Macrophage aggregates, increased	F26	Ovarian mineralization
M27	Seminoma, spermatocytic	F27	Atrophy
M28	Microsporidia	F28	Microsporidia
M29	Inflammation, mixed cells	F29	Inflammation, mixed cells

Severity Codes:	
1	minimal
2	mild
3	moderate
4	severe

Cell Type Codes:	
SPA	Spermatogonia
SPC	Spermatocytes
SPT	Spermatids
SPZ	Spermatozoa
STC	Sertoli cells
ISC	Interstitial cells (Leydig cells)
PNO	Perinucleolar oocytes
CAO	Cortical alveolar oocytes
EVO	Early vitellogenic oocytes
LVO	Late vitellogenic oocytes
MSO	Mature / spawning oocytes
PFC	Perifollicular (Granulosa/theca) cells
OOG	Oogonia

Maturation Codes:	
stgJ	Stage Juvenile
stg0	Stage 0
stg1	Stage 1
stg2	Stage 2
stg3	Stage 3
stg4	Stage 4
stg5	Stage 5
NR	Not remarkable
UTS	Unable to stage

APPENDIX B

FIGURES AND LEGENDS

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11, SSL No. 13784.6109
11/18/05 DRAFT

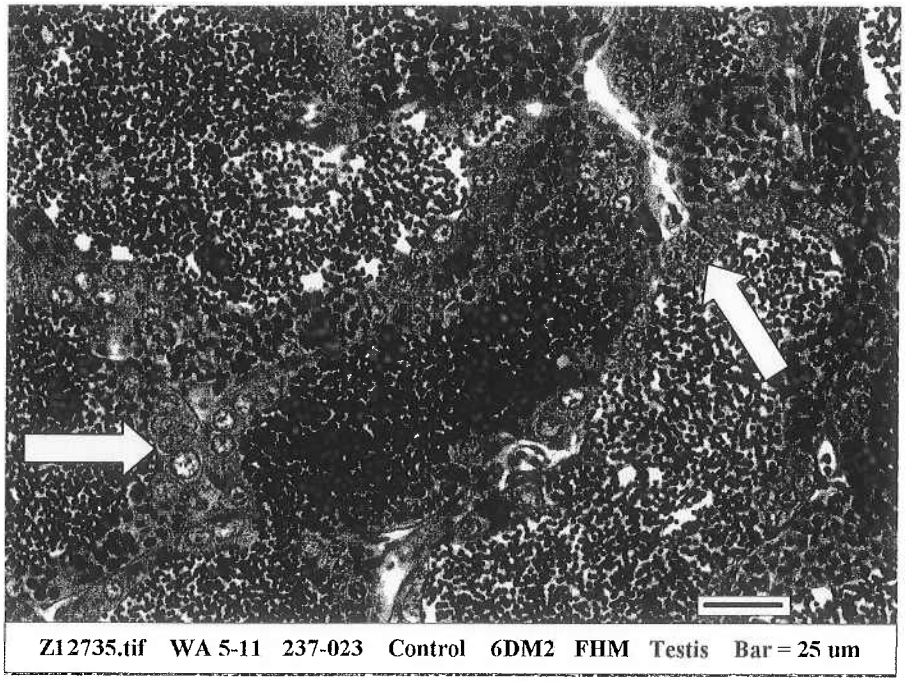

Figure 1 (Z12735). Testis from a control group male. There is a minimal (Grade 1) increase in the proportion of spermatogonia (arrows). Compare to germinal epithelium of testis in Fig. 5 in which only occasional spermatogonia are evident. H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11, SSL No. 13784.6109
11/18/05 DRAFT

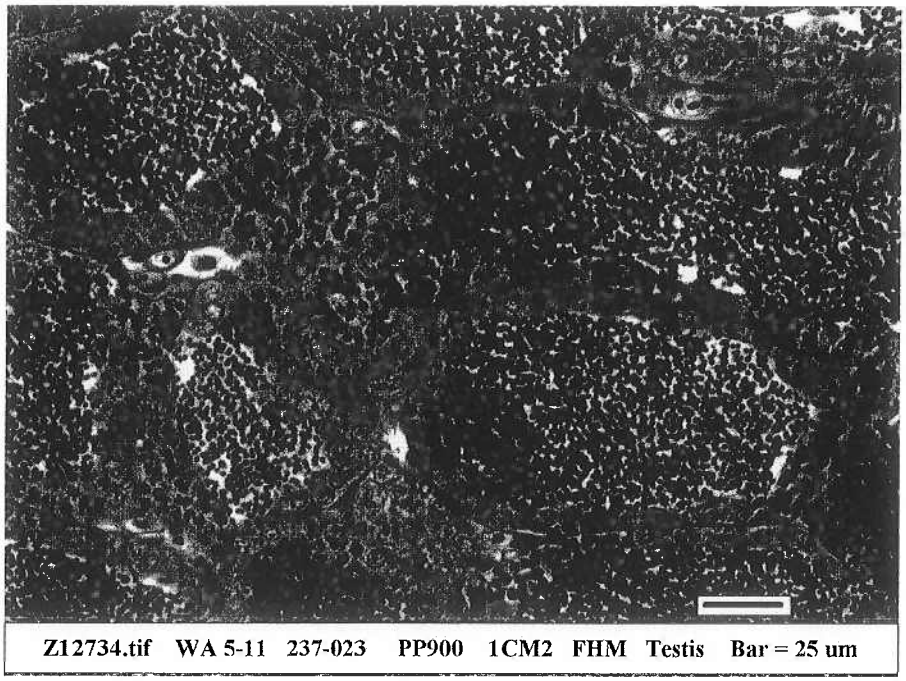

Figure 2 (Z12734). Testis from a 900 µg/L group male. There is a slight/mild (Grade 2) increase in the proportion of spermatogonia throughout the germinal epithelium. H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11, SSL No. 13784.6109
11/18/05 DRAFT

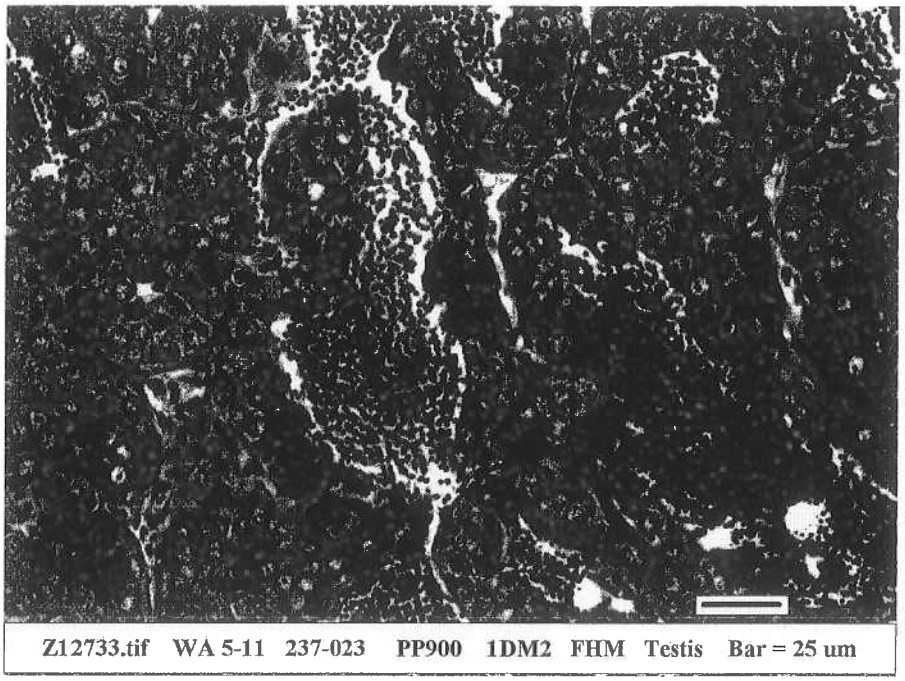

Figure 3 (Z12733). Testis from a 900 µg/L group male. There is a moderate (Grade 3) increase in the proportion of spermatogonia. H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11, SSL No. 13784.6109
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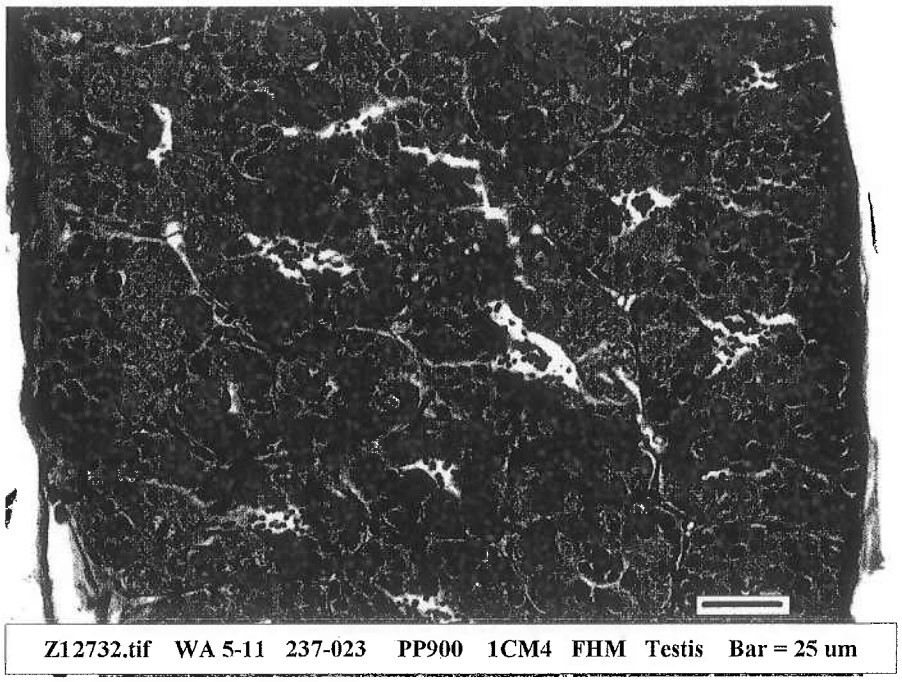


Figure 4 (Z12732). Testis from a 900 µg/L group male. There is a severe (Grade 4) increase in the proportion of spermatogonia. H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11, SSL No. 13784.6109
11/18/05 DRAFT

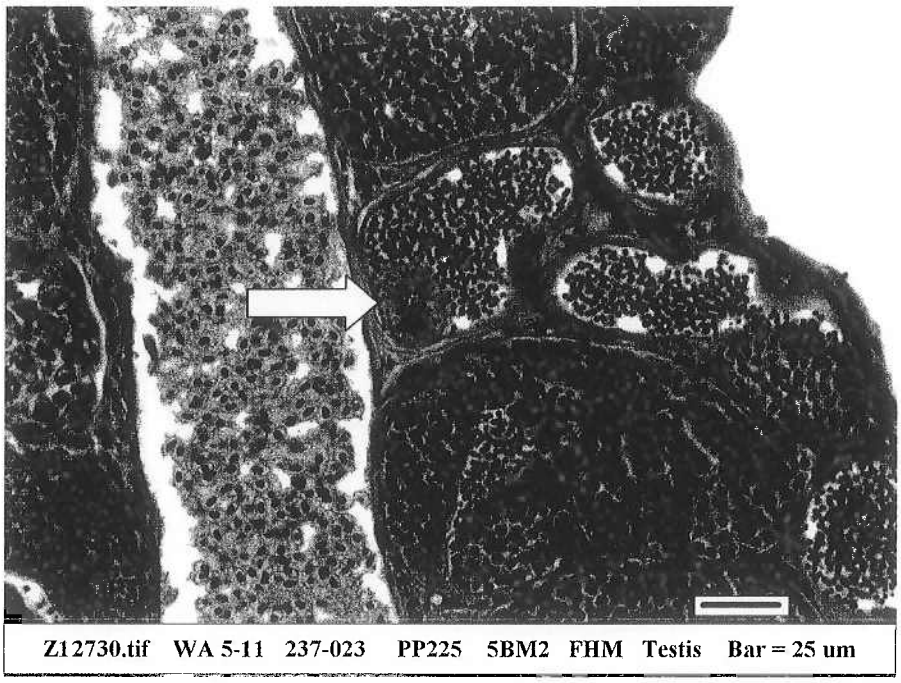


Figure 5 (Z12730). Testis from a 225 µg/L group male. There is a microsporidial cyst (arrow) within the lumen of one tubule. H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11, SSL No. 13784.6109
11/18/05 DRAFT

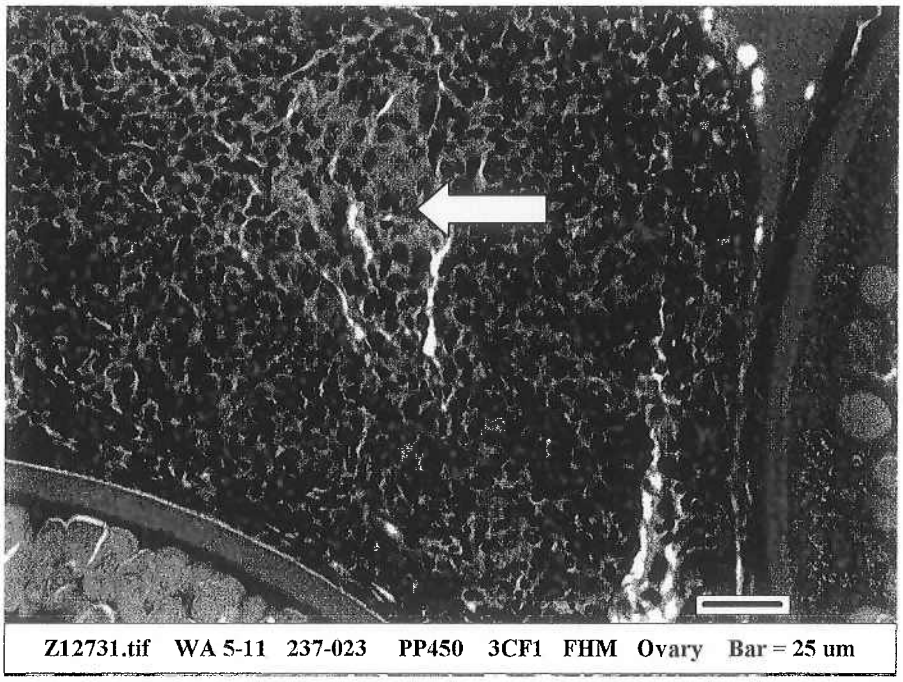


Figure 6 (Z12731). Ovary from a 450 µg/L group female. A small cluster of microsporidial spores (arrow) is within the cytoplasm of a macrophage in an interstitial area of granulomatous inflammation. H&E.

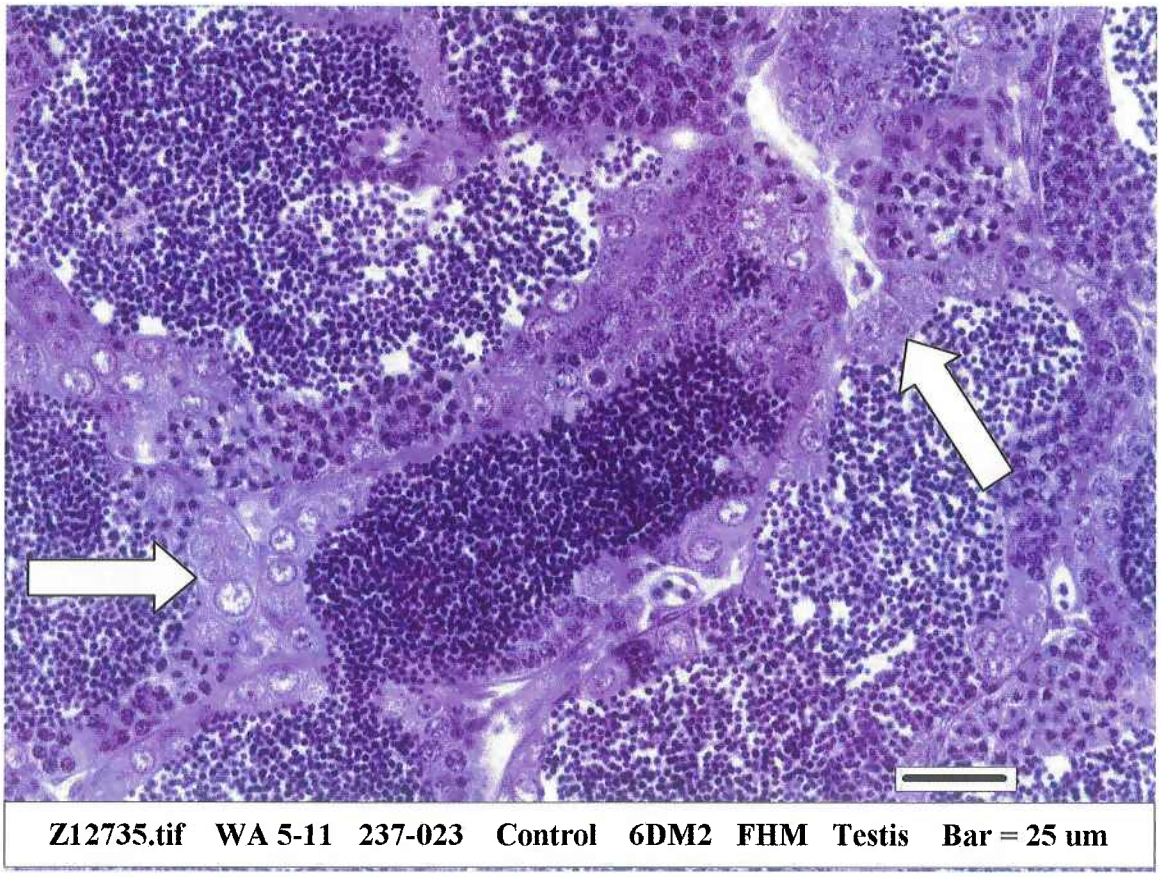
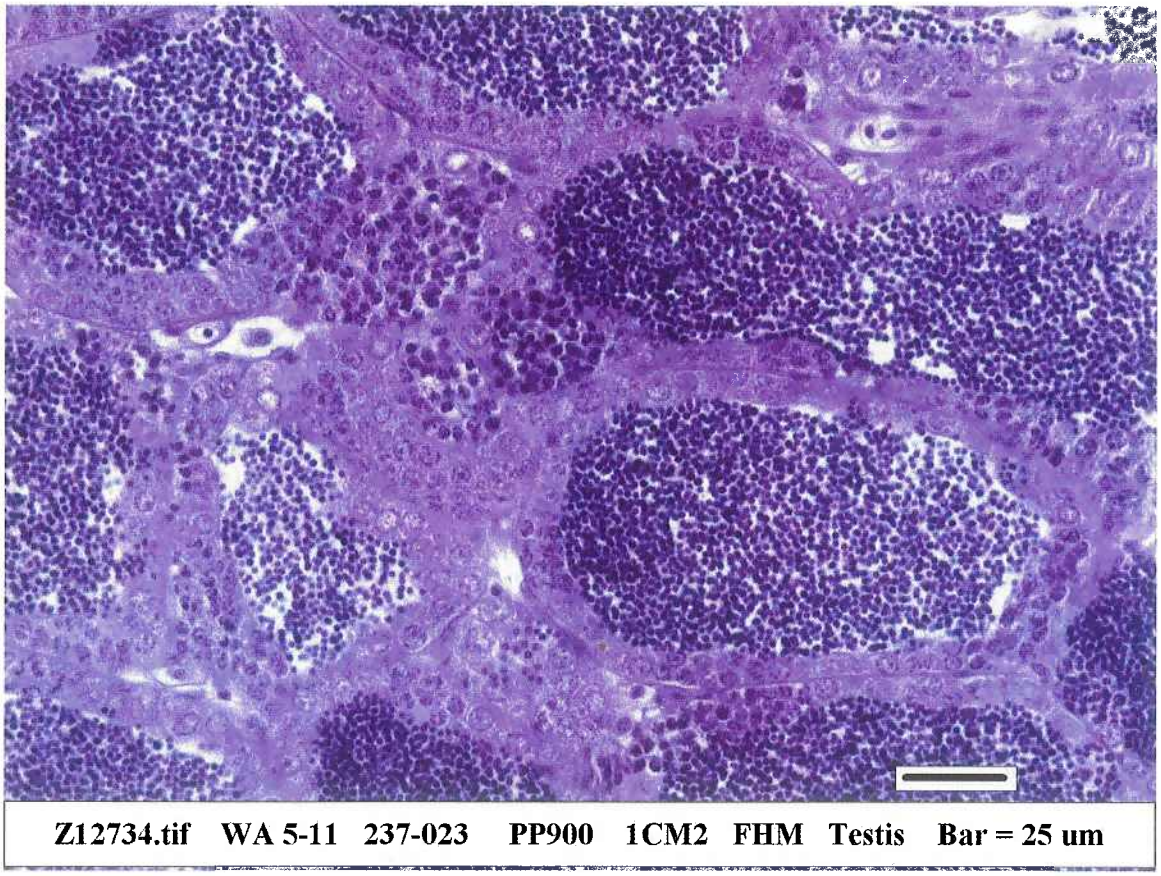


Figure 1 (Z12735). Testis from a control group male. There is a minimal (Grade 1) increase in the proportion of spermatogonia (arrows). Compare to germinal epithelium of testis in Fig. 5 in which only occasional spermatogonia are evident. H&E.



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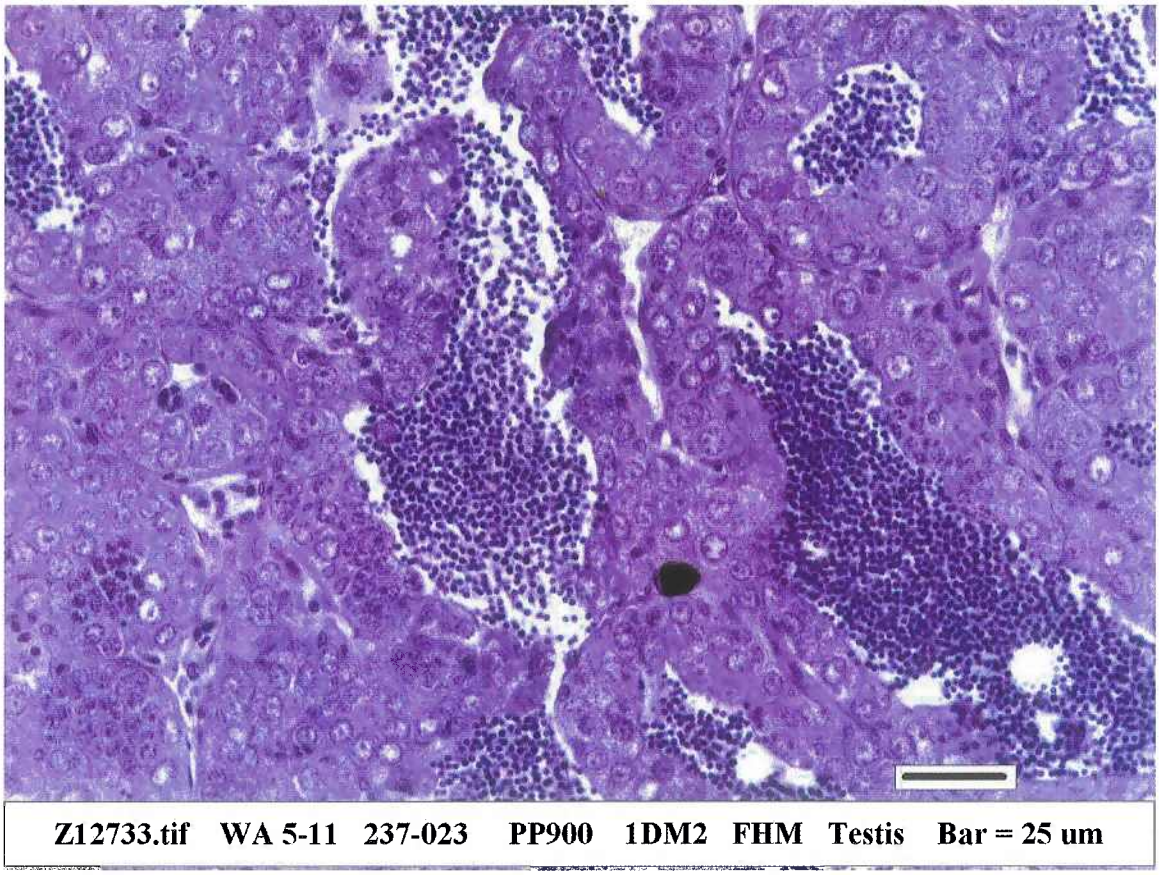

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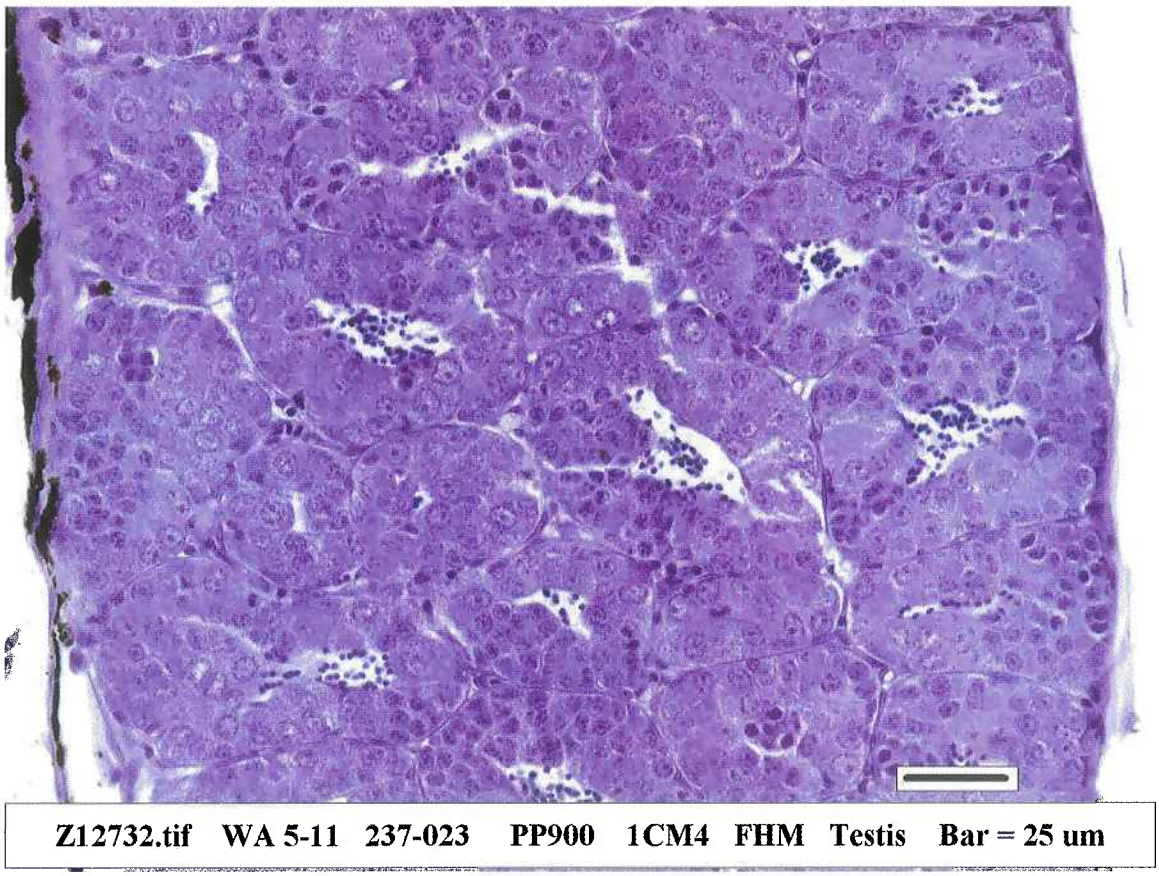

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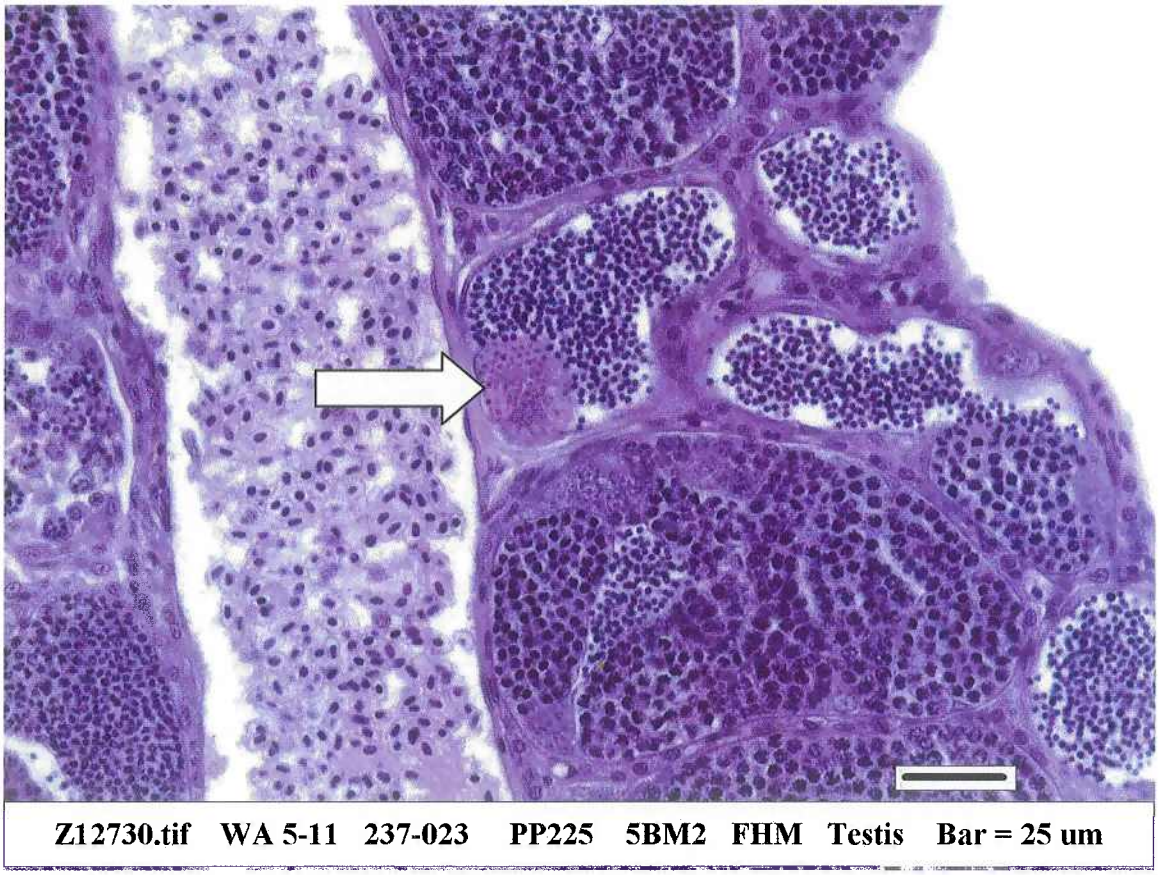

Figure 5 (Z12730). Testis from a 225 µg/L group male. There is a microsporidial cyst (arrow) within the lumen of one tubule. H&E.

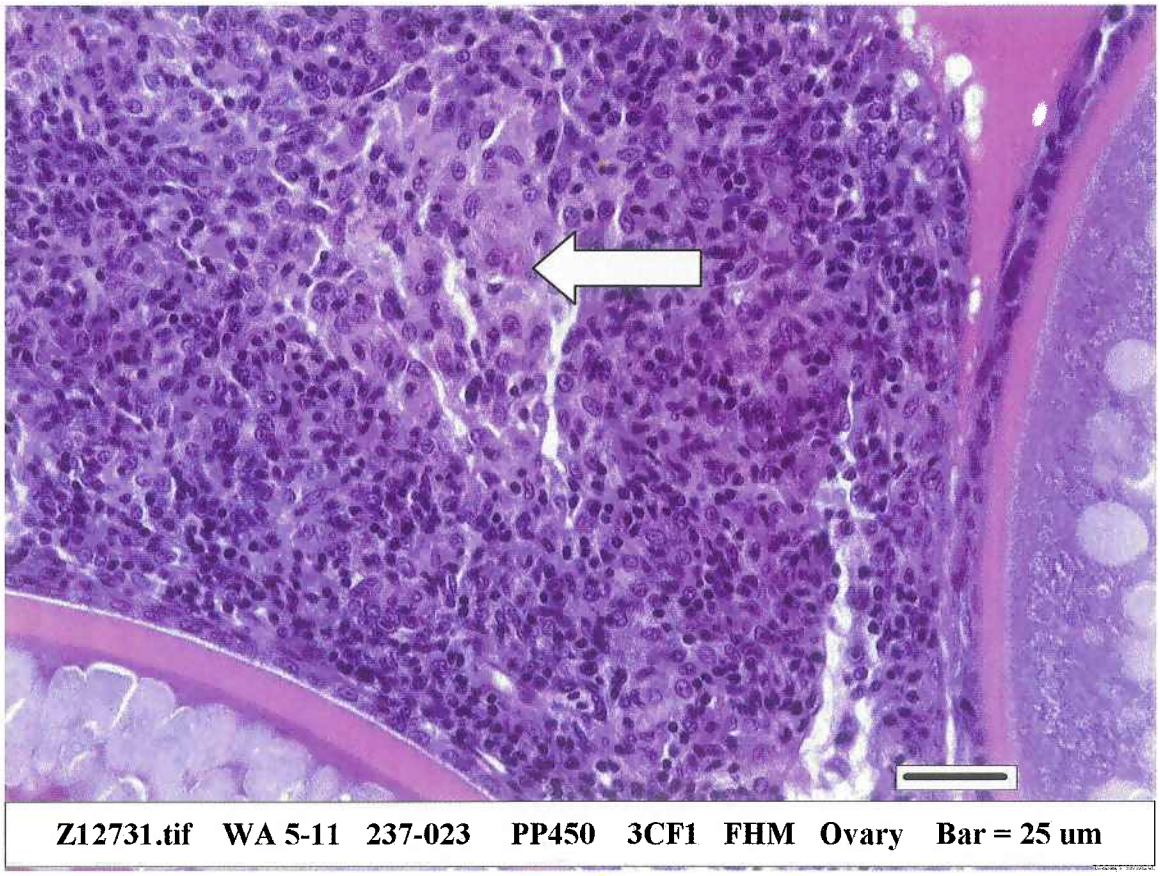

Figure 6 (Z12731). Ovary from a 450 µg/L group female. A small cluster of microsporidial spores (arrow) is within the cytoplasm of a macrophage in an interstitial area of granulomatous inflammation. H&E.

Ketoconazole

EPL® Experimental Pathology Laboratories, Inc.

BATTELLE PACIFIC NORTHWEST DIVISION,
BATTELLE MEMORIAL INSTITUTE
STUDY NUMBER WA 5-11
EPL PROJECT NUMBER 237-025

KETOCONAZOLE: PHASE 1B VALIDATION – FISH SCREENING ASSAY FOR ENDOCRINE ACTIVE SUBSTANCE WITH THE FATHEAD MINNOW (PIMEPHALES PROMELAS)

DRAFT PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.
P.O. Box 474
Herndon, VA 20172-0474
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Submitted to:

Battelle Pacific Northwest Division, Battelle Memorial Institute
Sequim, WA 98382

January 6, 2006

EPL® Experimental Pathology Laboratories, Inc.

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DRAFT PATHOLOGY SUMMARY

Experimental Pathology Laboratories, Inc.

BATTELLE PACIFIC NORTHWEST DIVISION,
BATTELLE MEMORIAL INSTITUTE
STUDY NUMBER WA 5-11
EPL PROJECT NUMBER 237-025

KETOCONAZOLE: PHASE 1B VALIDATION – FISH SCREENING ASSAY FOR ENDOCRINE ACTIVE SUBSTANCE WITH THE FATHEAD MINNOW (PIMEPHALES PROMELAS)

DRAFT PATHOLOGY SUMMARY

INTRODUCTION

The objective of this study was to determine the effects, if any, of ketoconazole administered via water bath on gonadal tissue of adult fathead minnows (FHM, *Pimephales promelas*).

The experimental design is presented in the following table:

Table 1. Experimental Design for Ketoconazole Study

Exposure Group	Ketoconazole	Male Replicates				Female Replicates			
		A	B	C	D	A	B	C	D
6	0 µg/L (Control)	2	2	2	2	4	4	3	4
5	25 µg/L	2	2	2	2	4	4	4	4
3	100 µg/L	2	2	2	2	4	3	4	3
1	400 µg/L	2	2	2	1	4	4	4	5

METHODS

The tissues were submitted by Springborn Smithers Laboratories, Inc. Unless otherwise indicated, histopathological procedures were performed according to the draft form of the "OECD Guidance Document for Performing Gonadal Histopathology in Small Fish." Briefly, following routine processing the left and right gonads were embedded horizontal to their long axis to allow for

longitudinal sectioning. During microtomy, the first section from each block was acquired at the point at which approximately half of the gonad had been cut away and the size of the section was maximized. The second and third sections were then obtained at 50 micron intervals. Sections were stained with hematoxylin and eosin, and mounted with glass coverslips. Labels included the EPL Project No. (237-025), the group/replicate designation (e.g., CONT/A), the study ID (WA 5-11), and the Animal No. (e.g., 1AM1).

The pathologist evaluated the slides by brightfield microscopy for changes that included, but were not limited to, the types of findings that are listed in the aforementioned guidance document. As per that document, severity grading of findings was performed according to the following scale: NR = not remarkable, Grade 1 = minimal, Grade 2 = mild, Grade 3 = moderate, Grade 4 = severe. Ovarian oocyte atresia was graded according to the following scale: Grade 1 = 3 to 5 atretic oocytes per ovary; Grade 2 = 6 to 9 atretic oocytes per ovary; Grade 3 = greater than 9 atretic oocytes per ovary, but less than the vast majority; and Grade 4 = the vast majority of oocytes were atretic. The pathologist recorded findings on a spreadsheet. This original spreadsheet as contained within the guidance document was modified slightly by the study pathologist to include the addition of a column in order to accommodate the animal numbers of the female fathead minnows (which were different from what appeared on the worksheet), and corrections of some of the animal numbers to correspond with the animal numbers that were submitted by the client. The data collection spreadsheet is incorporated into this report. Results were simultaneously recorded into EPL's Pathology Data Reporting System, and tabulated in the accompanying Histopathology Incidence Tables (HIT) and summarized in the Summary Incidence Tables (SIT).

RESULTS

Males

Based on incidence and/or severity data, the single finding that was substantially different in ketoconazole-exposed males as compared to control males was the presence of increased interstitial (Leydig) cells (minimal to mild) in the testes of the 25, 100, and 400 µg/L group males (Figs. 3-4). This finding appeared to be somewhat dose-responsive in terms of incidence and severity, although the incidence did not reach 100% in any of the ketoconazole-exposed groups. When compared to the interstitial cells of unaffected males, the interstitial cells of affected males also tended to have larger, more rounded nuclei and cytoplasm that was more densely-staining, and in the mildly affected individuals, there was a propensity for interstitial cell aggregates to fully occupy and expand some of the interstitial spaces.

Testicular stage scores of ketoconazole-exposed males were comparable to those of controls.

The incidence and severity of selected histopathologic results for male FHM are presented in the following table:

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Table 2. Combined Incidence and Severity of Selected Histopathologic Findings in Male Fathead Minnows

Exposure Group	6					5					3					1				
Ketoconazole Dose (µg/L)	0					25					100					400				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	2	2	2	2	8	2	2	2	2	8	2	2	2	2	8	2	2	2	1	7
Increased Cells, Interstitial Cells	0	0	0	0	0	1	1	2	0	4	1	1	1	1	4	1	1	2	1	5
Minimal	-	-	-	-	-	1	1	2	-	4	1	-	1	1	3	1	-	1	-	2
Mild	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	1	1	1	3
Moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testicular Stage																				
Stage 0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stage 1	-	-	-	1	1	-	-	2	-	2	-	-	-	1	1	-	-	1	-	1
Stage 2	2	2	2	1	7	2	2	-	2	6	2	1	2	1	6	2	2	1	-	5
Stage 3	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	-	-	1	1
Average	1.9					1.8					2.0					2.0				

*T = totals

Females

Based on incidence and/or severity data, there were no findings that were substantially different in ketoconazole-exposed females as compared to control females.

Minimal to moderate oocyte atresia was occasionally diagnosed in the ovaries of females in all exposure groups, and this generally involved resorption of senescent oocytes. A few ovaries featured distinctly different areas of gonadal development in which one area was characterized as ovarian Stage 2 and the other as ovarian Stage 4; this finding, diagnosed as "Asynchronous Development, Gonad", was also not related to ketoconazole exposure (Fig. 5).

Ovarian stage scores of ketoconazole-exposed females were comparable to those of controls.

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Study Number WA 5-11
1/6/06 DRAFT

The incidence and severity of selected histopathologic results for female fathead minnows are presented in the following table:

Table 3. Combined Incidence and Severity of Selected Histopathologic Findings in Female Fathead Minnows

Exposure Group	6					5					3					1				
Ketoconazole Dose (µg/L)	0					25					100					400				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	4	4	3	4	15	4	4	4	4	16	4	3	4	3	14	4	4	4	5	17
Oocyte Atresia	0	1	0	2	3	1	1	1	0	3	1	0	1	0	2	0	1	1	0	2
Minimal	-	-	-	-	-	1	1	1	-	3	1	-	-	-	1	-	-	-	-	-
Mild	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-
Moderate	-	1	-	2	3	-	-	-	-	-	-	-	-	-	-	-	1	1	-	2
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asynchronous Development, Gonad	1	0	0	2	3	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1
Minimal	1	-	-	2	3	-	1	-	-	1	-	1	-	-	1	-	-	1	-	1
Mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovarian Stage																				
Stage 0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stage 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stage 2	1	1	-	1	3	2	2	2	2	8	1	1	-	1	3	1	-	2	-	3
Stage 3	2	3	2	1	8	1	2	-	1	4	2	2	4	1	9	3	3	1	4	11
Stage 4	1	-	1	2	4	1	-	2	1	4	1	-	-	1	2	-	1	1	1	3
Average	3.1					2.8					2.9					3.0				

*T = totals

DISCUSSION

Ketoconazole is a weak imidazole that is used as a fungicide. Ketoconazole is also been demonstrated to disrupt hepatic and gonadal steroidogenesis in mammals, and this is thought to occur via pathways that may include interference with cytochrome P450 enzymes (Kan et al., 1985; Chaudhary and Stocco, 1989) and possibly aromatase inhibition (Doody et al). One consequence of this disrupted steroidogenesis is lowered serum testosterone levels in humans (Rajfer et al., 1986) and rats (Bhasin et al., 1986), which has been linked to decreased androgen production by the testis (Bhasin et al., 1986; Kan et al, 1985, Rajfer et al, 1986). Doubt has been shed on a previous theory that the decreased serum testosterone is due to ketoconazole-enhanced biotransformation and elimination by the liver (Wilson and LeBlanc, 2000). Prior studies of ketoconazole in fish have primarily involved the effects of this substance on cytochrome enzyme activity (Hasselberg et al, 2005; Hegelund et al, 2004).

The results of the present study appear consistent with the idea that ketoconazole interferes with androgen steroidogenesis to some degree, because interstitial cell proliferation could be interpreted to be a compensatory response to diminished androgen levels via a pathway involving the hypothalamic-pituitary-gonadal axis. The observation that the testes of ketoconazole-exposed male fathead minnows were otherwise morphologically comparable to controls suggests that endogenously circulating androgens were maintained in concentrations that were to some extent adequate for the support of spermatogenesis. Because an analogous response (e.g., granulosa cell hyperplasia/hypertrophy) was not observed in the ovaries of ketoconazole-exposed females, it can be presumed that exposure to the test article had minimal, if any, effect on estrogen production. In the males, the diagnosis of increased interstitial cells was based on a comparison between the testes of

control fish and those exposed to the test article. This is necessary because the abundance of testicular interstitial cells in control fish can vary (Figs. 1-2). The larger, more rounded nuclei and denser cytoplasm of the interstitial cells in the affected ketoconazole-exposed fish indicate that these cells were probably slightly hypertrophic in addition to being hyperplastic.

The finding of asynchronous development in the ovaries of occasional females of all treatment groups most likely represents the normal transition from Stage 4 to Stage 2 that occurs at or around the time of spawning.

Other findings in this study either occurred in comparable numbers of control and ketoconazole-exposed fish (background lesions), or as low-frequency (incidental) findings, and are therefore not considered to be exposure-related.

CONCLUSION AND SUMMARY

The sole histopathological finding that was attributable to ketoconazole exposure was the presence of increased interstitial (Leydig) cells in the testes of the 25, 100, and 400 µg/L group males as compared to controls. This finding appeared to be somewhat dose-responsive in terms of incidence and severity, although the incidence did not reach 100% in any of the ketoconazole-exposed male groups.

Other findings in this study either occurred in comparable numbers of control and ketoconazole-exposed fish (background lesions), or as low-frequency (incidental) findings, and are therefore not considered to be exposure-related.

JEFFREY C. WOLF, DVM, Diplomate, ACVP Date
Veterinary Pathologist

JCW/cb

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EPL® Experimental Pathology Laboratories, Inc.

1/6/06 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Ketoconazole: Phase 1B Validation – Fish Screening Assay for Endocrine Active Substance with the Fathead Minnow (Pimephales promelas)

Client Study: WA 5-11 EPL Project Coordinator: Dr. Jeffrey C. Wolf

EPL Project Number: 237-025 EPL Pathologist: Dr. Jeffrey C. Wolf

The following aspects of this study were inspected by the Quality Assurance Unit of Experimental Pathology Laboratories, Inc. Dates inspections were performed and findings reported to the EPL Project Coordinator and Management are indicated below.

Area Inspected	Dates	
	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management XXX

Date of last quarterly facility inspection 7/05

EPL Quality Assurance Unit Date

Form No. 6-2 (7/2/99)

SUMMARY INCIDENCE TABLES

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP CONT/A	GROUP CONT/B	GROUP CONT/C	GROUP CONT/D	GROUP 25/A	GROUP 25/B
TESTIS (NO. EXAMINED)	(2)	(2)	(2)	(2)	(2)	(2)
Atrophy				1		
Histiocytic Cells, Intraluminal					1	
Inflammation, Granulomatous					1	
Interstitial Cells, Increased Cells					1	1
Mineralization	1		1			1
Mineralization, Collecting Duct	2		1	1	1	1
Sperm Necrosis				1		
Spermatogonia, Increased Cells						
Stage 1				1		
Stage 2	2	2	2	1	2	2
Stage 3						

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 25/C	GROUP 25/D	GROUP 100/A	GROUP 100/B	GROUP 100/C	GROUP 100/D
TESTIS (NO. EXAMINED)	(2)	(2)	(2)	(2)	(2)	(2)
Atrophy						
Histiocytic Cells, Intraluminal						
Inflammation, Granulomatous						1
Interstitial Cells, Increased Cells	2		1	1	1	1
Mineralization						
Mineralization, Collecting Duct						
Sperm Necrosis						
Spermatogonia, Increased Cells		1				
Stage 1	2					1
Stage 2		2	2	1	2	1
Stage 3				1		

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 400/A	GROUP 400/B	GROUP 400/C	GROUP 400/D		
TESTIS (NO. EXAMINED)	(2)	(2)	(2)	(1)		
Atrophy						
Histiocytic Cells, Intraluminal						
Inflammation, Granulomatous						
Interstitial Cells, Increased Cells	1	1	2	1		
Mineralization						
Mineralization, Collecting Duct						
Sperm Necrosis						
Spermatogonia, Increased Cells						
Stage 1			1			
Stage 2	2	2	1			
Stage 3				1		

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP CONT/A	GROUP CONT/B	GROUP CONT/C	GROUP CONT/D	GROUP 25/A	GROUP 25/B
OVARY (NO. EXAMINED)	(4)	(4)	(3)	(4)	(4)	(4)
Asynchronous Development, Gonad	1			2		1
Inflammation, Granulomatous						
Oocyte Atresia, Increased		1		2	1	1
Stage 2	1	1		1	2	2
Stage 3	2	3	2	1	1	2
Stage 4	1		1	2	1	

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 25/C	GROUP 25/D	GROUP 100/A	GROUP 100/B	GROUP 100/C	GROUP 100/D
OVARY (NO. EXAMINED)	(4)	(4)	(4)	(3)	(4)	(3)
Asynchronous Development, Gonad				1		
Inflammation, Granulomatous						
Oocyte Atresia, Increased	1		1		1	
Stage 2	2	2	1	1		1
Stage 3		1	2	2	4	1
Stage 4	2	1	1			1

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 400/A	GROUP 400/B	GROUP 400/C	GROUP 400/D		
OVARY (NO. EXAMINED)	(4)	(4)	(4)	(5)		
Asynchronous Development, Gonad			1			
Inflammation, Granulomatous				1		
Oocyte Atresia, Increased		1	1			
Stage 2	1		2			
Stage 3	3	3	1	4		
Stage 4		1	1	1		

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP CONT/A		GROUP CONT/B		GROUP CONT/C		GROUP CONT/D		GROUP 25/A		GROUP 25/B		GROUP 25/C	
ANIMAL	6AM1	6AM2	6BM1	6BM2	6CM1	6CM2	6DM1	6DM2	5AM1	5AM2	5BM1	5BM2	5CM1	5CM2
TESTIS														
Atrophy								2						
Histiocytic Cells, Intraluminal									2					
Inflammation, Granulomatous									2					
Interstitial Cells, Increased Cells										1	1		1	1
Mineralization		1				1					2			
Mineralization, Collecting Duct	1	1				2	1			2	3			
Sperm Necrosis								1						
Spermatogonia, Increased Cells														
Stage 1							P						P	P
Stage 2	P	P	P	P	P	P		P	P	P	P	P		
Stage 3														

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II-1

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 25/D		GROUP 100/A		GROUP 100/B		GROUP 100/C		GROUP 100/D		GROUP 400/A		GROUP 400/B	
ANIMAL	5DM1	5DM2	3AM1	3AM2	3BM1	3BM2	3CM1	3CM2	3DM1	3DM2	1AM1	1AM2	1BM1	1BM2
TESTIS														
Atrophy														
Histiocytic Cells, Intraluminal														
Inflammation, Granulomatous									1					
Interstitial Cells, Increased Cells			1		2			1		1		1		2
Mineralization														
Mineralization, Collecting Duct														
Sperm Necrosis														
Spermatogonia, Increased Cells		1												
Stage 1										P				
Stage 2	P	P	P	P	P		P	P	P		P	P	P	P
Stage 3						P								

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Experimental Pathology Laboratories, Inc.

II-2

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 400/C		GROUP 400/D
ANIMAL	1CM1	1CM2	1DM1
TESTIS			
Atrophy			
Histiocytic Cells, Intraluminal			
Inflammation, Granulomatous			
Interstitial Cells, Increased Cells	2	1	2
Mineralization			
Mineralization, Collecting Duct			
Sperm Necrosis			
Spermatogonia, Increased Cells			
Stage 1	P		
Stage 2		P	
Stage 3			P

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II-3

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP CONT/A				GROUP CONT/B				GROUP CONT/C			GROUP CONT/D			
ANIMAL	6AF1	6AF2	6AF3	6AF4	6BF1	6BF2	6BF3	6BF4	6CF1	6CF2	6CF3	6DF1	6DF2	6DF3	6DF4
OVARY															
Asynchronous Development, Gonad	1											1	1		
Inflammation, Granulomatous															
Oocyte Atresia, Increased							3							3	3
Stage 2				P			P								P
Stage 3		P	P			P	P		P		P			P	
Stage 4	P									P		P	P		

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Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 25/A				GROUP 25/B				GROUP 25/C				GROUP 25/D			
ANIMAL	5A F 1	5A F 2	5A F 3	5A F 4	5B F 1	5B F 2	5B F 3	5B F 4	5C F 1	5C F 2	5C F 3	5C F 4	5D F 1	5D F 2	5D F 3	5D F 4
OVARY																
Asynchronous Development, Gonad							1									
Inflammation, Granulomatous						1					1					
Oocyte Atresia, Increased	1															
Stage 2		P	P		P		P				P	P			P	P
Stage 3	P					P		P					P			
Stage 4				P					P	P				P		

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 100/A				GROUP 100/B			GROUP 100/C				GROUP 100/D			
ANIMAL	3AF1	3AF2	3AF3	3AF4	3BF1	3BF2	3BF3	3CF1	3CF2	3CF3	3CF4	3DF1	3DF2	3DF3	
OVARY															
Asynchronous Development, Gonad						1									
Inflammation, Granulomatous															
Oocyte Atresia, Increased	1							2							
Stage 2	P					P								P	
Stage 3		P		P	P		P	P	P	P	P		P		
Stage 4			P									P			

EPL Experimental Pathology Laboratories, Inc.

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 400/A				GROUP 400/B				GROUP 400/C				GROUP 400/D				
ANIMAL	1AF1	1AF2	1AF3	1AF4	1BF1	1BF2	1BF3	1BF4	1CF1	1CF2	1CF3	1CF4	1DF1	1DF2	1DF3	1DF4	1DF5
OVARY																	
Asynchronous Development, Gonad												1					
Inflammation, Granulomatous														1			
Oocyte Atresia, Increased						3				3							
Stage 2			P							P	P						
Stage 3	P	P		P	P		P	P	P				P	P	P	P	
Stage 4						P						P					P

EPL Experimental Pathology Laboratories, Inc.

II-7

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=mild 3=moderate 4=severe
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

APPENDIX A

GONAD HISTOPATHOLOGY RESULTS WORKSHEET

Fish Endocrine Screening Assay Phase 1B OECD Protocol
Gonad Histopathology Results Worksheet

Fathead Minnow

LABORATORY'S NAME: Battelle Pacific Northwest Division, Battelle Memorial Institute

EPL Project No. 237-025

Study No. WA 5-11

VESSEL A

	Male		Female		Comments
Group CONT/A 0 µg/L	6AM1 slg2: M20-1		6AF3 slg4: F07-1		
	6AM2 slg2: M20-1; M19-1		6AF2 slg2		
			6AF3 slg3		
			6AF4 slg2		
Group 25/A 25 µg/L	5AM1 slg2: M15-2; M16-2		5AF1 slg3: F05-1		
	5AM2 slg2: M01-ISC-1; M20-2		5AF2 slg2		
			8AF3 slg2		
			5AF4 slg4		
Group 100/A 100 µg/L	3AM1 slg2: M01-ISC-1		3AF1 slg2: F05-1		
	3AM2 slg2		3AF2 slg3		
			3AF3 slg4		
			3AF4 slg7		
Group 400/A 400 µg/L	1AM1 slg2:		1AF1 slg3		
	1AM2 slg2: M01-ISC-1		1AF2 slg3		
			1AF3 slg2		
			1AF4 slg3		

VESSEL B

	Male		Female		Comments
Group CONT/B 0 µg/L	6BM1 slg2:		6BF1 slg3		
	6BM2 slg2		6BF2 slg3		
			6BF3 slg2: F05-3		
			6BF4 slg3		
Group 25/B 25 µg/L	5BM1 slg2: M01-ISC-1; M18-2; M20-3		5BF1 slg2		
	5BM2 slg2		5BF2 slg3: F05-1		
			5BF3 slg2: F07-1		
			5BF4 slg1		
Group 100/B 100 µg/L	3BM1 slg2: M01-ISC-2		3BF1 slg3		
	3BM2 slg3		3BF2 slg2: R05-1		
			3BF3 slg3		
Group 400/B 400 µg/L	1BM1 slg2:		1BF1 slg3		
	1BM2 slg2: M01-ISC-2		1BF2 slg4: F05-3		
			1BF3 slg3		
			1BF4 slg3		

VESSEL C

	Male		Female		Comments
Group CONT/C 0 µg/L	6CM1 slg2		6CF1 slg3		
	6CM2 slg2: M18-2; M19-1		6CF2 slg4		
			6CF3 slg3		
Group 25/C 25 µg/L	5CM1 slg1: M01-ISC-1		5CF1 slg4		
	5CM2 slg1: M01-ISC-1		5CF2 slg1		
			5CF3 slg2: F05-1		
			5CF4 slg2		
Group 100/C 100 µg/L	3CM1 slg2:		3CF1 slg3: F05-2		
	3CM2 slg2: M01-ISC-1		3CF2 slg3		
			3CF3 slg3		
			3CF4 slg3		
Group 400/C 400 µg/L	1CM1 slg1: M01-ISC-2		1CF1 slg3		
	1CM2 slg2: M01-ISC-1		1CF2 slg2: F05-3		
			1CF3 slg2		
			1CF4 slg4: F01-1		

VESSEL D

	Male	Female	Comments
Group CONT/D 0 µg/L	6DM1 stg3, M20-1	6DF1 stg4, F07-1	
	6DM2 stg2, M21-1, M39-2	6DF2 stg4, F07-2	
		6DF3 stg3, F06-1	
		6DF4 stg4, F06-2	
Group 25/D 25 µg/L	5DM1 stg2,	5DF1 stg3,	
	5DM2 stg2, M01-SPA-1	5DF2 stg4,	
		5DF3 stg2,	
		5DF4 stg2,	
Group 100/D 100 µg/L	3DM1 stg2, M15-1	3DF1 stg4,	
	3DM2 stg3, M04-ISC-1	3DF2 stg3,	
		3DF3 stg2,	
Group 400/D 400 µg/L	1DM1 stg3, M01-ISC-2	1DF1 stg3,	
		1DF2 stg3, F15-1	
		1DF3 stg3,	
		1DF4 stg3,	
		1DF5 stg4,	

Diagnosis Codes, Testis:		Diagnosis Codes, Ovary:	
M01	Increased cells (followed by Cell Type Code)	F01	Increased cells (followed by Cell Type Code)
M02	Decreased cells (followed by Cell Type Code)	F02	Decreased cells (followed by Cell Type Code)
M03	Tubule ectasia	F03	Ovarian cysts, multiple
M04	Tubule edema, mild	F04	Ovarian edema/necrosis
M05	Testicular degeneration, increased	F05	Hemorrhage, ovarian
M06	Atypical spermatogenesis, spermiogenesis	F06	Oocyte atresia, increased
M07	Asynchronous development, gonad	F07	Asynchronous development, gonad
M08	Asynchronous development, right & left gonads	F08	Asynchronous development, right & left gonads
M09	Proteinaceous fluid, intravascular	F09	Proteinaceous fluid, interstitial
M10	Proteinaceous fluid, interstitial	F10	Proteinaceous fluid, interstitial
M11	Interstitial fibrosis	F11	Interstitial fibrosis
M12	Seminiferous tubule atrophy	F12	Post-ovulatory follicles, increased
M13	Hypocellular, lymphocytes, increased	F13	Hypocellular, lymphocytes, decreased
M14	Nephropathy	F14	Nephropathy
M15	Granulomatous inflammation	F15	Granulomatous inflammation
M16	Histiocytic cells, mural/luminal	F16	Macrophage aggregates, increased
M17	Retained peritoneal attachments	F17	Oocyte membrane fusing
M18	Mineralization	F18	Egg debris, oviduct
M19	Renal tubules, mineralization	F19	Renal tubules, mineralization
M20	Mineralization, collecting duct	F20	Skeletal muscle degeneration
M21	Sperm granuloma	F21	Damaged vitellogenesis
M22	Hypertrophy (followed by Cell Type)	F22	Hypertrophy (followed by Cell Type)
M23	Renal tubules, dilatation	F23	Renal tubules, dilatation
M24	Kidney, granulomatous inflammation	F24	Small ovulatory follicles, decreased
M25	Exfoliated germ cells, increased	F25	Ovarian cyst
M26	Macrophage aggregates, increased	F26	Ovarian mineralization
M27	Inflammation, Mixed Cells	F27	Inflammation, Mixed Cells
M28	Microsporidia	F28	Microsporidia
M29	Atrophy	F29	Ectopic neural tissue

Severity Codes:	
1	minimal
2	mild
3	moderate
4	severe
Cell Type Codes:	
SPA	Spermatogonia
SPC	Spermatocytes
SPT	Spermatids
SPZ	Spermatozoa
STC	Sertoli cells
ISC	Interstitial cells (Leydig cells)
PNO	Perinucleolar oocytes
CAO	Cortical alveolar oocytes
EVO	Early vitellogenic oocytes
LVO	Late vitellogenic oocytes
MSO	Multiple / spawning oocytes
PFC	Purkinje cells (Granular/neural cells
OOG	Oogonia

Maturation Codes:	
stg1	
stg0	Stage Juvenile
stg1	Stage 0
stg2	Stage 1
stg3	Stage 2
stg4	Stage 3
stg5	Stage 4
	Stage 5
NR	Not remarkable
UTS	Unable to stage

APPENDIX B

FIGURES AND LEGENDS

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Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/6/06 DRAFT

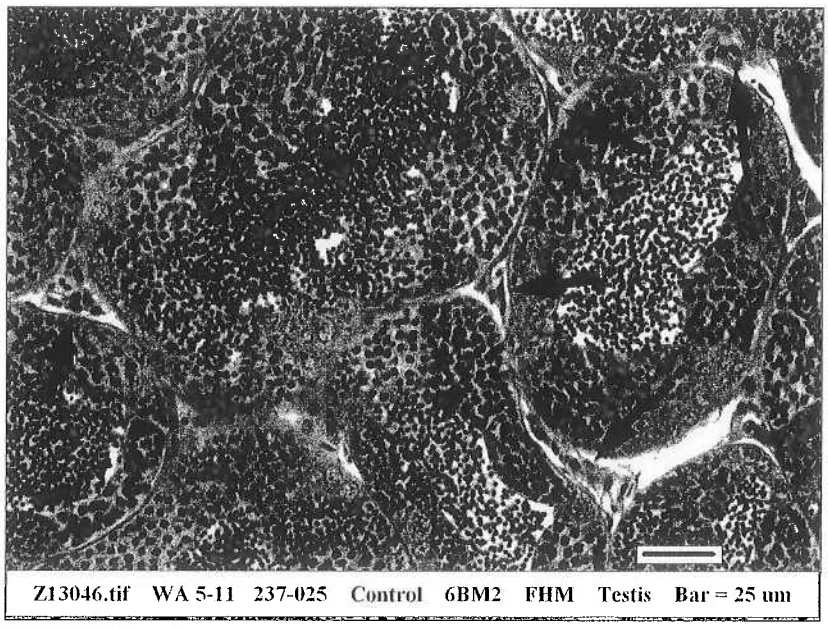

Figure 1 (Z13046). Testis from a control group male. Interstitial areas contain small aggregates of interstitial (Leydig) cells (arrows). Most interstitial cells have wispy, pale cytoplasm. H&E.

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Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/6/06 DRAFT

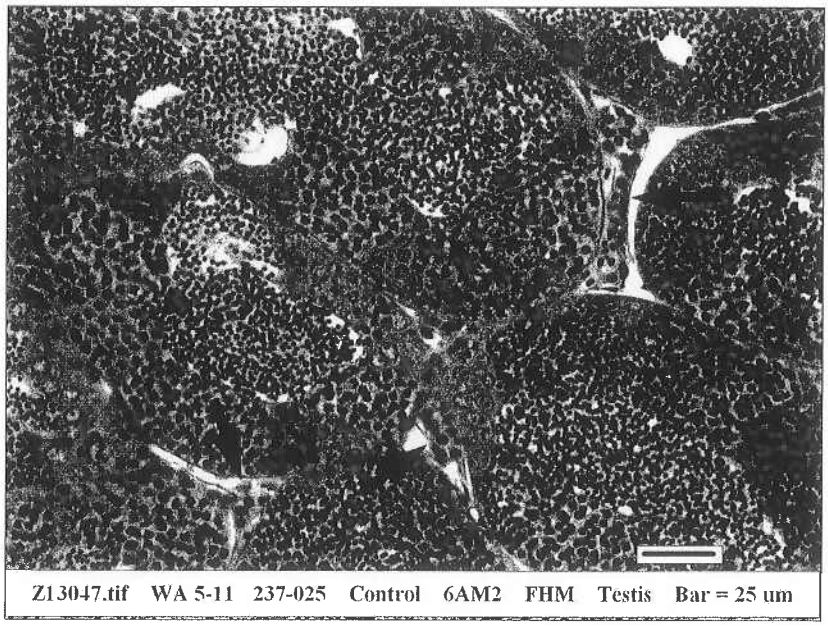

Figure 2 (Z13047). Testis from a control group male. Interstitial cell aggregates (arrows) in the testis of this fish are slightly larger as compared to Fig. 1. This essentially represents the upper extent of interstitial cell proliferation observed in control fish. H&E.

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Study Number WA 5-11
1/6/06 DRAFT

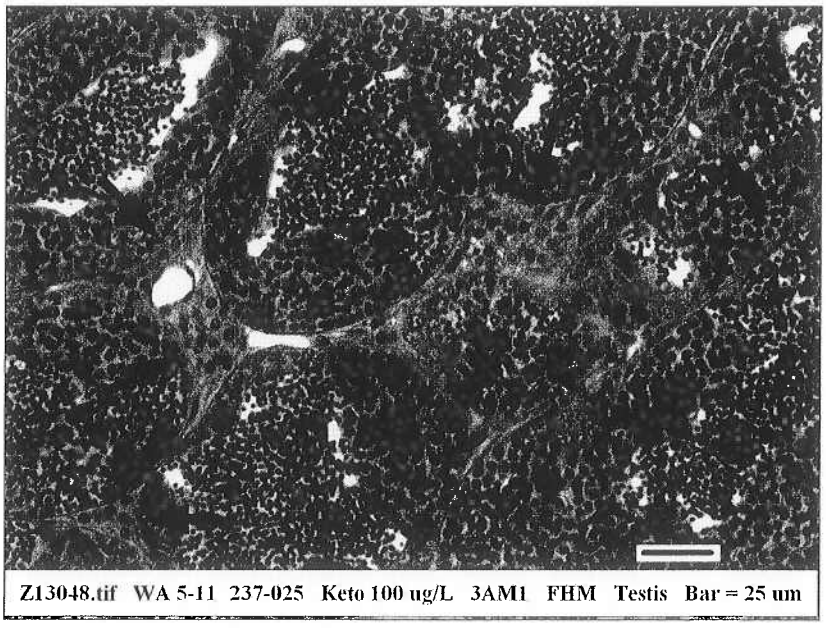

Figure 3 (Z13048). Testis from a 100 µg/L ketoconazole group male. Interstitial cell aggregates (arrows) in the testis of this fish are larger than in control fish, and the cytoplasm of these cells is slightly more dense. This was diagnosed as Increased Cells, Interstitial Cells, Grade 1 (minimal) severity. H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/6/06 DRAFT

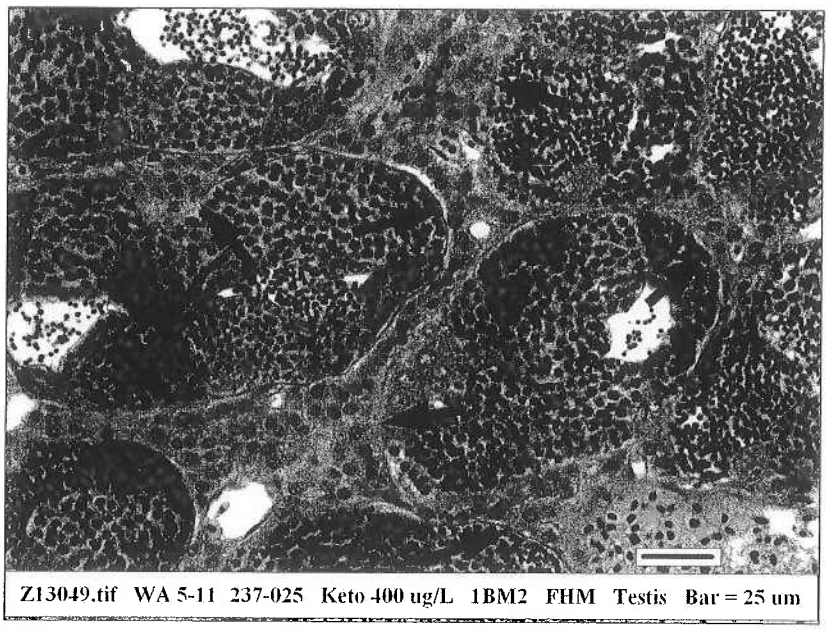

Figure 4 (Z13049). Testis from a 400 µg/L ketoconazole group male. Interstitial cell aggregates (arrows) in the testis of this fish are larger than in Fig. 2, and the cells tend to fill and expand the interstitial spaces. This was diagnosed as Increased Cells, Interstitial Cells, Grade 2 (mild) severity. H&E.

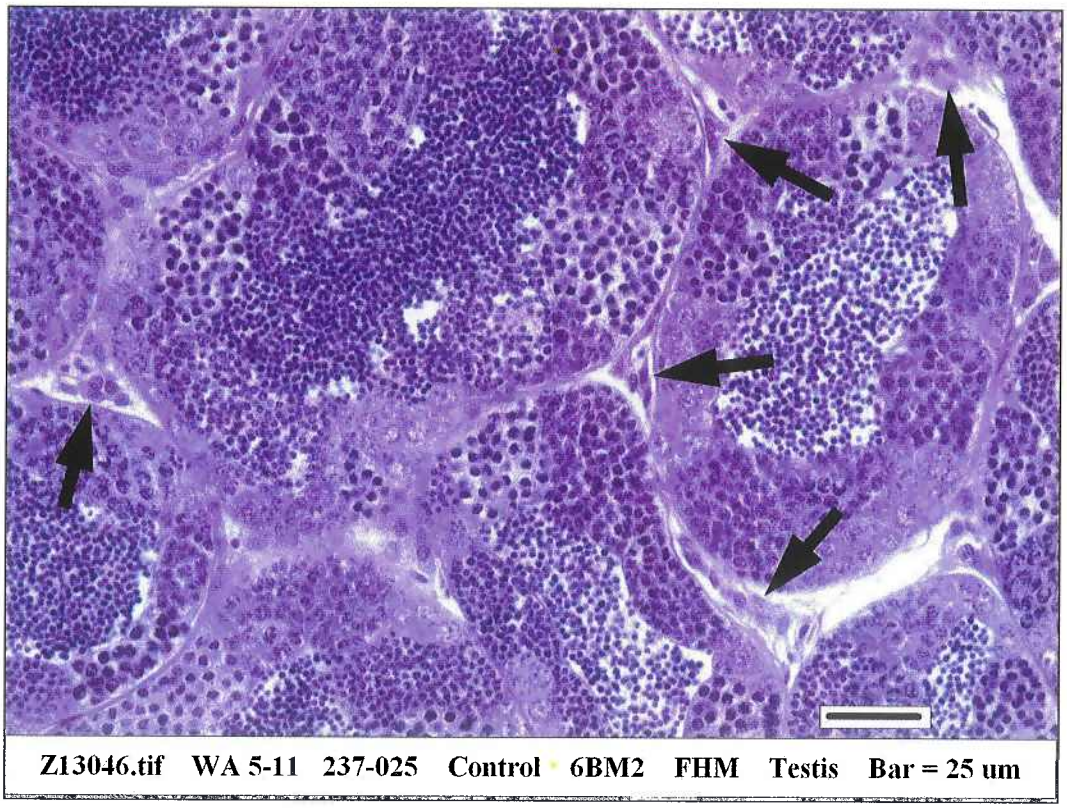

Figure 1 (Z13046). Testis from a control group male. Interstitial areas contain small aggregates of interstitial (Leydig) cells (arrows). Most interstitial cells have wispy, pale cytoplasm. H&E.

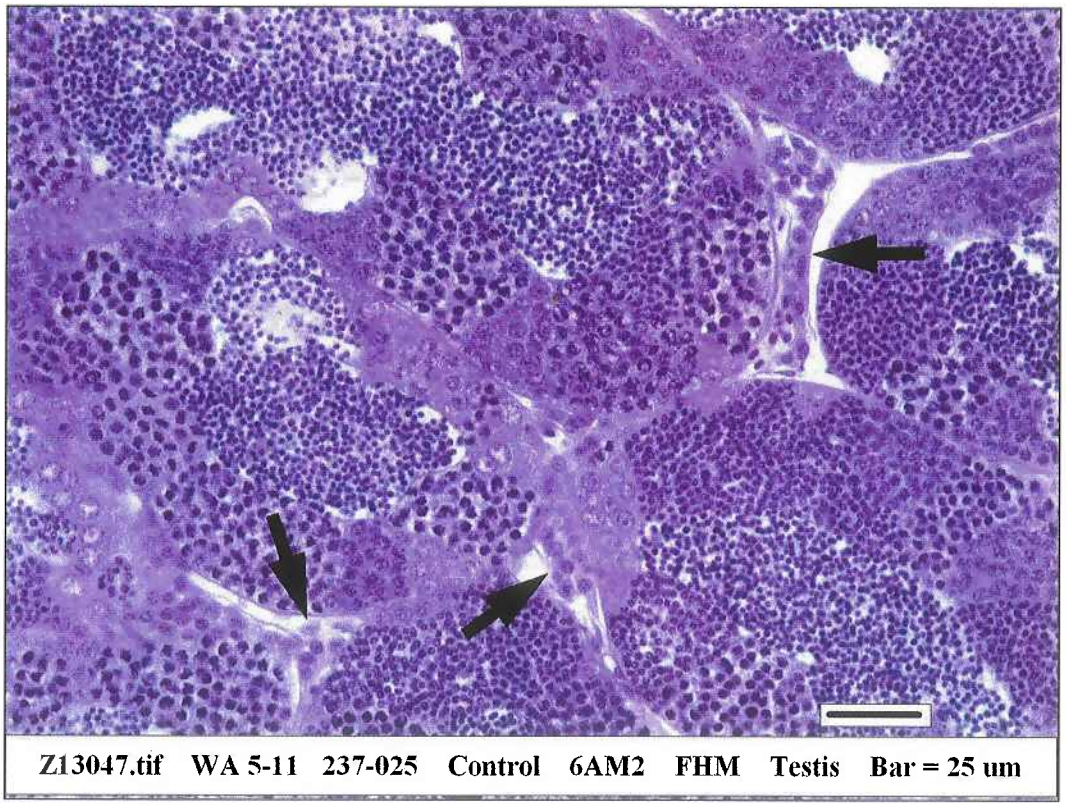

Figure 2 (Z13047). Testis from a control group male. Interstitial cell aggregates (arrows) in the testis of this fish are slightly larger as compared to Fig. 1. This essentially represents the upper extent of interstitial cell proliferation observed in control fish. H&E.

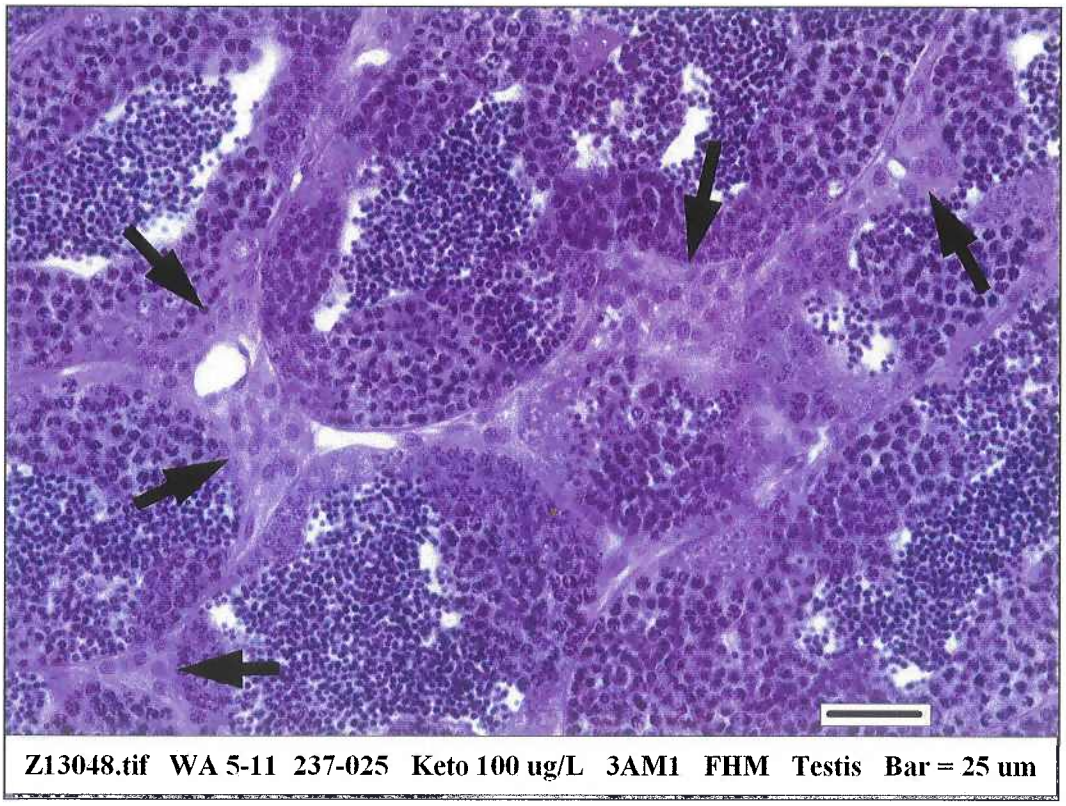

Figure 3 (Z13048). Testis from a 100 µg/L ketoconazole group male. Interstitial cell aggregates (arrows) in the testis of this fish are larger than in control fish, and the cytoplasm of these cells is slightly more dense. This was diagnosed as Increased Cells, Interstitial Cells, Grade 1 (minimal) severity. H&E.

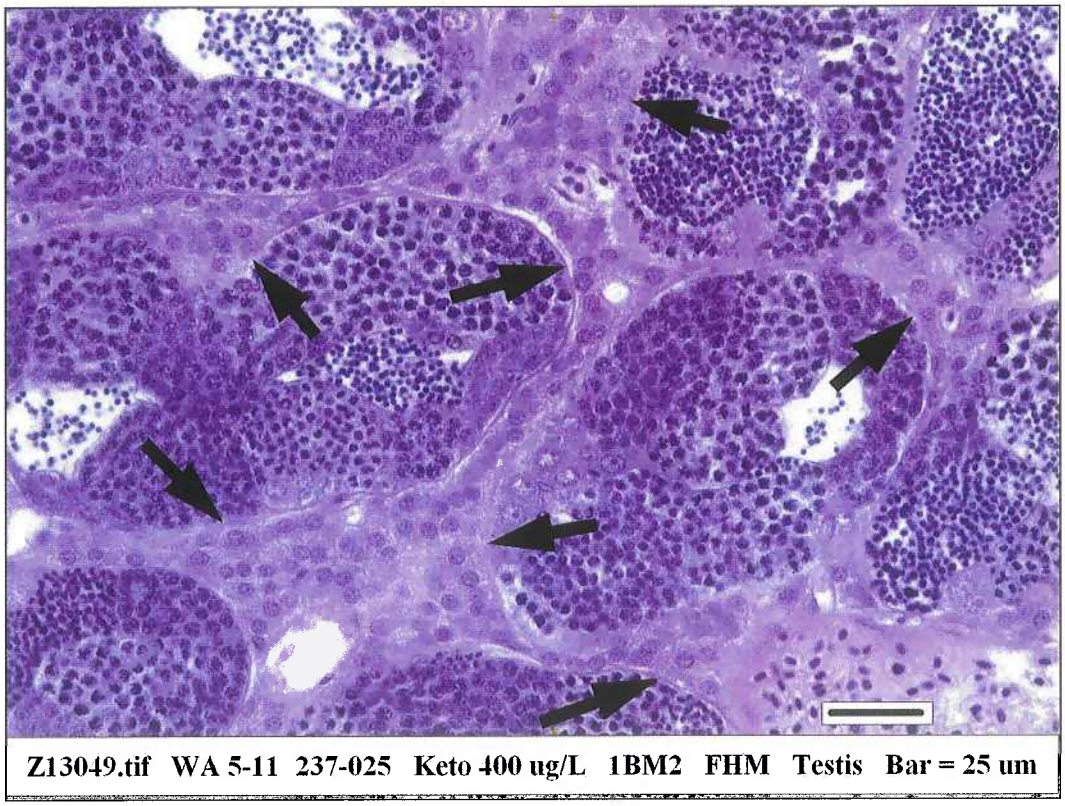

Figure 4 (Z13049). Testis from a 400 µg/L ketoconazole group male. Interstitial cell aggregates (arrows) in the testis of this fish are larger than in Fig. 2, and the cells tend to fill and expand the interstitial spaces. This was diagnosed as Increased Cells, Interstitial Cells, Grade 2 (mild) severity. H&E.

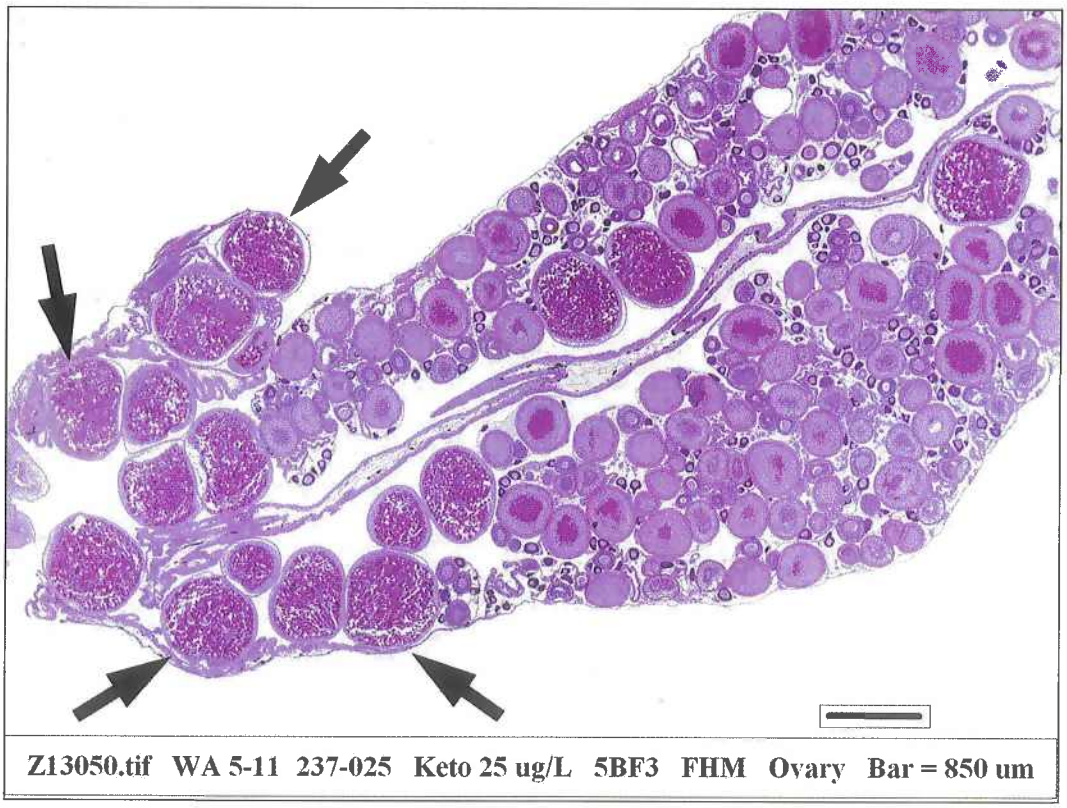

Figure 5 (Z13050). Ovary from a 25 µg/L ketoconazole group female. A number of mature/spawning oocytes (arrows) are present in an otherwise Stage 2 (early to mid-vitellogenesis) ovary. This was seen occasionally in controls and all other exposure groups, and it most likely represents the normal transition from ovarian Stage 4 to Stage 2 that occurs at or around spawning. This finding was designated as Asynchronous Development. H&E.

Appendix C – Wildlife International Report

FLUTAMIDE: PHASE 1B FOLLOW-UP – FISH SCREENING ASSAY FOR ENDOCRINE ACTIVE SUBSTANCE WITH THE FATHEAD MINNOW (*Pimephales promelas*)

REVISED DRAFT REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 607A-101

Based on
OECD Phase 1B Protocol with Modifications to Endpoints
Battelle Protocol: Fish: Screening Assay OECD Phase 1B Follow-up

AUTHOR:

Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: August 10, 2005

STUDY COMPLETION DATE: _____, 2006

SUBMITTED TO:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2693

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(410) 822-8600

FLUTAMIDE: PHASE 1B FOLLOW-UP – FISH SCREENING ASSAY FOR ENDOCRINE ACTIVE SUBSTANCE WITH THE FATHEAD MINNOW (*Pimephales promelas*)

SPONSOR:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2693
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REPORT APPROVAL

STUDY DIRECTOR	DATE
LABORATORY MANAGEMENT	DATE
SPONSORS REPRESENTATIVE	DATE

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SUMMARY

SPONSOR: Battelle Memorial Institute

TITLE: Flutamide: Phase 1b Follow-Up – Fish Screening Assay For Endocrine Active Substance With The Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 607A-101

TEST DATES:

Study Initiation:	August 10, 2005
Experimental Start (EPA):	September 2, 2005
Biological Termination:	September 23, 2005

LENGTH OF EXPOSURE: 21 Days

TEST ORGANISMS: Fathead Minnow *(Pimephales promelas)*

SOURCE OF TEST ORGANISMS: Springborn-Smithers Laboratories

AGE OF TEST ORGANISMS: Adults in Reproductive Condition

TEST CONCENTRATIONS:

Nominal	Mean Measured
Negative Control	< LOQ
100 µg/L	89 µg/L
500 µg/L	388 µg/L
1000 µg/L	822 µg/L

RESULTS:

Endpoint	LOEC (µg/L)	NOEC (µg/L)
Female Body Length	>1000	1000
Male Body Length	>1000	1000
Female Body Weight	>1000	1000
Male Body Weight	500	100
Female GSI	>1000	1000
Male GSI	500	100
Female VTG	>1000	1000
Male VTG	>1000	1000
Male Fatpad Score	1000	500
Male Fatpad Index	1000	500
Male Tubercle Count	500	100
Male Tubercle Score	100	<100
Mean Number of Eggs per Rep. Day	1000	500
Mean Number of Fertile Eggs per Rep. Day	1000	500
Mean Number of Eggs	1000	500
Mean Number of Fertile Eggs	1000	500
Mean Number of Infertile Eggs	>1000	1000

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Battelle Memorial Institute at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the definitive test was conducted from August 10 to September 23, 2005. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 607A-101 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to detect the estrogenic activity and other observed effects of flutamide, an endocrine active test substance, on the fathead minnow *(Pimephales promelas)* during a 14-day pre-exposure period followed by a 21-day exposure period under flow-through test conditions. Survival, body weights, and observations of abnormal behavior were monitored. Endocrine mediated endpoints that were evaluated at the end of the test included fecundity, fertility, observations of secondary sex characteristics (e.g., tubercles and fatpad measurements), measurement of vitellogenin, histopathology of gonads, and gonadosomatic index (GSI).

EXPERIMENTAL DESIGN

Fathead minnows *(Pimephales promelas)* (first-time spawners) were exposed to three test concentrations (100, 500, and 1000 µg/L) and a negative control (dilution water) for 21 days. Prior to the exposure period, active spawning in all groups was established during a pre-exposure period of 14 days in the same size tanks used for the 21-day exposure period. The original 14-day pre-exposure period was extended to 23 days in order for fish to meet the acceptance requirement of the Sponsor.

The fish used in this study were 160 days of age at the beginning of the exposure period. During the first 14 days of the pre-exposure period, some mortality occurred from Day 12 through Day 15. After discussions with Battelle and EPA it was decided to replace dead fish in tanks on Day 16. The additional fish were from the same lot of fish maintained under the same conditions as study fish. From Day 16 through Day 23, monitoring of fecundity was continued.

Prior to Day 16, tanks were cleaned on pre-exposure Days 7 and 13, approximately once per week. After Day 16, which was a Friday, tanks were cleaned three times per week, on Monday, Wednesday, and Friday throughout the remainder of the study. The cleaning procedure involved transferring fish from an "old tank" into a newly cleaned tank. After this procedure was initiated there were no mortalities or observations of abnormal fish for the remainder of the study.

During the pre-exposure period, there were 24 test chambers maintained with 6 fathead minnows (2 males/4 females) in each chamber. Three spawning tiles were maintained in the tanks during pre-exposure. Fecundity was estimated (semi-quantitatively) during the pre-exposure period. Both the frequency of spawning and number of eggs produced were used as the selection criteria for selecting the final 16 tanks for inclusion in the exposure period. Tanks were ranked from highest to lowest based on the estimated number of eggs produced from Days 16-22. Since one day was needed to prepare for the exposure phase, Day 23 pre-exposure egg production data could not be used as part of the ranking procedure. The first four of the 16 tanks were randomly assigned to the control and three treatment replicates, followed by the second, third and fourth set of tanks.

During the 21-day exposure period, four replicate test chambers were maintained in each treatment and control group, with 6 fathead minnows (2 males/4 females) in each chamber for a total of 24 fathead minnows (8 males/16 females) per test concentration. Three tiles were placed in each tank to monitor fecundity and fertility throughout the exposure period.

Nominal test concentrations (100, 500, and 1000 µg/L) were indicated by study sponsor based on results of the OECD Phase 1B results. Water samples from each test chamber were collected at specified intervals for analysis of the test substance. Results of analyses were used to calculate mean measured test concentrations.

To control bias, fathead minnows in spawning condition were impartially assigned to exposure chambers at test initiation. There were no other potential sources of bias expected to affect the results of the study. Observations of mortality, fecundity, fertility, and other clinical signs were made throughout the 23-day pre-exposure and 21-day exposure periods. The six core endpoints that were used as indicators of endocrine disruptor activity were fecundity, fertility, gross morphology (particularly male secondary sexual characteristics of tubercles and fatpad), vitellogenin (VTG) concentrations, gonadal histology, and gonadosomatic index (GSI).

Observations and measurements from the effects of flutamide were analyzed in order to define the LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) for each of the core endocrine endpoints.

MATERIALS AND METHODS

The study was performed based on the Battelle protocol: Fish Screening Assay OECD Phase 1B Follow-up and on procedures in OECD Protocol for Phase 1B – Fish Screening Assay for Endocrine Active Substances – Annex 1 with modifications to measurement endpoints, Document ENV/JM/TG/EDTA(2004)REV2.

Test and Reference Substances

The test substance used to prepare the test solutions, the analytical calibration standards, and the analytical matrix fortification samples for the study was received from Battelle on May 6, 2005. It was assigned Wildlife International, Ltd. identification number 7177 upon receipt and was stored under ambient conditions. The test substance, a solid, was identified as Flutamide (Sigma F9397); lot number 121K1083; CAS number 13311-84-7. The reported purity was 100% with an expiration date of April 1, 2006.

The reference substance used during the vitellogenin assays was received from Battelle on September 22, 2005. It was assigned Wildlife International, Ltd. identification number 7360 upon receipt and was stored at -80°C. The reference substance, a liquid, was identified as Fathead Minnow VTG, 1.86 mg/mL (AAA), 50% G.AP 4.1.04.

Test Organism

Fathead minnows, *Pimephales promelas*, were used in this test. This species is representative of an important group of organisms and was selected for use in the test based upon past use and ease of handling in the laboratory. The identity of the species was verified by the supplier, or by Wildlife

International, Ltd. personnel using appropriate taxonomic keys, such as Eddy (1). Loading of test tanks, defined as the total wet weight of fish per liter of test solution, did not exceed 0.5 grams of fish per liter of solution that passed through a tank in 24 hours. Instantaneous loading did not exceed 5 grams of fish per liter of test solution present in the test tank at any given time.

During the holding period, the test fish were fed at least twice a day with brine shrimp or trout chow (or equivalent). Fish were acclimated for 7 days prior to the test at 25 ± 2°C. During the pre-exposure and exposure period fish were fed twice a day with live brine shrimp. The feeding amounts and frequency were documented in the raw data. Feeding rates were based on initial wet weights of a representative sub-sample from same lot of fish used for the pre-exposure phase. Fish were not fed within 12 hours of the end of the test. Specifications for acceptable levels of contaminants in fish diets have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test. Five females and five males were collected, preserved, and shipped to Experimental Pathology Laboratories, Inc. (EPL) for a determination of general health prior to the beginning of the exposure period.

Dilution Water

The water used for holding and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water is characterized as moderately hard water.

The well water was passed through a sand filter to remove particles greater than approximately 25 µm, and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to delivery to the diluter system, the water again was filtered (0.45 µm), and then passed through an ultraviolet (UV) sterilizer to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water are presented in Appendix 1.

Preparation of Test Concentrations

One stock solution was prepared for each of the three concentrations tested. Stock solutions were prepared twice weekly during the definitive test. The primary stock was prepared by dissolving the test substance in well water at a concentration of 20 mg/L. The stock was sonicated, and mixed overnight using an electric top-down mixer, and appeared clear and colorless after mixing. Aliquots of the primary stock solution were proportionally diluted with well water to prepare additional stocks at concentrations of 2.0 and 10 mg/L. The stock solutions were injected into the diluter mixing chambers (at a rate of 8.5 mL/minute) where they were mixed with dilution water (at a rate of 95 mL/minute) to achieve the nominal test concentrations of 100, 500, and 1000 µg/L. All of the test solutions appeared clear and colorless in the mixing chambers and test chambers at test initiation and termination.

Test Apparatus

A continuous-flow diluter was used to deliver each concentration of the test substance and a negative (well water) control. Peristaltic pumps (Cole-Parmer Instrument Company, Chicago, Illinois) were used to deliver the three test substance stock solutions into mixing chambers indiscriminately assigned to each treatment. The pumps were calibrated prior to the test and at approximately weekly intervals thereafter. The stock solutions were diluted with well water in the mixing chambers in order to

obtain the desired test concentrations. The flow of dilution water to the mixing chambers was controlled by rotameters, which were calibrated prior to test initiation and at approximately weekly intervals thereafter. The flow of test water from each mixing chamber was split and allowed to flow into four replicate test chambers. The proportion of the test water that was split into each replicate was checked prior to the test, and at approximately weekly intervals thereafter to ensure that flow rates varied by no more than ±10% of the mean for the four replicates. The diluter flow rate was adjusted to provide approximately six volume additions of test water in each test chamber per day. The general operation of the diluter was checked visually at least two times per day during the test and at least once at the end of the test. During the test, the diluter system was periodically cleaned to prevent the buildup of bacterial/fungal growth. Test chambers and mixing chambers were periodically removed and replaced with freshly cleaned components.

The test was conducted in a temperature-controlled environmental chamber designed to maintain the target test temperature throughout the test period. The test chambers were 18-L glass aquaria filled with approximately 10 L of test solution. The depth of the test water in a representative test chamber was approximately 17.9 cm. Test chambers were labeled with the project number, test concentration and replicate.

Analytical Sampling

Prior to test initiation (Day –2), a sample of the test water was collected from each treatment and control group to evaluate diluter performance. On Days 0, 3, 7, 10, 14 and 21 (test termination), water samples were collected from alternating replicate test chambers of each treatment and control group to measure concentrations of the test substance. The samples were collected at mid-depth from each test chamber and placed in glass vials for immediate analysis.

Analytical Method

The method used for the analyses of flutamide in freshwater was developed by Wildlife International, Ltd. Samples were diluted with freshwater, as needed, and analyzed by high performance liquid chromatography (HPLC) using variable wavelength detection set at 293 nm.

Concentrations of flutamide in the samples were determined using an Agilent Series 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector. Chromatographic separations were achieved using a Zorbax phenyl column (250 mm x 4.6 mm, 5-µm particle size). A method flowchart for the analysis of flutamide is presented in Appendix 3.1 and typical instrumental parameters are summarized in Appendix 3.2.

Five calibration standards of flutamide, ranging in concentration from 40.0 to 400 µg/L, were prepared in freshwater using a stock solution of flutamide in methanol (Appendix 3.3). Fresh calibration standards were prepared and analyzed with each sample set. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of flutamide in the samples was determined by substituting the peak area responses of the samples into the applicable linear regression equation and multiplying by the dilution factor. An example of the calculations for a representative sample is included in Appendix 3.4.

The method limit of quantitation (LOQ) for the analysis of flutamide in freshwater was set at 40.0 µg/L, calculated as the product of the concentration of the lowest calibration standard (40.0 µg/L) and the dilution factor of the matrix blank samples (1.00). Six matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses (Appendix 3.5).

Samples of freshwater were fortified at 100 and 1000 µg/L using a stock solution of flutamide in methanol (Appendix 3.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 95.1 to 102% of nominal concentrations (Appendix 3.5).

A representative calibration curve is presented in Appendix 3.6. Representative chromatograms of low- and high-level calibration standards are presented in Appendices 3.7 and 3.8, respectively. A representative chromatogram of a matrix blank sample is presented in Appendix 3.9 and a representative chromatogram of a matrix fortification sample is presented in Appendix 3.10. A representative chromatogram of a test sample is presented in Appendix 3.11.

Environmental Conditions

Fluorescent light bulbs that emit wavelengths similar to natural sunlight (Colortone® 50) were used for illumination of the test chambers. A photoperiod of 16 hours of light and 8 hours of dark was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity was measured weekly at the surface of the water in five arbitrarily selected positions in the environmental chamber using a SPER Scientific Model 840006C light meter. Readings ranged from 225 to 909 lux.

The target test temperature during the test was 25 ± 1°C. Temperature was measured in each test chamber at the beginning and end of the test and at weekly intervals during the test using a liquid-in-glass thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder which was verified prior to test initiation using a liquid-in-glass thermometer.

Dissolved oxygen was measured in alternating replicates of each treatment and control group at the beginning and end of the test, daily during the first seven days of the test and at weekly intervals thereafter during the test. Measurements of pH were made in alternating replicates of each treatment and control group at the beginning and end of the test and at weekly intervals thereafter during the test. Measurements of dissolved oxygen were made using a Thermo Orion Model 850Aplus dissolved oxygen meter and pH was measured using a Thermo Orion Model 720Aplus pH meter.

Hardness, alkalinity, and specific conductance were measured in alternating replicates of the negative (dilution water) control and the highest concentration treatment group at the beginning of the test, at weekly intervals during the test and at test termination. Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness and alkalinity were measured by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* (2).

Endpoints

Observations of biological responses were conducted daily and included survival, spawning, and behavior. Fish were examined for external abnormalities (e.g. hemorrhage and discoloration). No

replacement fish were added at any time during the exposure period of the test. Fish behavior was noted for such signs as hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical feeding habits. Characteristics such as body color, coloration patterns, and body shape (head and pectoral region) were assessed daily. Tiles were checked daily and if eggs were present, fecundity estimates were determined. Tiles were then removed from test chambers and placed in incubation tanks for up to three days so that fertilization could be evaluated. After fertilization was determined, eggs were sacrificed.

Endpoints that were assessed included fertility, fecundity, observations of secondary sex characteristics (tubercles and fatpad), measurement of vitellogenin, histopathology of gonads, and gonadosomatic index (GSI). At test termination, all fish were removed from tanks and euthanized in a buffered MS-222 solution. For each test chamber, all male fish were sacrificed prior to female fish to minimize the possibility of contamination of blood samples with vitellogenin (normal males do not have any vitellogenin, or have very low levels). As soon as gill movement had ceased, the fish was blotted dry, weighed to the nearest 0.1 mg and measured for length to the nearest 0.1 mm using a graduated scale. Immediately after measurement, the caudal peduncle was severed and blood samples were collected from the caudal vein/artery. Gonads were then surgically removed and weighed. Finally, the fatpad was removed and weighed. The remaining tissues were preserved and at a later date tubercles were mapped and scored.

Fecundity - Egg production was determined daily. Because fathead minnows spawn within a few hours after the lights are turned on, they were not disturbed (except for feeding) until late morning. This allowed time for spawning and fertilization to be completed and for eggs to water-harden. The spawning substrates were removed from the tanks to enumerate any eggs that were present. If no embryos were present, the substrate was returned to the tank; new substrates were added to replace any that were removed. Fecundity was expressed on the basis of surviving females per reproductive (test) day per replicate, as cumulative eggs laid over the test, and as total number of eggs laid through the exposure period. Semi-quantitative fecundity measurements were measured by visual estimation using a matrix, with exact numbers being listed for estimates less than 10, after which numbers were estimated as 25, 50, 100, 150, 200, 250 and estimates above 250 in increments of 50. Semi-quantitative counts were performed by only person and exact counts by another to evaluate if estimates were as good as the more labor intensive counts.

Fertilization Success - After the spawning substrate was removed from the tank, the substrate was transferred to a tank of clean dilution water for up to three days at which time fertile embryos (eyed embryos) were counted under appropriate magnification. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated; viable embryos remain clear for 36 to 48 hours until reaching the eyed stage.

Measurement of Vitellogenin - At test termination, all fish were anaesthetized with MS-222. At least two blood samples were collected, if possible, from the caudal vein/artery with heparinized microhematocrit capillary tubes. After collection, the plasma was separated using a centrifuge. The plasma was then mixed with a protease inhibitor and stored frozen at approximately -80°C until analyzed for vitellogenin. Analysis for vitellogenin was conducted with a commercially available Enzyme-Linked Immunosorbent Assay (ELISA) system (Amersham Biosciences RPN5942), a microplate washer/shaker (Columbus Washer model F109201) and a microplate reader (Rainbow Reader model F039346). To cover the possible range of results, samples were serially diluted and analyzed in duplicate. Samples that were out of range for the ELISA were diluted as necessary and reanalyzed. The procedures used to collect, prepare, and analyze the plasma samples were based upon methodology provided by the ELISA system

manufacturer and those presented by the US EPA (A Short-Term Test Method for Assessing the Reproductive Toxicity of Endocrine Disrupting Chemicals Using the Fathead Minnow (*Pimephales promelas*) 2002, EPA/600/R-01/067) (3).

A comparison was made between the vitellogenin (VTG) kit standards and the standard provided by Battelle. Based on the absorbance criteria for the standard, only the Battelle standard was within acceptable range. The VTG standards used in the analysis were 3.91, 7.81, 15.6, 31.3, 62.5, 125 and 250 ng VTG/mL, along with a blank. The internal standard provided with the ELISA kits produced low absorbance values (optical density). For the high standard, 250 ng/mL, absorbencies ranged from 0.186 to 0.392 units over four trials. For the 250 ng/mL standard made from the VTG provided by Battelle, absorbencies were 1.864 and 2.107 units in two trials. Plasma samples from untreated female fathead minnows actively producing eggs were analyzed along with the two sets of standards. The plasma was serially diluted to a total of 518,400 fold, which slightly exceeded the 480K dilution recommended in the EPA method. The values for the blood plasma generally exceeded the curve produced with the ELISA kit standard but were within the range of the curve generated with the standard provided by Battelle. Therefore, the Battelle standard was used for the vitellogenin analysis.

Necropsy, Histology, and Histopathology

Necropsy - Immediately following sacrifice, each fish was placed in right lateral recumbency on the stage of a dissecting microscope. Utilizing microdissection tools throughout the procedure, the left abdominal body wall was excised, taking care to free it from any attachments to the left gonad. The mid-portion of the swimbladder was then grasped with forceps, reflected cranially, severed from its cranial attachment, if necessary, and removed. The caudal-most segment of the intestine was isolated and severed a few millimeters cranial to the anus, and the abdominal portion of the gastrointestinal tract was reflected cranially and ventrally, and freed from the gonads by severing any existing mesenteric attachments. A few drops of Bouin's solution were applied to the gonads prior to their removal. The left and right gonads were then excised together by grasping them at their caudal-most extent using forceps, and by severing all mesenteric attachments as the gonads were reflected cranially. Gonads were then gently lifted from the solution, and placed in a tared weigh boat for weighing. Following weighing, the gonads were positioned in a tissue cassette that was then placed in an individual container of fixative (Bouin's solution), the volume of which was at least 10-fold greater than the approximated tissue volume. The fixative container was then gently agitated for five seconds to dislodge air bubbles from the cassette. After a period of time ranging from 6 to 12 hours, each fixed specimen was rinsed in 70% ethanol and then maintained in an individual container of neutral buffered formalin for at least 48 hours prior to being shipped to EPL for tissue processing and analysis.

Histology and Histopathology – Gonads removed from fish were sent to EPL (Experimental Pathology Laboratories, Inc.) for analysis. The procedures used to process and analyze samples are described in the EPL report presented in Appendix 4. Tissues were examined by a board-certified veterinary pathologist who had experience in the evaluation of fish gonads for changes caused by endocrine-active substances.

Gonadosomatic Index – An assessment of reproductive status can be determined by the gonadosomatic index (GSI). The GSI is the weight of the ovaries or testes relative to the total body weight of the fish (GSI = 100 x gonad weight (g)/ body weight (g)).

Male Secondary Sex Characteristics – Male secondary sex characteristics were evaluated for every fish. At test termination each fish was initially examined for the presence or absence of three male features: 1) A black spot on the dorsal fin, 2) Tubercles on the nose or upper lip, and 3) Pigmentation on the nose or upper lip. If none of these were present the fish was considered to be a female. Additionally, the development of the dorsal pad and the number and size of breeding tubercles were evaluated.

Dorsal fatpad – The following scoring scheme was used to assess the dorsal fatpad. Scores ranged from 1-5, with 1= no fatpad visible; 2= small fatpad evident; 3= fatpad clearly visible and just above the body surface; 4= fatpad prominent and clearly above the body surface but not "overhanging"; and 5= fatpad very prominent and starting to "overhang" the body surface. A fatpad index was also determined as the fatpad weight divided by the total wet body weight.

Tubercle presence and size – were quantitatively scored for each fish as: 1-present; 2-enlarged; and 3-pronounced. Rating 1 (present) was identified as any tubercle having a single point whose height was nearly equivalent to its radius (diameter). Rating 2 (enlarged) was identified by tissue resembling an asterisk in appearance, usually with a large radial base with grooves or furrows emerging from the center. Tubercle height is often more jagged but can be somewhat rounded at times. Rating 3 (pronounced) was usually quite large and rounded with less definition in structure.

Data Analyses

Statistical analyses were performed on the following data: 1) fecundity; 2) fertility; 3) survival; 4) total length and wet weight of fish; 5) secondary sex characteristics (fatpad and tubercles); 6) vitellogenin; 7) histopathology; and 8) gonadosomatic index. The histopathology of gonads included assessment, sex ratio, and an assessment of the intermixing of testicular and ovarian cells in gonads. If responses appeared to be fundamentally monotonic, the differences between test concentrations were evaluated using a step-down approach based on the Jonckheere-Terpstra trend test as described in draft OECD guidance on statistical testing in ecotoxicology and "A short term test method for assessing reproductive toxicity of endocrine disrupting chemicals using the fathead minnow (*Pimephales promelas*)" (EPA 600/R-01/067, June 2002) (3).

If responses were strongly non-monotonic, dichotomous and categorical data (e.g., live/dead,) were evaluated using Fisher's Exact tests. Analyses of non-monotonic responses in continuous variables such as growth, vitellogenin, and reproductive endpoints were evaluated for normality and homogeneity of variances. If the data were deemed normal with homogeneous variances, hypothesis testing using analysis of variance (ANOVA) and multiple means tests (e.g., Dunnett's, Bonferroni, Scheffe, etc.) were used. All continuous-variable data were evaluated for normality using Shapiro-Wilk's test, and for homogeneity of variance using Levene's test ($p = 0.05$). If the non-monotonic data failed the tests for normality or homogeneity, then the transformation of the data was tried in order to correct the condition. When data transformations failed to correct for non-normality or heterogeneity of variances, nonparametric procedures were used, if appropriate, to identify statistically significant differences between the treatment and control groups. All statistical tests were performed using a personal computer with SAS (5).

Pathology results from EPL confirmed that four fish that were originally identified as females at the beginning of the pre-exposure period were actually males (testes present). The pathology report was considered the definitive determination of sex. The misidentification was attributed to poorly developed and ambiguous secondary sex characteristics that resulted in males having the appearance of females. All

four fish had poorly developed secondary sex characteristics at test initiation and appeared to be female. One of the four was assigned to Replicate A of the Negative Control, one to Replicate A of the 500 µg/L group, and two to Replicate C of the 500 µg/L group. An experienced biologist who routinely determines the phenotypic sex of fathead minnows performed the procedure used to assign fish to the test tanks at the beginning of the test.

All four fish were dropped from analysis of the male variables that included length, weight, fatpad score, fatpad index, tubercle count, tubercle score, gonadosomatic index (GSI), and vitellogenin. There were no differences in the interpretation of the results for males if the four fish were included or excluded in the analysis, although there were some differences that were no longer statistically significant. This is because inclusion of the four poorly developed males adds variability to the results. For example, the tubercle scores of the three males in Replicate A of the 500 µg/L treatment group were 29, 20 and 0. If the all three fish are included in the analysis, the mean number of tubercles is 16.3 compared to 24.5 if the three fish are dropped.

The incorrect assignment of the four fish resulted in sex ratios that were shifted from 2 males and 4 females to 3 males and 3 females in Replicate A of the Negative Control and Replicate A of the 500 µg/L treatment group, and to 4 males and 2 females in Replicate C of the 500 µg/L treatment group. The shifted sex ratio showed no clear patterns or obvious effects upon fecundity.

Finally, these data bring up an interesting point regarding the use of groups or pairs when designing endocrine studies with fathead minnows or other fish. When assigning groups there is always a small chance of having males with poorly defined secondary sex characteristics. In this study 4of 96 fish or approximately 4% of the fish were incorrectly assigned. In paired designs with pre-exposure assessments of reproduction there are no questions as to whether this assignment is correct, provided fertility is assessed at least once for each replicate during pre-exposure.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Nominal concentrations used in the 21-day study were 100, 500, and 1000 µg /L. Stock solutions were analyzed to verify mixing procedures and are presented in Table 1. The measured stock concentrations ranged from 89.8 to 98.1 % of nominal.

Table 1. Measured Stock Concentrations of Flutamide

Nominal Test Concentration (µg/L)	Sample Number (607A-101-)	Sampling Time (Days)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]	Mean Measured Concentration (µg/L)	Mean Measured Percent of Nominal
2000	S-1	0	1942	97.1	1946	97
	S-4	3	1936	96.8		
	S-7	10	1961	98.1		
10000	S-3	0	9540	95.4	9395	94
	S-5	3	9567	95.7		
	S-9	10	9078	90.8		
20000	S-2	0	18571	92.9	18368	92
	S-6	3	17956	89.8		
	S-8	10	18576	92.9		

[1] The limit of quantitation (LOQ) was 40.0 µg/L, calculated as the product of the concentration of the lowest calibration standard (40.0 µg/L) and the dilution factor of the matrix blank samples (1.00).
[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Results of analyses to measure concentrations of flutamide in water samples collected during the test are presented in Table 2 below. The measured concentrations ranged from 69.6 to 99.0% of nominal in the 100 µg/L treatment group, 59.3 to 94.6% of nominal in the 500 µg/L treatment group, and from 63.5 to 97.6% of nominal in the 1000 µg/L treatment group. When the measured concentrations of the samples collected during the test were averaged for each treatment group, the mean measured concentrations were 89, 388, and 822 µg/L, which represented 89, 78, and 82% of nominal concentrations, respectively. The results of the study were based on nominal test concentrations.

Since a solvent was not used in this study, the flow rates used in this study pushed the limits of our peristaltic pumps. The pump used in this study was calibrated on Day –1 and on Days 4 and 6. The measured concentrations on Day 7 were lower than Day 0 or Day 3. It was suspected that our pump tubing may have lost some its initial properties (e.g., increased flexibility of the wall of the tubing) which resulted in slightly reduced flow rates. Therefore, from Day 10 onward, pump tubing was changed daily and pumps were calibrated daily. The increase in measured concentrations from Day 10 through the rest of the study indicates that this modification appeared to have corrected any problem that may have existed.

Table 2. Measured Concentrations of Flutamide in Freshwater Samples

Nominal Test Concentration (µg/L)	Sample Number (607A-101-)	Replicate	Sampling Time (Days)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]	Mean Measured Concentration (µg/L)	Mean Measured Percent of Nominal
0.0 (Negative Control)	1	A	0	< LOQ	--	--	--
	2	C	0	< LOQ	--		
	9	B	3	< LOQ	--		
	10	D	3	< LOQ	--		
	17	A	7	< LOQ	--		
	18	C	7	< LOQ	--		
	25	B	10	< LOQ	--		
	26	D	10	< LOQ	--		
	33	B	10	< LOQ	--		
	34	D	10	< LOQ	--		
	41	A	14	< LOQ	--		
	42	C	14	< LOQ	--		
	49	B	21	< LOQ	--		
	50	D	21	< LOQ	--		
100	3	A	0	98.6	98.6	89	89
	4	C	0	99.0	99.0		
	11	B	3	84.3	84.3		
	12	D	3	82.6	82.6		
	19	A	7	69.6	69.6		
	20	C	7	69.9	69.9		
	27	B	10	91.2	91.2		
	28	D	10	92.0	92.0		
	35	B	10	98.6	98.6		
	36	D	10	98.5	98.5		
	43	A	14	96.5	96.5		
	44	C	14	96.6	96.6		
	51	B	21	86.7	86.7		
	52	D	21	85.6	85.6		
500	7	A	0	420	84.0	388	78
	8	C	0	416	83.3		
	13	B	3	339	67.8		
	14	D	3	345	69.0		
	21	A	7	309	61.7		
	22	C	7	314	62.8		
	29	B	10	447	89.4		
	30	D	10	458	91.6		
	37	B	10	463	92.7		
	38	D	10	473	94.6		
	45	A	14	422	84.5		
	46	C	14	425	85.0		
	53	B	21	296	59.3		
	54	D	21	300	59.9		

Table 2. (Continued) Measured Concentrations of Flutamide in Freshwater Samples

Nominal Test Concentration (µg/L)	Sample Number (607A-101-)	Replicate	Sampling Time (Days)	Measured Concentration (µg/L)[1]	Percent of Nominal[2]	Mean Measured Concentration (µg/L)	Mean Measured Percent of Nominal
1000	5	A	0	789	78.9	822	82
	6	C	0	801	80.1		
	15	B	3	635	63.5		
	16	D	3	641	64.1		
	23	A	7	660	66.0		
	24	C	7	663	66.3		
	31	B	10	965	96.5		
	32	D	10	976	97.6		
	39	B	10	921	92.1		
	40	D	10	958	95.8		
	47	A	14	863	86.3		
	48	C	14	869	86.9		
	55	B	21	884	88.4		
	56	D	21	889	88.9		

[1] The limit of quantitation (LOQ) was 40.0 µg/L, calculated as the product of the concentration of the lowest calibration standard (40.0 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.

Physical and Chemical Measurements of Water

Temperature, dissolved oxygen, and pH in the test chambers were measured during the test. Temperatures remained within the desired range of 25 ± 1°C throughout the pre-exposure and exposure phases of the test. Dissolved oxygen concentrations remained ≥69% of saturation (5.7 mg/L) and measurements of pH ranged from 8.1 to 8.3 throughout the pre-exposure and exposure phases of the test. The specific conductance of dilution water ranged between 320 and 350 µmhos/cm, alkalinity measurements of dilution water ranged between 182 to 188 mg/L as $CaCO_3$, and hardness ranged between 120 to 140 mg/L as $CaCO_3$ throughout the pre-exposure and exposure phases of the test.

Survival of Fish During the Test

There were no mortalities observed during the exposure phase of the study. There were a few mortalities during the pre-exposure period but these were considered incidental. No or low mortalities were to be expected in this test because concentrations were selected for chronic exposure.

Body Length

Body lengths of females used in the 21-day screening assay ranged from 48 to 60 mm in standard length. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There were no statistically significant differences in mean length among treatments (Dunnett's test $p>0.05$, step-down Jonckheere-Terpstra trend test $p>0.05$, and Kruskal-Wallis test $p>0.05$).

Table 3. Summary Statistics for Female Body Length for the 21-Day Flutamide Assay

Level	N	Mean (mm)	SD	CV (%)
CTRL	4	54.4	0.79	1.45
Low	4	54.2	1.39	2.57
Middle	4	54.3	1.66	3.06
High	4	53.9	1.96	3.64

One fish in Negative Control Replicate A, and three fish in the 500 µg/L treatment group (one fish in Replicate A and two fish in Replicate C) were incorrectly identified as females when fish were assigned to groups due to ambiguous secondary sex characteristics, and therefore, were excluded from analysis of the data.

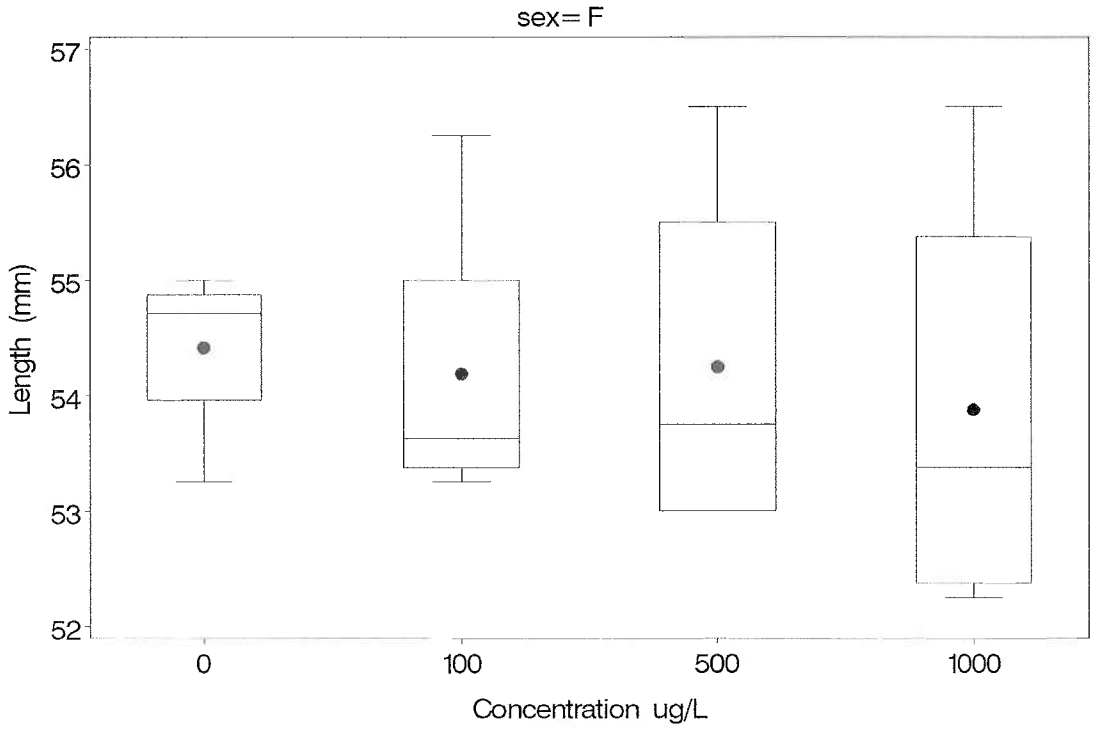


Figure 1. **Box Plot of Female Body Length by Treatment for the 21-Day Flutamide Assay** (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

The body lengths of males used in the 21-day screening assay ranged from 63 to 76 mm in standard length. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There were no statistically significant differences in mean length among treatments (Dunnett's test $p>0.05$, step-down Jonckheere-Terpstra trend test $p>0.05$, and Kruskal-Wallis test $p>0.05$).

Table 4. Summary Statistics for Male Body Length for the 21-Day Flutamide Assay

Level	N	Mean (mm)	SD	CV (%)
CTRL	4	70.0	2.48	3.55
Low	4	71.0	2.12	2.99
Middle	4	67.1	1.55	2.31
High	4	69.1	2.29	3.31

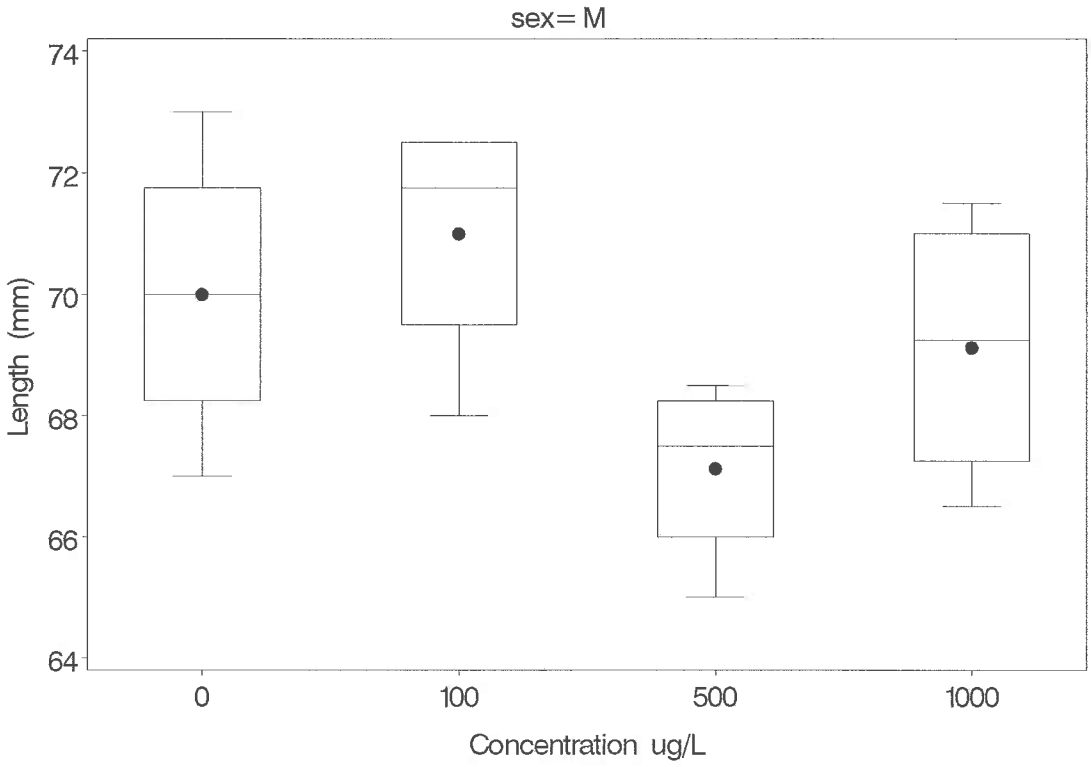


Figure 2. Box Plot of Male Body Length by Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

Body Weight

Body weights of females used in the 21-day screening assay ranged from 1.01 to 2.20 g in standard weight. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There were no statistically significant differences in mean weight among treatments (Dunnett's test $p>0.05$, step-down Jonckheere-Terpstra trend test $p>0.05$, and Kruskal-Wallis test $p>0.05$).

Table 5. Summary Statistics for Female Body Weight for the 21-Day Flutamide Assay

Level	N	Mean (g)	SD	CV (%)
CTRL	4	1.61	0.09	5.71
Low	4	1.62	0.08	5.01
Middle	4	1.58	0.10	6.60
High	4	1.55	0.23	14.7

One fish in Negative Control Replicate A, and three fish in the 500 µg/L treatment group (one fish in Replicate A and two fish in Replicate C) were incorrectly identified as females when fish were assigned to groups due to ambiguous secondary sex characteristics and therefore were excluded from analysis of the data.

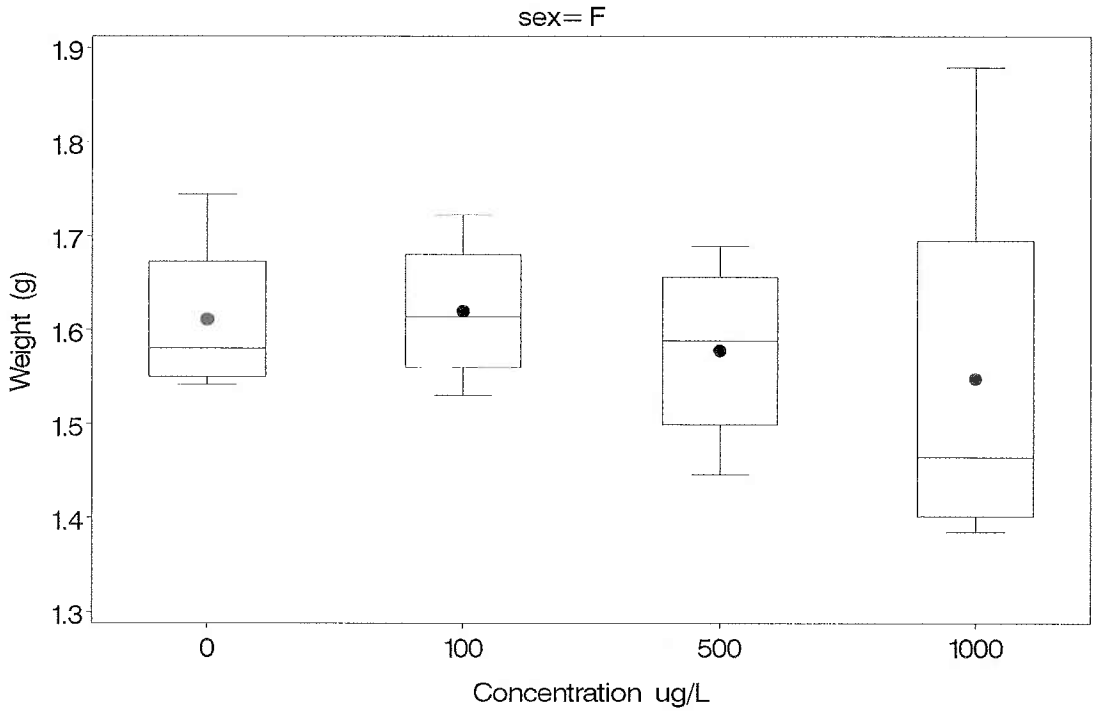


Figure 3. Box Plot of Female Body Weight by Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

The body weights of males used in the 21-day screening assay ranged from 3.01 to 5.44 g in standard weight. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There was a statistically significant difference between the mean weight in controls and the 500 µg/L treatment group using the Dunnett's test ($p<0.05$). Both the 1000 and 500 µg/L treatment groups were effect levels based on the step-down Jonckheere-Terpstra trend test ($p<0.05$). There were statistically significant differences among treatments using the Kruskal-Wallis test ($p<0.05$).

Table 6. Summary Statistics for Male Body Weight for the 21-Day Flutamide Assay

Level	N	Mean (g)	SD	CV (%)
CTRL	4	4.31	0.25	3.54
Low	4	4.59	0.29	4.12
Middle	4	3.52 [1,2]	0.13	3.56
High	4	3.88 [2]	0.47	12.0

[1] Statistically significant difference from the negative control using Dunnett's test ($p < 0.05$)
[2] Statistically significant effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$).

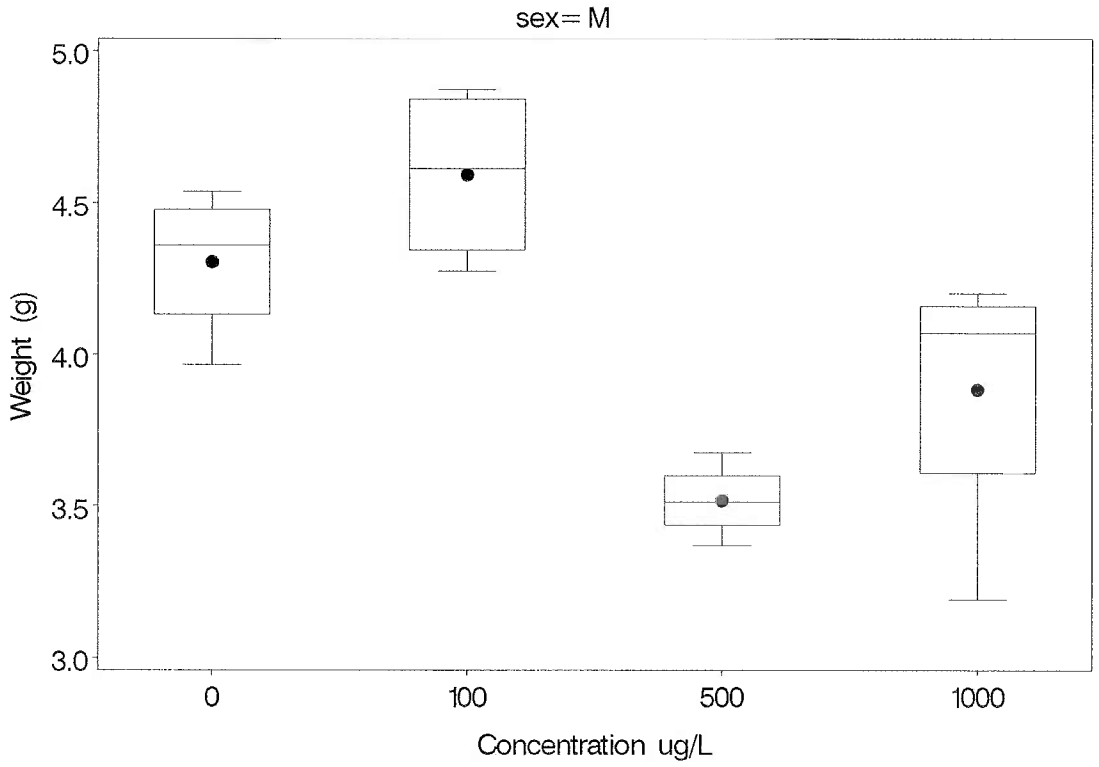


Figure 4. **Box Plot of Male Body Weight by Treatment for the 21-Day Flutamide Assay** (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

Gonadosomatic Index (GSI)

The GSI of females used in the 21-day screening assay ranged from 3.20 to 23.3. The data failed to meet the assumption of homogeneity of variance (Levene's test $p<0.05$), but did meet the assumption of normality (Shapiro-Wilkes test $p>0.05$). Therefore Dunnett's test was not used for analysis of this data set. There were no statistically significant differences in mean GSI among treatments using the step-down Jonckheere-Terpstra trend test ($p>0.05$) or Kruskal-Wallis test ($p>0.05$).

Table 7. Summary Statistics for Female GSI for the 21-Day Flutamide Assay

Level	N	Mean GSI	SD	CV (%)
CTRL	4	9.9	1.15	11.7
Low	4	12.1	1.50	12.4
Middle	4	11.2	3.37	30.2
High	4	7.7	1.13	14.6

One fish in Negative Control Replicate A, and three fish in the 500 µg/L treatment group (one fish in Replicate A and two fish in Replicate C) were incorrectly identified as females when fish were assigned to groups due to ambiguous secondary sex characteristics and therefore were excluded from analysis of the data.

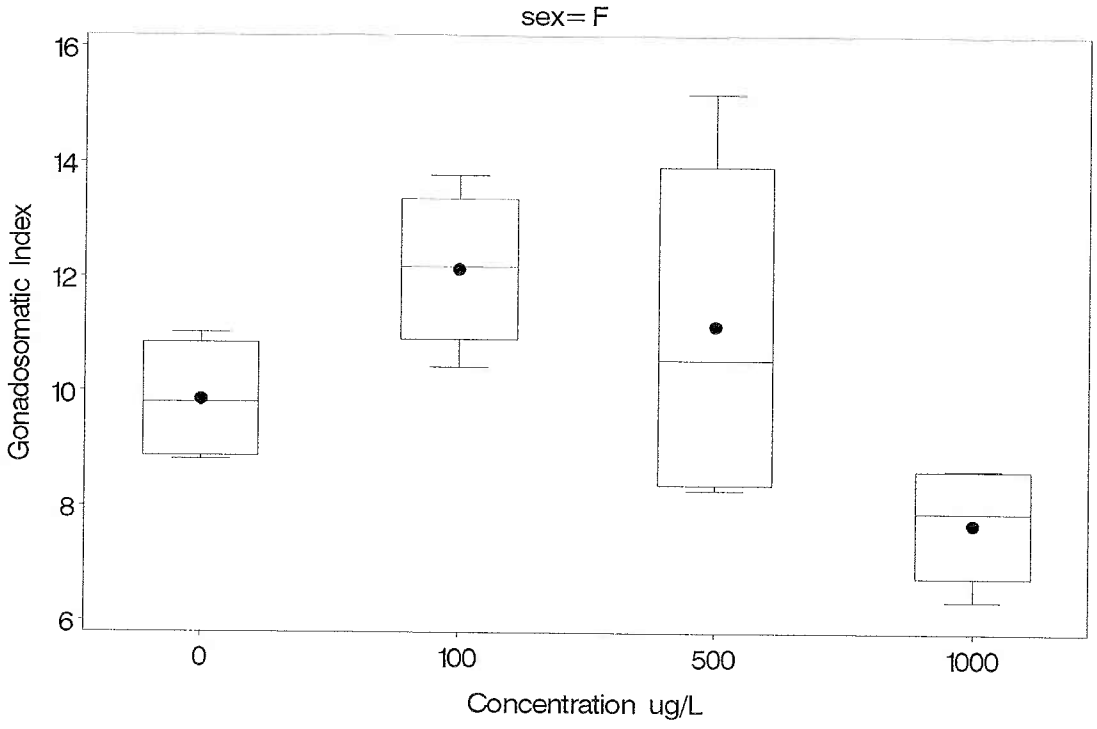


Figure 5. **Box Plot of Female GSI by Treatment for the 21-Day Flutamide Assay** (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

The GSI of males used in the 21-day screening assay ranged from 0.82 to 2.05. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There was a statistically significant difference between the mean GSI in controls and the 500 µg/L treatment group using the Dunnett's test ($p<0.05$). Both the 1000 and 500 µg/L treatment groups were effect levels based on the step-down Jonckheere-Terpstra trend test ($p<0.05$). There were statistically significant differences among treatments using and the Kruskal-Wallis test ($p<0.05$).

Table 8. Summary Statistics for Male GSI for the 21-Day Flutamide Assay

Level	N	Mean GSI	SD	CV (%)
CTRL	4	1.3	0.24	19.3
Low	4	1.3	0.11	8.4
Middle	4	1.7 [1, 2]	0.24	14.1
High	4	1.5 [2]	0.26	17.2

[1] Statistically significant difference from the negative control using Dunnett's test ($p < 0.05$)
[2] Statistically significant effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$).

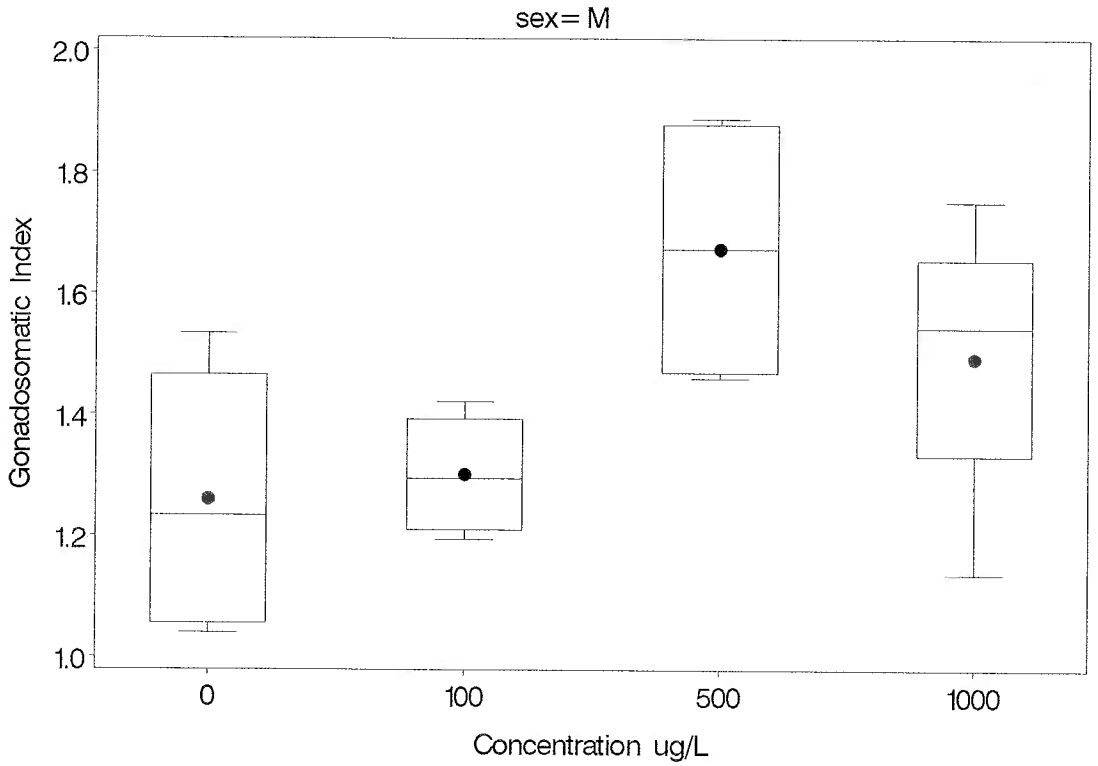


Figure 6. **Box Plot of Male GSI by Treatment for the 21-Day Flutamide Assay** (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

Vitellogenin (VTG)

The VTG levels of females used in the 21-day screening assay ranged from 3.26×10^5 to 6.48×10^7 ng/ml of plasma. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There were no statistically significant differences in mean VTG among treatments (Dunnett's test $p>0.05$, step-down Jonckheere-Terpstra trend test $p>0.05$, and Kruskal-Wallis test $p>0.05$).

Table 9. Summary Statistics for Female VTG for the 21-Day Flutamide Assay

Level	N	Mean (ng/ml)	SD	CV (%)
CTRL	4	1.46×10^7	3.58×10^6	24.5
Low	4	1.89×10^7	1.03×10^7	54.3
Middle	4	1.82×10^7	1.46×10^7	80.7
High	4	1.75×10^7	8.89×10^6	50.7

One fish in Negative Control Replicate A, and three fish in the 500 µg/L treatment group (one fish in Replicate A and two fish in Replicate C) were incorrectly identified as females when fish were assigned to groups due to ambiguous secondary sex characteristics and therefore were excluded from analysis of the data.

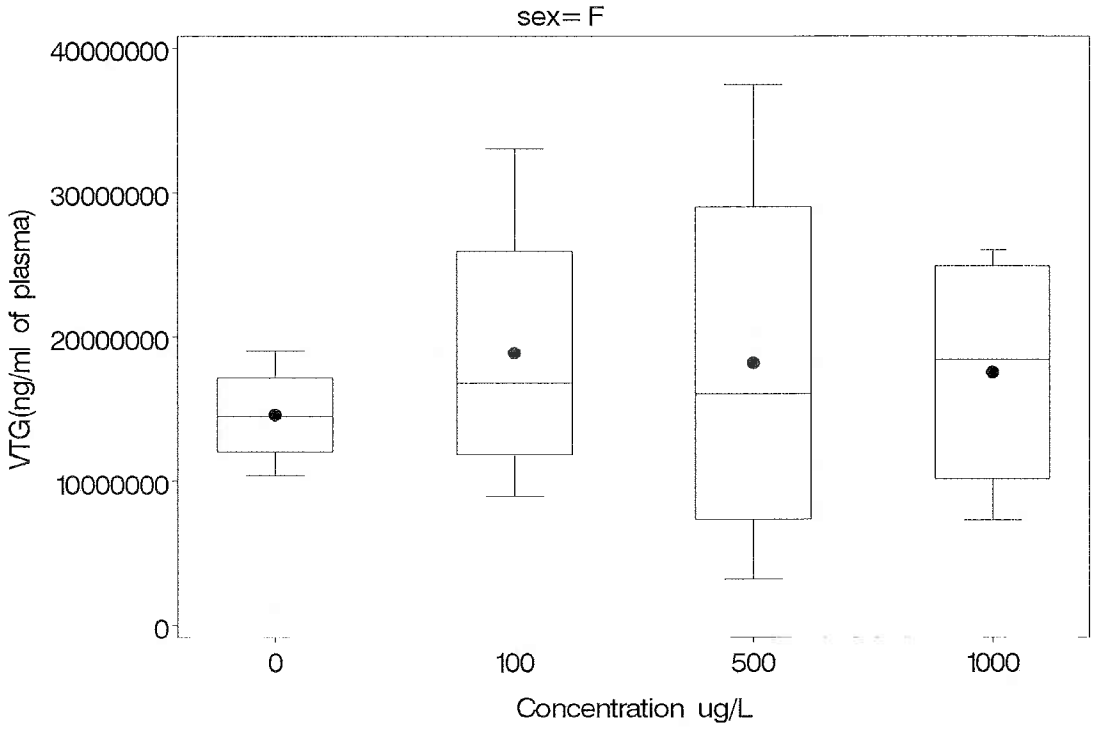


Figure 7. **Box Plot of Female VTG by Treatment for the 21-Day Flutamide Assay** (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

The VTG levels of males used in the 21-day screening assay ranged from 96 to 3.78 x 10^4 ng/ml of plasma. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There were no statistically significant differences in mean VTG among treatments (Dunnett's test $p>0.05$, step-down Jonckheere-Terpstra trend test $p>0.05$, and Kruskal-Wallis test $p>0.05$).

Table 10. Summary Statistics for Male VTG for the 21-Day Flutamide Assay

Level	N	Mean (ng/ml)	SD	CV (%)
CTRL	4	6.27 x 10^3	5.04 x 10^3	80.3
Low	4	3.20 x 10^3	3.06 x 10^3	95.7
Middle	4	7.73 x 10^3	7.27 x 10^3	94.0
High	4	5.68 x 10^3	8.85 x 10^3	156

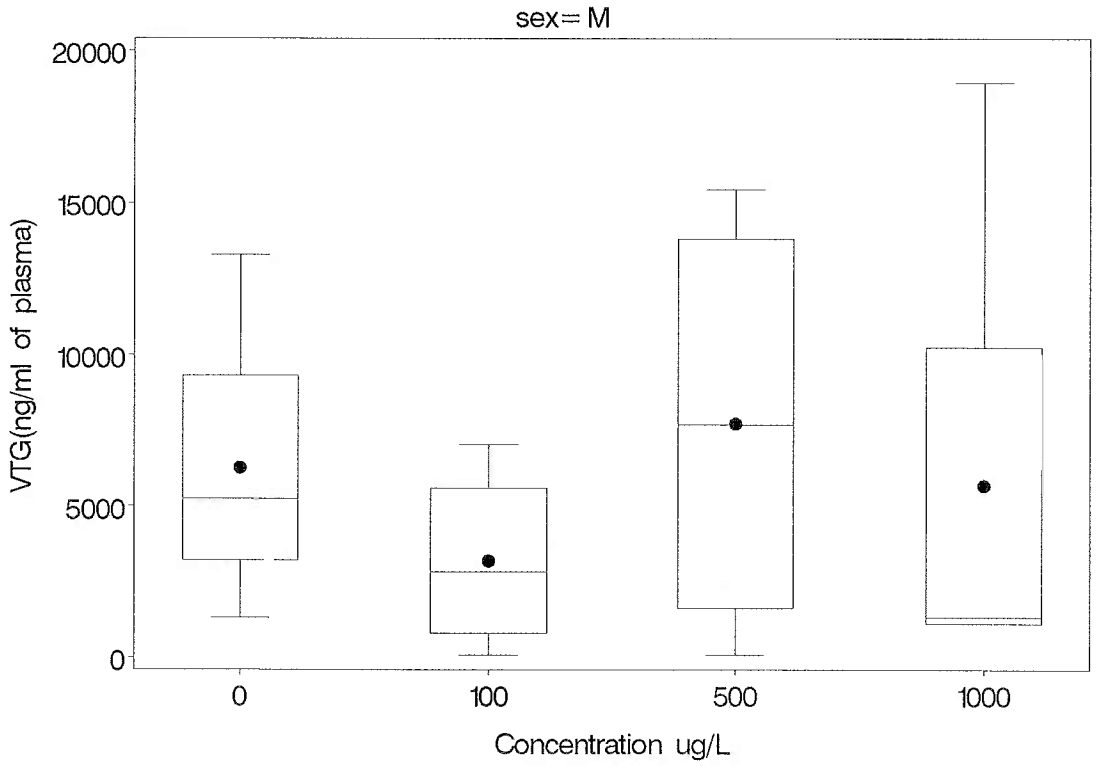


Figure 8. **Box Plot of Male VTG by Treatment for the 21-Day Flutamide Assay** (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

Secondary Sex Characteristics in Males

Several male secondary sex characteristics were evaluated in this study. They included fatpad score, fatpad index, tubercle count, and tubercle score. Fatpad scores in males used in the 21-day screening assay ranged between 1 and 5. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There were no statistically significant differences between the mean fatpad score in controls and any of the treatment groups using the Dunnett's test ($p>0.05$). The 1000 µg/L treatment group was an effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$). There were no statistically significant differences among treatments using and the Kruskal-Wallis test ($p>0.05$).

Table 11. Summary Statistics for Male Fatpad Score for the 21-Day Flutamide Assay

Level	N	Mean Score	SD	CV (%)
CTRL	4	3.13	0.63	20.1
Low	4	3.00	0.41	13.6
Middle	4	2.63	1.03	39.3
High	4	2.38 [1]	0.48	20.2

[1] Statistically significant effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$).

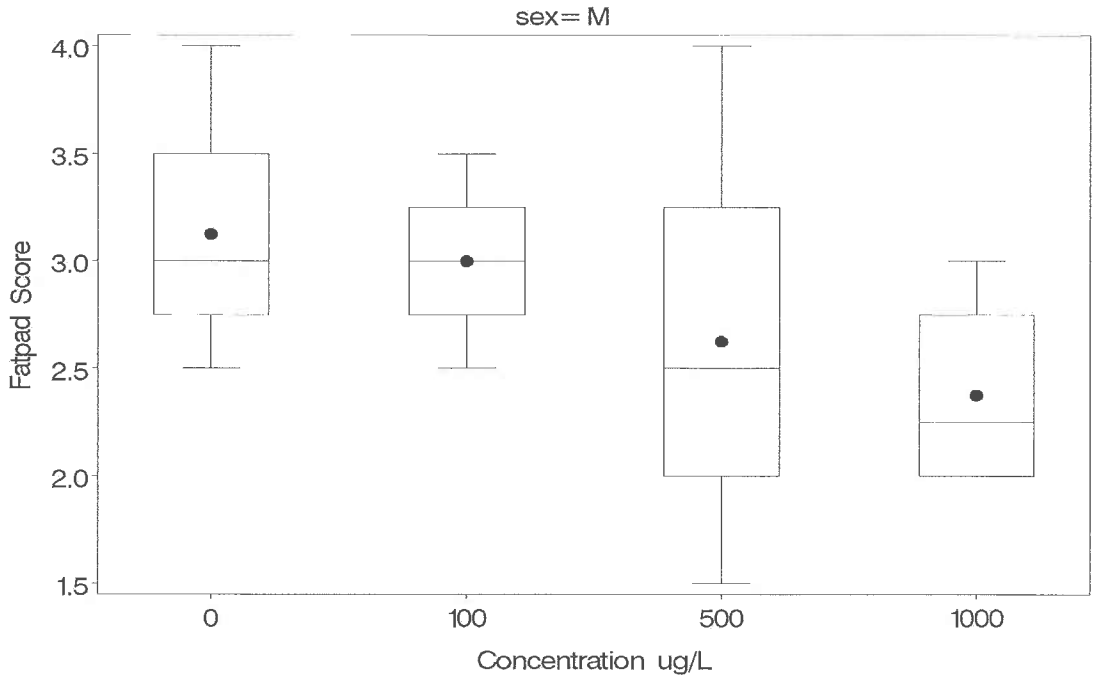


Figure 9. Box Plot of Male Fatpad Score by Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

The fatpad index is the fatpad weight divided by the weight of the fish multiplied by 100. Fatpad index values in males used in the 21-day screening assay ranged between 0 and 7.5. The assumption of the homogeneity of variance (Levene's test $p>0.05$) was met, but the assumption of normality was not met (Shapiro-Wilkes test $p=0.05$). Therefore Dunnett's test ($p<0.05$) was not used to evaluate the data. Only the 1000 µg/L treatment group was an effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$). There were no statistically significant differences among treatments using the Kruskal-Wallis test ($p<0.05$).

Table 12. Summary Statistics for Male Fatpad Index for the 21-Day Flutamide Assay

Level	N	Mean Index	SD	CV (%)
CTRL	4	2.97	0.77	25.9
Low	4	3.24	1.20	37.1
Middle	4	2.87	2.44	85.2
High	4	1.56 [1]	0.833	53.5

[1] Statistically significant effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$).

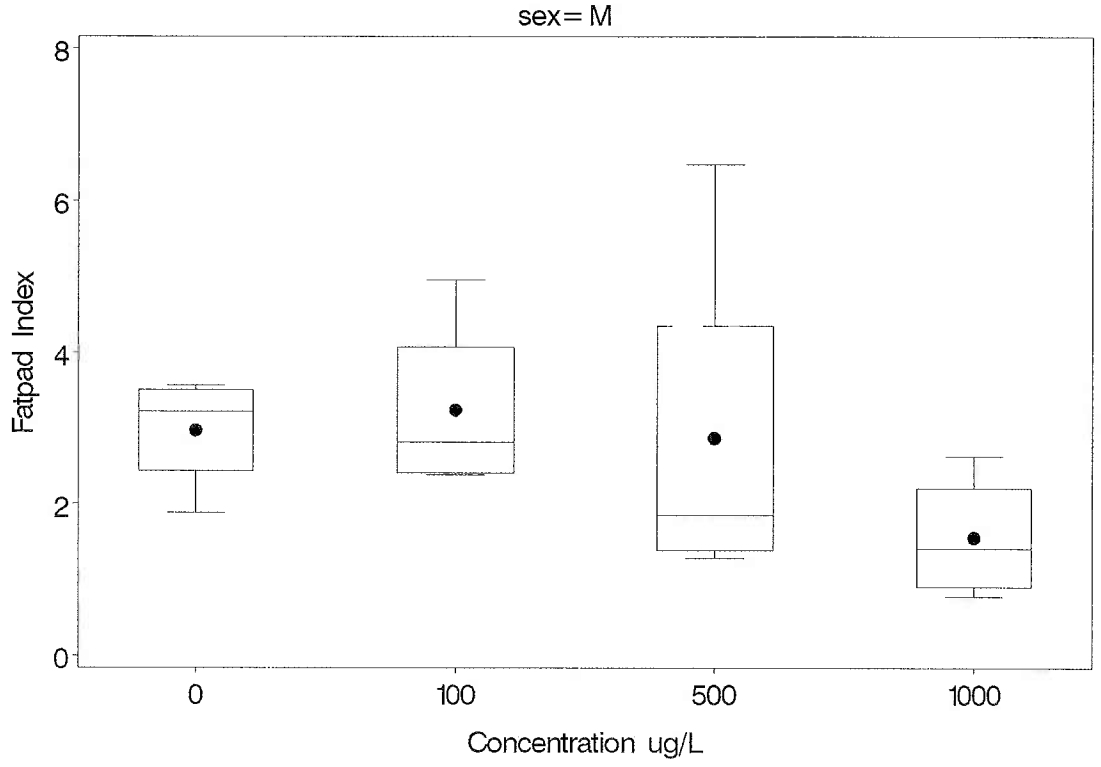


Figure 10. Box Plot of Male Fatpad Index by Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

Tubercle count values in males used in the 21-day screening assay ranged between 0 and 43. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There was a statistically significant difference between the mean tubercle count in controls and the 1000 µg/L treatment group using the Dunnett's test ($p<0.05$). Both the 1000 and 500 µg/L treatment groups were effect levels based on the step-down Jonckheere-Terpstra trend test ($p<0.05$). There were statistically significant differences among treatments using the Kruskal-Wallis test ($p<0.05$).

Table 13. Summary Statistics for Male Tubercle Count for the 21-Day Flutamide Assay

Level	N	Mean Count	SD	CV (%)
CTRL	4	24.6	5.51	22.4
Low	4	22.0	2.12	9.64
Middle	4	18.3 [2]	1.55	8.52
High	4	12.1 [1, 2]	7.17	59.1

[1] Statistically significant difference from the negative control using Dunnett's test ($p < 0.05$)
[2] Statistically significant effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$).

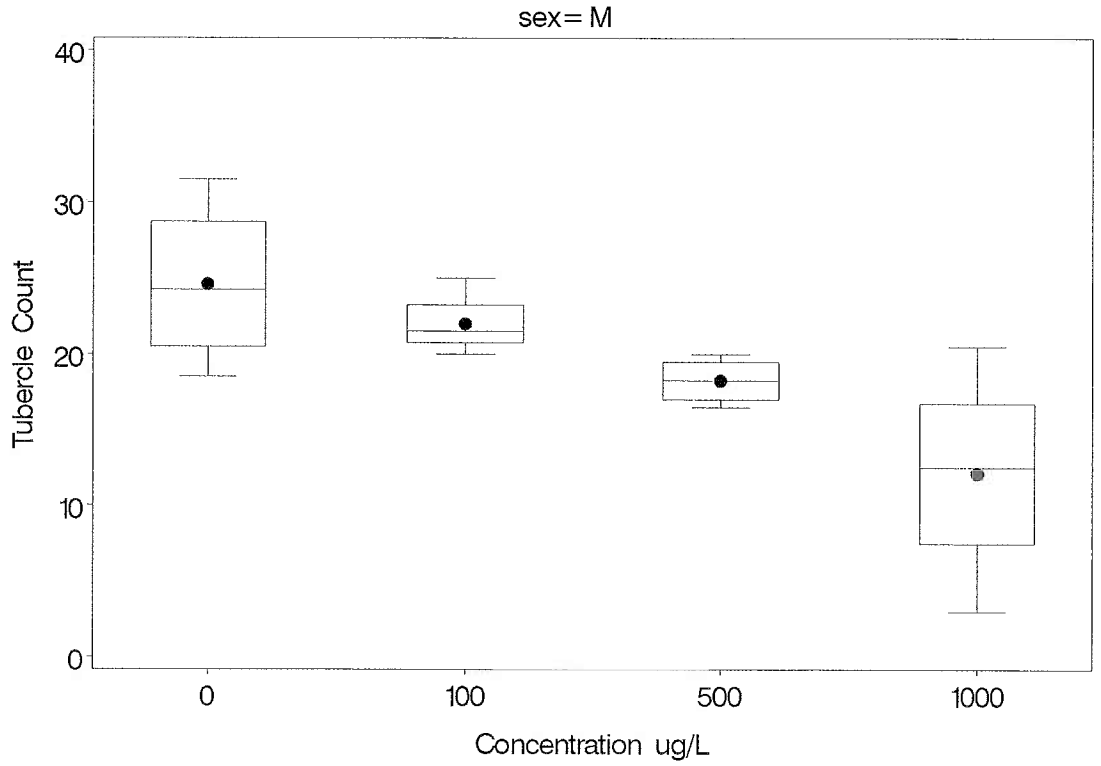


Figure 11. Box Plot of Male Tubercle Count by Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

The tubercle score values in males used in the 21-day screening assay ranged between 0 and 52. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There was a statistically significant difference between the mean tubercle score in controls and the 500 and 1000 µg/L treatment groups using the Dunnett's test ($p<0.05$). All treatment groups (100, 500, and 1000 µg/L) were effect levels based on the step-down Jonckheere-Terpstra trend test ($p<0.05$). There were statistically significant differences among treatments using the Kruskal-Wallis test ($p<0.05$).

Table 14. Summary Statistics for Male Tubercle Score for the 21-Day Flutamide Assay

Level	N	Mean Score	SD	CV (%)
CTRL	4	39.4	6.22	15.8
Low	4	30.3 [2]	4.77	15.8
Middle	4	22.8 [1, 2]	3.97	17.4
High	4	17.6 [1, 2]	10.9	62.0

[1] Statistically significant difference from the negative control using Dunnett's test ($p < 0.05$)
[2] Statistically significant effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$).

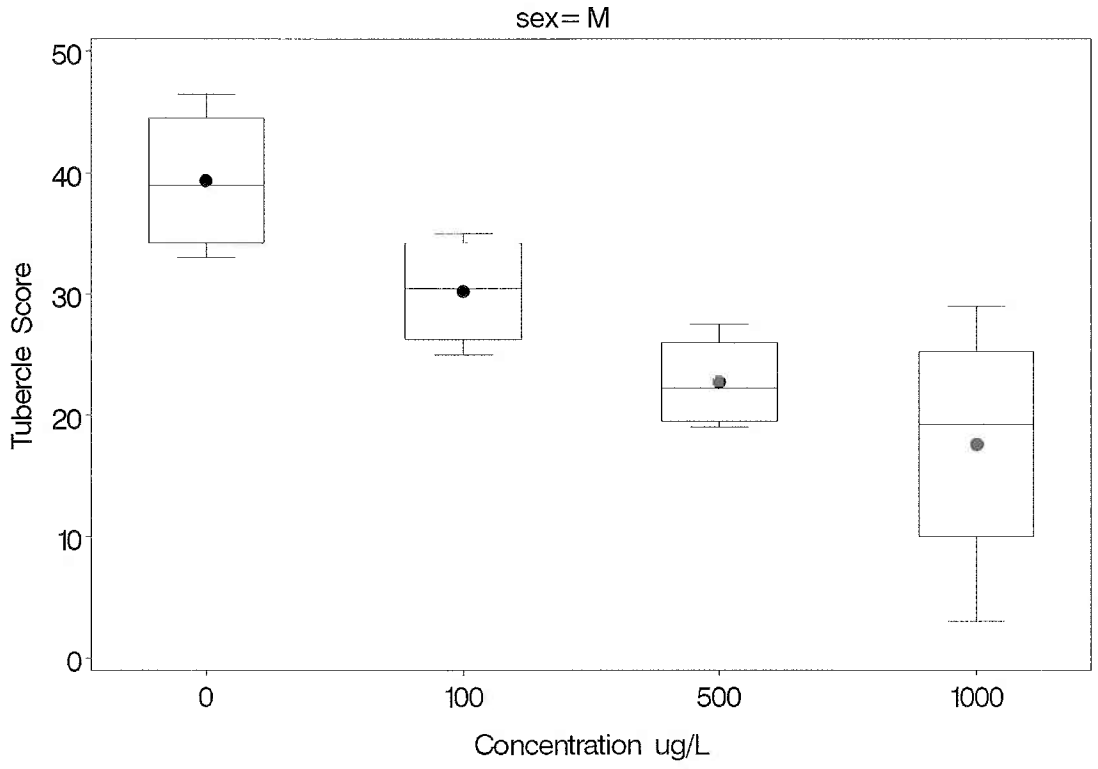


Figure 12. Box Plot of Male Tubercle Score by Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

Reproductive Endpoints

Several reproductive endpoints were evaluated in this study, including fecundity (the mean number of eggs per female per reproductive day) and fertility (the mean number of fertile eggs per female per reproductive day). The number of eggs and the number of fertile eggs for each replicate during the exposure period of the test are presented in Appendices 2.1 and 2.2, respectively. Additionally, the mean number of eggs per treatment, the mean number of fertile eggs per treatment, and the mean number of infertile eggs per treatment were evaluated. Cumulative fecundity is presented below to show relationship of fecundity over time.

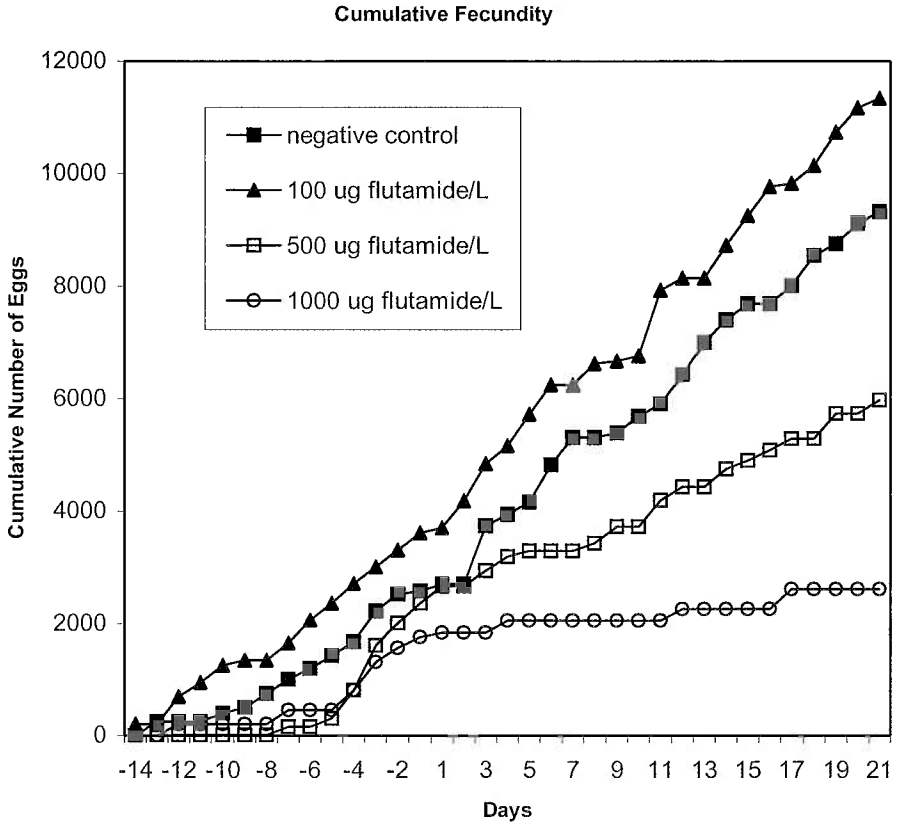


Figure 13. Plot of Cumulative Fecundity by Treatment for the 21-Day Flutamide Assay (Treatment groups diverge at day 0 with the medium and high treatment groups diverging from the control in a dose responsive pattern. The low treatment group is greater than control.)

Cumulative fertility is presented below to show relationship of fertility over time during the exposure period only, since fertility was not monitored during the pre-exposure period.

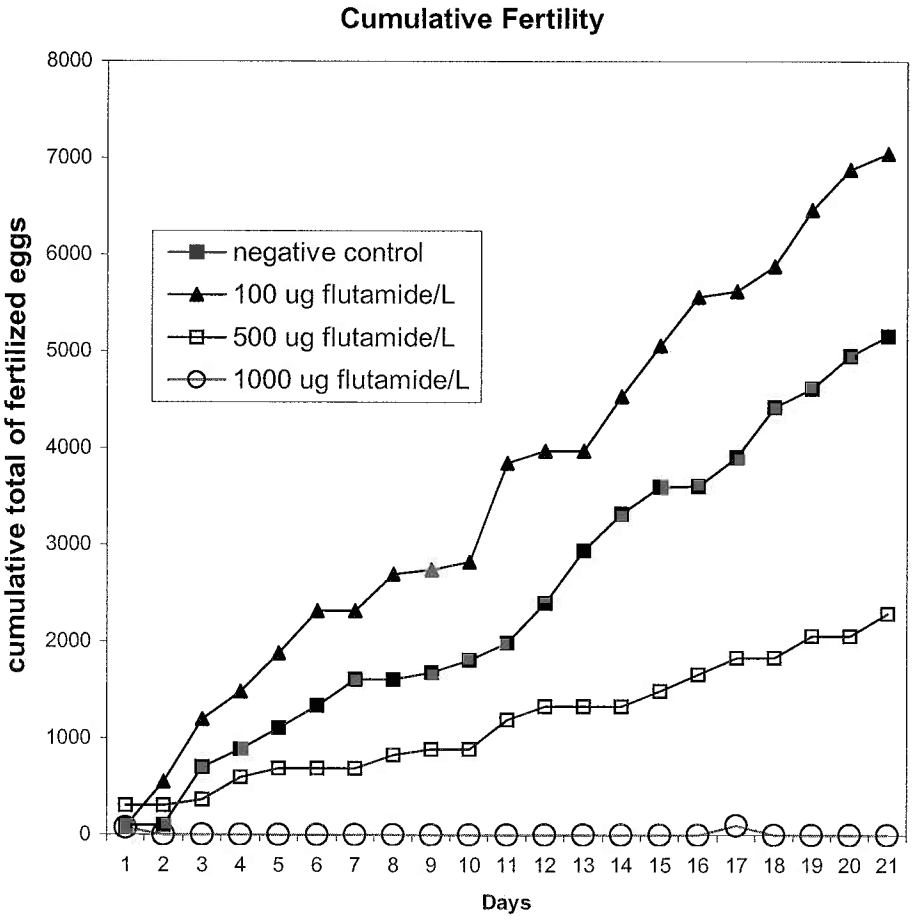


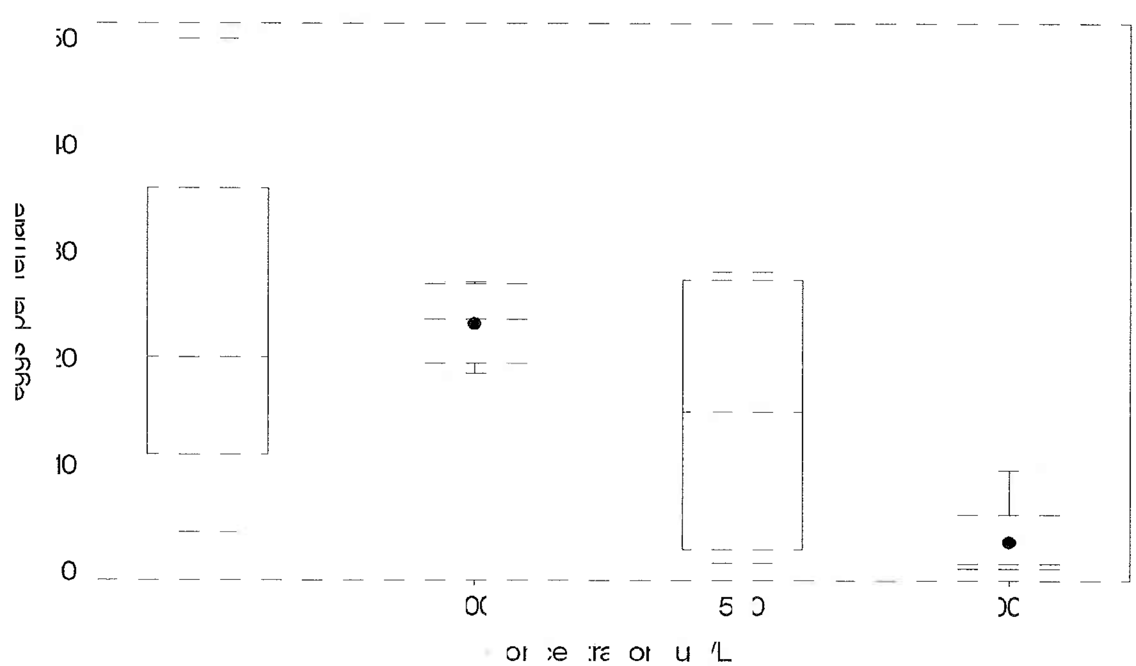
Figure 14. **Plot of Cumulative Fertility by Treatment for the 21-Day Flutamide Assay** (Treatment groups diverge at day 0 with the medium and high treatment groups diverging from the control in a dose responsive pattern. The low treatment group is greater than control.)

ec 1d y, x es :d s e m ei of gg p fi na : r r or lu :iv d. ', ng :d et ee 0 a 1: .3 m ng all e ic: es A u: pt n: of e .o og ne y f v ri: c (L /e :': :e: p:).( ) d n m lit (: a: rc W ke te t p 0 5) we e e T re w e o a ti ll si i: a : (ffe en es b w n e ne n n e of gg p fi na r r pr lu ti d / i c r ls d n: of he re m nt g u u ng hi D m t': :e: (p 0. 5) Tl 1)C g t at e g u wa a: ef ct :v b: ec on e st - w J nc he re e st t n te (:0 5 e v re o ta sti all si ni ca t ffe en es a or t at er s in th K s al- Va is st p:).( ).

T bl 1: S m a y :a sti s 1 r e Ie n u b o E gs e Fe a r e o c ve I y or h 21 D: I ut m e .s y

L ve	I	le n eg s/ m le a	D	V %
C R	[illegible]	.3	9.	8 .5
l w	[illegible]	.3	.3	1 .0
M ld	[illegible]	4.	4.	1 1
I gl	[illegible]	.5	.4	1 7

[1] at ti ll si if a e ec le l s c th s p- ov 1 J nc hc re e st t n te: (:0 5)



F u : 1 . 3o P t f t e le 1 ur be o E s er e a p 1 ep oc ct e a b r at e f t e - ay h a id A sa (t x p: se ts e t qu rt: r g v sk s pi se tl c ta n e, e or o al n is e e ar va e, he ir e he ne n alt , d e st is r or er p si e ut er .

Fertility expressed as the number of fertile eggs per female per reproductive day ranged between 0 and 29.3 among all replicates. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There was a statistically significant difference between the mean number of fertile eggs per female per reproductive day in controls and the 1000 µg/L treatment group using the Dunnett's test ($p<0.05$). The 1000 µg/L treatment group also was an effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$). There were statistically significant differences among treatments using the Kruskal-Wallis test ($p<0.05$).

Table 16. Summary Statistics for the Mean Number of Fertile Eggs per Female per Reproductive Day for the 21-Day Flutamide Assay

Level	N	Mean (Fertile Eggs/Female/Day)	SD	CV (%)
CTRL	4	17.2	10.7	62.4
Low	4	21.0	4.40	21.0
Middle	4	7.3	10.1	151
High	4	0.5 [1, 2]	0.62	118

[1] Statistically significant difference from the negative control using Dunnett's test ($p < 0.05$)
[2] Statistically significant effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$).

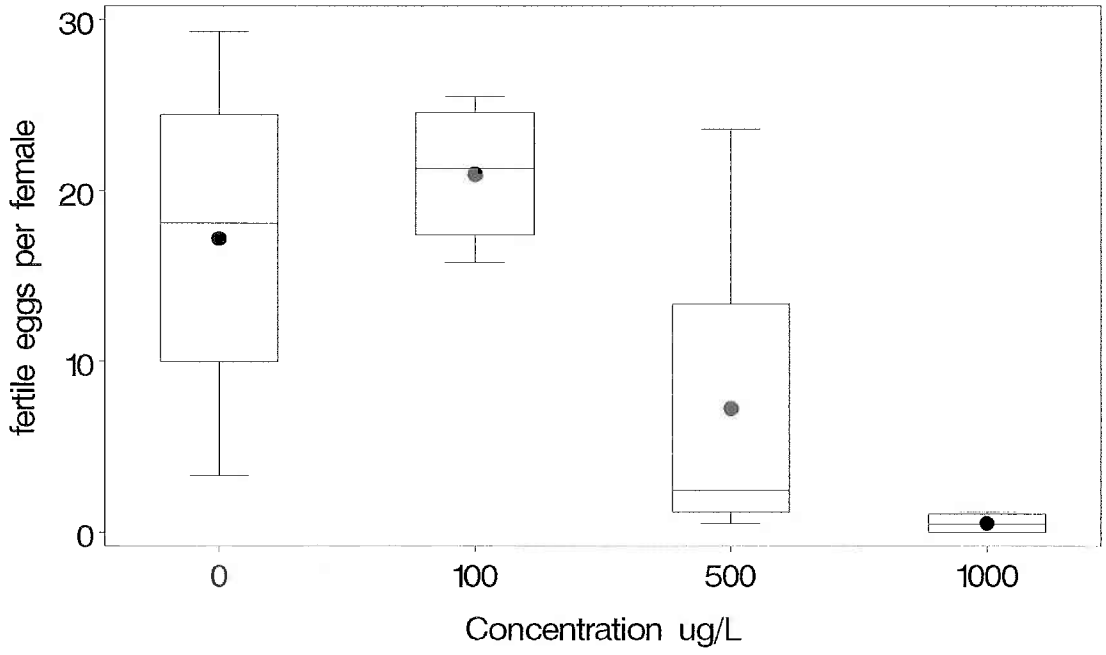


Figure 16. Box Plot of the Mean Number of Fertile Eggs per Female per Reproductive Day by Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

The number of eggs per treatment group ranged between 0 and 3133 among all replicates. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There were no statistically significant differences between the mean number of eggs per treatment in controls and any of the treatment groups using the Dunnett's test ($p>0.05$). The 1000 µg/L treatment group was an effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$). There were statistically significant differences among treatments using the Kruskal-Wallis test ($p<0.05$).

Table 17. Summary Statistics for the Mean Number of Eggs per Treatment for the 21-Day Flutamide Assay

Level	N	Mean (Eggs/Treatment)	SD	CV (%)
CTRL	4	1687	1169.6	69.3
Low	4	1931	368.4	19.1
Middle	4	905	1000.9	111
High	4	212 [1]	375.0	177

[1] Statistically significant effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$).

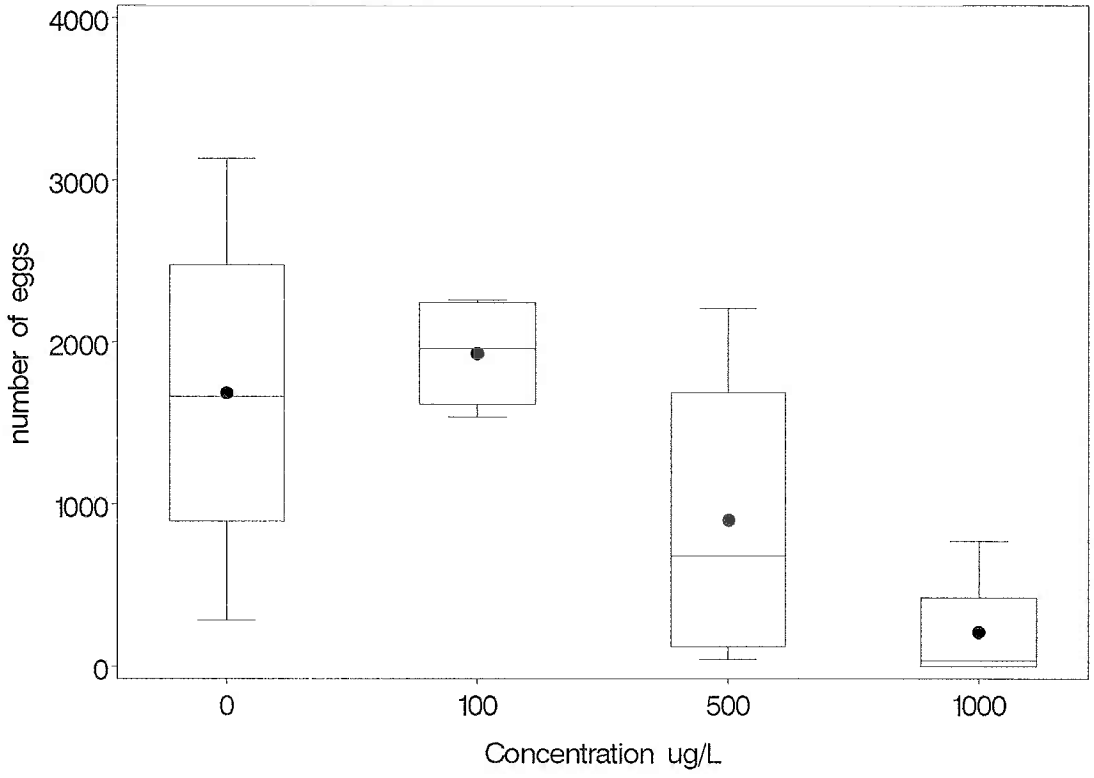


Figure 17. Box Plot of the Mean Number of Eggs per Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

The number of fertile eggs per treatment group ranged between 0 and 2140 between all replicates. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There was a statistically significant difference between the mean number of fertile eggs in controls and the 1000 µg/L treatment group using the Dunnett's test ($p<0.05$). The 1000 µg/L treatment group also was an effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$). There were statistically significant differences among treatments using the Kruskal-Wallis test ($p<0.05$).

Table 18. Summary Statistics for the Mean Number of Fertile Eggs per Treatment for the 21-Day Flutamide Assay

Level	N	Mean (Fertile Eggs/Treatment)	SD	CV (%)
CTRL	4	1290	702.1	54.5
Low	4	1761	369.8	21.0
Middle	4	573	941.6	164
High	4	44 [1, 2]	51.5	118

[1] Statistically significant difference from the negative control using Dunnett's test ($p < 0.05$)
[2] Statistically significant effect level based on the step-down Jonckheere-Terpstra trend test ($p<0.05$).

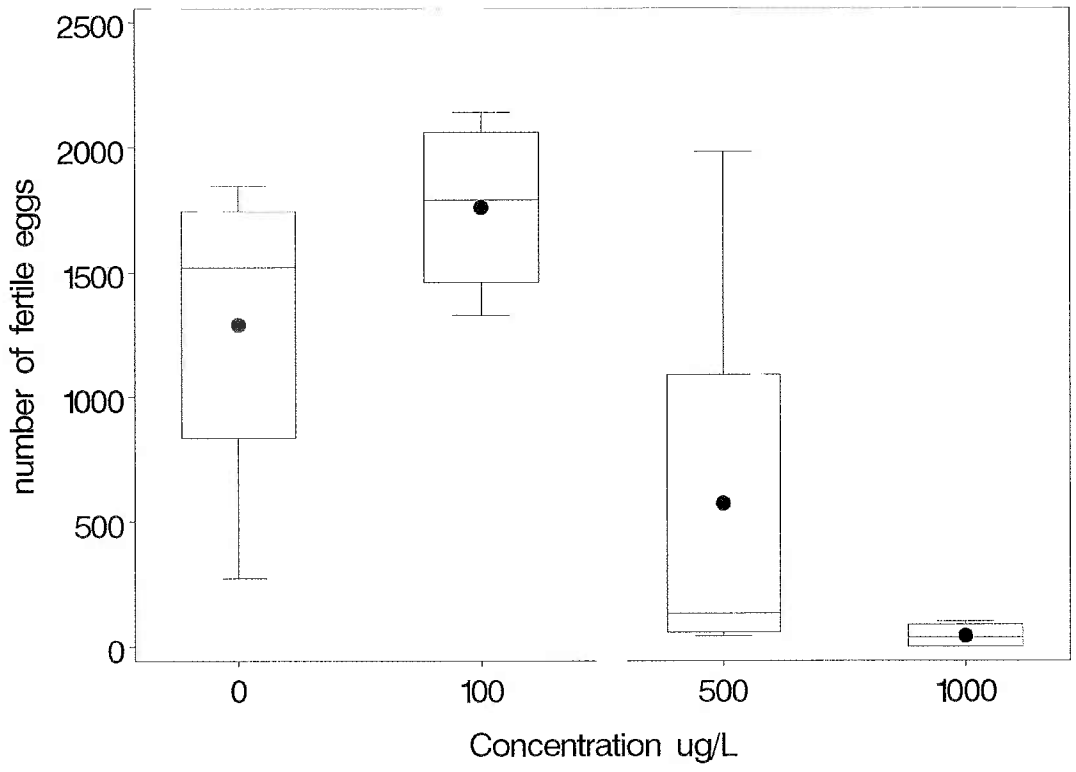


Figure 18. Box Plot of the Mean Number of Fertile Eggs per Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

The mean number of infertile eggs per treatment group ranged between 0 and 1288 among all replicates. Assumptions of the homogeneity of variance (Levene's test $p>0.05$) and normality (Shapiro-Wilkes test $p>0.05$) were met. There were no statistically significant differences in mean number of infertile eggs among treatments (Dunnett's test $p>0.05$, step-down Jonckheere-Terpstra trend test $p>0.05$, and Kruskal-Wallis test $p>0.05$).

Table 19. Summary Statistics for the Mean Number of Infertile Eggs per Treatment for the 21-Day Flutamide Assay

Level	N	Mean (Infertile Eggs/Treatment)	SD	CV (%)
CTRL	4	398	597.3	150
Low	4	170	91.0	53.4
Middle	4	331	518.4	156
High	4	168	335.0	199

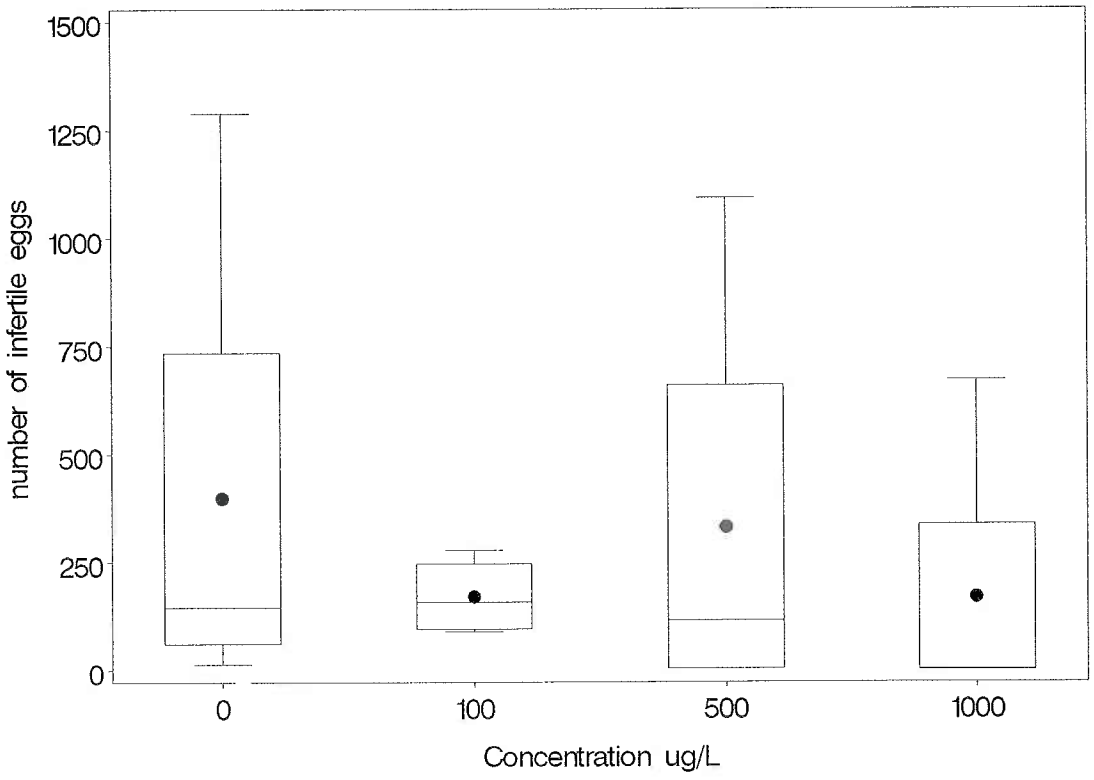


Figure 19. Box Plot of the Mean Number of Infertile Eggs per Treatment for the 21-Day Flutamide Assay (box represents the interquartile range, whiskers represent the data range, the horizontal line is the median value, the circle is the mean value, and the asterisks represent possible outliers)

Evaluation of the Variability in Endpoints

The variability of study endpoints were evaluated by calculating the mean of the percent coefficient of variation of all treatments. The control group is reported but not used in the calculation of the mean. Endpoints have been ranked from high to low in the table below.

Table 20. Coefficient of Variance (% CV) Comparison for the Endpoints Determined During the 21-Day Exposure of Fathead Minnow (*Pimephales promelas*) to Flutamide.

Endpoint	% CV Control	% CV Low Treatment	% CV Medium Treatment	% CV High Treatment	% CV Mean of all Treatments
Male Length	3.55	2.99	2.31	3.31	**2.87**
Female Length	1.45	2.57	3.06	3.64	**3.09**
Male Weight	3.54	4.12	3.56	12.0	**6.56**
Female Weight	5.71	5.01	6.6	14.66	**8.76**
Male GSI	19.3	8.4	14.1	17.2	**13.2**
Female GSI	11.7	12.4	30.2	14.6	**19.1**
Male Fatpad Score	20.1	13.6	39.3	20.2	**24.4**
Male Tubercle Count	22.4	9.62	8.52	59.1	**25.7**
Male Tubercle Score	15.81	15.77	17.44	62.0	**31.7**
Male Fatpad Index	25.9	37.1	85.2	53.5	**58.6**
Female VTG	24.5	54.3	80.7	50.7	**61.9**
Fertile Eggs per Female	62.4	21.0	151	118	**96.7**
Eggs per Female	83.5	19.0	101	177	**99.0**
Number of Fertile Eggs	54.4	21.0	164	118	**101**
Number of Eggs	69.3	19.1	111	177	**102**
Male VTG	80.3	95.7	94.0	155	**115**
Number of Infertile Eggs	150	53.4	156	199	**136**

Evaluating Estimation versus Actual Counts of Egg Data

During the exposure period, both estimates and actual counts of eggs were recorded. Since the ability to estimate is not a function of concentration, all data were pooled in order to compare estimated values to actual values. The numbers of eggs both estimated and counted were plotted against the actual counts. Many of the estimates fell below the actual counts. As the figure below shows, there was a tendency to underestimate actual counts with the error rate increasing with larger numbers of eggs. The estimated counts differed quite a bit from the actual counts; sometimes estimates were lower than 50 percent of the actual count. Therefore estimates could be misleading.

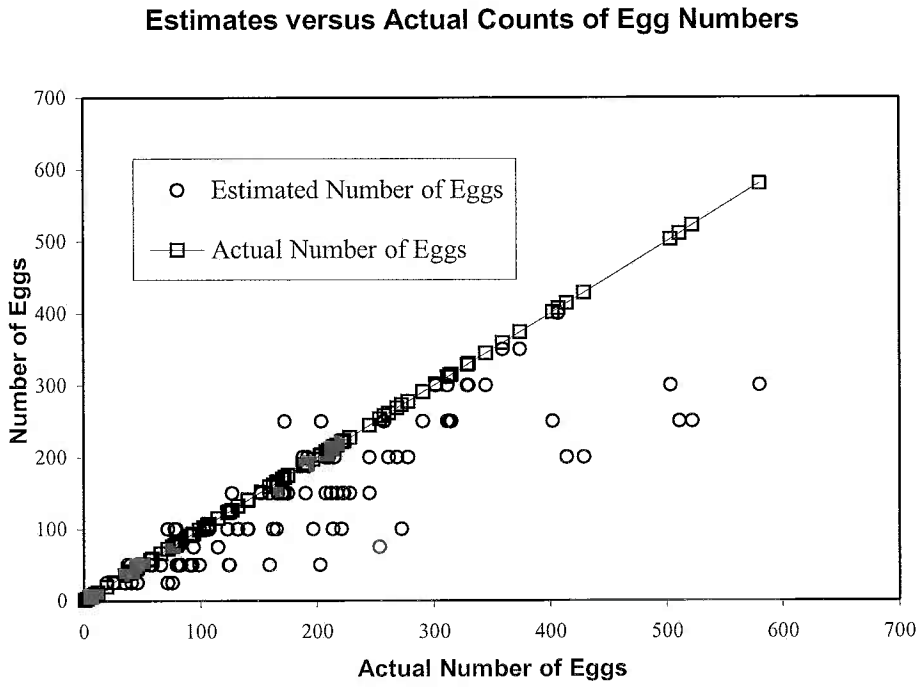


Figure 20. **Plot of the Number of Eggs Estimated and Actually Counted versus the Actual Egg Counts for the 21-Day Flutamide Assay**

Pathology and Histology Report of Gonadal Tissues

Gonadal tissues were examined at Experimental Pathology Laboratories, Inc. (EPL). Their report is presented in Appendix 4. Results for males showed an increase in the testicular stage score in the 1000 µg/L treatment group relative to the controls. A slight increase in the incidence and severity of "increased cells, spermatogonia" was also observed in the 500 and 1000 µg/L treatment groups relative to the controls. Increased spermatogonia and increased testicular stage score were also observed in fathead minnows exposed to flutamide in the OECD Phase 1B validation.

Results for females showed an increase in both the incidence and severity of oocyte atresia in the 1000 µg/L treatment group relative to the controls. Microsporidia were also observed in some females in the controls and 500 and 1000 µg/L treatment groups. The presence of microsporidia was not considered to be a confounding factor. Increased oocyte atresia of the ovaries was also reported in one of the studies for flutamide in the OECD Phase 1B validation.

CONCLUSIONS

Reproductive groups of fathead minnows (4 females and 2 males) were exposed to three flutamide concentrations (100, 500 and 1000 µg/L) and a negative control. There were no apparent effects on female length, weight, GSI, or vitellogenin observed in any of the test concentrations. There were apparent treatment-related effects on fertility and fecundity at the 1000 µg/L test concentration, the highest concentration tested, that were statistically significant. The effects observed were consistent with the pathology report, which indicated an increase in both the incidence and severity of oocyte atresia in the 1000 µg/L treatment group relative to the controls. In males, there were no apparent effects on length or vitellogenin. There were apparent treatment-related effects on male weight, GSI, and secondary sex characteristics (fatpad score, fatpad index, tubercle count, and tubercle score) at the 1000 µg/L test concentration, the highest concentration tested, that were statistically significant. Effects on weight, tubercle count and score, and GSI were also apparent and statistically significant at 500 µg/L, the middle test concentration. Only effects on tubercle score were apparent at the 100 µg/L test concentration, the lowest concentration tested. The effects observed were consistent with the pathology report, which indicated an increase in spermatogonia in both the 500 and 1000 µg/L treatment groups and an increase in testicular stage score in the 1000 µg/L treatment group relative to the controls.

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2 **APHA, AWWA, WPCF.** 1985. *Standard Methods for the Examination of Water and Wastewater.* 16th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

3 **United States Environmental Protection Agency.** 2002. A Short-term Test Method For Assessing Reproductive Toxicity Of Endocrine Disrupting Chemicals Using The Fathead Minnow (*Pimephales Promelas*). EPA 600/R-01/067.

4 **West, Inc. and D.D. Gulley.** 1996. TOXSTAT Release 3.5. Western EcoSystems Technology, Inc. Cheyenne, Wyoming.

5 **SAS System for Windows.** 2001. Version 8.2. SAS Institute, Inc. Cary, North Carolina.

Appendix 1

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]

Pesticides and Organics			
Component	Measured Concentration (µg/L)	Component	Measured Concentration (µg/L)
Aldrin	< 0.0099	Heptachlor Epoxide	< 0.0099
Alpha BHC	< 0.0099	Malathion	< 2.0
Beta BHC	< 0.040	Merphos	< 2.0
Bolstar	< 2.0	Methoxychlor	< 0.099
Chlordane	< 0.50	Methyl Parathion	< 2.0
Coumaphos	< 3.0	Mevinphos	< 2.0
Delta BHC	< 0.0099	Mirex	< 0.050
Demeton-O	< 2.0	Naled	< 3.0
Demeton-S	< 2.0	o,p-DDD	< 0.020
Diazinon	< 2.0	o,p-DDE	< 0.020
Dichlorvos	< 2.0	o,p-DDT	< 0.020
Dieldrin	< 0.020	p,p-DDD	< 0.020
Disulfoton	< 2.0	p,p-DDE	< 0.020
Dursban (Chlorpyrifos)	< 2.0	p,p-DDT	< 0.025
Endosulfan I	< 0.0099	PCB-1016	< 0.50
Endosulfan II	< 0.042	PCB-1221	< 1.2
Endosulfan Sulfate	< 0.020	PCB-1232	< 0.89
Endrin	< 0.020	PCB-1242	< 0.50
EPN	< 4.0	PCB-1248	< 0.50
Ethion	< 2.0	PCB-1254	< 0.50
Ethoprop	< 2.0	PCB-1260	< 0.50
Ethyl Parathion	< 2.0	Phorate	< 2.0
Famphur	< 2.0	Ronnel	< 2.0
Fensulfothion	< 4.0	Stirophos	< 2.0
Fenthion	< 2.0	Telodrin	< 0.0099
Gamma BHC – Lindane	< 0.0099	Tokuthion	< 2.0
Guthion (Azinphos-methyl)	< 4.0	Toxaphene	< 0.99
HCB	< 0.099	Trichloronate	< 2.0
Heptachlor	< 0.0099	Trithion	< 2.0

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2004.

Appendix 1 (Continued)

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]

Metals			
Component	Measured Concentration (mg/L)	Component	Measured Concentration (mg/L)
Aluminum	< 0.200	Magnesium	12.7
Antimony	< 0.0200	Manganese	< 0.0050
Arsenic	< 0.0100	Mercury	< 0.00020
Barium	< 0.0050	Nickel	< 0.0100
Beryllium	< 0.0050	Nitrate Nitrogen	< 0.50
Bromide	< 2.5	Nitrite Nitrogen	< 0.50
Cadmium	< 0.0050	Potassium	6.64
Calcium	31.1	Selenium	< 0.0100
Chloride	6.9	Silver	< 0.0050
Chromium	< 0.0050	Sodium	19.7
Cobalt	< 0.0050	Sulfate	5.5
Copper	< 0.0100	Thallium	< 0.0200
Fluoride	< 0.50	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0200		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2004.

Appendix 2.1

Number of Eggs Counted

	Negative Control															
	Replicate A				Replicate B				Replicate C				Replicate D			
Day	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total
1	--	--	132	132	--	--	--	0	--	--	--	0	--	--	--	0
2	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
3	--	--	522	522	--	511	--	511	--	--	--	0	--	--	--	0
4	--	--	--	0	--	1	--	1	--	--	162	162	41	--	--	41
5	--	--	--	0	--	--	--	0	--	--	222	222	--	--	--	0
6	--	--	503	503	--	79	--	79	38	36	--	74	--	--	--	0
7	--	--	269	269	--	228	--	228	--	--	--	0	--	--	--	0
8	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
9	--	--	--	0	--	78	--	78	--	--	--	0	--	--	--	0
10	--	291	--	291	--	--	--	0	--	--	--	0	--	--	--	0
11	--	--	--	0	--	--	--	0	--	1	217	218	--	--	--	0
12	--	107	51	158	--	--	--	0	--	8	359	367	--	--	--	0
13	--	312	--	312	--	--	172	172	--	26	59	85	--	--	--	0
14	--	--	--	0	--	407	--	407	--	--	--	0	--	--	--	0
15	--	278	--	278	--		--	0	--	--	--	0	--	--	--	0
16	--	--	--	0	--	5	--	5	--	--	--	0	--	--	--	0
17	--	--	--	0	--	40	--	40	--	172	--	172	107	--	--	107
18	--	330	80	410	--	--	--	0	--	--	--	0	123	--	--	123
19	--	--	--	0	--	--	--	0	--	--	204	204	--	--	--	0
20	--	258	--	258	--	91	--	91	--	--	--	0	--	6	10	16
21	--	--	--	0	214	--	--	214	--	--	--	0	--	--	--	0
Total:	0	1576	1557	3133	214	1440	172	1826	38	243	1223	1504	271	6	10	287

Appendix 2.1 (Continued)

Number of Eggs Counted

	100 µg/L															
	Replicate A				Replicate B				Replicate C				Replicate D			
Day	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total
1	--	--	--	0	94	--	--	94	--	--	--	0	--	--	--	0
2	46	--	--	46	--	--	--	0	--	--	429	429	--	--	--	0
3	125	--	--	125	124	--	--	124	--	--	--	0	414	--	--	414
4	--	--	--	0	57	--	--	57	262	--	--	262	--	--	--	0
5	--	--	165	165	--	190	--	190	--	--	--	0	99	--	106	205
6	--	--	--	0	--	--	--	0	--	315	--	315	210	--	--	210
7	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
8	--	212	--	212	--	170	--	170	--	--	--	0	--	--	--	0
9	--	--	--	0	--	20	--	20	--	--	--	0	26	--	--	26
10	--	--	--	0	--	--	--	0	--	--	--	0	--	83	--	83
11	3	--	--	3	--	402	--	402	--	580	--	580	6	175	--	181
12	--	--	--	0	--	215	--	215	--	--	--	0	--	--	--	0
13	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
14	--	82	--	82	--	--	--	0	175	--	--	175	329	--	--	329
15	--	254	--	254	203	--	--	203	--	--	--	0	72	--	--	72
16	--	127	--	127	374	--	--	374	--	7	--	7	--	--	--	0
17	--	--	--	0	--	--	--	0	--	--	57	57	--	--	--	0
18	--	223	--	223	--	--	--	0	93	--	--	93	--	--	--	0
19	302	--	--	302	--	--	159	159	--	--	--	0	140	--	--	140
20	--	--	--	0	188	--	--	188	--	208	--	208	36	--	--	36
21	--	--	--	0	--	--	66	66	103	--	--	103	--	--	--	0
			--													
Total:	476	898	165	1539	1040	997	225	2262	633	1110	486	2229	1332	258	106	1696

Appendix 2.1 (Continued)

Number of Eggs Counted

	500 µg/L															
	Replicate A				Replicate B				Replicate C				Replicate D			
Day	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total
1	--	197	--	197	115	--	--	115	--	--	--	0	--	--	--	0
2	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
3	--	--	--	0	--	--	--	0	273	--	--	273	--	--	--	0
4	--	--	--	0	--	245	--	245	--	--	--	0	--	--	--	0
5	--	--	--	0	--	103	--	103	--	--	--	0	--	--	--	0
6	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
7	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
8	--	--	--	0	--	141	--	141	--	--	--	0	--	--	--	0
9	--	--	--	0	72	--	--	72	--	--	222	222	--	--	--	0
10	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
11	--	--	--	0	314	--	--	314	152	--	--	152	--	--	--	0
12	--	--	--	0	245	--	--	245	--	--	--	0	--	--	--	0
13	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
14	--	--	--	0	--	--	--	0	312	--	--	312	--	--	--	0
15	--	--	--	0	159	--	--	159	--	--	--	0	--	--	--	0
16	--	--	--	0	172	--	--	172	--	--	--	0	--	--	--	0
17	--	--	--	0	--	210	--	210	--	--	--	0	--	--	--	0
18	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
19	--	--	--	0	192	--	--	192	--	--	208	208	45	--	--	45
20	--	--	--	0	--	--	--	0	--	--	--	0	1	--	--	1
21	--	--	--	0	12	228	--	240	--	--	--	0	--	--	--	0
Total:	0	197	0	197	1281	927	0	2208	737	0	430	1167	46	0	0	46

Appendix 2.1 (Continued)

Number of Eggs Counted

	1000 µg/L															
	Replicate A				Replicate B				Replicate C				Replicate D			
Day	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total
1	--	--	--	0	--	--	--	0	--	--	--	0	76	--	--	76
2	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
3	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
4	--	--	221	221	--	--	--	0	--	--	--	0	--	--	--	0
5	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
6	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
7	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
8	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
9	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
10	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
11	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
12	--	40	166	206	--	--	--	0	--	--	--	0	--	--	--	0
13	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
14	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
15	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
16	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
17	--	--	345	345	--	--	--	0	--	--	--	0	--	--	--	0
18	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
19	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
20	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
21	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
Total:	0	40	732	772	0	0	0	0	0	0	0	0	76	0	0	76

Appendix 2.2

Number of Fertilized Embryos

	Negative Control															
	Replicate A				Replicate B				Replicate C				Replicate D			
Day	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total
1	--	--	98	98	--	--	--	0	--	--	--	0	--	--	--	0
2	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
3	--	--	143	143	--	460	--	460	--	--	--	0	--	--	--	0
4	--	--	--	0	--	1	--	1	--	--	152	152	37	--	--	37
5	--	--	--	0	--	--	--	0	--	--	219	219	--	--	--	0
6	--	--	82	82	--	72	--	72	37	35	--	72	--	--	--	0
7	--	--	50	50	--	225	--	225	--	--	--	0	--	--	--	0
8	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
9	--	75	--	75	--	--	--	0	--	--	--	0	--	--	--	0
10	--	121	--	121	--	--	--	0	--	--	--	0	--	--	--	0
11	--	--	--	0	--	--	--	0	--	1	181	182	--	--	--	0
12	--	29	38	67	--	--	--	0	--	8	333	341	--	--	--	0
13	--	301	--	301	--	--	168	168	--	25	55	80	--	--	--	0
14	--	--	--	0	--	377	--	377	--	--	--	0	--	--	--	0
15	--	275	--	275	--	--	--	0	--	--	--	0	--	--	--	0
16	--	--	--	0	--	5	--	5	--	--	--	0	--	--	--	0
17	--	--	--	0	--	37	--	37	--	161	--	161	104	--	--	104
18	--	321	77	398	--	--	--	0	--	--	--	0	119	--	--	119
19	--	--	--	0	--	--	--	0	--	--	189	189	--	--	--	0
20	--	235	--	235	--	90	--	90	--	--	--	0	--	4	9	13
21	--	--	--	0	209	--	--	209	--	--	--	0	--	--	--	0
Total:	0	1357	488	1845	209	1267	168	1644	37	230	1129	1396	260	4	9	273

Appendix 2.2 (Continued)

Number of Fertilized Embryos

	100 µg/L															
	Replicate A				Replicate B				Replicate C				Replicate D			
Day	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total
1	--	--	--	0	87	--	--	87	--	--	--	0	--	--	--	0
2	43	--	--	43	--	--	--	0	--	--	423	423	--	--	--	0
3	124	--	--	124	114	--	--	114	--	--	--	0	408	--	--	408
4	--	--	--	0	46	--	--	46	238	--	--	238	--	--	--	0
5	--	--	25	25	--	178	--	178	--	--	--	0	97	--	101	198
6	--	--	--	0	--	--	--	0	--	279	--	279	158	--	--	158
7	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
8	--	210	--	210	--	169	--	169	--	--	--	0	--	--	--	0
9	--	--	--	0	--	18	--	18	--	--	--	0	23		--	23
10	--	--	--	0	--	--	--	0	--	--	--	0	--	80	--	80
11	3	--	--	3	--	398	--	398	--	456	--	456	6	162	--	168
12	--	--	--	0	--	--	--	0	--	121	--	121	--	--	--	0
13	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
14	--	76	--	76	--	--	--	0	169	--	--	169	322	--	--	322
15	--	253	--	253	201	--	--	201	--	--	--	0	71	--	--	71
16	--	126	--	126	371	--	--	371	--	7	--	7	--	--	--	0
17	--	--	--	0	--	--	--	0	--	--	55	55	--	--	--	0
18	--	171	--	171	--	--	--	0	90	--	--	90	--	--	--	0
19	295	--	--	295	--	--	152	152	--	--	--	0	137	--	--	137
20	--	--	--	0	187	--	--	187	--	203	--	203	30	--	--	30
21	--	--	--	0	--	--	63	63	99	--	--	99	--	--	--	0
Total:	465	836	25	1326	1006	763	215	1984	596	1066	478	2140	1252	242	101	1595

Appendix 2.2 (Continued)

Number of Fertilized Embryos

	500 µg/L															
	Replicate A				Replicate B				Replicate C				Replicate D			
Day	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total
1	--	194	--	194	109	--	--	109	--	--	--	0	--	--	--	0
2	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
3	--	--	--	0	--	--	--	0	62	--	--	62	--	--	--	0
4	--	--	--	0	--	230	--	230	--	--	--	0	--	--	--	0
5	--	--	--	0	--	97	--	97	--	--	--	0	--	--	--	0
6	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
7	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
8	--	--	--	0	--	138	--	138	--	--	--	0	--	--	--	0
9	--	--	--	0	57	--	--	57	--	--	2	2	--	--	--	0
10	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
11	--	--	--	0	298	--	--	298	9	--	--	9	--	--	--	0
12	--	--	--	0	138	--	--	138	--	--	--	0	--	--	--	0
13	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
14	--	--	--	0	--	--	--	0	1	--	--	1	--	--	--	0
15	--	--	--	0	158	--	--	158	--	--	--	0	--	--	--	0
16	--	--	--	0	170	--	--	170	--	--	--	0	--	--	--	0
17	--	--	--	0	--	170	--	170	--	--	--	0	--	--	--	0
18	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
19	--	--	--	0	184	--	--	184	--	--	0	0	42	--	--	42
20	--	--	--	0	--	--	--	0	--	--	--	0	0	--	--	0
21	--	--	--	0	12	221	--	233	--	--	--	0	--	--	--	0
Total:	0	194	0	194	1126	856	0	1982	72	0	2	74	42	0	0	42

Appendix 2.2 (Continued)

Number of Fertilized Embryos

	1000 µg/L															
	Replicate A				Replicate B				Replicate C				Replicate D			
Day	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total	Tile 1	Tile 2	Tile 3	Total
1	--	--	--	0	--	--	--	0	--	--	--	0	73	--	--	73
2	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
3	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
4	--	--	1	1	--	--	--	0	--	--	--	0	--	--	--	0
5	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
6	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
7	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
8	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
9	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
10	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
11	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
12	--	1	1	2	--	--	--	0	--	--	--	0	--	--	--	0
13	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
14	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
15	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
16	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
17	--	--	98	98	--	--	--	0	--	--	--	0	--	--	--	0
18	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
19	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
20	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
21	--	--	--	0	--	--	--	0	--	--	--	0	--	--	--	0
Total:	0	1	100	101	0	0	0	0	0	0	0	0	73	0	0	73

Appendix 3.1

Analytical Method Flowchart for the Analysis of Flutamide in Freshwater

METHOD OUTLINE FOR THE ANALYSIS OF FLUTAMIDE IN FRESHWATER

Prepare calibration standards in freshwater using volumetric flasks and gas-tight syringes.

↓

Prepare matrix fortification samples in freshwater using volumetric flasks, volumetric pipettes, culture tubes and gas-tight syringes.

↓

Dilute samples with freshwater using volumetric pipettes, culture tubes and gas-tight syringes such that all samples fall within the calibration range.

↓

Transfer samples and standards to autosampler vials for analysis by HPLC/UV.

Appendix 3.2

Typical HPLC Operational Parameters

INSTRUMENT:	Agilent Series 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector
ANALYTICAL COLUMN:	Zorbax phenyl (250 x 4.6 mm, 5-µm particle size)
STOP TIME:	15 minutes
FLOW RATE:	1.000 mL/min
SOLVENT A:	0.1% H_3PO_4
SOLVENT B:	CH_3CN

GRADIENT ELUTION PROFILE:

Time (min)	%A	%B	Flow (mL/min)
0.01	90.0	10.0	1.000
1.00	90.0	10.0	1.000
9.00	5.0	95.0	1.000
10.00	5.0	95.0	1.000
10.10	90.0	10.0	1.000
15.00	90.0	10.0	1.000

OVEN TEMPERATURE:	40°C
INJECTION VOLUME:	100 µL
FLUTAMIDE RETENTION TIME:	Approximately 12 minutes
WAVELENGTH:	293 nm

Appendix 3.3

Analytical Stocks and Standards Preparation

A stock solution of flutamide was prepared by accurately weighing 0.1002 g of the test substance on an analytical balance. The test substance was transferred to a 100-mL volumetric flask and was brought to volume with methanol. The stock (1.00 mg/mL) was diluted with methanol to produce 0.100 and 0.0100 mg/mL stock solutions. The 0.100 and 0.0100 mg/mL stock solutions were used to fortify the quality control samples. Calibration standards were prepared in freshwater using the 0.0100 mg/mL stock solution. The following shows the dilution scheme for a set of standards:

Stock Concentration (mg/mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (µg/L)
0.0100	40.0	10.0	40.0
0.0100	100	10.0	100
0.0100	200	10.0	200
0.0100	300	10.0	300
0.0100	400	10.0	400

Appendix 3.4

Example Calculations for a Representative Sample

The analytical result and percent recovery for sample number 607A-101-3, with a nominal concentration of 100 µg/L, were calculated using the following equations:

$$\text{Concentration of flutamide µg/L in sample} = \frac{\text{Peak area - (Y-intercept)}}{\text{Slope}} \text{ X Dilution factor}$$

$$\text{Percent of nominal concentration} = \frac{\text{Measured concentration of sample (µg/L)}}{\text{Nominal concentration of sample (µg/L)}} \text{ x } 100$$

Peak area = 15.57452
Y-intercept = -0.26705
Slope = 0.1607
Dilution Factor = 1.00

$$\text{Concentration of flutamide µg/L in sample} = \frac{15.57452 + 0.26705}{0.1607} \text{ X } 1.00$$

Concentration of flutamide in sample µg/L = 98.6

$$\text{Percent of nominal concentration} = \frac{98.6 \text{ (µg/L)}}{100 \text{ (µg/L)}} \text{ X } 100$$

Percent of nominal concentration = 98.6%

Appendix 3.5

Quality Control Samples of Flutamide in Freshwater

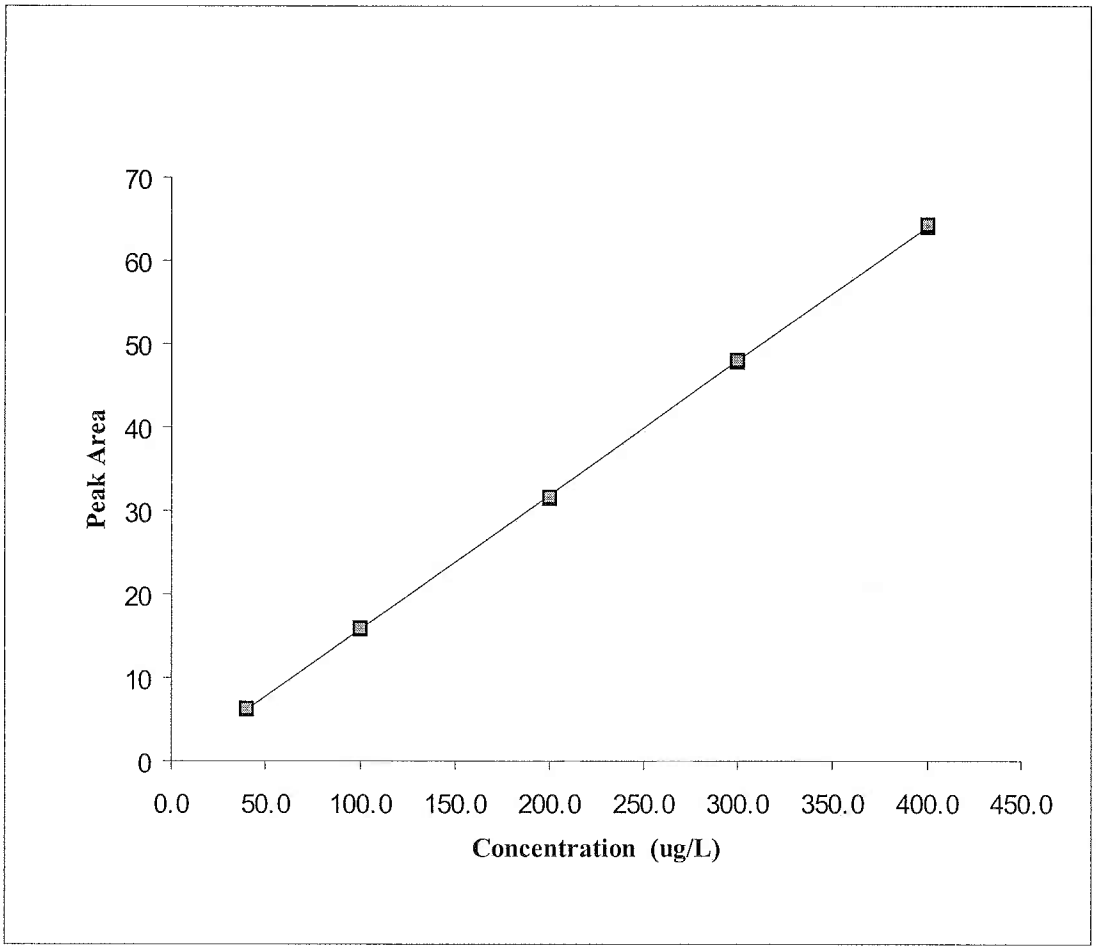
Sample Number (607A-101-)	Sample Type	Concentration of Flutamide		Percent Recovery[2]
		Fortified (µg/L)	Measured[1] (µg/L)	
MAB-1	Matrix Blank	0.0	< LOQ	--
MAB-2	Matrix Blank	0.0	< LOQ	--
MAB-3	Matrix Blank	0.0	< LOQ	--
MAB-4	Matrix Blank	0.0	< LOQ	--
MAB-5	Matrix Blank	0.0	< LOQ	--
MAB-6	Matrix Blank	0.0	< LOQ	--
MAS-1	Matrix Fortification	100	101	101
MAS-2	Matrix Fortification	1000	958	95.8
MAS-3	Matrix Fortification	100	102	102
MAS-4	Matrix Fortification	1000	951	95.1
MAS-5	Matrix Fortification	100	100	100
MAS-6	Matrix Fortification	1000	976	97.6
MAS-7	Matrix Fortification	100	102	102
MAS-8	Matrix Fortification	1000	961	96.1
MAS-9	Matrix Fortification	100	100	100
MAS-10	Matrix Fortification	1000	976	97.6
MAS-11	Matrix Fortification	100	101	101
MAS-12	Matrix Fortification	1000	968	96.8
			Mean =	98.8
			Standard Deviation =	2.52
			CV =	2.55%

[1] The limit of quantitation (LOQ) was 40.0 µg/L, calculated as the product of the concentration of the lowest calibration standard (40.0 µg/L) and the dilution factor of the matrix blank samples (1.00).

[2] Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

Appendix 3.6

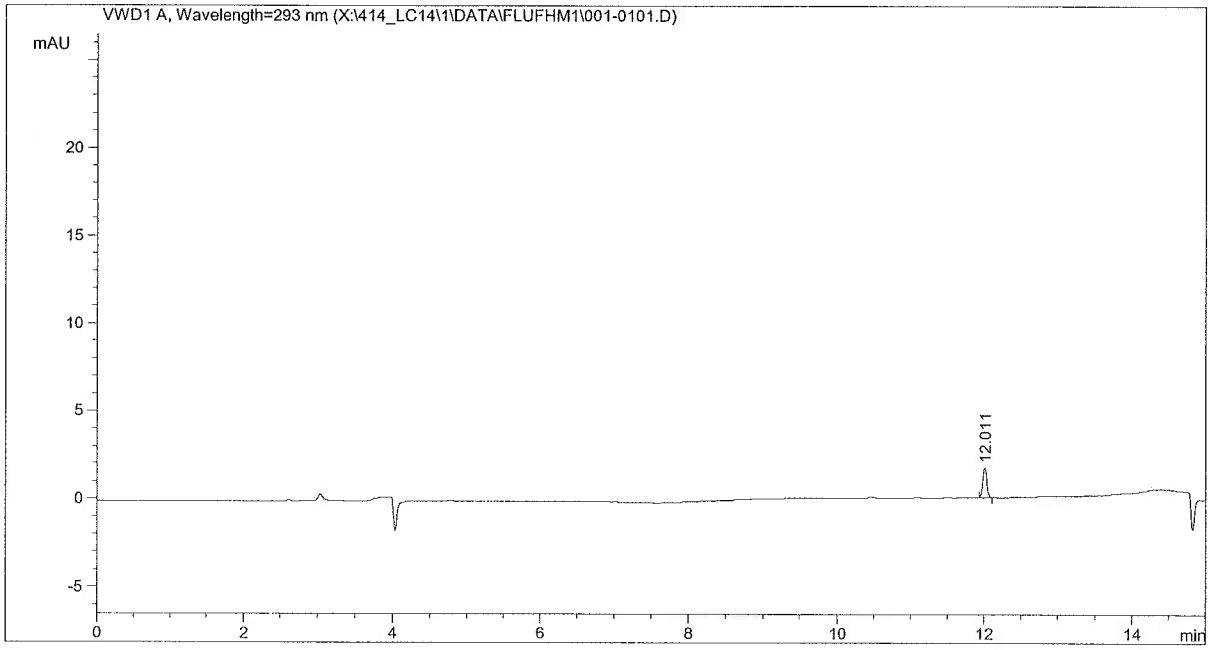
Representative Calibration Curve for Flutamide



Slope = 0.1607; Y-Intercept = -0.26705; R^2 = 0.9999

Appendix 3.7

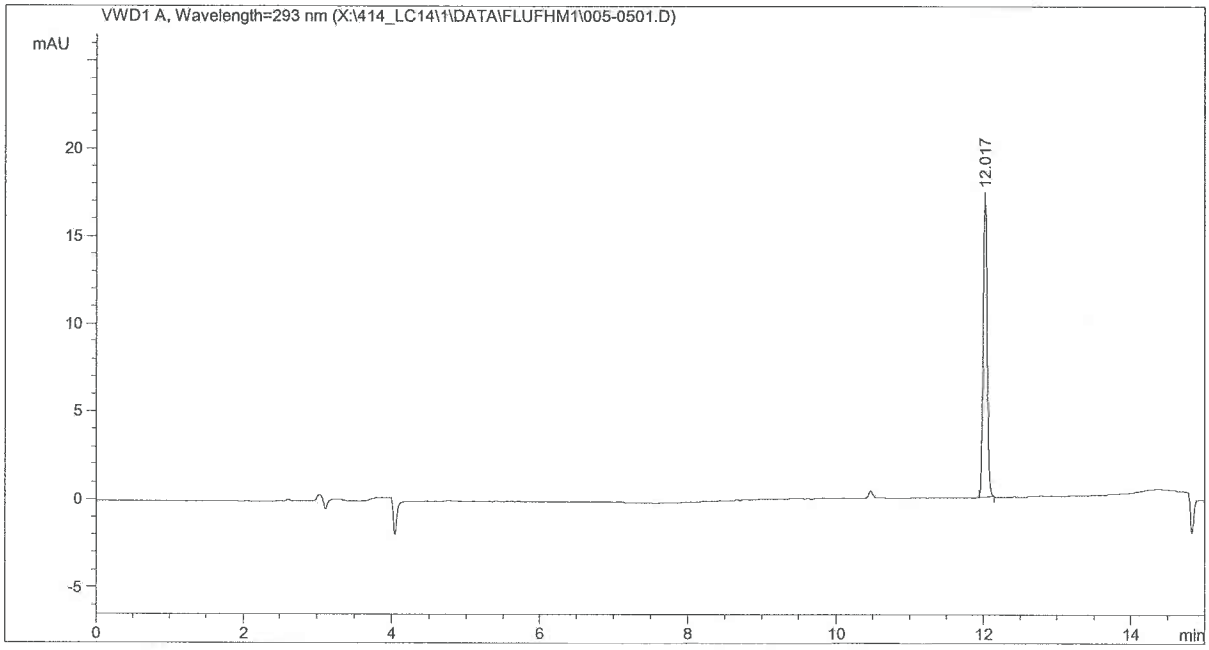
Representative Chromatogram of a Low-level Flutamide Calibration Standard



Standard concentration: 40.0 µg/L

Appendix 3.8

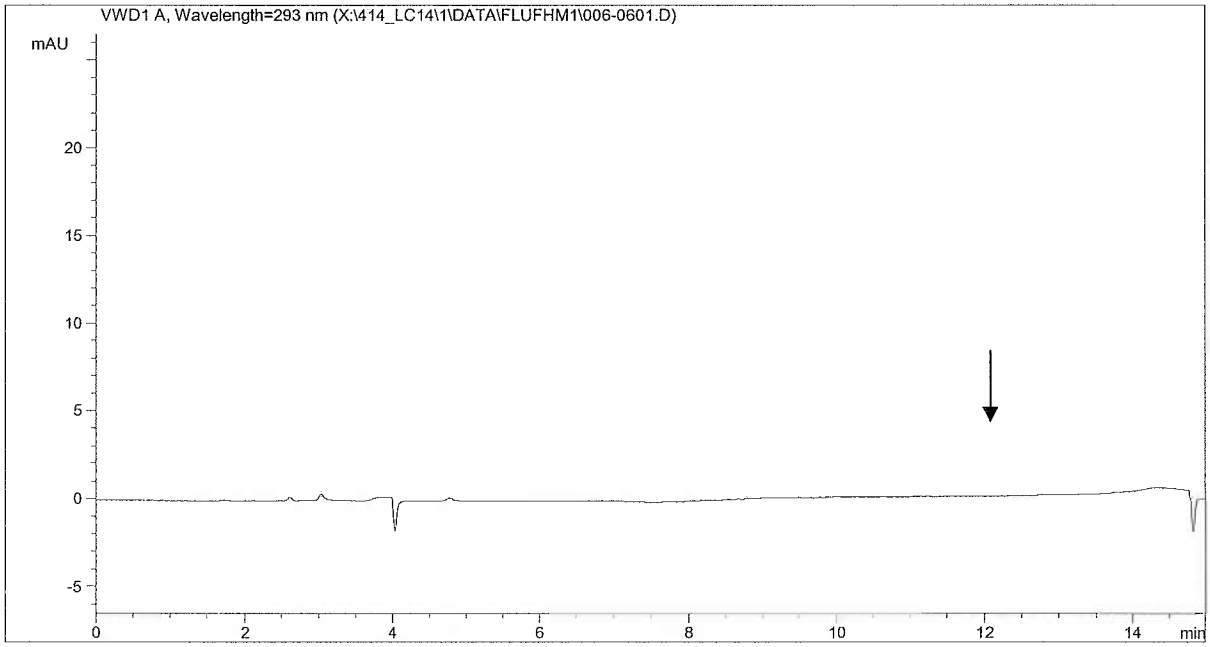
Representative Chromatogram of a High-level Flutamide Calibration Standard



Standard concentration: 400 µg/L

Appendix 3.9

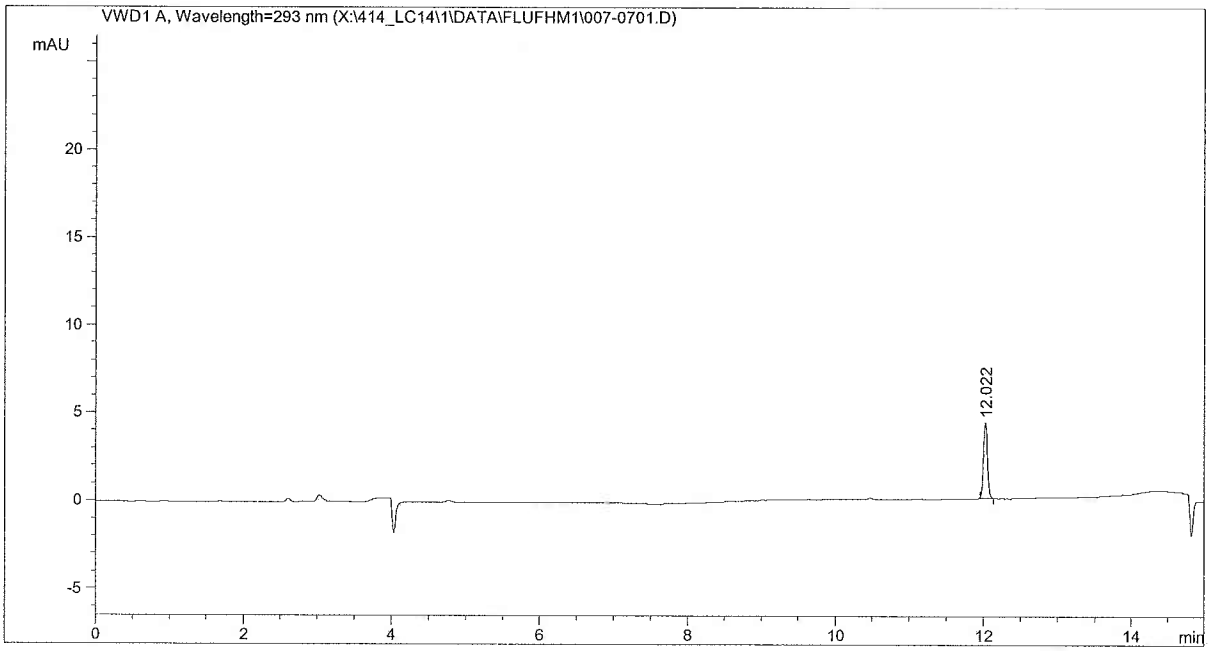
Representative Chromatogram of a Matrix Blank Sample



Sample number 607A-101-MAB-1. The arrow indicates the approximate retention time of flutamide.

Appendix 3.10

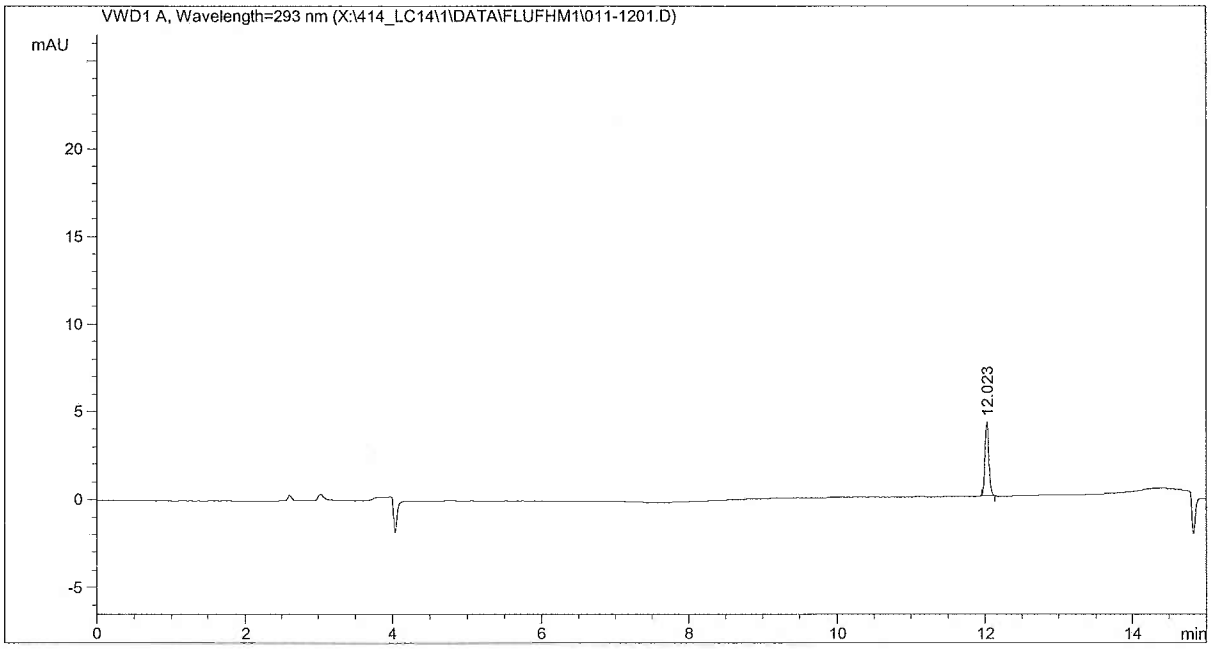
Representative Chromatogram of a Matrix Fortification Sample



Sample number: 607A-101-MAS-1, nominal concentration 100 µg/L.

Appendix 3.11

Representative Chromatogram of a Test Sample



Sample number: 607A-101-3, 0 Hour, nominal concentration 100 µg/L.

Appendix 4

EPL Pathology Report

EPL® Experimental Pathology Laboratories, Inc.

BATTELLE PACIFIC NORTHWEST DIVISION,
BATTELLE MEMORIAL INSTITUTE
STUDY NUMBER WA 5-11
EPL PROJECT NUMBER 237-024

FLUTAMIDE: PHASE 1B VALIDATION – FISH SCREENING ASSAY FOR ENDOCRINE ACTIVE SUBSTANCE WITH THE FATHEAD MINNOW (PIMEPHALES PROMELAS)

DRAFT PATHOLOGY REPORT

Submitted by:

Experimental Pathology Laboratories, Inc.
P.O. Box 474
Herndon, VA 20172-0474
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Submitted to:

Battelle Pacific Northwest Division, Battelle Memorial Institute
Easton, MD 21601

January 13, 2006

EPL® Experimental Pathology Laboratories, Inc.

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DRAFT PATHOLOGY SUMMARY

EPL® Experimental Pathology Laboratories, Inc.

BATTELLE PACIFIC NORTHWEST DIVISION,
BATTELLE MEMORIAL INSTITUTE
STUDY NUMBER WA 5-11
EPL PROJECT NUMBER 237-024

FLUTAMIDE: PHASE 1B VALIDATION – FISH SCREENING ASSAY FOR ENDOCRINE ACTIVE SUBSTANCE WITH THE FATHEAD MINNOW (PIMEPHALES PROMELAS)

DRAFT PATHOLOGY SUMMARY

INTRODUCTION

The objective of this study was to determine the effects, if any, of flutamide administered via water bath on gonadal tissue of adult fathead minnows (FHM, *Pimephales promelas*).

The experimental design is presented in the following table:

Table 1. Experimental Design for Flutamide Study

Flutamide	Male Replicates				Female Replicates			
	A	B	C	D	A	B	C	D
0 µg/L (Control)	3	2	2	2	3	4	4	4
100 µg/L	2	2	2	2	4	4	4	4
500 µg/L	3	2	4	2	3	4	2	4
1000 µg/L	2	2	2	2	4	4	4	4

METHODS

Unless otherwise indicated, histopathological procedures were performed according to the draft form of the "OECD Guidance Document for Performing Gonadal Histopathology in Small Fish." Briefly, following routine processing the left and right gonads were embedded horizontal to their long axis to allow for longitudinal sectioning. During microtomy, the first section from each block was

acquired at the point at which approximately half of the gonad had been cut away and the size of the section was maximized. The second and third sections were then obtained at 50 micron intervals. Sections were stained with hematoxylin and eosin, and mounted with glass coverslips. Labels included the EPL Project No. (237-024), the group/replicate designation (e.g., Flu1000/D), the study ID (WA 5-11), and the Animal No. (e.g., 1AM1).

The pathologist evaluated the slides by brightfield microscopy for changes that included, but were not limited to, the types of findings that are listed in the aforementioned guidance document. As per that document, severity grading of findings was performed according to the following scale: NR = not remarkable, grade 1 = minimal, grade 2 = mild, grade 3 = moderate, grade 4 = severe. Ovarian oocyte atresia was graded according to the following scale: Grade 1 = 3 to 5 atretic oocytes per ovary; Grade 2 = 6 to 9 atretic oocytes per ovary; Grade 3 = greater than 9 atretic oocytes per ovary, but less than the vast majority; and Grade 4 = the vast majority of oocytes were atretic. The pathologist recorded findings on a spreadsheet. This original spreadsheet as contained within the guidance document was modified slightly by the study pathologist to include the addition of a column in order to accommodate the animal numbers of the female fathead minnows (which were different from what appeared on the worksheet), and corrections of some of the animal numbers to correspond with the animal numbers that were submitted by the client. The data collection spreadsheet is incorporated into this report. Results were simultaneously recorded into EPL's Pathology Data Reporting System, and tabulated in the accompanying Histopathology Incidence Tables (HIT) and summarized in the Summary Incidence Tables (SIT).

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RESULTS

Males

Based on incidence and/or severity data, the single finding that was substantially different in flutamide-exposed males as compared to control males was an increase in the testicular stage score in the in the 1000 µg/L male group (Figs 1 and 2). There was also a slight increase in the incidence and severity of "Increased Cells, Spermatogonia" in the 500 µg/L and 1000 µg/L male groups as compared to the control males (Figs 1 and 2). The difference in incidence alone is unlikely to be statistically significant; however, it is possible that the combination increase in incidence and severity in the 1000 µg/L male group could be significant.

The incidence and severity of selected histopathologic results for male FHM are presented in the following table:

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Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/13/06 DRAFT

Table 2. Combined Incidence and Severity of Selected Histopathologic Findings in Male Fathead Minnows

Flutamide Dose (µg/L)	0					100					500					1000				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	3	2	2	2	9	2	2	2	2	8	3	2	4	2	11	2	2	2	2	8
Increased Cells, Spermatogonia	1	0	0	1	2	1	0	0	0	1	2	0	3	0	5	1	2	2	0	5
Minimal	-	-	-	1	1	1	-	-	-	1	1	-	1	-	2	1	-	2	-	3
Mild	1	-	-	-	1	-	-	-	-	-	1	-	1	-	2	-	1	-	-	1
Moderate	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	1	-	-	1
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testicular Degeneration, Increased	1	0	1	0	2	0	0	0	0	0	1	0	2	0	3	0	2	0	0	2
Minimal	-	-	1	-	1	-	-	-	-	-	-	-	1	-	1	-	1	-	-	1
Mild	1	-	-	-	1	-	-	-	-	-	1	-	1	-	2	-	1	-	-	1
Moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Increased Cells, Interstitial Cells	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Minimal	-	-	1	-	1	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-
Mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testicular Stage																				
Stage 0	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-
Stage 1	1	-	1	-	2	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-
Stage 2	1	2	1	2	6	1	1	2	1	5	2	2	2	-	6	-	-	-	-	-
Stage 3	1	-	-	-	1	1	-	-	1	2	1	-	1	2	4	2	2	2	2	8
Average	1.9					2.1					2.2					3.0				

*T = totals

Females

Based on incidence and/or severity data, the single finding that was substantially different in flutamide-exposed females as compared to control females was "Oocyte Atresia, Increased" in the 1000 µg/L dose group (Figs 3 and 4). Note that both the incidence and severity of this finding are markedly increased in the 1000 µg/L dose group.

Microsporidia were evident within areas of granulomatous inflammation in the ovarian interstitium of few females (Fig. 5) and in one female (Animal No. C-17), they were observed within the ooplasm of an atretic oocyte (Figs. 6 and 7). Conversely, there were no microsporidia observed in the testes.

The incidence and severity of selected histopathologic results for female fathead minnows are presented in the following table:

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/13/06 DRAFT

Table 3. Combined Incidence and Severity of Selected Histopathologic Findings in Female Fathead Minnows

Flutamide Dose (µg/L)	0					100					500					1000				
Replicate	A	B	C	D	T*	A	B	C	D	T	A	B	C	D	T	A	B	C	D	T
No. Examined	3	4	4	4	15	4	4	4	4	16	3	4	2	4	13	4	4	4	4	16
Oocyte Atresia, Increased	0	1	0	0	1	2	0	0	2	4	1	0	0	1	2	1	3	3	3	10
Minimal	-	1	-	-	1	1	-	-	1	2	-	-	-	1	1	-	1	1	-	2
Mild	-	-	-	-	-	1	-	-	-	1	1	-	-	-	1	-	1	1	-	2
Moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	3	6
Severe	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-
Microsporidia	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1	1	0	1	0	2
Minimal	-	-	1	-	1	-	-	-	-	-	-	-	-	1	1	1	-	1	-	2
Mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Granulomatous, Inflammation	0	1	1	0	2	0	0	0	0	0	1	0	0	1	2	1	0	1	1	3
Minimal	-	1	1	-	2	-	-	-	-	-	1	-	-	1	2	-	-	1	1	2
Mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1
Moderate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovarian Stage																				
Stage 0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stage 1	-	-	-	1	1	-	-	-	-	-	-	-	-	1	1	-	1	-	1	2
Stage 2	1	3	1	-	5	-	3	1	-	4	1	1	-	1	3	2	1	1	2	6
Stage 3	2	1	3	3	9	3	-	2	3	8	2	2	2	1	7	1	-	2	1	4
Stage 4	-	-	-	-	-	1	1	1	1	4	-	1	-	1	2	1	2	1	-	4
Average	2.5					3.0					2.8					2.6				

*T = totals

DISCUSSION

Flutamide is a non-steroidal antiandrogen which inhibits androgen uptake and/or nuclear binding of androgen in target tissues (Sufrin and Coffey, 1976). The effects of flutamide on adult gonadal histopathology have been investigated for several fishes including zebrafish (*Danio rerio*), male guppies (*Poecilia reticulata*) and Japanese medaka (*Oryzias latipes*). Findings in flutamide-exposed male zebrafish (up to 1000 µg/L flutamide in water) included an increase in interstitial cells, nuclear hypertrophy of Sertoli cells, an increase in the proportion of spermatogonia, and a decrease in the proportion of spermatocytes (Wester, et al., 2003). There were no histopathologic findings for exposed adult female zebrafish in that study. Histopathologic findings attributed to flutamide exposure in male guppies (up to 100 µg/mg of feed) included a reduced number of spermatogenic cysts and increased numbers of spermatozeugmata in ducts (Kinnberg and Toft, 2003).

Fathead minnows in the previous Phase 1B experiments, which were exposed to the same nominal doses as the present study, did not exhibit consistent responses to flutamide ("Revised Draft Report: Phase 1B of the Validation of the 21-Day Fish Assay for the Detection of Endocrine Active Substances"). However, increased spermatogonia and an increase in the testicular stage score were among the findings reported for fathead minnow males in the Phase 1B studies. Similarly, increased oocyte atresia of the ovaries was reported for female fathead minnows in at least one of those studies.

The presence of microsporidia in the ovaries of some fish is not considered to be a confounding factor in this study because the incidence and severity of the parasite infection, and the severity granulomatous inflammation that tended to accompany this infection, were all quite low.

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Other findings in this study either occurred in comparable numbers of control and flutamide-exposed fish (background lesions), or as low-frequency (incidental) findings, and are therefore not considered to be exposure-related.

CONCLUSION AND SUMMARY

Histopathological findings that were attributable to flutamide exposure included an increase in the testicular stage score in the testes of 1000 µg/L males and increased oocyte atresia in the ovaries of 1000 µg/L females. A potential exposure-related finding was a slight increase in the incidence and severity of increased spermatogonia in the testes of fish in the 500 µg/L and 1000 µg/L male groups as compared to the control males. The difference in incidence alone is unlikely to be statistically significant; however, it is possible that the combination increase in incidence and severity of this finding could be significant.

Other findings in this study either occurred in comparable numbers of control and flutamide-exposed fish (background lesions), or as low-frequency (incidental) findings, and are therefore not considered to be exposure-related.

JEFFREY C. WOLF, DVM, Diplomate, ACVP Date
Veterinary Pathologist

JCW/cb

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REFERENCES

Kinnberg K, Toft G. (2003). Effects of estrogenic and antiandrogenic compounds on the testis structure of the adult guppy (*Poecilia reticulata*). Ecotoxicology and Environmental Safety, 54(1):16-24.

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Sufrin G, Coffey DS. (1976). Flutamide. Mechanism of action of a new nonsteroidal antiandrogen. Invest Urol, 13(6):429-34.

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1/13/06 DRAFT

QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: Flutamide: Phase 1B Validation – Fish Screening Assay For Endocrine Active Substance With The Fathead Minnow (Pimephales promelas)

Client Study: WA 5-11 EPL Project Coordinator: Dr. Jeffrey C. Wolf

EPL Project Number: 237-024 EPL Pathologist: Dr. Jeffrey C. Wolf

The following aspects of this study were inspected by the Quality Assurance Unit of Experimental Pathology Laboratories, Inc. Dates inspections were performed and findings reported to the EPL Project Coordinator and Management are indicated below.

	Dates	
Area Inspected	Inspection	Reporting
EPL Project Sheets		
Project Setup		
Histology Setup		
Data Review		
Draft Report		
Final Report		

Date reported to Study Director/Management XXX

Date of last quarterly facility inspection 7/05

EPL Quality Assurance Unit Date

Form No. 6-2 (7/2/99)

SUMMARY INCIDENCE TABLES

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP Cont/A	GROUP Cont/B	GROUP Cont/C	GROUP Cont/D	GROUP 100/A	GROUP 100/B
TESTIS (NO. EXAMINED)	(3)	(2)	(2)	(2)	(2)	(2)
Granulomatous Inflammation				1		
Histiocytic Cells, Intraluminal						
Increased Cells, Interstitial Cells (Leydig Cells)			1			
Increased Cells, Spermatogonia	1			1	1	
Inflammation, Mixed Cells						
Mineralization			2			
Mineralization, Collecting Duct						
Stage 0						
Stage 1	1		1			1
Stage 2	1	2	1	2	1	1
Stage 3	1				1	
Testicular Degeneration, Increased	1		1			

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 100/C	GROUP 100/D	GROUP 500/A	GROUP 500/B	GROUP 500/C	GROUP 500/D
TESTIS (NO. EXAMINED)	(2)	(2)	(3)	(2)	(4)	(2)
Granulomatous Inflammation			1			
Histiocytic Cells, Intraluminal			1			
Increased Cells, Interstitial Cells (Leydig Cells)		1				
Increased Cells, Spermatogonia			2		3	
Inflammation, Mixed Cells		1			1	
Mineralization						
Mineralization, Collecting Duct			1		1	
Stage 0					1	
Stage 1						
Stage 2	2	1	2	2	2	
Stage 3		1	1		1	2
Testicular Degeneration, Increased			1		2	

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 1000/A	GROUP 1000/B	GROUP 1000/C	GROUP 1000/D		
TESTIS (NO. EXAMINED)	(2)	(2)	(2)	(2)		
Granulomatous Inflammation				1		
Histiocytic Cells, Intraluminal						
Increased Cells, Interstitial Cells (Leydig Cells)						
Increased Cells, Spermatogonia	1	2	2			
Inflammation, Mixed Cells		1				
Mineralization		1		1		
Mineralization, Collecting Duct		1		1		
Stage 0						
Stage 1						
Stage 2						
Stage 3	2	2	2	2		
Testicular Degeneration, Increased		2				

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP Cont/A	GROUP Cont/B	GROUP Cont/C	GROUP Cont/D	GROUP 100/A	GROUP 100/B
OVARY (NO. EXAMINED)	(3)	(4)	(4)	(4)	(4)	(4)
Asynchronous Development, Gonad						2
Asynchronous Development, Right and Left Gonads						
Granulomatous Inflammation		1	1			
Inflammation, Mixed Cells	1	1	1			1
Microsporidia			1			
Oocyte Atresia, Increased		1			2	
Stage 1				1		
Stage 2	1	3	1			3
Stage 3	2	1	3	3	3	
Stage 4					1	1

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 100/C	GROUP 100/D	GROUP 500/A	GROUP 500/B	GROUP 500/C	GROUP 500/D
OVARY (NO. EXAMINED)	(4)	(4)	(3)	(4)	(2)	(4)
Asynchronous Development, Gonad						
Asynchronous Development, Right and Left Gonads						
Granulomatous Inflammation			1			1
Inflammation, Mixed Cells			1	2		2
Microsporidia						1
Oocyte Atresia, Increased		2	1			1
Stage 1						1
Stage 2	1		1	1		1
Stage 3	2	3	2	2	2	1
Stage 4	1	1		1		1

SUMMARY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 1000/A	GROUP 1000/B	GROUP 1000/C	GROUP 1000/D		
OVARY (NO. EXAMINED)	(4)	(4)	(4)	(4)		
Asynchronous Development, Gonad						
Asynchronous Development, Right and Left Gonads	1					
Granulomatous Inflammation	1		1	1		
Inflammation, Mixed Cells						
Microsporidia	1		1			
Oocyte Atresia, Increased	1	3	3	3		
Stage 1		1		1		
Stage 2	2	1	1	2		
Stage 3	1		2	1		
Stage 4	1	2	1			

HISTOPATHOLOGY INCIDENCE TABLES

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

ANIMAL	GROUP Cont/A			GROUP Cont/B		GROUP Cont/C		GROUP Cont/D		GROUP 100/A		GROUP 100/B	
	A-1	A-2	A-9	B-3	B-4	C-5	C-6	D-7	D-8	A-25	A-26	B-27	B-28
TESTIS			m										
Granulomatous Inflammation									3				
Histiocytic Cells, Intraluminal													
Increased Cells, Interstitial Cells (Leydig Cells)							1						
Increased Cells, Spermatogonia			2						1		1		
Inflammation, Mixed Cells													
Mineralization						1	1						
Mineralization, Collecting Duct													
Stage 0													
Stage 1			P			P						P	
Stage 2		P		P	P		P	P	P	P			P
Stage 3	P										P		
Testicular Degeneration, Increased			2				1						

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II-1

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac /death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 100/C			GROUP 100/D			GROUP 500/A			GROUP 500/B			GROUP 500/C				GROUP 500/D		
ANIMAL	C-29	C-30		D-31	D-32		A-49	A-50	A-57	B-51	B-52		C-53	C-54	C-65	C-67		D-55	D-56
TESTIS																			
Granulomatous Inflammation							1												
Histiocytic Cells, Intraluminal							2												
Increased Cells, Interstitial Cells (Leydig Cells)					1														
Increased Cells, Spermatogonia								1	2				1		3	2			
Inflammation, Mixed Cells				1										1					
Mineralization																			
Mineralization, Collecting Duct								1						2					
Stage 0															P				
Stage 1																			
Stage 2	P	P		P			P	P		P	P			P		P			
Stage 3					P				P				P					P	P
Testicular Degeneration, Increased									2						1	2			

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II-2

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac /death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Male Pimephales promelas

	GROUP 1000/A			GROUP 1000/B			GROUP 1000/C			GROUP 1000/D		
ANIMAL	A-73	A-74		B-75	B-76		C-77	C-78		D-79	D-80	
TESTIS												
Granulomatous Inflammation										1		
Histiocytic Cells, Intraluminal												
Increased Cells, Interstitial Cells (Leydig Cells)												
Increased Cells, Spermatogonia		1		3	2		1	1				
Inflammation, Mixed Cells					1							
Mineralization					1						1	
Mineralization, Collecting Duct					2						1	
Stage 0												
Stage 1												
Stage 2												
Stage 3	P	P		P	P		P	P		P	P	
Testicular Degeneration, Increased				1	2							

EPL Experimental Pathology Laboratories, Inc.

II-3

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP Cont/A				GROUP Cont/B				GROUP Cont/C				GROUP Cont/D			
ANIMAL	A-10	A-11	A-12		B-13	B-14	B-15	B-16	C-17	C-18	C-19	C-20	D-21	D-22	D-23	D-24
OVARY																
Asynchronous Development, Gonad																
Asynchronous Development, Right and Left Gonads																
Granulomatous Inflammation						1			1							
Inflammation, Mixed Cells			1		1					1						
Microsporidia									1							
Oocyte Atresia, Increased					1											
Stage 1													P			
Stage 2		P			P	P		P		P						
Stage 3	P		P				P		P		P	P		P	P	P
Stage 4																

EPL
Experimental Pathology Laboratories, Inc.

II-4

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 100/A					GROUP 100/B					GROUP 100/C					GROUP 100/D				
ANIMAL	A-33	A-34	A-35	A-36		B-37	B-38	B-39	B-40		C-41	C-42	C-43	C-44		D-45	D-46	D-47	D-48	
OVARY																				
Asynchronous Development, Gonad							2	1												
Asynchronous Development, Right and Left Gonads																				
Granulomatous Inflammation																				
Inflammation, Mixed Cells									1											
Microsporidia																				
Oocyte Atresia, Increased	1	2														4	1			
Stage 1																				
Stage 2							P	P	P				P							
Stage 3		P	P	P								P		P		P	P	P		
Stage 4	P					P					P								P	

EPL Experimental Pathology Laboratories, Inc.

II-5

Key: X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

	GROUP 500/A				GROUP 500/B				GROUP 500/C			GROUP 500/D							
ANIMAL	A-58	A-59	A-60		B-61	B-62	B-63	B-64	C-66	C-68		D-69	D-70	D-71	D-72				
OVARY																			
Asynchronous Development, Gonad																			
Asynchronous Development, Right and Left Gonads																			
Granulomatous Inflammation			1												1				
Inflammation, Mixed Cells			1		1			1					1		1				
Microsporidia															1				
Oocyte Atresia, Increased		2													1				
Stage 1														P					
Stage 2			P			P									P				
Stage 3	P	P					P	P		P	P		P						
Stage 4					P							P							

EPL Experimental Pathology Laboratories, Inc.

Key. X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

HISTOPATHOLOGY INCIDENCE TABLE

WA 5-11
Terminal Sacrifice
Female Pimephales promelas

ANIMAL	GROUP 1000/A					GROUP 1000/B					GROUP 1000/C					GROUP 1000/D				
	A-81	A-82	A-83	A-84		B-85	B-86	B-87	B-88		C-89	C-90	C-91	C-92		D-93	D-94	D-95	D-96	
OVARY																				
Asynchronous Development, Gonad																				
Asynchronous Development, Right and Left Gonads			3																	
Granulomatous Inflammation				2										1			1			
Inflammation, Mixed Cells																				
Microsporidia				1										1						
Oocyte Atresia, Increased				3		1		3	2		1	3	2			3	3	3		
Stage 1									P									P		
Stage 2	P			P			P							P			P		P	
Stage 3		P									P		P			P				
Stage 4			P			P		P				P								

EPL Experimental Pathology Laboratories, Inc.

Key X=Not Remarkable N=No Section I=Incomplete A=Autolysis
1=minimal 2=slight/mild 3=moderate 4=moderately severe 5=severe/high
P=Present B=Benign M=Malignant
m=missing one paired organ u=unscheduled sac./death

APPENDIX A
GONAD HISTOPATHOLOGY RESULTS SPREADSHEET

Animal	Sex	Group	Days	Fate	Examed	Tumors	Lcode	Lesion	Tcode	Tissue	WOD	TumorType	COD	TisGrade	LesGrade	Records
A-1	M	0	.	1	0	1001	Stage 3	1510	TESTIS	.	.	2	P	.	1	1
A-2	M	0	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
A-9	M	0	.	1	0	1004	Stage 1	1510	TESTIS	.	.	2	P	.	4	4
A-9	M	0	.	1	0	1005	Testicular Degeneration, Increased	1510	TESTIS	.	.	2	.	2	4	4
A-9	M	0	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS	.	.	2	.	2	4	4
A-9	M	0	.	1	0	-1	Missing one of pair	1510	TESTIS	.	.	.	.	2	4	4
B-3	M	0	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
B-4	M	0	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
C-5	M	0	.	1	0	1004	Stage 1	1510	TESTIS	.	.	2	P	.	2	2
C-5	M	0	.	1	0	1003	Mineralization	1510	TESTIS	.	.	2	.	1	2	2
C-6	M	0	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	4	4
C-6	M	0	.	1	0	1003	Mineralization	1510	TESTIS	.	.	2	.	1	4	4
C-6	M	0	.	1	0	1005	Testicular Degeneration, Increased	1510	TESTIS	.	.	2	.	1	4	4
C-6	M	0	.	1	0	1006	Increased Cells, Interstitial Cells (Leyig Cells)	1510	TESTIS	.	.	2	.	1	4	4
D-7	M	0	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
D-8	M	0	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	3	3
D-8	M	0	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS	.	.	2	.	1	3	3
D-8	M	0	.	1	0	1007	Granulomatous Inflammation	1510	TESTIS	.	.	2	.	3	3	3
A-25	M	4	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
A-26	M	4	.	1	0	1001	Stage 3	1510	TESTIS	.	.	2	P	.	2	2
A-26	M	4	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS	.	.	2	.	1	2	2
B-27	M	1	.	1	0	1004	Stage 1	1510	TESTIS	.	.	2	P	.	1	1
B-28	M	1	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
C-29	M	2	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
C-30	M	2	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
D-31	M	3	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	2	2
D-31	M	3	.	1	0	1010	Inflammation, Mixed Cells	1510	TESTIS	.	.	2	.	1	2	2
D-32	M	3	.	1	0	1001	Stage 3	1510	TESTIS	.	.	2	P	.	2	2
D-32	M	3	.	1	0	1006	Increased Cells, Interstitial Cells (Leyig Cells)	1510	TESTIS	.	.	2	.	1	2	2
A-49	M	5	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	3	3
A-49	M	5	.	1	0	1007	Granulomatous Inflammation	1510	TESTIS	.	.	2	.	1	3	3
A-49	M	5	.	1	0	1012	Histiocytic Cells, Intraluminal	1510	TESTIS	.	.	2	.	2	3	3
A-50	M	5	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	3	3
A-50	M	5	.	1	0	1009	Mineralization, Collecting Duct	1510	TESTIS	.	.	2	.	1	3	3
A-50	M	5	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS	.	.	2	.	1	3	3
A-57	M	5	.	1	0	1001	Stage 3	1510	TESTIS	.	.	2	P	.	3	3
A-57	M	5	.	1	0	1005	Testicular Degeneration, Increased	1510	TESTIS	.	.	2	.	2	3	3
A-57	M	5	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS	.	.	2	.	2	3	3
B-51	M	6	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
B-52	M	6	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	1	1
C-53	M	7	.	1	0	1001	Stage 3	1510	TESTIS	.	.	2	P	.	2	2
C-53	M	7	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS	.	.	2	.	1	2	2
C-54	M	7	.	1	0	1002	Stage 2	1510	TESTIS	.	.	2	P	.	3	3
C-54	M	7	.	1	0	1009	Mineralization, Collecting Duct	1510	TESTIS	.	.	2	.	2	3	3
C-54	M	7	.	1	0	1010	Inflammation, Mixed Cells	1510	TESTIS	.	.	2	.	1	3	3

Animal	Sex	Group	Days	Fate	Examed	Tumors	Lcode	Lesion	Tcode	Tissue	WOD	TumorType	COD	TisGrade	LesGrade	Records
C-65	M	7	.	1	0	1013	Stage 0	1510	TESTIS				2	P		3
C-65	M	7	.	1	0	1005	Testicular Degeneration, Increased	1510	TESTIS				2		1	3
C-65	M	7	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS				2		3	3
C-67	M	7	.	1	0	1002	Stage 2	1510	TESTIS				2	P		3
C-67	M	7	.	1	0	1005	Testicular Degeneration, Increased	1510	TESTIS				2		2	3
C-67	M	7	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS				2		2	3
D-55	M	8	.	1	0	1001	Stage 3	1510	TESTIS				2	P		1
D-56	M	8	.	1	0	1001	Stage 3	1510	TESTIS				2	P		1
A-73	M	11	.	1	0	1001	Stage 3	1510	TESTIS				2	P		1
A-74	M	11	.	1	0	1001	Stage 3	1510	TESTIS				2	P		2
A-74	M	11	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS				2		1	2
B-75	M	9	.	1	0	1001	Stage 3	1510	TESTIS				2	P		3
B-75	M	9	.	1	0	1005	Testicular Degeneration, Increased	1510	TESTIS				2		1	3
B-75	M	9	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS				2		3	3
B-76	M	9	.	1	0	1001	Stage 3	1510	TESTIS				2	P		6
B-76	M	9	.	1	0	1009	Mineralization, Collecting Duct	1510	TESTIS				2		2	6
B-76	M	9	.	1	0	1003	Mineralization	1510	TESTIS				2		1	6
B-76	M	9	.	1	0	1005	Testicular Degeneration, Increased	1510	TESTIS				2		2	6
B-76	M	9	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS				2		2	6
B-76	M	9	.	1	0	1010	Inflammation, Mixed Cells	1510	TESTIS				2		1	6
C-77	M	10	.	1	0	1001	Stage 3	1510	TESTIS				2	P		2
C-77	M	10	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS				2		1	2
C-78	M	10	.	1	0	1001	Stage 3	1510	TESTIS				2	P		2
C-78	M	10	.	1	0	1008	Increased Cells, Spermatogonia	1510	TESTIS				2		1	2
D-79	M	12	.	1	0	1001	Stage 3	1510	TESTIS				2	P		2
D-79	M	12	.	1	0	1007	Granulomatous Inflammation	1510	TESTIS				2		1	2
D-80	M	12	.	1	0	1001	Stage 3	1510	TESTIS				2	P		3
D-80	M	12	.	1	0	1009	Mineralization, Collecting Duct	1510	TESTIS				2		1	3
D-80	M	12	.	1	0	1003	Mineralization	1510	TESTIS				2		1	3
A-10	F	0	.	1	0	1001	Stage 3	1520	OVARY				2	P		1
A-11	F	0	.	1	0	1002	Stage 2	1520	OVARY				2	P		1
A-12	F	0	.	1	0	1001	Stage 3	1520	OVARY				2	P		2
A-12	F	0	.	1	0	1010	Inflammation, Mixed Cells	1520	OVARY				2		1	2
B-13	F	0	.	1	0	1002	Stage 2	1520	OVARY				2	P		3
B-13	F	0	.	1	0	1011	Oocyte Atresia, Increased	1520	OVARY				2		1	3
B-13	F	0	.	1	0	1010	Inflammation, Mixed Cells	1520	OVARY				2		1	3
B-14	F	0	.	1	0	1002	Stage 2	1520	OVARY				2	P		2
B-14	F	0	.	1	0	1007	Granulomatous Inflammation	1520	OVARY				2	P	1	2
B-15	F	0	.	1	0	1001	Stage 3	1520	OVARY				2	P		1
B-16	F	0	.	1	0	1002	Stage 2	1520	OVARY				2	P		1
C-17	F	0	.	1	0	1001	Stage 3	1520	OVARY				2	P		3
C-17	F	0	.	1	0	1007	Granulomatous Inflammation	1520	OVARY				2		1	3
C-17	F	0	.	1	0	1018	Microsporidia	1520	OVARY				2		1	3
C-18	F	0	.	1	0	1002	Stage 2	1520	OVARY				2	P		2
C-18	F	0	.	1	0	1010	Inflammation, Mixed Cells	1520	OVARY				2		1	2

Animal	Sex	Group	Days	Fate	Examed	Tumors	Lcode	Lesion	Tcode	Tissue	WOD	TumorType	COD	TisGrade	LesGrade	Records
C-19	F	0	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	P	.	1	1
C-20	F	0	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	P	.	1	1
D-21	F	0	.	1	0	1004	Stage 1	1520	OVARY	.	.	2	P	.	1	1
D-22	F	0	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	P	.	1	1
D-23	F	0	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	P	.	1	1
D-24	F	0	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	P	.	1	1
A-33	F	4	.	1	0	1016	Stage 4	1520	OVARY	.	.	2	P	.	2	2
A-33	F	4	.	1	0	1011	Oocyte Atresia, Increased	1520	OVARY	.	.	2	.	1	2	2
A-34	F	4	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	P	.	2	2
A-34	F	4	.	1	0	1011	Oocyte Atresia, Increased	1520	OVARY	.	.	2	.	2	2	2
A-35	F	4	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	1	1
A-36	F	4	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	1	1
B-37	F	1	.	1	0	1016	Stage 4	1520	OVARY	.	.	2	.	P	1	1
B-38	F	1	.	1	0	1002	Stage 2	1520	OVARY	.	.	2	.	P	.	2
B-38	F	1	.	1	0	1019	Asynchronous Development, Gonad	1520	OVARY	.	.	2	.	.	2	2
B-39	F	1	.	1	0	1002	Stage 2	1520	OVARY	.	.	2	.	P	.	2
B-39	F	1	.	1	0	1019	Asynchronous Development, Gonad	1520	OVARY	.	.	2	.	.	2	2
B-40	F	1	.	1	0	1002	Stage 2	1520	OVARY	.	.	2	.	P	.	2
B-40	F	1	.	1	0	1010	Inflammation, Mixed Cells	1520	OVARY	.	.	2	.	.	2	2
C-41	F	2	.	1	0	1016	Stage 4	1520	OVARY	.	.	2	.	P	.	1
C-42	F	2	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	1	1
C-43	F	2	.	1	0	1002	Stage 2	1520	OVARY	.	.	2	.	P	.	1
C-44	F	2	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	1	1
D-45	F	3	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	.	2
D-45	F	3	.	1	0	1011	Oocyte Atresia, Increased	1520	OVARY	.	.	2	.	.	2	2
D-46	F	3	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	4	2
D-46	F	3	.	1	0	1011	Oocyte Atresia, Increased	1520	OVARY	.	.	2	.	.	2	2
D-47	F	3	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	1	2
D-48	F	3	.	1	0	1016	Stage 4	1520	OVARY	.	.	2	.	P	.	1
A-58	F	8	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	1	1
A-59	F	8	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	.	2
A-59	F	8	.	1	0	1011	Oocyte Atresia, Increased	1520	OVARY	.	.	2	.	.	2	2
A-60	F	8	.	1	0	1002	Stage 2	1520	OVARY	.	.	2	.	P	.	3
A-60	F	8	.	1	0	1010	Inflammation, Mixed Cells	1520	OVARY	.	.	2	.	.	1	3
A-60	F	8	.	1	0	1007	Granulomatous Inflammation	1520	OVARY	.	.	2	.	.	1	3
B-61	F	5	.	1	0	1016	Stage 4	1520	OVARY	.	.	2	.	P	.	2
B-61	F	5	.	1	0	1010	Inflammation, Mixed Cells	1520	OVARY	.	.	2	.	.	1	2
B-62	F	5	.	1	0	1002	Stage 2	1520	OVARY	.	.	2	.	P	.	1
B-63	F	5	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	.	1
B-64	F	5	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	.	1
B-64	F	5	.	1	0	1010	Inflammation, Mixed Cells	1520	OVARY	.	.	2	.	.	1	2
C-66	F	6	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	.	1
C-68	F	6	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	.	1
D-69	F	7	.	1	0	1016	Stage 4	1520	OVARY	.	.	2	.	P	.	1
D-70	F	7	.	1	0	1001	Stage 3	1520	OVARY	.	.	2	.	P	.	2

Animal	Sex	Group	Days	Fate	Examed	Tumors	Lcode	Lesion	Tcode	Tissue	WOD	TumorType	COD	TisGrade	LesGrade	Records
D-70	F	7	.	1	0	1	1010 Inflammation, Mixed Cells	1520	OVARY	.	.	.	2.	.	1	2
D-71	F	7	.	1	0	1	1004 Stage 1	1520	OVARY	.	.	.	2.	P	.	1
D-72	F	7	.	1	0	1	1002 Stage 2	1520	OVARY	.	.	.	2.	P	.	5
D-72	F	7	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	.	1	5
D-72	F	7	.	1	0	1	1010 Inflammation, Mixed Cells	1520	OVARY	.	.	.	2.	.	1	5
D-72	F	7	.	1	0	1	1007 Granulomatous Inflammation	1520	OVARY	.	.	.	2.	.	1	5
D-72	F	7	.	1	0	1	1018 Microsporidia	1520	OVARY	.	.	.	2.	.	1	5
A-81	F	11	.	1	0	1	1002 Stage 2	1520	OVARY	.	.	.	2.	P	.	1
A-82	F	11	.	1	0	1	1001 Stage 3	1520	OVARY	.	.	.	2.	P	.	1
A-83	F	11	.	1	0	1	1016 Stage 4	1520	OVARY	.	.	.	2.	P	.	2
A-83	F	11	.	1	0	1	1017 Asynchronous Development, Right and Left	1520	OVARY	.	.	.	2.	.	3	2
A-84	F	11	.	1	0	1	1002 Stage 2	1520	OVARY	.	.	.	2.	P	.	4
A-84	F	11	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	.	3	4
A-84	F	11	.	1	0	1	1007 Granulomatous Inflammation	1520	OVARY	.	.	.	2.	.	2	4
A-84	F	11	.	1	0	1	1018 Microsporidia	1520	OVARY	.	.	.	2.	.	1	4
B-85	F	9	.	1	0	1	1016 Stage 4	1520	OVARY	.	.	.	2.	P	.	2
B-85	F	9	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	.	1	2
B-86	F	9	.	1	0	1	1002 Stage 2	1520	OVARY	.	.	.	2.	P	.	1
B-87	F	9	.	1	0	1	1016 Stage 4	1520	OVARY	.	.	.	2.	P	.	2
B-87	F	9	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	P	3	2
B-88	F	9	.	1	0	1	1004 Stage 1	1520	OVARY	.	.	.	2.	P	.	2
B-88	F	9	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	P	2	2
C-89	F	10	.	1	0	1	1001 Stage 3	1520	OVARY	.	.	.	2.	P	.	2
C-89	F	10	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	P	1	2
C-90	F	10	.	1	0	1	1016 Stage 4	1520	OVARY	.	.	.	2.	P	.	2
C-90	F	10	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	P	3	2
C-91	F	10	.	1	0	1	1001 Stage 3	1520	OVARY	.	.	.	2.	P	.	2
C-91	F	10	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	P	2	2
C-92	F	10	.	1	0	1	1002 Stage 2	1520	OVARY	.	.	.	2.	P	.	3
C-92	F	10	.	1	0	1	1007 Granulomatous Inflammation	1520	OVARY	.	.	.	2.	.	1	3
C-92	F	10	.	1	0	1	1018 Microsporidia	1520	OVARY	.	.	.	2.	.	1	3
D-93	F	12	.	1	0	1	1001 Stage 3	1520	OVARY	.	.	.	2.	P	.	2
D-93	F	12	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	P	3	2
D-94	F	12	.	1	0	1	1002 Stage 2	1520	OVARY	.	.	.	2.	P	.	3
D-94	F	12	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	.	3	3
D-94	F	12	.	1	0	1	1007 Granulomatous Inflammation	1520	OVARY	.	.	.	2.	.	1	3
D-95	F	12	.	1	0	1	1004 Stage 1	1520	OVARY	.	.	.	2.	P	.	2
D-95	F	12	.	1	0	1	1011 Oocyte Atresia, Increased	1520	OVARY	.	.	.	2.	P	3	2
D-96	F	12	.	1	0	1	1002 Stage 2	1520	OVARY	.	.	.	2.	P	.	1

APPENDIX B
FIGURES AND LEGENDS

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/13/06 DRAFT

FIGURES

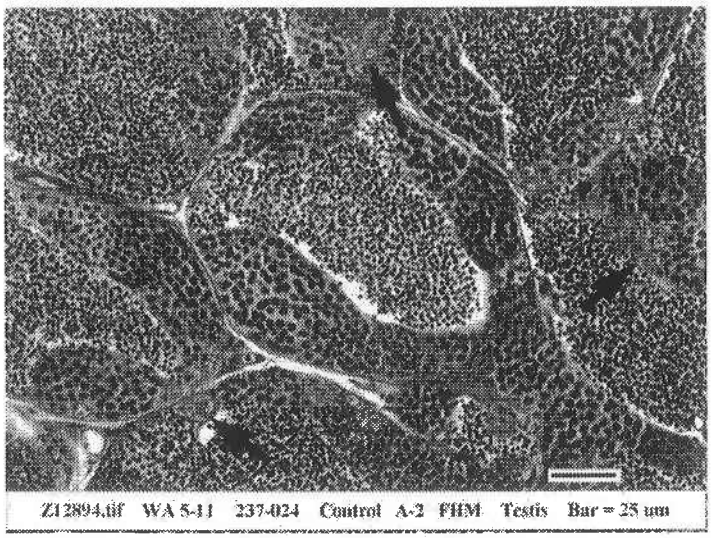

Figure 1 (Z12894). Stage 2 testis from a control group male. The germinal epithelium is moderately thick, the different spermatogenic cell types and spermatozoa are more or less equally represented, and spermatogonia are present occasionally in small clusters (arrows). H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/13/06 DRAFT

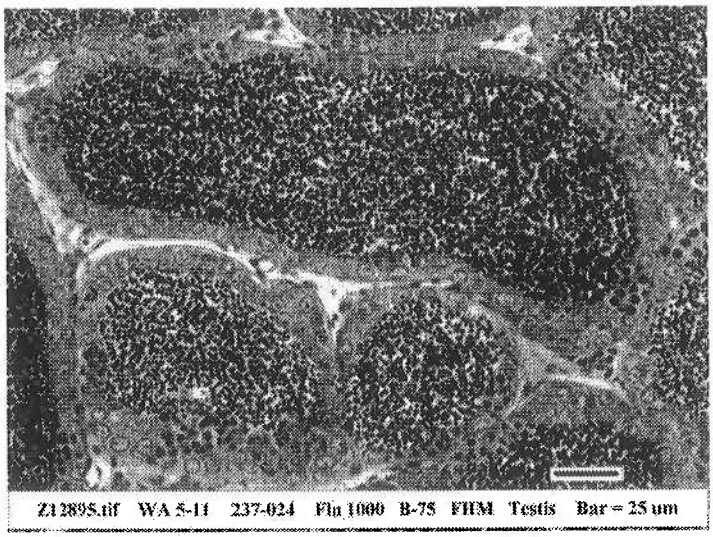

Figure 2 (Z12895). Stage 3 testis from a 1000 µg/L group male. Compared to Fig. 1, the germinal epithelium is thinner and is dominated by spermatogonia. H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/13/06 DRAFT

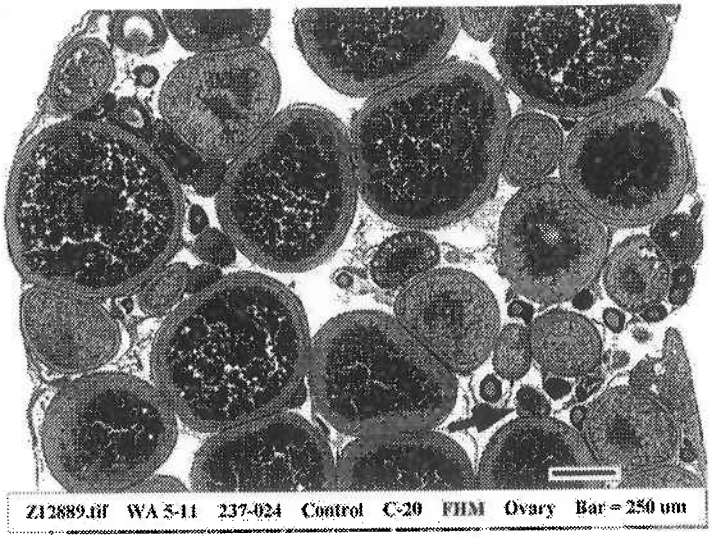

Figure 3 (Z12889). Ovary from a control group female. There is a remnant of a single atretic oocyte in this photographic field (arrow). H&E.

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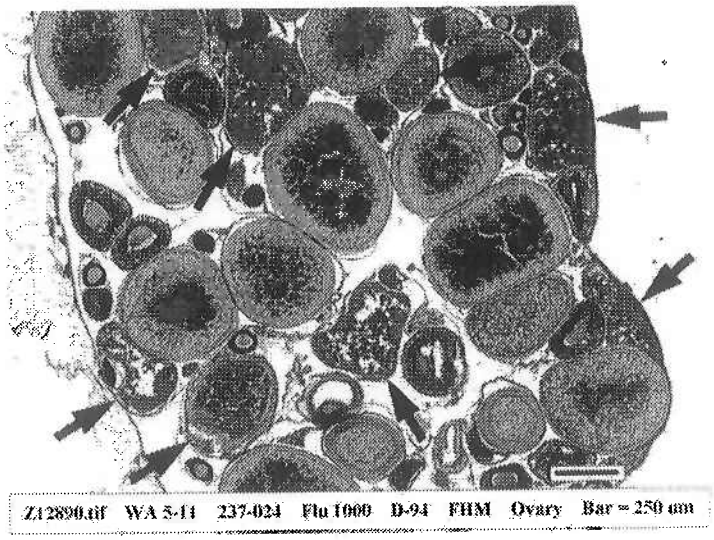


Figure 4 (Z12890). Ovary from a 1000 µg/L group female. Numerous atretic oocytes are evident (arrows). H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/13/06 DRAFT

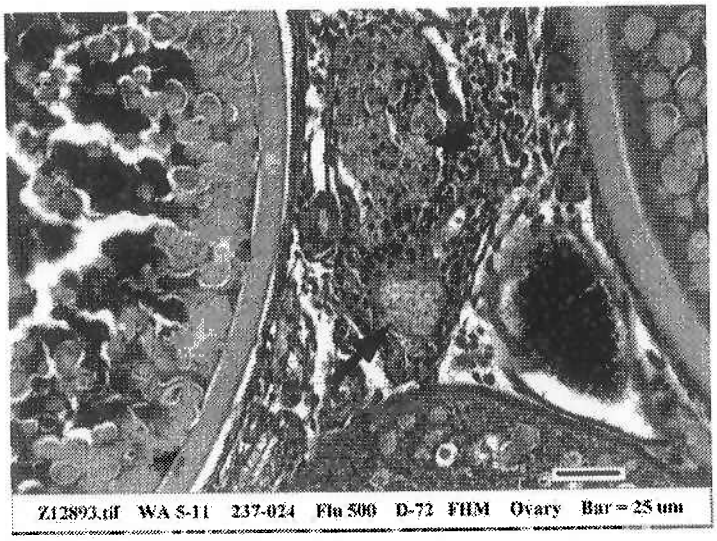


Figure 5 (Z12893). Ovary from a 500 µg/L group female. A microsporidian cyst (arrow) is present within an area of granulomatous inflammation in the ovarian interstitium. Microsporidia are additionally evident within individual macrophages (arrowhead). H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/13/06 DRAFT

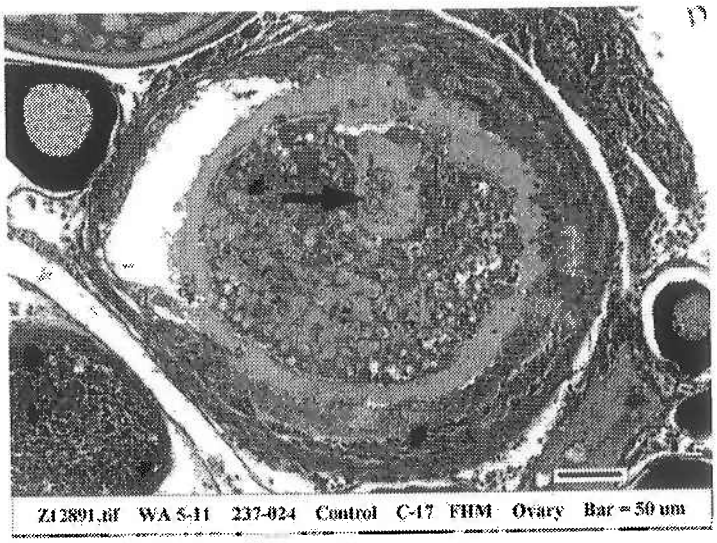


Figure 6 (Z12891). Ovary from a control group female. A microsporidian cyst (arrow) is visible within the ooplasm of an atretic oocyte. H&E.

EPL® Experimental Pathology Laboratories, Inc.

Battelle Pacific Northwest Division, Battelle Memorial Institute
Study Number WA 5-11
1/13/06 DRAFT

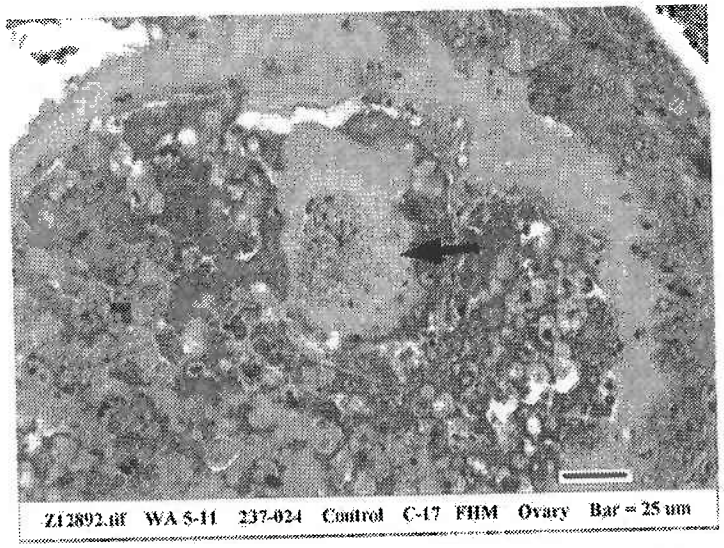


Figure 7 (Z12892). Higher magnification of Fig 6. The parasites appear to be walled off by a dense irregular layer of tissue that resembles the chorion (arrow). H&E.

FIGURES

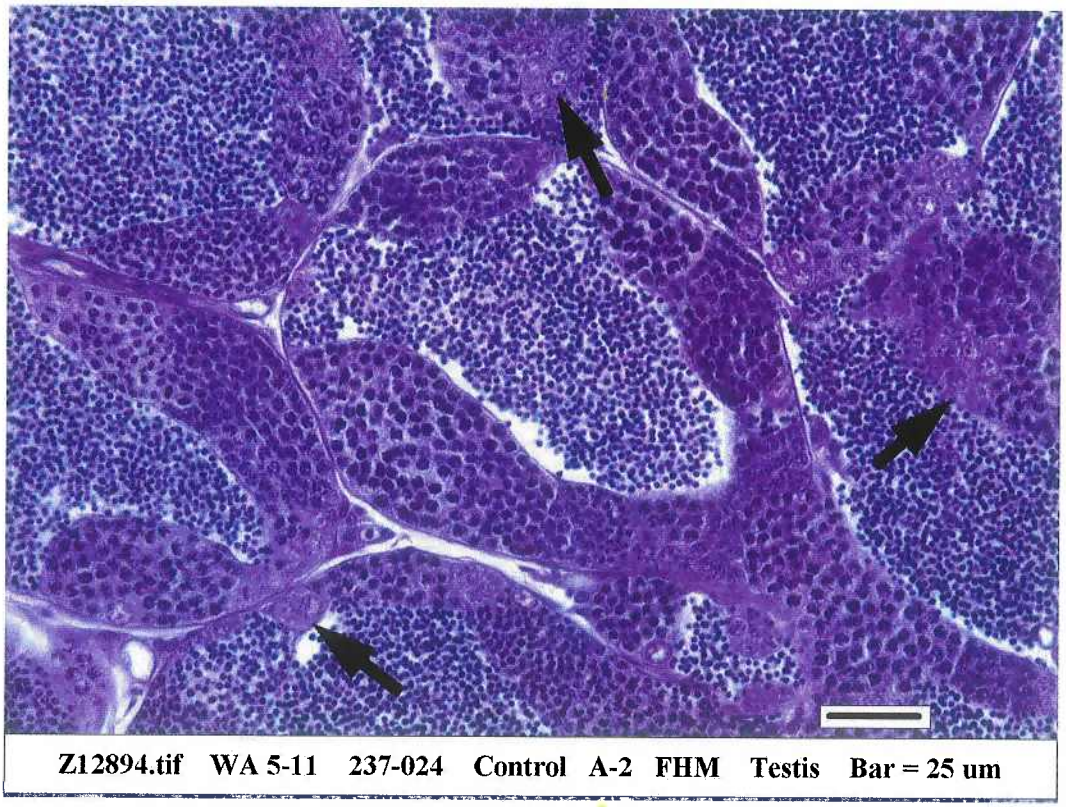


Figure 1 (Z12894). Stage 2 testis from a control group male. The germinal epithelium is moderately thick, the different spermatogenic cell types and spermatozoa are more or less equally represented, and spermatogonia are present occasionally in small clusters (arrows). H&E.

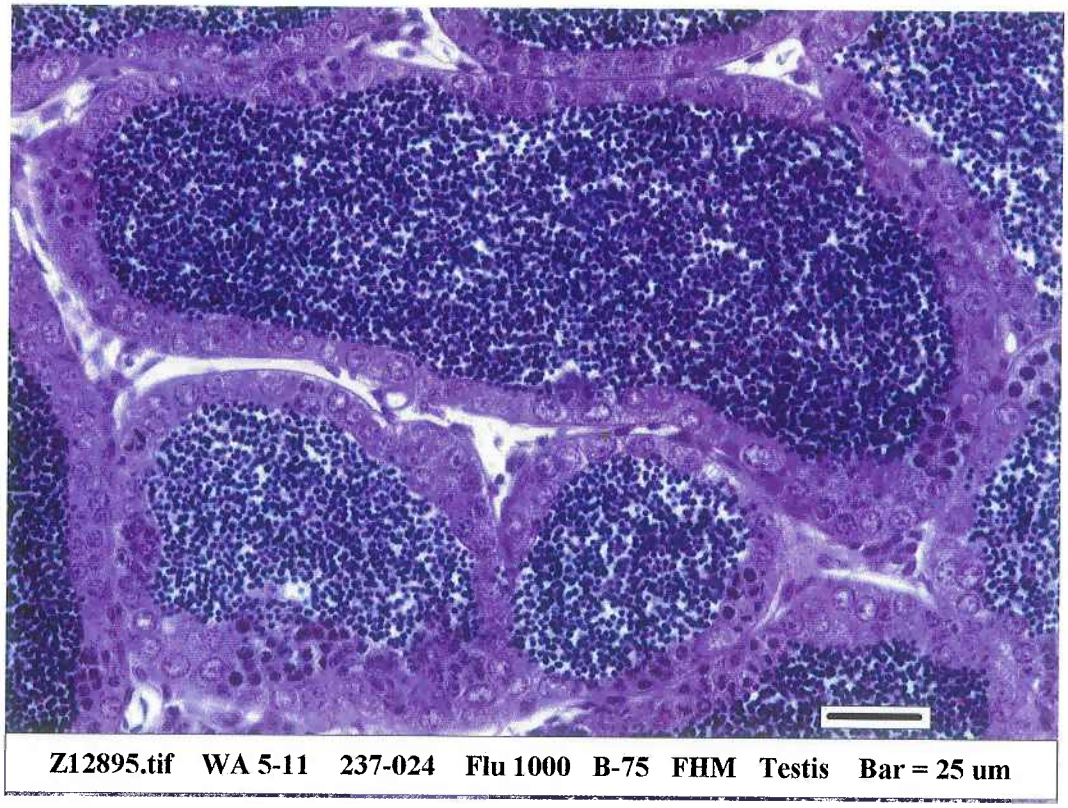


Figure 2 (Z12895). Stage 3 testis from a 1000 µg/L group male. Compared to Fig. 1, the germinal epithelium is thinner and is dominated by spermatogonia. H&E.

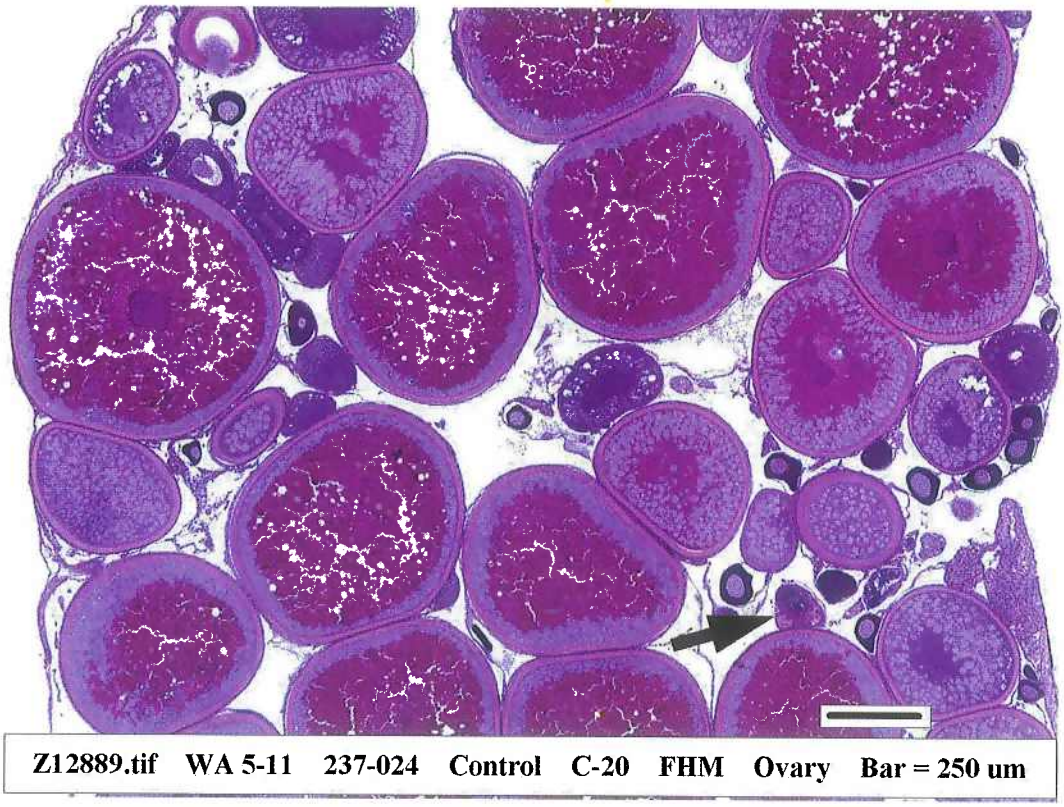


Figure 3 (Z12889). Ovary from a control group female. There is a remnant of a single atretic oocyte in this photographic field (arrow). H&E.

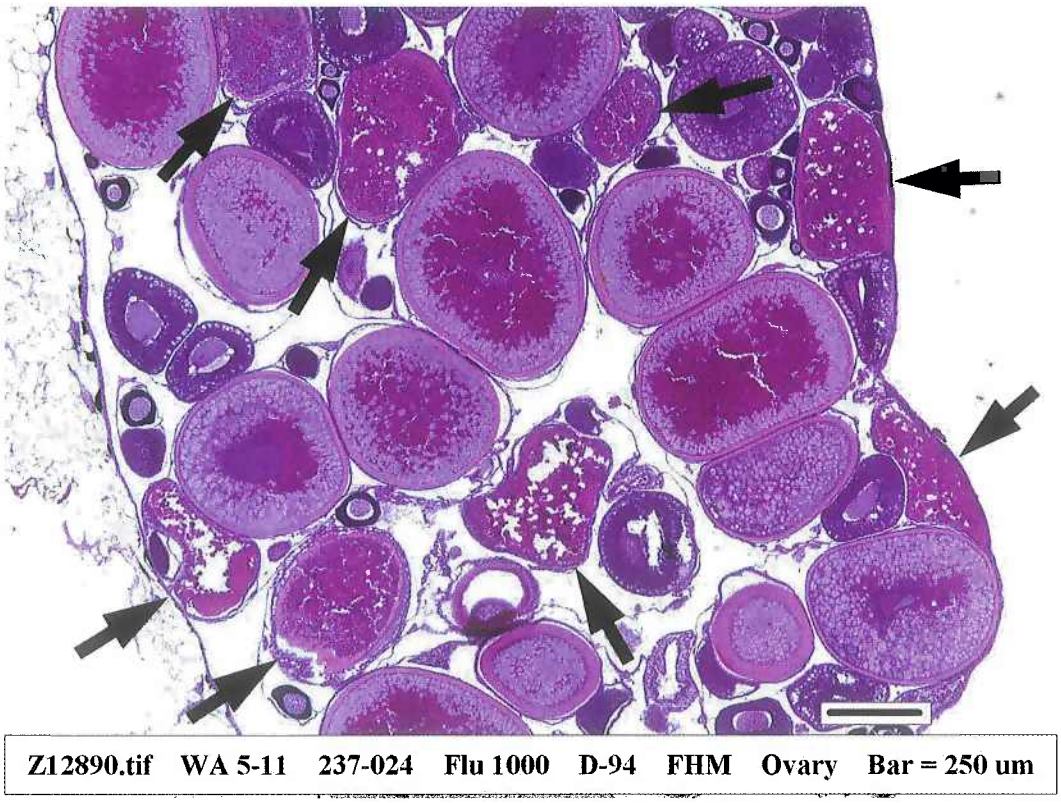


Figure 4 (Z12890). Ovary from a 1000 µg/L group female. Numerous atretic oocytes are evident (arrows). H&E.

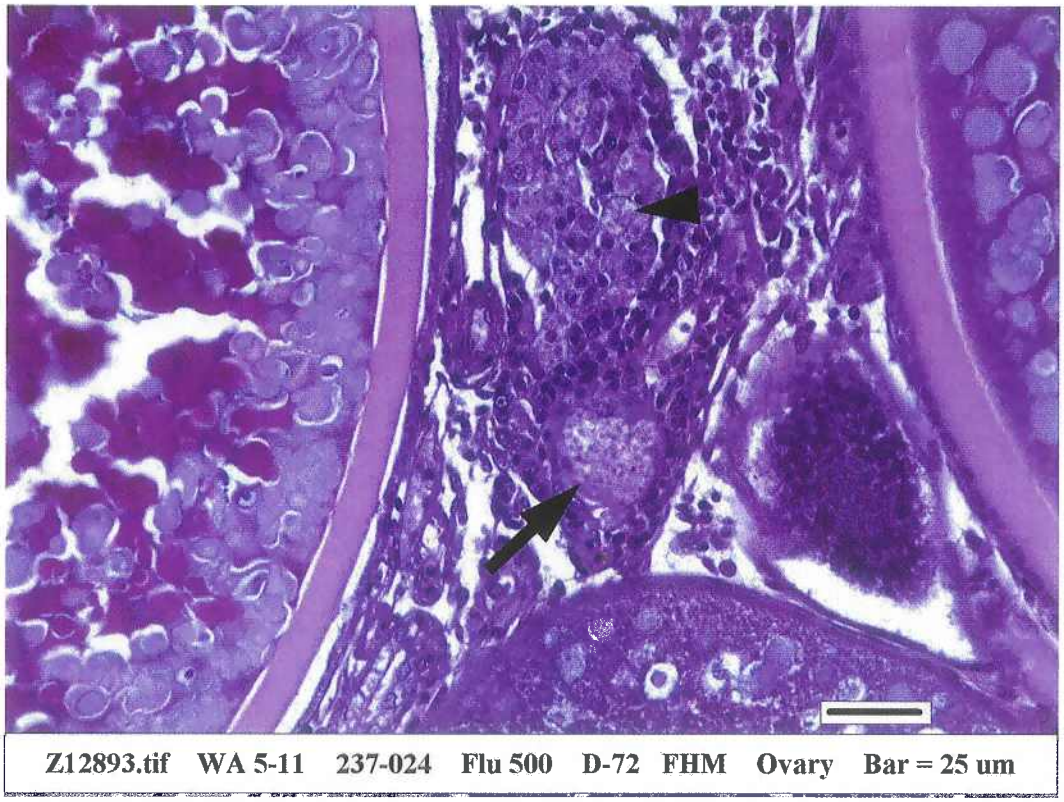
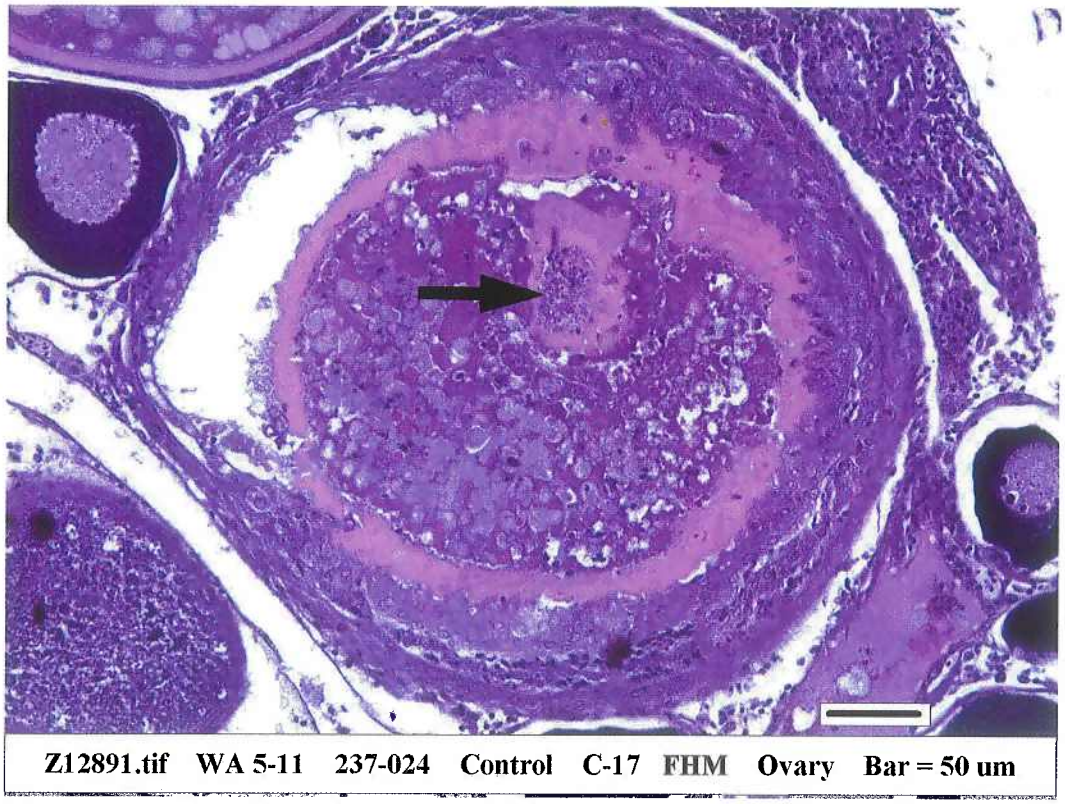


Figure 5 (Z12893). Ovary from a 500 µg/L group female. A microsporidian cyst (arrow) is present within an area of granulomatous inflammation in the ovarian interstitium. Microsporidia are additionally evident within individual macrophages (arrowhead). H&E.



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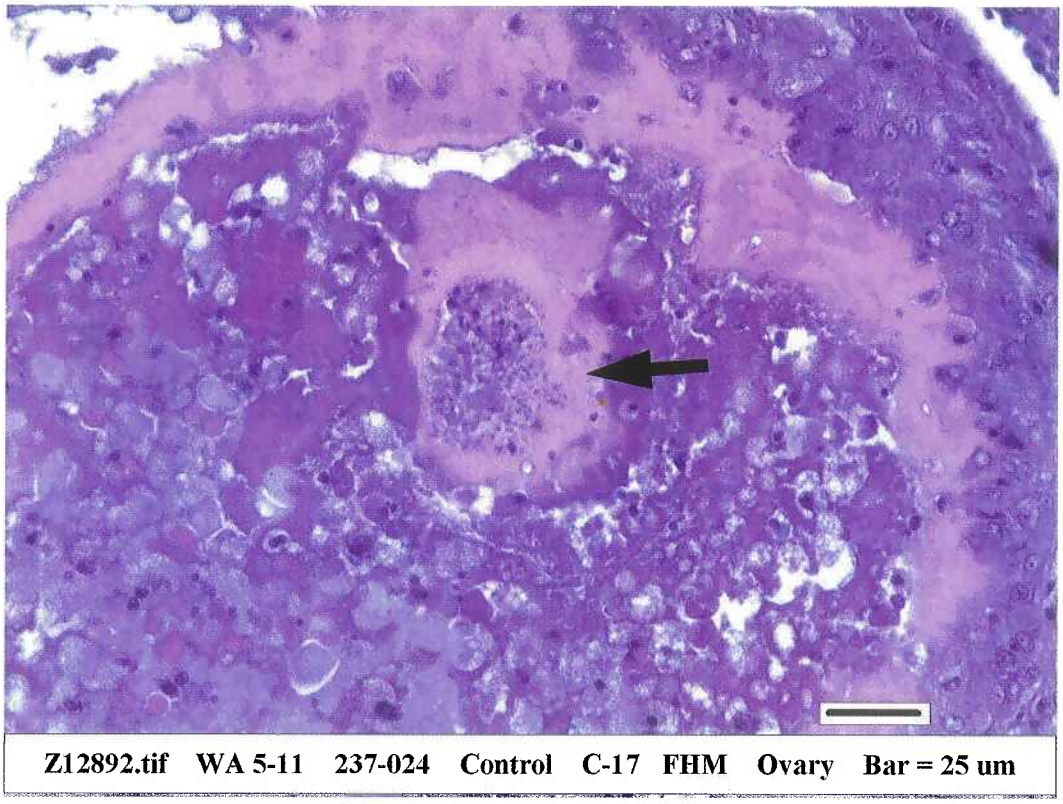

Figure 7 (Z12892). Higher magnification of Fig 6. The parasites appear to be walled off by a dense irregular layer of tissue that resembles the chorion (arrow). H&E.

Appendix D – Chemical Repository Analysis of Test Substances

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Chemical Repository Services for the EDSP
EPA Contract No. 68-W-01-023

1.0 TITLE PAGE

Study Title: Analysis of Test Substances for Work Assignment 5-11

Authors: Tim Fortman, Michael Cobb

Study Initiation Date: May 26, 2005

Study Completion Date: January 3, 2006

Performing Lab: EDSP Chemical Repository,
Battelle Marine Sciences Laboratory,
1529 West Sequim Bay Road,
Sequim, WA 98382

Study Number: EDSP.511-01

Data Requirement: 40 CFR Part 160.105, 160.113

Submitted To: Dr. David P. Houchens,
EDSP Program Manager
Battelle Columbus Operations,
505 King Avenue,
Columbus, OH, 43201-2693

Total Number of Pages: 48

2.0 STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentially is made for any information contained in this study on the basis of its falling within the scope of the United States Environmental Protection Agency Federal Insecticide, Fungicide, and Rodenticide Act Section 10(d) (1)(A), (B), or (C).

Company: Battelle

Company Agent: David P. Houchens, Ph.D.

Title: EDSP Program Manager

Signature: David P. Houchens Date: 12/22/05

3.0 STATEMENT OF COMPLIANCE

This study meets the requirements for 40 CFR Part 160, EPA FIFRA Good Laboratory Practices with the following exceptions:
The method for trenbolone was tested prior to signing the study protocol.
Purity for trenbolone was verified by the chemical repository previously during a non-GLP study.

Note: Protocol, amendments, and deviations are provided in Appendix B of this report.
Method deviations are described in Appendix F of this report.

Study Director: _____ 1/03/06
Michael Cobb
Battelle – Marine Sciences Laboratory
Date

Sponsor's Representative _____ 12/22/05
David Houchens, Ph.D.
Battelle Columbus Operations
Date

Submitter: _____ 12/22/05
Date

4.0 QUALITY ASSURANCE

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160, 17 August 1989. The dates of all audits and inspections and the dates of any findings were reported to the Study Director and Test Facility Management as follows:

ACTIVITY	DATE CONDUCTED	DATE REPORTED TO:	
		STUDY DIRECTOR	MANAGEMENT
Technical Systems Audit - Preparation and Analysis of stability samples	August 3, 2005	August 3, 2005	August 3, 2005
Audit of Data Quality, Ketoconazole data	Nov. 11 & 15, 2005	November 15, 2005	November 15, 2005
Audit of Data Quality, Draft Report and Trenbolone data	December 19, 2005	December 19, 2005	December 19, 2005
Audit of Data Quality, Final Report	December 22, 2005	December 22, 2005	December 22, 2005

Mary E. Lynn
Quality Assurance

12/22/05
Date

5.0 APPROVALS PAGE

Study Title: Analysis of Test Substances for Work Assignment 5-11

Submitted by: Battelle Marine Sciences Laboratory
Address: 1529 West Sequim Bay Road
Sequim, WA 98382

Prepared by:

12-22-05

Timothy Fortman — Date
Senior Chemistry Analyst
Battelle – Marine Sciences Laboratory

Approved by:

01/03/06

Michael E. Cobb — Date
EDSP Chemical Repository Study Director
Battelle – Marine Sciences Laboratory

Approved by:

1/3/06

Eric Crecelius — Date
Manager, EDSP Chemical Repository
Battelle – Marine Sciences Laboratory

Personnel participating in this study:

Analyst: Rebecca Wood, Tim Fortman

Chemical Repository Study Director: Michael Cobb

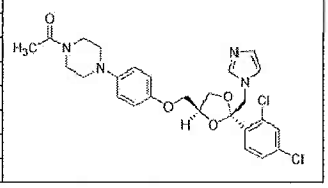
Experimental Start: May 17, 2005

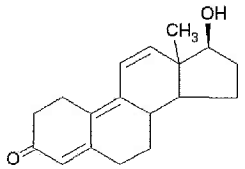
Experimental Termination: August 23, 2005

6.0 EXECUTIVE SUMMARY

Analysis of Test Substances for Work Assignment 5-11

Table 1. Study Test and Reference Substance

Parameter	Test and ReferenceSubstance	Ketoconazole
Compound Name	Ketoconazole	
CAS #	65277-42-1	
Central File No.	1850-2B	
Initial Receipt Date	9/3/2002	
Expiration Date	3/31/2006	
Supplier	Spectrum	
Lot Number	QL0352	
Method	EDSP.H5-032	

Parameter	Test and Reference Substance	Trenbolone
Compound Name	Trenbolone	
CAS #	10161-33-8	
Central File No.	1817-1a thru 1e	
Initial Receipt Date	05/17/02	
Expiration Date	05/01/06	
Supplier	Sigma-Aldrich	
Lot Number	029H3923	
Method	EDSP.H5-031	

Executive Summary

Work Assignment (WA) 5-11 of the Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP) describes a fish screening assay phase 1B follow-up study. The pre-start chemistry formulation and characterization for the test substance ketoconazole is documented in the present report. Method adaptation and validation was carried out for the test substance trenbolone. The Chemical Repository (CR) has the responsibility for carrying out the purity, formulation preparation and analysis development and validation, and formulation stability determinations of selected study test substances for EDSP studies.

The test substances of interest are ketoconazole and trenbolone. The purity for these test substances, as determined by the supplier and confirmed in the CR, is provided in Table 2.

Table 2. Test and Reference Substance Purity

TEST SUBSTANCE	SUPPLIER REPORTED PURITY	LOT NUMBER	CR DETERMINED PURITY[1]
Ketoconazole	99.73%	QL0352	98.83%
Trenbolone	98.7%	029H3923	99.1%[2]

Due to minimal water solubility of the test substance ketoconazole, a saturator column was employed for preparation of the formulation. A saturated solution was prepared by dissolving a known mass of ketoconazole in acetone that was then placed into a saturator column, a stainless steel tube packed with glass wool. The acetone was evaporated off using vacuum. The column was connected to a pump and water from a 55 gallon barrel was pumped through the saturator column until the concentration of ketoconazole reached equilibrium in the water. Water was

[1] Calculations for purity are: area of compound of interest divided by the total area where the total area is adjusted by subtracting a blank area.
[2] Trenbolone purity was determined for a previous work assignment (2-18).

pumped through the saturator column for 7 days to reach equilibration. The concentration of ketoconazole in the water is reported in Table 3.

Table 3. Formulation Concentration (Day 0)

Test Substance	Measured Concentration (average of 3 replicates)
Ketoconazole	2723.5 µg/L

Stability of the formulation prepared in the saturator column was determined from day 0 to day 15. The formulation remained within the ±10% recovery target, defined as stable for this study.

7.0 TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

8.0 INTRODUCTION

The goal of the Battelle-Sequim, Marine Sciences Laboratory (MSL) Chemical Repository for the Endocrine Disruptor Screening Program (EDSP) is to provide the participating laboratory with requested chemicals of documented quality and if required, at concentrations in a matrix appropriate for different toxicological tests. The EDSP Chemical Repository (CR) provides the supplier's information regarding purity and stability, the material safety data sheet (MSDS) chemical information, and independent analysis of purity, formulation preparation and analysis development and validation, and stability in a matrix specified by the Study Protocol: Analysis of Test Substances for Work Assignment 5-11 [EDSP Study Number: EDSP.511-01], made in collaboration with the requesting Study Director. Under Work Assignment (WA) 5-11, the Environmental Protection Agency (EPA) contracted with the CR for formulation, purity verification, and stability of the formulated test substance (Table 5), ketoconazole. In addition, the CR was charged with the adaptation and validation of the trenbolone analytical method.

9.0 GENERAL METHODS

Methods of standard operation of the CR are currently addressed in MSL SOPs numbered R-001 through R-017. These procedures address chemical procurement including procurement of controlled substances, when applicable, which have unique permitting, ordering, handling, inventory, and storage requirements; chemical receipt and chain of custody, chemical log-in and labeling, inventory, chemical storage; stock solution preparation, documentation and archiving; test solution preparation, documentation and shipping; chemical disposal, and CR maintenance over time. The quality assurance (QA) requirements for procurement of chemicals for use in the CR are addressed in the Quality Assurance Project Plan (QAPP) for EDSP Chemical Repository.

9.1 TEST SUBSTANCE PROCUREMENT

As requested by EPA for WA 5-11, ketoconazole, (CAS No. 65277-42-1), and trenbolone, (CAS No. 10161-33-8) were transferred from a previous work assignment (WA 2-18). The trenbolone was used for method adaptation and validation. No further studies were carried out on the trenbolone as the purity and stability of the test substance were evaluated in a previous study. The ketoconazole was used for purity, formulation preparation, analysis development and validation, stability analysis, as specified in 8.0 above, and both ketoconazole and trenbolone were shipped to the participating laboratories as the test substances for use in the *Fish Screening Assay Phase 1b Follow-Up*. The chemicals were originally purchased from two separate suppliers (Table 4). The chemicals were previously logged into the Chemical Management System (CMS) (at purchase) and were given unique CMS barcodes and unique log-in (central file) numbers as per the QAPP for the EDSP Chemical Repository. The chemicals have been stored per supplier recommendations in the Chemical Repository inventory.

Table 4. Study Test and Reference Substance

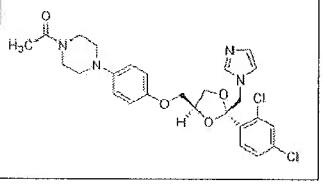
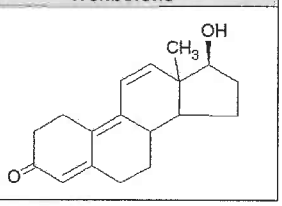
Parameter	Test and Reference Substance	Ketoconazole
Compound Name	Ketoconazole	
CAS #	65277-42-1	
Central File No.	1850-2B	
Initial Receipt Date	9/3/2002	
Expiration Date	3/31/2006	
Supplier	Spectrum	
Lot Number	QL0352	
Method	EDSP.H5-032	

Table 4. Study Test and Reference Substance (continued)

Parameter	Test and Reference Substance	Trenbolone
Compound Name	Trenbolone	
CAS #	10161-33-8	
Central File No.	1817-1a thru 1e	
Initial Receipt Date	05/17/02	
Expiration Date	05/01/06	
Supplier	Sigma-Aldrich	
Lot Number	029H3923	
Method	EDSP.H5-031	

9.2 TEST SUBSTANCE PURITY

Purity of the lot of trenbolone employed in this study was carried out for a previous work assignment, (WA 2-18), and reported in this study. Ketoconazole purity was determined using high performance liquid chromatographic analysis (HPLC) with fluorescence detection. Purity verification for this test substance was conducted by making a solution of about 150 ug/L of ketoconazole in 75% acetonitrile, 25% water. This matrix was then run on the HPLC using a fluorescence detector. A 75% acetonitrile, 25% water blank was also analyzed on the system. The purity was determined by comparing the area of the peak associated with the substance of interest with the total area of all the peaks in the chromatogram. The areas associated with peaks common to the blank were eliminated by subtraction. The percentage associated with the largest peak represented the purity of the test substance. This result was compared to the supplier's certificate of analysis/purity (Appendix A). The HPLC was set up with a Perkin Elmer 250 pump, with a Gilson 294 liquid autosampler with fluorescence detector (Waters 474) set at an excitation wavelength of 245 nm and an emission wavelength of 370 nm. The column employed was a Phenomenex SYNERGI 4 μ Hydro-RP80A 250 X 4.6 mm, serial number 258206-4. The pump used was a binary gradient pump with one reservoir filled with a 75% methanol, 25% water eluent. One replicate was analyzed.

The supplier determined purity for the test/reference substances utilized for calibration and calibration verification are listed in Table 5.

Table 5. Supplier Determined Test/Reference Substance Purity

Reference Substance	Lot Number	Supplier Reported Purity
Ketoconazole	QL0352	99.73%
Trenbolone	029H3923	98.7%

9.3 FORMULATION PREPARATION AND STABILITY DETERMINATIONS

The study plan for formulation preparation, analysis development and validation, and stability testing, based on the Technical Work Plan for WA 5-11, was developed and documented in the Study Protocol: *Analysis of Test Substances for Work Assignment 5-11, EDSP Study Number: EDSP 511-01.* This protocol is presented in Appendix B.

A saturated solution of ketoconazole was prepared on 08/01/05 for determining stability (Table 6). Briefly, a column packed with glass wool was coated with the test substance, ketoconazole, water from a 55 gallon barrel was passed through this column until the ketoconazole level in the water reached steady state. The saturated solution was analyzed on 08/03/05 in triplicate for calculation of a mean concentration and relative standard deviation (RSD). The solution had not reached saturation on 08/03/05, so it was reanalyzed on 08/08/05 (time zero for stability).

The barrel containing the saturated solution was stored in the wet lab at conditions mimicking test conditions. This formulation was sampled on day zero, day 4, and day 15. The stability study was originally planned for 21 days but shortened to 15 days[3] based on a communication from the work assignment leader (WAL) indicating in-life test volume requirements would mandate making up new solution every couple of weeks.

Table 6. Formulation Prepared for Stability Testing

Test Substance	Target Conc.	Nominal Conc.	Stock Matrix
Ketoconazole	saturated	2723 ug/L	water

9.4 ANALYTICAL METHODS

The analytical method adapted (from WA 2-18) and validated for trenbolone is attached in Appendix E. Results of method testing for trenbolone are in Appendix C. Solution stability, purity, and accuracy of ketoconazole were evaluated using the method described below. The frequency of determinations and the duration of testing were selected by the WAL and the chemists based on *a priori* knowledge of the stability of this chemical in water and usage requirements appropriate to the study demands.

9.4.1 Ketoconazole Formulation Sampling

For ketoconazole, 0.025 mL of the saturated solution was pipetted into a 1.8 mL autosampler vial. 0.975 ml of a solution of 70% acetonitrile, 30% deionized water was then added to the vial. The vial was then capped and was ready for analysis. All solutions are run on the HPLC. All sampling was done in triplicate.

9.4.2 Analysis of Ketoconazole with HPLC with Fluorescence Detection

Analysis of stability and purity samples for ketoconazole were done using HPLC with fluorescence detection. System setup parameters are described in Table 7.

Table 7. HPLC System Setup - Ketoconazole

Instrument	Battelle MSL ID, HPLC1
Column	Reverse Phase, 250 X 4.6 mm Synergi 4 μ RP80A (Phenomenex Torrance, CA)
Eluent	75% Acetonitrile, 25% Water
Eluent Flow Rate	1 mL/minute
Detector Type	fluorescence, excitation wavelength set at 245 mn, emission wavelength set at 370 nm
Column Oven Temp	Ambient
Injector loop size	100 ul
Injection Volume	100 ul
Run Time	18 minutes

Calibration of the HPLC was done using 5 calibration standards. To start, a stock is made at a concentration of about 100 μg/mL. Approximately 0.0100 grams is weighed into a 100 mL volumetric flask and diluted to the mark with methanol. Then, 0.5 mL of this stock is diluted to 50 mL with methanol. The intermediate stock is then serially diluted to make standards ranging from about 150 ng/mL down to 5 ng/mL using a solution that will mimic the eluent, 75% acetonitrile, 25% water.

Calibration linearity specifications were an R^2 value of greater than or equal to 0.995. Initial and continuing calibration verification standards (ICV and CCV) were run where the ICV was a solution made from an independent standard and diluted to be within the calibration range of the standards. The CCV was a mid point calibration standard run every 10 samples to verify the

[3] See protocol deviation in Appendix B – this covers the shortened stability study and the deviation from the originally specified testing intervals.

HPLC remained calibrated for the entire run. Both ICV and CCV specification require that results remain within 10% of target concentrations. Matrix spikes and blanks were run for method validation and with each sampling. A matrix spike was prepared prior to the start of the test.

10.0 RESULTS

10.1 TEST SUBSTANCE PURITY

The purities of ketoconazole and trenbolone determined by the CR were 98.83% and 99.1% respectively (Table 8), within the protocol set accuracy window of ±3% of the values provided on the certificates of analysis as specified by the suppliers. As previously noted, the purity for trenbolone was carried out for work assignment (WA) 2-18 and reported herein. Chromatograms for the purity determination of ketoconazole are provided as Figures 1 and 2, a blank and the test substance respectively.

Table 8. Summary of Test Substance Purity

TEST SUBSTANCE	SUPPLIER REPORTED PURITY	LOT NUMBER	CR DETERMINED PURITY
Ketoconazole	99.73%	QL0352	98.83%
Trenbolone	98.7%	029H3923	99.1%

10.2 FORMULATION ANALYSIS RESULTS

Table 9 shows the results of the stability formulation tests on day zero (0).

Table 9. Nominal & Actual (Day 0) Formulation Concentration Comparisons

Test Substance	Nominal Conc.	Avg. Measured Conc.	RSD (%) for replicate measurements	PD* (%), nominal versus measured
Ketoconazole	N/A	2723 ug/L	14.52%	N/A

*PD = percent deviation. Note, the nominal concentration was a saturated solution, PD and nominal concentration not applicable

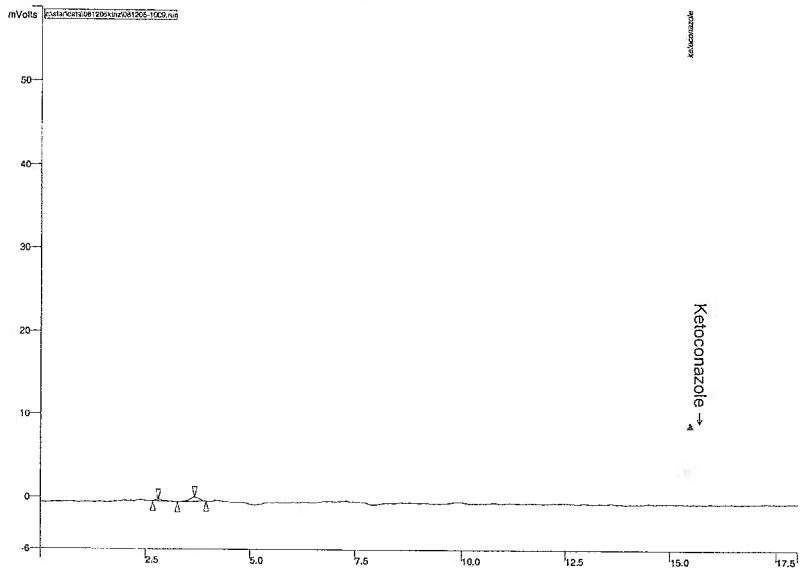


Figure 1. Typical Chromatogram for WA 5-11 HPLC Analysis of Ketoconazole Blank

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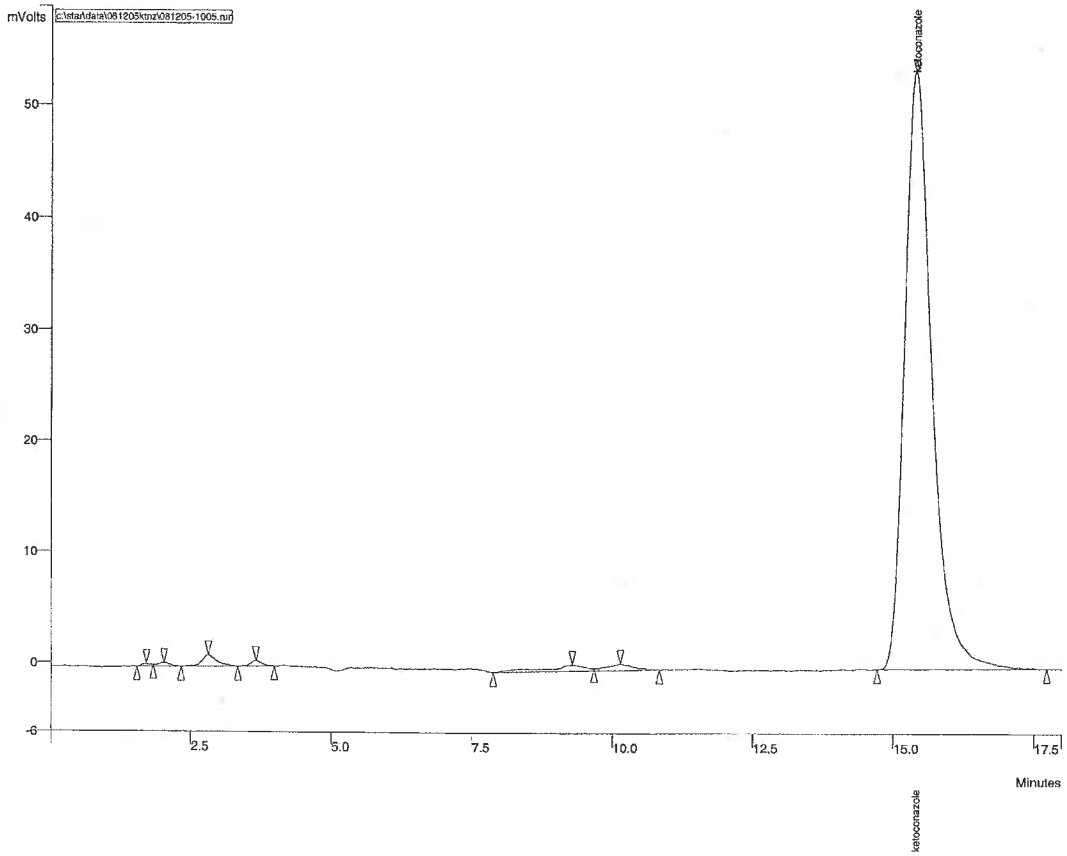

Figure 2. Typical Chromatogram for WA 5-11 HPLC Analysis of Ketoconazole

10.3 FORMULATION STABILITY RESULTS

Formulation stability as a percent of time 0 values is tabulated in Table 10 and plotted in Figure 3.

Table 10. Solution Stability Results

Test Substance	Test Duration	Time 0 Conc.	Percent of Time 0
Ketoconazole	15 days	2723 ug/L	93.5% to 105%

Method detection limits (MDL) and ICV/CCV recovery ranges for ketoconazole is provided in Table 11. The analytical and quality control (QC) results are presented in Appendix C.

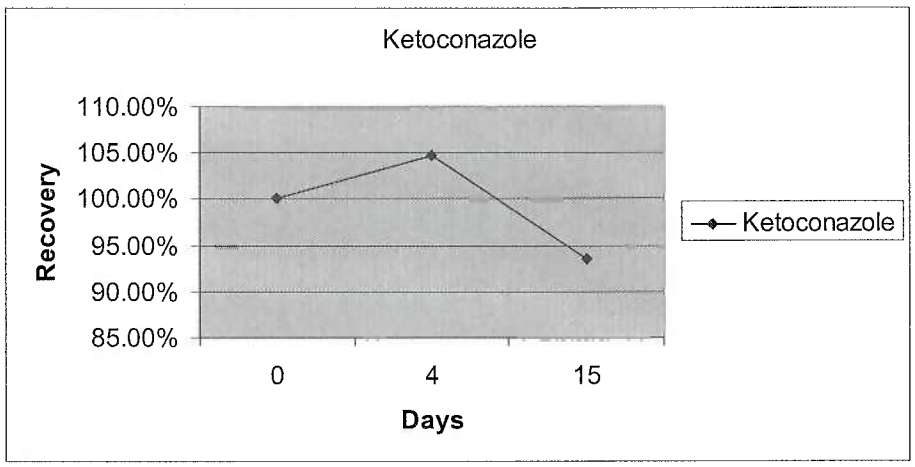

Figure 3. Recoveries of Ketoconazole Plotted Against Time

Table 11. MDL and ICV/CCV Recovery Ranges

Test Substance	Method Detection Limit	ICV/CCV Recoveries
Ketoconazole	1.48 µg/L	95.0% to 109.6%

Calibration curves all met the R^2 criteria of 0.995, see table 12. Blanks and matrix spikes were analyzed with every batch for QC purposes. All blanks were less than 3 times the detection limit for all the compounds.

Table 12. Calibration Acceptance

Calibration Curve Date	R^2 Value for Ketoconazole
7/22/05	0.999961
8/3/05	0.999951
8/8/05	0.999947
8/12/05	0.999942
8/23/05	0.999957

11.0 CONCLUSIONS

11.1 TEST SUBSTANCE PURITY

Purity determinations for trenbolone and ketoconazole, carried out by the CR, compared favorably (within 1%) to the supplier's reported results.

11.2 FORMULATION ANALYSIS

Comparisons of the nominal and actual concentrations of the ketoconazole formulations was not possible due to the time 0 value used as a nominal value.

11.3 FORMULATION STABILITY

Stability of the ketoconazole solutions remained within 90% of the nominal concentration for the 15 days that the stability study was run. Stability was terminated prior to the set 21 days due to a decision that the solutions will need to be generated more frequently and 15 days would be sufficient for demonstrating stability.

11.4 ARCHIVING

Archive samples of the test substances employed in this study will be maintained in the EDSP Chemical Repository for the shelf life indicated on the chemical label.

The protocol, any amendments, all records and the final report generated as a result of this study will be transported to and maintained for archival purposes at the following address:

PNNL Records Management
540 Fifth Street
Richland, WA 99352
PH: 509.375.2340

APPENDIX A

SUPPLIER'S CERTIFICATES OF TEST SUBSTANCE ANALYSIS/PURITY

06/19/2002 03:14PM PAGE 002 OF 002

CERTIFICATE OF ANALYSIS

Date: 06/19/02 Page 1

PRODUCT: KETOCONAZOLE USP

CATALOG NO: K1149 ** CUSTOMER P.O.: 2581
LOT NO: QL0352

CUSTOMER NO: GSA772
COUNTRY OF ORIGIN: USA
MANUFACTURER: SPECTRUM

DESCRIPTION	LIMIT MIN.	LIMIT MAX.	RESULT
ASSAY (DRIED BASIS)	98.0	102.0 %	99.73 %
MELTING RANGE	148	152 C	149 - 150 C
SPECIFIC ROTATION $[a]_D$	-1 to	+1	-0.786
LOSS ON DRYING	--	0.5 %	0.22 %
RESIDUE ON IGNITION	--	0.1 %	0.05 %
HEAVY METALS	--	0.002 %	<0.002 %
CHROMATOGRAPHIC IMPURITIES	-- TO PASS TEST		PASSES TEST
ORGANIC VOLATILE IMPURITIES	-- TO PASS TEST		PASSES TEST
IDENTIFICATION	-- TO PASS TEST		PASSES TEST
EXPIRATION DATE			09/30/2005

EXTENDED TO 3-31-06 AS PER ATTACHED LETTER
AK 10-25-05

APPROVED BY: Lilian D. Casabar
LILIAN D. CASABAR
CofA COORDINATOR
New Brunswick, N.J. Plant

encl 194

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Spectrum Laboratory Products, Inc.

Corporate Headquarters:	*East Coast Plant:*
14422 S. San Pedro St.	755 Jersey Ave.
Gardena, CA 90248	New Brunswick, NJ 08901

(310) 516-8000 • Fax: (310) 516-9843 • (732) 214-1300

FROM FAXCOM TO 6/19/2002 3:17 PM Page 2

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Trenbolone, ≥98%
Product Number	T3925
Product Brand	
CAS Number	10161-33-8
Molecular Formula	$C_{18}H_{22}O_2$
Molecular Weight	270.37

TEST	SPECIFICATION	LOT 029H3923 RESULTS
APPEARANCE	WHITE TO YELLOW POWDER	YELLOW POWDER
SOLUBILITY	CLEAR FAINT YELLOW TO YELLOW-GREEN SOLUTION AT 50MG/ML IN CHLOROFORM	CLEAR YELLOW
PROTON NMR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS
CARBON		79.8%
PURITY BY HPLC	MINIMUM 95%	98.7%
QC ACCEPTANCE DATE		JUNE 1999

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

APPENDIX B

STUDY PROTOCOL, AMENDMENTS, AND DEVIATIONS

Study Protocol:
Analysis of Test Substances for Work Assignment 5-11
EDSP Study Number: EDSP.511-01

Study Objective:

The following tasks will be carried out for the (2) two test *Chemicals* as specified in Table 2:

1. Prepare and test an analytical method as required for each of the test substances over the concentration ranges needed to measure the target stock concentrations.
2. Determine the stability of ketoconazole dissolved in the specified carrier (at the concentrations specified in Table 2), over a 21 day period.
3. Provide a report documenting the results on the above tasks.

This study is in support of EPA contract number 68-W-01-023, MSL Work Assignment Number 5-11, *Fish Screening Assay Phase 1B Follow-Up.*

Address of Testing Facility:	**Address of Sponsor's Representative**
Battelle – Marine Research Operations	Battelle
1529 West Sequim Bay Road	550 King Avenue
Sequim, Washington 98382	Columbus, Ohio 43201-2693
Ph: (360) 681-4580	Ph: (614) 424-3564
FAX (360) 681-3699	FAX (614) 458-3564
Email: michael.cobb@pnl.gov	Email: houchensd@battelle.org

Proposed experimental start and termination dates:

Start Date – May 25, 2005
Termination Date – August 31, 2005

Definitions:

Test Substance: The test substances are the 2 chemicals listed in Table 2. The test substances are the subject chemicals of the tasks described in this protocol.

Reference Substance: The reference substances are identical chemicals to the test substances and may be from the same manufacturer and lot, or purchased as different lots and/or possibly from separate manufacturers than the test substances. The source, purity, and lot number of reference substances will be documented in the data and reported. Regardless of the source, the reference substance solutions will be made up separately from the test substance solutions. The reference substances are used for the calibration standards in the analytical methods referenced in Table 2. A reference substance can also be a material used to facilitate the analysis of the test substance, such as, an internal standard.

TABLE 1
Test Substance Abbreviations:

Chemical	Abbreviation
Ketoconazole	**Ktcnz**
Trenbolone	**Trnbln**

TABLE 2
Test Substance Specifications:

Chemical Name	Ktcnz	Trnbln
Manufacturer	Spectrum	Sigma
CAS #	65277-42-1	10161-33-8
Lot #	QL0352	17129CA
Supplier Purity requirement	≥ 97%	≥ 97%
Supplier Purity Claim	99.73%	98.7%
Target Concentration Stock Solution	TBA[1]	1 mg/L
Duration Stability Study	21 Days	NA[2]
Concentrations for Stability Study	TBA	NA[2]
Carrier (Vehicle)	Well Water	Well Water
Analytical Method	EDSP.H5-032	EDSP.H5-031

[1]TBA = to be amended
[2] Will use data from previous EDSP Chemical Repository study

Experimental Design:

- Analytical methods will be tested for each of the test substances.
- Purity of ketoconazole will be verified using HPLC, GC/MS, GC/FID or other method as appropriate, and should be within ±3% of the value provided on the Certificate of Analysis by the manufacturer. To use substances with values that fall outside this ±3% range or are less than 97% pure, written pre-approval must be secured from the designated EPA work assignment manager.
- Solubility of ketoconazole will be assessed visually in the carrier at the stock formulation concentration (see Table 2). The accuracy of attaining the target concentration for the formulations will be verified in triplicate using the analytical methods referenced in Table 2. Acceptable accuracy will be ±10 percent of the target concentration.
- Stability test solutions – Stability testing of ketoconazole will be carried out at the stock concentration level and the low exposure concentration (as specified in Table 2), stored in the dark (i.e., same storage conditions of solutions employed in the in-life studies of WA 5-11) at room temperature. Nominal concentrations to be tested in are delineated

in Table 2, but the actual concentrations used for testing will be within ±10 percent of the target concentration.

- Storage and Labeling Requirements of Formulations – Stock formulations will be stored at room temperature. Minimally, containers will be uniquely labeled with the name of the test substance, the date of preparation, the formulation concentration, and the study number.
- Testing Schedule – Samples will be analyzed the day of collection from the test formulation
- Replicates – 3 aliquots per sample tested at each analysis time point.
- Sampling schedule. – Samples will be collected for analysis at initiation of the stability study (on day of formulation preparation), then on days 7, 14, and 21 of storage (if a test date falls on a weekend or holiday, testing scheduled for that date will be carried out on the closest work day).
- For details of the analytical methods see the substance specific method cited in Table 2.

Data Analysis:

The stability data collected on days 0, 7, 14, and 21 (average of triplicate determinations) will be compared to the nominal test concentration prepared for the study. Percent variation from the nominal concentration will be used to determine instability. If a solubility problem is encountered with ketoconazole, and a saturator column is required, stability data will be compared to the day zero results and for the acceptance criteria day zero will replace nominal in the calculations.

Acceptance Criteria:
Acceptable stability will be defined as the concentration not varying more than 10 percent from the nominal concentration over the 21 day stability period. The Work Assignment Study Director/Principal Investigator will be consulted for a recommended course of action for any data found outside the ±10% acceptance range. If needed, more frequent preparation of stock solutions will be recommended for in-life studies, and in-life sampling and testing will be coordinated to insure testing is carried out within the viable sample stability window.

Regulatory requirements:

This study will be conducted in compliance with EPA FIFRA Good Laboratory Practices (40 CFR, Part 160). An EDSP QA representative will inspect the study at least once while in-progress and will audit the data and final report.

Report:

A final report covering the following information for both chemicals (where applicable) will be issued to the Sponsor Representative (Dr. David Houchens, EDSP Program Manager), who will then forward the report to the testing laboratories:

Title Page
Executive Summary
Table of Contents
Introduction
General Methods
    Chemical Procurement
    Purity

Formulation Preparation (Methods)
Stability Testing Plan Design and Detail
Analytical Method

Results
- Purity
- Formulation Analysis
- Analytical Method Validation
- Formulation Stability

Conclusions
Appendix
- Manufacturer's Certificates of Analysis
- Stability Testing Protocol
- List of Protocol Amendments
- Analytical Results of Stability Testing

Records to be maintained:

All records, including the protocol, any amendments, and the data and final reports, generated as a result of analysis of the two test substances evaluated for this study, will be transported to and maintained for archival purposes at the following address:

PNNL Records Management
540 Fifth Street
Richland, WA 99352
PH: 509.375.2340

Approval:

Chemical Repository Study Director _____ 5/26/05
Michael Cobb **Date**

Chemical Repository Manager _____ 5/26/05
Eric Crecelius, Ph.D. **Date**

Sponsor Representative _____ 5/25/05
David Houchens, Ph. D. **Date**

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.511-01
AMENDMENT NUMBER: A-1

ENDOCRINE DISRUPTOR SCREENING PROGRAM AMENDMENT REPORT

STUDY NUMBER: EDSP.511-01			DATE: October 13, 2005		
AMENDMENT NUMBER: A-1			WAL/STUDY DIRECTOR:		
NOTEBOOK NUMBER: N/A			Michael Blanton/Michael Cobb		
TITLE OF STUDY: Analysis of Test Substances for Work Assignments 5-11: EDSP Study Number: EDSP.511-01					
QAPP/PROTOCOL ID: Work Assignment 5-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[ ]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

All protocol details that will be amended are indicated in bold, underlined, and in a Georgia font.

TABLE 2
Test Substance Specifications:

Chemical Name	Ktcnz	Trnbln
Manufacturer	Spectrum	Sigma
CAS #	65277-42-1	10161-33-8
Lot #	QL0352	17129CA
Supplier Purity requirement	≥ 97%	≥ 97%
Supplier Purity Claim	99.73%	98.7%
Target Concentration Stock Solution	**TBA**[1]	1mg/L
Duration Stability Study	21 Days	NA[2]
Concentrations for Stability Study	**TBA**	NA[2]
Carrier (Vehicle)	Well Water	Well Water
Analytical Method	EDSP.H5-032	EDSP.H5-031

[1] **TBA = To Be Amended**
[2] Will use data from previous EDSP Chemical Repository study

PROTOCOL AMENDMENT
STUDY NUMBER: EDSP.511-01
AMENDMENT NUMBER: A-1

AMENDMENT:

TABLE 2
Test Substance Specifications:

Chemical Name	Ktcnz	Trnbln
Manufacturer	Spectrum	Sigma
CAS #	65277-42-1	10161-33-8
Lot #	QL0352	17129CA
Supplier Purity requirement	≥ 97%	≥ 97%
Supplier Purity Claim	99.73%	98.7%
Target Concentration Stock Solution	≥ 400 µg/L	1mg/L
Duration Stability Study	21 Days	NA[1]
Concentrations for Stability Study	2723.49[2] µg/L	NA[1]
Carrier (Vehicle)	Well Water	Well Water
Analytical Method	EDSP.H5-032	EDSP.H5-031

[1] Will use data from previous EDSP Chemical Repository study
[2] This concentration was achieved through use of a saturator column

REASON FOR CHANGES:

Concentration of ketoconazole was determined during formulation work carried out at beginning of study. Changed footnote number two to footnote number one when first footnote was eliminated. Added a new footnote for clarification.

Approvals:

Work Assignment Leader _____ Date 11/30/05

Study Director _____ Date 10-21-05

EDSP QA Representative _____ Date 10/21/05

MSL Laboratory Director _____ Date 21 OCT 05

EDSP Program Management _____ Date 10/18/05

EDSP Battelle QAM _____ Date 10-17-05

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

DATE: August 18, 2005

PROTOCOL DEVIATION
STUDY NUMBER: EDSP.511-01
DEVIATION NUMBER: D-1
DATE: December 15, 2005
Page 1 of 2

ENDOCRINE DISRUPTOR SCREENING PROGRAM DEVIATION FORM

STUDY NUMBER: EDSP.511-01			DATE: December 15, 2005		
AMENDMENT NUMBER: D-1			WAL/STUDY DIRECTOR:		
NOTEBOOK NUMBER: N/A			Michael Blanton/Michael Cobb		
TITLE OF STUDY: Analysis of Test Substances for Work Assignment 5-11					
QAPP/PROTOCOL ID: Work Assignment 5-11					
AMENDMENT RELATING TO:					
[ ]	QAPP	[ ]	QMP	[x]	Protocol
[]	SOP	[ ]	Method		

ORIGINAL DOCUMENT SPECIFICATIONS:

1. **Experimental Design**:
   - Stability test solutions – Stability testing of ketoconazole will be carried out at the stock concentration level and the low exposure concentration (as specified in Table 2), stored in the dark (i.e., same storage conditions of solutions employed in the in-life studies of WA 5-11) at room temperature.
2. **Experimental Design**:
   - Sampling schedule. – Samples will be collected for analysis at initiation of the stability study (on day of formulation preparation), then on days 7, 14, and 21 of storage (if a test date falls on a weekend or holiday, testing scheduled for that date will be carried out on the closest work day).

DEVIATION:

1. The stability study was carried out at the stock concentration only.
2. Sample collection and testing was done on day zero, day 4, and day 15. The study was terminated at day 15.

REASON/IMPACT:

1. The analytical chemist referenced a draft protocol rather than the final signed copy. The draft protocol specified carrying out stability testing on the stock solution only. No impact, the stock indicated the ketoconazole was stable.
2. The Work Assignment Leader authorized termination of the test at day 15 since the in-life testing required volumes of stock be made up at intervals shorter than 15 days. The initial stability measurement was made at 4 days to provide the in-life labs immediate information on the stability of the stock since in-life was starting during the stability testing period. Once stability was demonstrated at 4 days, the 7 day test interval was dropped as being unnecessary. No impact.

PROPOSED CORRECTIVE ACTION AND SCHEDULE FOR COMPLETION:

None, beyond this documentation.

ACTIONS TO PREVENT RECURRENCE:

Study Director will ensure that a signed copy of the appropriate protocol is in each Chemical Characterization Task Notebook.

PROTOCOL DEVIATION
STUDY NUMBER: EDSP.511-01
DEVIATION NUMBER: D-1
DATE: December 15, 2005
Page 2 of 2

Approval:

Work Assignment Leader		Date 12-20-05
Study Director		Date 12-22-05
EDSP QA Representative	Mary E Ryan	Date 12/22/05
MSL Laboratory Director	RM Tobin	Date 12/20/05
EDSP Program Management		Date 12/6/05
EDSP Battelle QAM		Date 12-15-05

cc: Send final approved copies to:
MSL QA Manager
EDSP Battelle QAM

APPENDIX C

ANALYTICAL RESULTS

(Note: Calculations were conducted at full precision in a spreadsheet.)

Table C1. Diluter Stability Results in Water Vehicle for Ketoconazole (ug/L)

Sample ID	Conc. µg/L (T_0)	Analysis Date	Results	Average (T_d)	Recovery T_0/T_d	RSD	Average, all data	Recovery, all data	RSD, all data
080805Ar1	2723.49	8/8/2005	2627.10	2653.1	97.42%	2.41%	2723.49	NA	14.52%
080805Ar2	2723.49	8/8/2005	2725.98						
080805Ar3	2723.49	8/8/2005	2606.30						
080805Br1	2723.49	8/8/2005	2535.51	3076.4	112.96%	17.30%			
080805Br2	2723.49	8/8/2005	3599.48						
080805Br3	2723.49	8/8/2005	3094.10						
080805Cr1	2723.49	8/8/2005	2283.65	2441.0	89.63%	6.33%			
080805Cr2	2723.49	8/8/2005	2446.66						
080805Cr3	2723.49	8/8/2005	2592.67						
081205Ar1	2723.49	8/12/2005	2509.72	2585.2	94.92%	2.57%	2853.13	104.76%	16.28%
081205Ar2	2723.49	8/12/2005	2634.44						
081205Ar3	2723.49	8/12/2005	2611.36						
081205Br1	2723.49	8/12/2005	2506.96	2654.2	97.46%	4.91%			
081205Br2	2723.49	8/12/2005	2754.21						
081205Br3	2723.49	8/12/2005	2701.54						
081205Cr1	2723.49	8/12/2005	3604.09	3320.0	121.90%	17.77%			
081205Cr2	2723.49	8/12/2005	2641.79						
081205Cr3	2723.49	8/12/2005	3714.09						
082305ABottom r1	2723.49	8/23/2005	2303.53	2357.9	86.58%	2.95%	2547.21	93.53%	8.50%
082305ABottom r2	2723.49	8/23/2005	2436.23						
082305ABottom r3	2723.49	8/23/2005	2333.92						
082305BMiddle r1	2723.49	8/23/2005	2472.24	2522.2	92.61%	1.72%			
082305BMiddle r2	2723.49	8/23/2005	2545.55						
082305BMiddle r3	2723.49	8/23/2005	2548.76						
082305CTop r1	2723.49	8/23/2005	2996.76	2761.6	101.40%	8.67%			
082305CTop r2	2723.49	8/23/2005	2518.27						
082305CTop r3	2723.49	8/23/2005	2769.67						

Table C2. Calibration Verification Data for Ketoconazole

Sample Name	Date	Expected Ketoconazole (ng/mL)	Measured Ketoconazole (ng/mL)	Recovery
WA511ktcnz-6 ICV	7/22/2005	19.8	19.87	100.34%
WA511ktcnz-3D CCV	7/22/2005	21.6	22.73	105.23%
WA511ktcnz-3D CCV	7/22/2005	21.6	22.75	105.34%
WA511ktcnz-3D CCV	7/22/2005	21.6	22.66	104.89%

Table C2. Calibration Verification Data for Ketoconazole (continued)

Sample Name	Date	Expected Ketoconazole (ng/mL)	Measured Ketoconazole (ng/mL)	Recovery
WA511ktcnz-3D CCV	7/22/2005	21.6	21.67	100.33%
WA511ktcnz-6 ICV	8/3/2005	19.8	20.65	104.27%
WA511ktcnz-3D CCV	8/3/2005	21.6	21.03	97.35%
WA511ktcnz-3D CCV	8/3/2005	21.6	21.62	100.11%
WA511ktcnz-3D CCV	8/3/2005	21.6	20.52	95.01%
WA511ktcnz-6 ICV	8/8/2005	19.8	20.48	103.45%
WA511ktcnz-3D CCV	8/8/2005	21.6	22.16	102.58%
WA511ktcnz-3D CCV	8/8/2005	21.6	23.68	109.64%
WA511ktcnz-3D CCV	8/8/2005	21.6	21.01	97.25%
WA511ktcnz-3D CCV	8/8/2005	21.6	22.06	102.13%
WA511ktcnz-6 ICV	8/12/2005	19.8	20.53	103.69%
WA511ktcnz-3D CCV	8/12/2005	21.6	21.51	99.59%
WA511ktcnz-3D CCV	8/12/2005	21.6	22.21	102.80%
WA511ktcnz-3D CCV	8/12/2005	21.6	22.60	104.61%
WA511ktcnz-6 ICV	8/23/2005	19.8	20.83	105.20%
WA511ktcnz-3C CCV	8/23/2005	54.0	54.82	101.52%
WA511ktcnz-3C CCV	8/23/2005	54.0	57.27	106.06%
WA511ktcnz-3C CCV	8/23/2005	54.0	56.85	105.28%

Table C3. Spike Recovery Data for Ketoconazole Stability Analyses

Compound	Nominal Conc. (ng/mL)	Sample ID	Date	Measured (ng/mL)	Recovery
Ketoconazole	432	Spike R-1	7/22/2005	389.64	90.19%
Ketoconazole	432	Spike R-2	7/22/2005	402.64	93.20%
Ketoconazole	432	Spike R-3	7/22/2005	393.65	91.12%
Ketoconazole	432	Spike R-4	7/22/2005	418.25	96.82%
Ketoconazole	432	Spike R-5	7/22/2005	411.01	95.14%
Ketoconazole	432	Spike R-6	8/3/2005	392.05	90.75%
Ketoconazole	432	Spike R-7	8/8/2005	374.28	86.64%
Ketoconazole	432	Spike R-8	8/12/2005	384.18	88.93%
Ketoconazole	432	Spike R-9	8/23/2005	396.84	91.86%

Table C4. MDL and ICV/CCV Recovery Ranges

Test Substance	Method Detection Limit	ICV/CCV Recoveries
Trenbolone	0.2 µg/L	105.2% to 108.8%

Table C5. Summary of Test Substance Purity

TEST SUBSTANCE	LOT NUMBER	CR DETERMINED PURITY
Trenbolone	029H3923	99.1%

Table C6. Calibration Verification Data for Trenbolone

Sample Name	Date	Expected Trenbolone (ng/mL)	Measured Trenbolone (ng/mL)	Recovery*
WA511tren-6 ICV	5/17/2005	2.60	2.74	105.21%
WA511tren-4C CCV	5/17/2005	2.59	2.77	107.13%
WA511tren-4C CCV	5/17/2005	2.59	2.81	108.60%
WA511tren-4C CCV	5/17/2005	2.59	2.81	108.77%

*calculations were done in a spreadsheet at full precision

Table C7. Spike Recovery Data for Trenbolone Analyses

Compound	Nominal Conc. (ng/mL)	Sample ID	Date	Measured (ng/mL)	Recovery*
Trenbolone	0.52	Spike R-1	5/17/2005	0.54	104.39%
Trenbolone	0.52	Spike R-2	5/17/2005	0.56	108.54%
Trenbolone	0.52	Spike R-3	5/17/2005	0.58	112.18%
Trenbolone	0.52	Spike R-4	5/17/2005	0.52	101.09%
Trenbolone	0.52	Spike R-5	5/17/2005	0.43	83.64%
Trenbolone	0.52	Spike R-6	5/17/2005	0.47	90.56%
Trenbolone	0.52	Spike R-7	5/17/2005	0.57	110.41%

*calculations were done in a spreadsheet at full precision

APPENDIX D

NEAT CHEMICAL, VEHICLE, AND FORMULATION STORAGE RECOMMENDATIONS

1. Neat Chemical Storage
   a. Ketoconazole: Keep tightly closed, store in a cool dry place – room temperature.
   b. Trenbolone: Keep tightly closed, store at 2-8°C.

2. Formulation Storage
   Per the protocol, stock formulations were stored at room temperature.

APPENDIX E

ANALYTICAL METHODS EMPLOYED BY THE CHEMICAL REPOSITORY FOR WA 5-11

NOTE 1: The following method documents were originally developed, validated, and utilized within the Chemical Repository for the generation of the study results documented in this report. These documents were also provided to the outside laboratories for analysis of the test substances.

NOTE 2: Data collections sheets were not reproduced for inclusion with the methods in this report.

EDSP Work Assignment 5-11
August 18, 2005

Battelle
The Business of Innovation

EDSP Chemical Repository

EFFECTIVE DATE: 08-18-05

Method # EDSP.H5-032-01

FORMULATION PREPARATION AND ANALYSIS METHODS FOR KETOCONAZOLE IN WELL WATER USING HPLC WITH FLUORESCENCE DETECTION

Approvals:		
AUTHOR: Linda Bingler	Eric Crecelius for	8/18/05
	Signature	Date
TECHNICAL REVIEWER: Tim Fortman		8-18-05
	Signature	Date
TECHNICAL GROUP MANAGER: Michael Cobb		8-18-05
	Signature	Date

ANALYSIS OF KETOKONAZOLE IN WELL WATER USING HPLC WITH FLUORESCENCE DETECTION

1.0 SCOPE AND APPLICATION

The U.S. Environmental Protection Agency (EPA) has agreed to sponsor several chemicals and contract laboratories in a follow-on study (Work Assignment 5-11), to the OECD Phase 1B fish screening assay validation activity, based on features in the short-term reproduction assay with the fathead minnow as described in EPA (2001).

The Endocrine Disruptor Screening Program (EDSP) Chemical Repository (CR) shall be responsible for preparing and determining the stability of the stock formulations for each chemical identified under Work Assignment 5-2 to this contract and making appropriate arrangements for distribution to the sub-contracted laboratories.

This specific method document relates to studies to be carried out employing the test substance ketoconazole (CAS # 65277-42-1). Method development and validation for the test substance was carried out at EDSP CR. The CR then conducted purity and stability studies on this compound as specified in study protocol: *Analysis of Test Substances for Work Assignment 5-11 - EDSP Study Number: EDSP.511-01.* This chemistry document describes the formulation preparation and analysis methods for ketoconazole. The formulation vehicle is well water. The analytical method employs high performance liquid chromatography (HPLC) with fluorescence detection.

2.0 DEFINITIONS

Initial Calibration Verification	A standard made from a neat material independently prepared from the solutions made up for the calibration standards. Used to verify the calibration solutions.
Continuing Caliibration Verification (CCV)	A mid level calibration standard run after every 10 samples to ensure the instrument is in calibration.

3.0 RESPONSIBLE STAFF

Researcher/Technician – sample preparation.
Analyst – analysis, calculations.
QA Manager or Representative – data verification.

4.0 REAGENTS and APPARATUS

4.1 **Saturator Column**

- 1 inch OD (outside diameter) seamless steel tubing T-316 alloy
- Swagelok reducing unions, reduces 1 inch to ¼ inch
- Stainless Steel 90 micron inline filter to fit ¼ inch tubing
- Glass wool
- Teflon-lined 55-gallon container

- Teflon tubing, sufficient to complete the circuit with input and output at different depths, $^{3}/_{16}$" ID, ¼" OD, $^{1}/_{32}$" wall
- Aspirator vacuum pump (Büchi of Switzerland, model B-169, 110V, or equivalent)
- Acetone, HPLC grade
- Well water
- Ketoconazole, 97% purity or better

4.2 Analytical System

- Balance capable of weighing to 0.0001 g
- High Performance liquid chromatograph [(Perkin Elmer 250 pump, with a Gilson 294 liquid autosampler) with fluorescence detector (Waters 474) set at an excitation wavelength of 245nm and an emission wavelength of 370nm] or equivalent.
- Phenomenex SYNERGI 4µ Hydro-RP 80A 250 X 4.6 mm HPLC column Serial # 302074-13 or equivalent.
- Methanol, HPLC grade or better.
- Ketoconazole, 97% purity or better.
- Sample vials.
- Software for collection of data from fluorescence detector, Varian Star Software, vers. 6.2 or equivalent.
- Variable positive displacement pipettors.

5.0 METHOD

5.1 HPLC Mobile Phase (Eluent)

5.1.1 The pump used is a binary gradient pump. One reservoir is filled with a 75% Methanol, 25% water eluent. The pump is programmed to use 100% of this eluent solution.

5.2 Calibration Solution

5.2.1 A 6-point curve is used to calibrate the HPLC over a range that will bracket the concentration in the stability tests. To begin, a stock is made at a concentration of 100,000 µg/L. Approximately, 0.0100 grams is weighed into a 100 mL volumetric flask and diluted to the mark with methanol. Record exact information on an appropriate form and give the solution a unique identifying label (include contents, date prepared, prepared by, work assignment number). Pour the solution into an appropriate size vial with Teflon-lined cap. Stability of the calibration solutions should be verified at the end of the test by the analysis of a new (freshly made) solution prepared from the neat material and compared to the calibration solutions.

5.2.1 typo MAB 12/20/05

5.2.2 Prepare an intermediate standard of 1000 µg/L by removing 0.5 mL of the solution prepared in ~~4.3.1~~ and placing into a 50 mL volumetric flask and diluting to the mark with methanol. Record all information on an appropriate form.

5.2.2 typo MAB 12/20/05

5.2.3 Serially dilute the solution prepared in ~~4.3.2~~ to make standards ranging from 5 µg/mL to 150 µg/L using a solution that will mimic the eluent, 75% methanol, 25% water and record information on an appropriate form.

5.3 HPLC Setup

5.3.1 The HPLC pump flow rate is set at 1.0 mL /min. The mobile phase (eluent) is purged using helium (He) for about 15 minutes prior to running the system (other degassing systems are acceptable). The pump is primed as per instrument instructions and the flow directed thru the HPLC system. The pump run time should be set to 18 minutes. Alternatively, the HPLC should be set up according to manufacturer instructions.

5.3.2 The autosampler is programmed to inject 100 µl. A 100 µl loop is installed. See instrument manual for programming details. The autosampler is then programmed to flush the contaminated surfaces with methanol.

5.3.3 The column used is a Phenomenex Synergi 4µ hydro RP 80A 250 X 4.6 mm HPLC column Serial # 302074-13, or equivalent.

5.3.4 The detector is a fluorescence detector set to an emission wavelength of 370 nm and an excitation wavelength of 245 nm.

5.3.5 The run time is set at 18 minutes. Calibration samples are run prior to analysis and the software is used to calculate the sample results.

5.4 Stability

5.4.1 Saturator Column

5.4.1.1 Stability studies for ketoconazole were conducted for 21 days using a saturator column. A saturated solution is prepared by dissolving a known mass of ketoconazole in a volatile solvent that is then passed through a saturator column under normal laboratory conditions. The solvent is evaporated off and replaced by water, which collects the ketoconazole as it passes through the column. The saturated column must be allowed 7 days to equilibrate. The details for the stability are contained in the Study Protocol. Target concentration is 2500 ng/mL. Sampling and analysis will be performed four times over the course of the stability study at 0, 7 14 and 21 days after the stability solution is created. The saturator system is created as follows:

5.4.1.2 The saturator column consists of one-inch stainless steel tubing packed with glass wool. The glass wool should be packed firmly, but not tight. If the glass wool is packed too loosely, there is a risk of the ketaconazole slurry being pulled into the vacuum line and pump. If the wool is packed too tightly, there is a risk of the slurry running back into the tubing cap when the tubing is tilted. The aspirator vacuum pump is attached to one end. Approximately 2g of ketoconazole is dissolved in 50 mL of acetone and introduced into the column. The ketoconazole should be introduced to the end of the column that will be capped. The column is then tilted back and forth and rotated to distribute the mixture throughout the glass wool. The acetone is evaporated off using the aspirator vacuum, leaving the ketoconazole dried onto the glass wool. The vacuum pump is attached to one end of the column and the other end is capped (eg: a knotted piece of silicone tubing or other flexible tubing). When the vacuum is started, slightly elevate the end of the tubing attached to the pump. The amount of vacuum applied is controlled by placing a small clamp on the vacuum hose attached to the column. The clamp on the vacuum hose should be completely closed before starting the pump. Once the vacuum is established, the clamp can be opened enough to allow flow but not enough to pull the ketoconazole slurry into the pump. Compression of

the flexible tubing used as an end cap and evaporative cooling effect on the stainless tubing can help determine the amount of vacuum being applied. After ~40 min, the vacuum can be increased by opening the clamp, again being careful to have only enough vacuum to allow flow. When the vacuum has run for ~1.5 to 2 hours the clamp and knotted tubing end cap can be removed if the condensation has dissipated and the stainless tubing is near room temperature.

5.4.1.3 The saturator system consists of a Teflon-lined 55 gallon container filled to within about 7.5 cm of the top with tap water. Teflon tubing is run beneath the surface of the water into a pump that pushes the water through stainless steel tubing into the saturator column, where it collects the ketoconazole. The ketoconazole solution passes though a filter at the top of the saturator column prior to re-entry into the 55-gallon container through Teflon tubing. The lid of the 55-gallon container is placed lightly on top of the setup to reduce evaporation and exposure to light.

5.4.1.4 Samples of the saturated solution are collected and submitted for analysis of ketoconazole by HPLC.

5.4.2 Sample Preparation

5.4.2.1 Samples are prepared by removing a 0.025 mL aliquot of the sample and diluting with 0.975 mL mobile phase (~~see 4.2.1~~). 5.1.1 typo MMB 12/20/05

5.4.2.2 Samples should be analyzed on the day of sampling, but if this is not possible, samples should be stored at 4° C. until analysis. If samples are not analyzed on the day of sampling, the actual analysis date and storage conditions shall be documented.

5.5 Analysis

5.5.1 Solutions used for analysis by the HPLC should have similar composition to the eluent. For example, the solution for ketoconazole is prepared by pipetting 0.25 mL of the ketoconazole solution and 0.75 mL of methanol into a glass vial.

5.5.2 Prior to the analysis of samples, linearity must be demonstrated. The instrument is calibrated using a 5 or 6-point curve (minimum of a 4 point curve is needed). An r^2 value of greater than 0.995 is necessary before analysis can begin.

5.5.3 If possible, the calibration should be verified with an initial calibration verification sample (ICV). An independent solution is prepared using ketoconazole neat material and diluted to the appropriate concentration to be within the calibration range. This solution is analyzed and the value obtained should be within ±10% of the expected value.

5.5.4 Following initial calibration verification, a continuing calibration verification (CCV) sample is analyzed. Usually a mid-level calibration solution is used for the CCV. The value obtained should be within ±10% of the expected value. A CCV should be run after every 10 samples.

5.5.5 A blank should be prepared with each sampling by pipetting 0.025 mL of well water and 0.75 mL of eluent (methanol). Blanks should be prepared and analyzed under the same conditions as a sample. The blank should be < 3x the method detection limit (MDL).

0.975mL typo MMB 12/20/05

5.5.6 The MDL is determined by preparation and analysis of a low-level sample using techniques similar to those used to prepare and analyze the stability solution. Seven replicates are prepared and analyzed and the MDL is calculated as:

A x B = MDL

where
A = standard deviation of the number of replicates analyzed
B = Students T value

An MDL study should be performed prior to the analysis of samples.

6.0 DATA ANALYSIS AND CALCULATIONS

6.1 Prior to analysis of samples, the instrument is calibrated with a minimum of a 4-point curve. External standard calculations will be performed. All calculations are done by the software. Calibration curve fits can be set to non-linear (quadratic fit) or linear fit.

7.0 QUALITY CONTROL

7.1 A blank is prepared with each sampling, this blank is tap water processed identically to the stability solution. If background levels are sufficiently high (i.e., greater than 3 x MDL), this value may be subtracted from the values obtained for samples analyzed with that batch. Spike samples are optional. When available, an initial calibration verification standard (ICV) (prepared from a different source than the calibration standards) will be analyzed after the calibration curve. Continuing calibration verification standards (CCVs) will be analyzed after every 10 samples. If CCV variation exceeds a ±10% difference from expected, samples will be re-analyzed within acceptable calibration verification.

Table1. Summary of Data Quality Objectives and Corrective Actions

Quality Control Sample Type	Data Quality Objective[a] (DQO)	Corrective Action
Procedural Blank 1 per batch	<3 x MDL	Re-extract and analyze sample batch. If batch can't be re-extracted and analyzed, "B" flag all samples that are in the batch. Investigate sources of blank contamination.
Calibration curve acceptability	r^2 values greater than or equal to 0.995	If r^2 value is outside of criterion, re-analyze calibration standards, if r^2 is still out, perform instrument maintenance and/or remake calibration standards and rerun calibration samples.
Initial calibration verification (ICV) standard; 1 per batch	±10 % of true value	Re-calibrate. Must meet DQO in order to continue processing samples.
Continuing calibration verification standards; 1 per 10 sample analyzed	±10 % of true value	Re-run CCV, if still not acceptable, re-calibrate and reanalyze affected samples.
Replicate sample precision; triplicates will be analyzed	Precision: 30% as relative standard deviation (RSD)	If RSD is not acceptable, resample and reanalyze. If reanalysis data are still not acceptable, then "*" flag the values.
Blank or Matrix Spike and spike duplicate, one set per batch	±15% of true value	If recoveries are unacceptable, check the spike solution to ensure it has not degraded, also check pipettes to

		ensure they are delivering accurate volumes.

[a]DQO is based on limited sample analysis as part of method development, and may require adjustment when more experience with the method is available.

8.0 SAFETY

All analysts following this procedure should be aware of routine laboratory safety concerns and safety protocols regarding use of chemicals, including the following:

- Gloves, protective clothing and safety glasses should be worn when handling samples and chemicals.

9.0 TRAINING REQUIREMENTS

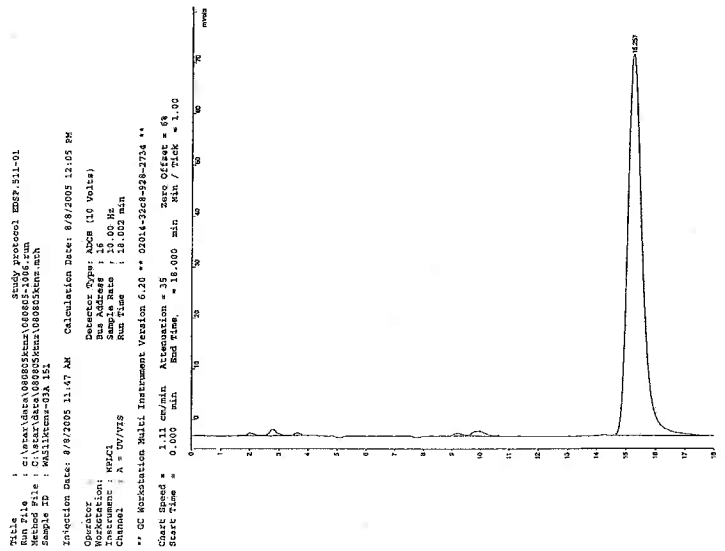
9.1 All staff performing this analysis should first read this procedure and conduct their first analysis under the supervision of a staff member who has had previous experience conducting the procedure. Staff should demonstrate proficiency in the process prior to performing the work. Training will be documented.

9.2 All staff should receive training in the handling of chemicals and the use of fume hoods.

10.0 REFERENCES

Federal Register (CFR Part 136 Appendix B).

EDSP Work Assignment 5-11
August 18, 2005



Typical Chromatogram

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Marine Sciences Laboratory

EFFECTIVE DATE: 05-17-05

Method # EDSP.H5-031-00

Battelle Pacific Northwest National Laboratories
Marine Sciences Laboratory

ANALYSIS OF TRENBOLONE IN WELL WATER USING HPLC WITH FLUORESCENCE DETECTION

Approvals:		
AUTHOR: Timothy Fortman	Timothy Fortman	5-17-05
	Signature	Date
TECHNICAL REVIEWER: Rebecca Wood	R. Wood	5/17/05.
	Signature	Date
Chemical Repository Study Director: Michael Cobb		5/17/05
	Signature	Date

ANALYSIS OF TRENBOLONE IN WELL WATER USING HPLC WITH FLUORESCENCE DETECTION

1.0 SCOPE AND APPLICATION

This method describes the determination of trenbolone in well water using fluorescence detection. The method was developed for use in the analysis of trenbolone for the EDSP program. The eluent used is an acetonitrile/water solution.

2.0 DEFINITIONS

Initial Calibration Verification (ICV)	A standard made from a neat material different from the material used to make the calibration standards. Used to verify the calibration solutions.
Continuing Calibration Verification (CCV)	A mid level calibration standard run after every 10 samples to ensure the instrument is in calibration.

3.0 RESPONSIBLE STAFF

Researcher/Technician - sample preparation.
Analyst - analysis, calculations
QA Manager or Representative - data verification

4.0 ANALYSIS

4.1 Hardware and Reagents

- Balance capable of weighing to 0.0001 g
- High performance liquid chromatograph (Perkin Elmer 250 pump, with a Gilson 294 liquid autosampler) with fluorescence detector (Waters 486 detector) set at an emission wavelength of 458 nm and an excitation wavelength of 359 nm.
- Phenomenex Synergi 4µ hydro-RP 80A 250 X 4.6 mm HPLC column Serial # 258206-4.
- Methanol, HPLC grade or better.
- Trenbolone, 97% purity or better.
- 1.8 mL vials
- Autosampler vial for Gilson Autosampler.
- Helium for sparging eluents.
- Software for collection of data from fluorescence detector, Varian Star Software, vers. 6.2.
- 1 liter amber bottle with Teflon lined lid.
- Variable positive displacement Pipettors
- 20 ml culture tube
- Methyl tert butyl ether (MTBE) HPLC grade or better
- Sodium Chloride

4.2 HPLC Mobile Phase (Eluent)

4.2.1 The pump used is a binary gradient pump, one reservoir is filled with methanol while the other is filled with DI water, the 75:25 eluent is then made by programming the pump to take 75% methanol and 25% water.

4.3 Calibration Solution

4.3.1 A 4 or 5 point curve is used to calibrate the HPLC over a range that will bracket the concentration in the stability tests. To start, a stock is made at a concentration of about 200 ug/mL. Approximately 0.02 grams is weighed into a 100 mL volumetric flask and diluted to the mark with methanol. Record exact information on the standards preparation form (attachment 1) and give the solution a unique identifying label (include contents, date prepared, prepared by, work assignment number). Pour the solution into an appropriate size amber vial with a Teflon lined lid. Stability of the calibration solutions should be verified at the end of the test by the analysis of a new (freshly made) solution prepared from the neat material and compared to the calibration solutions.

4.3.2 Dilute the solution made in 4.3.1 to make an intermediate standard of about 200 ng/mL by taking 0.1 ml of the solution made in 4.3.1 and placing it into a 100 ml volumetric flask and diluting to the mark with 75% methanol:25%Di water, (use Mobile Phase Preparation form to document the preparation of the eluent [diluent] see attachment 2 for example form). Record all information in the standards preparation form (see attachment 1)

4.3.3 Serially dilute the solution made in 4.3.2 to make standards ranging from 0.4 ng/mL to 10 ng/mL using a solution that will mimic the eluent, 75% methanol, 25% water (use Mobile Phase Preparation form to document the preparation of the eluent [diluent] see attachment 2 for example form). Record all information in the standards preparation form (see attachment 1).

4.4 HPLC Setup

4.4.1 The HPLC equipment has 5 main components, a pump, autosampler, HPLC column, detector and data system. The pump is set up to pump at 1.0 mL/min. The mobile phase (eluent) is purged using helium (H_2) for about 15 minutes prior to running the system. The pump is primed as per instrument instructions and the flow directed thru the HPLC system. The pump run time should be set to 9 minutes.

4.4.2 The autosampler is set up to inject 450 ul. A 500 ul loop is installed. See instrument manual for setup details. The autosampler is then set to flush the contaminated surfaces with methanol.

4.4.3 The column used is a Phenomenex Synergi 4µ hydro RP 80A 250 X 4.6 mm HPLC column Serial # 258206-4. Pressure limit on the column is 3000 PSI, adjust pump so pressure limit will shut the pump off prior to damaging the column.

4.4.4 The detector is a fluorescence detector set to an emission wavelength of 458 nm and an excitation wavelength of 359 nm. The detector is attached to the data collection system by way of the analog output from the 1 volt full scale (integrator) terminal.

The data system used is Varian's Star software, version 6.2. The system is set up to start automatically with the injection of the sample. The run time is set to 10 minutes. Data is collected at 10 Hz. Calibration samples are run prior to analysis and the software is used to calculate the unknowns. See software manuals for setup.

4.4.5 This system is composed of several different components from different manufacturers. The method printouts will only contain information pertaining to the calibration and quantitation of the unknowns, in order to ensure that the conditions used are recorded, an EDSP HPLC Analysis Form should be filled out (attachment 3). Also, much of this information should be included in the "notes" tab on the Varian Star method.

4.5 HPLC Analysis

4.5.1 Solutions run on the HPLC should have similar composition to the eluent. For example, the trenbolone samples are extracted with MTBE and evaporated to dryness, then reconstituted in a solution that mimics the mobile phase for 75% methanol, 25% water. Using a transfer pipette, the solution is mixed then transferred to a 1 mL autosampler vial designed for the Gilson autosampler.

4.5.2 Prior to the analysis of any samples, linearity must be demonstrated. A 4 or 5 point curve is run (minimum of a 4 point curve is needed). An r^2 value of greater than 0.995 is necessary before analysis can begin.

4.5.3 Once the calibration is done, if possible, it should be verified with an initial calibration verification sample (ICV). An independent solution is made using a another standard of the trenbolone and diluted to the proper concentration so that it is within the calibration range. This solution is run and the value obtained should be within +/−20% of the expected value.

4.5.4 After the calibration is verified, a continuing calibration verification (CCV) sample is run. This sample is usually one of the mid level calibration solutions. The value obtained should be within +/−15% of the expected value. A CCV should be run after every 10 samples.

4.5.5 A blank should be prepared with each sampling. The blank is hose tap water processed as a sample. The blank should be < 3X the detection limit.

4.5.6 The detection limit can be determined several ways, one way, by preparing a sample at a low concentration, using similar techniques as used to analyze the stability solution. This is done 7 times and the the detection limit is the students T (3.143 for 7 replicates) times the standard deviation of the seven replicate runs. This is an MDL. Alternately the lowest standard that can be detected by the system and analyzed as a sample can be used as the detection limit. This is the IDL. An MDL or should be performed prior to the analysis of any sample for trenbolone. Samples with no peak or less than the MDL or IDL will be reported as not detected.

5.0 Sample Preparation

5.1 Samples received from the wet lab are received in a 20 ml culture tube. 10 ml of each sample has been accurately placed in the culture tube. Extraction for the diluter samples

are done in this culture tube. Stock solutions are at a much higher concentration, an aliquot is removed for analysis.

5.2 2.0 grams of sodium chloride is added to each diluter sample, followed by 1.0 ml of MTBE. A blank and blank spike are prepared by pipetting 10 ml of house tap water into a culture tube and adding the salt and MTBE as done for the diluters. The blank spike has 0.005 ml of a 1 mg/L spike solution added.

5.3 Culture tubes are agitated vigorously by hand for 1 minute. The tubes are then allowed to sit until the MTBE separates from the aqueous phase (approximately 5 minutes).

5.4 The MTBE is carefully removed, avoiding the water and added to a 1.8 ml vial. 0.75 ml of the MTBE is accurately transferred (use a 1 ml syringe) to another 1.8 ml vial. The vials are then evaporated to dryness under a gentle stream of nitrogen. This takes about 15 minutes.

5.5 0.75 ml of 75% methanol, 25% water is accurately placed in the 1.8 ml vial (a syringe is used). The Solution is mixed with a transfer pipette and transferred to an autosampler vial that works with the autosampler.

5.4 Samples should be analyzed on the day of sampling, but if this is not possible, samples should be stored at 4 deg. C. until analysis. If samples are not analyzed in the day of sampling, the actual analysis date and storage conditions shall be documented.

6.0 DATA ANALYSIS AND CALCULATIONS

6.1 Prior to analysis of any samples, the instrument is calibrated with a minimum of a 4 point curve. External standard calculations will be performed. All calculations will be done using Varian's Star chromatography software, version 6.2. This software allows the input of a multiplier, so that any dilutions will be included with the software calculations. For example, for trenbolone stability, 0. 01 mL of the stock is diluted with 0. 99 mL of 75% methanol, 25% water and a multiplier of 100 is used so that the output from the software will give values that reflect the concentration in the stability solution. Calibration curve fits can be set to non-linear (quadratic fit) or a linear fit.

6.2 Prior to the tabulation of the data, each chromatogram and report printed by the software will be initialed and dated for GLP compliance.

7.0 QUALITY CONTROL

A blank is prepared with each sampling, this blank is tap water processed identically to the stability solution. If background levels are sufficiently high (i.e., greater than 3 x detection limit), this value may be subtracted from the values obtained for samples analyzed with that batch. Processing of these samples is very straight forward, therefore spikes are optional. Whenever available, an initial calibration verification (ICV) standard (made from a second source, not the same source as the calibration standards) will be analyzed following the calibration curve. Continuing calibration verification standards (CCVs) will be analyzed after every 10 samples. If CCV variation exceeds a +/-15% difference from expected, samples will be re-run with acceptable calibration criteria.

Table1. Summary of Data Quality Objectives and Corrective Actions

Quality Control Sample Type	Data Quality Objective[a] (DQO)	Corrective Action
Procedural Blank one/batch	Less than 3 x MDL	Re-extract and analyze sample batch. If batch can not be re-extracted and analyzed, "B" flag all samples that are in the batch. Investigate sources of blank contamination.
Calibration curve acceptability	r^2 values greater than or equal to 0.995	If r^2 value is outside of criterion, re-analyze calibration standards, if r^2 is still out, perform instrument maintenance and/or remake calibration standards and rerun calibration samples.
Initial calibration verification (ICV) standard; one/batch	+/−20 % of true value	Re-calibrate. Must meet DQO in order to continue processing samples.
Continuing calibration verification standards; one every 10th sample analyzed	+/−15 % of true value	Re-run CCV, if still not acceptable, re-calibrate and reanalyze affected samples.
Replicate sample precision; if triplicates are analyzed	Precision: 30% as relative standard deviation (RSD)	If RSD is not acceptable, resample and reanalyze. If reanalysis data are still not acceptable, then "*" flag the values.
Blank or Matrix Spike and spike duplicate, one set per batch (optional)	+/−20% of true value	If recoveries are unacceptable, check the spike solution to ensure it has not degraded, also check pipettes to ensure they are delivering accurate volumes.

[a] DQO is based on limited sample analysis as part of method development experience, and may require adjustment when more experience with the method is available.

Table 2. Data Qualifiers[a]

U	The analyte was detected below the MDL. Note: Samples with no peaks are reported as zero.
B	Samples associated with procedural blank contamination.
*	QC sample data that does not meet the DQO acceptability criterion.
Q	The data are questionable.
D	Sample diluted for analysis. (note: this procedure outlines the dilution of the samples, data will not be D flagged unless diluted other than indicated in this SOP)

[a] Additional data qualifiers may be added as necessary.

8.0 SAFETY

All analysts following this procedure should be aware of routine laboratory safety concerns, including all safety protocols regarding use of chemicals, including the following:

- Gloves, protective clothing and safety glasses should be worn when handling samples and chemicals.

9.0 TRAINING REQUIREMENTS

9.1 All staff performing this analysis should first read this procedure and conduct their first analysis under the supervision of a staff member who has had previous experience conducting the procedure. Staff should demonstrate proficiency in the process prior to performing the work. Documentation of training will be performed in accordance with MSL-A-006, Marine Sciences Laboratory Training.

9.2 All staff should have received training in the handling of chemicals and the use of fume hoods.

10.0 REFERENCES

MSL-A-006 Marine Sciences Laboratory Training

MSL-Q-007-04 Procedure for Determining Method Detection Limits

Federal Register (CFR Part 136 Appendix B).

APPENDIX F

METHOD DEVIATIONS

The following method deviation was filed:

1. EDSP.H5-032-01-D1 - Method deviation D-1 discusses the elimination of the final calibration verification run. The chemist felt that with the reduced term of the stability study that the final calibration verification run was unnecessary. It was concluded that this would have no impact on the data or study outcome.

Appendix E- Battelle Statistical Analysis

Endocrine Disruptor Screening Program
Work Assignment 6-3 Technical Directive No. 2

Carry Out Statistical Reanalysis of Studies for Work Assignment 5-11

Fathead Minnow Fish Screening Assay Phase 1b Follow-up

May 17, 2006

for

U. S. Environmental Protection Agency
Endocrine Disruptor Program
Washington, D. C.

by

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Statistical Methods

Table 0 summarizes the occurrences of significant differences from the controls for each of the test procedures considered, as displayed in Tables 10-14 in the results section. Tables 0 and 10-14 indicate the relative sensitivities of the statistical tests that were applied to the five laboratory-test chemical combinations. These tables indicate:

Parametric tests

The linear trend and the Williams ordered alternatives step down tests were more sensitive than the F and Dunnett's general alternatives tests. Thus step down tests should be used unless there is statistical or toxicological indication that the dose response trend is not monotonic. The linear trend test had equivalent sensitivity to the Williams test for the three Springborn Smithers data sets and greater sensitivity for the ABC and Wildlife International data sets and should be preferred.

Nonparametric tests

The linear trend and the Jonckheere-Terpstra step down tests were more sensitive than the Kruskal-Wallis and the Wilcoxon Mann Whitney (with Bonferroni's adjustment) general alternatives tests. Thus step down tests should be used unless there is statistical or toxicological indication that the dose response trend is not monotonic. The Wilcoxon-Mann-Whitney test with Bonferroni's adjustment was very insensitive and is not recommended for use with future data sets. The linear trend test had equivalent or greater sensitivity than the Jonkheere-Terpstra test for four of the five data sets. For the Springborn Smithers (flutamide) data set none of the statistical tests considered identified many significant effects. The linear trend test should be preferred.

The parametric and the nonparametric step down tests were about equally sensitive.

Recommendation

Carry out preliminary outlier detection procedures based on a heterogeneous variance generalization of Grubbs screening test. Determine which screened values, if any, to delete. After outliers have been deleted, for all endpoints other than survival or percent (in)fertile eggs if the dose group means vary over more than an order of magnitude then carry out a log transformation. For survival or percent (in)fertile eggs carry out logit transformations.

For endpoints other than survival, first carry out the Shapiro-Wilk normality test and Levene's heterogeneity of variance test to determine whether there are departures from the normality and homogeneity of variance assumptions. If the data are compatible with both of these assumptions the parametric procedures will be used. If one or both of these assumptions are violated then the nonparametric procedures will be used.

If parametric procedures are to be used, the F-test will first be carried out. The linear trend step down test procedure will be carried out irrespective of whether the F-test

is significant since this is more sensitive than the F-test. If the linear trend test does not identify any significant differences from the control and the F-test is not significant then stop. If the linear trend test does not identify any significant differences from the control and the F-test is significant then carry out Dunnett's general alternatives test to detect possible non monotonic alternatives.

If nonparametric procedures are to be used, the Kruskal-Wallis test will first be carried out. The linear trend step down test procedure will be carried out irrespective of whether the Kruskal-Wallis test is significant since this is more sensitive than the Kruskal-Wallis test. If the linear trend test does not identify any significant differences from the control and the Kruskal-Wallis test is not significant then stop. If the linear trend test does not identify any significant differences from the control and the Kruskal-Wallis test is significant then carry out Steele's many one rank test or Dunnett's test on the rank transformation of the data to detect possible non monotonic alternatives.

For the survival endpoint carry out tests based on males and females combined due to the small number of males per tank. Carry out a preliminary test of homogeneity of survival rates among tanks within dose groups by the Cochran Mantel Haenszel test. If the preliminary test is not significant combine data across tanks within dose groups. Test for dose response trends in survival by the exact Cochran-Armitage test in a step down fashion. If the preliminary test is significant, carry out logit transformations of the survival rates in each tank and treat the logit transformed survival rates in the same manner as the non survival endpoints.

Table 0. Summary of Significant Results[1]

	Linear-Parametric			Williams			Linear-Nonparametric			Jonckheere-Terpstra		
	L	M	H	L	M	H	L	M	H	L	M	H
S-S Flutamide	0/11	0/11	0/11	0/11	0/11	0/11	0/19	0/19	1/19	0/19	0/19	5/19
S-S Ketoconazole	0/10	2/10	2/10	0/10	2/10	2/10	0/19	1/19	5/19	0/19	1/19	4/19
S-S Pot Perman	0/12	2/12	2/12	0/12	2/12	2/12	0/19	2/19	2/19	0/19	1/19	1/19
ABC Pot Perman	0/12	7/12	7/12	0/12	2/12	2/12	2/21	13/21	15/21	2/21	12/21	15/21
Wildlife International Flutamide	0/15	3/15	6/15	0/15	2/15	5/15	0/20	4/20	10/20	0/20	3/20	9/20

	Dunnett			WMW-Bonferroni			F-Test	Kruskal-Wallis Test
	L	M	H	L	M	H		
S-S Flutamide	0/11	0/11	0/11	0/19	0/19	0/19	0/11	0/19
S-S Ketoconazole	0/10	0/10	2/10	0/19	0/19	0/19	2/10	3/19
S-S Pot Perman	0/12	2/12	1/12	0/19	0/19	0/19	2/12	2/19
ABC Pot Perman	0/12	2/12	1/12	0/21	0/21	0/21	4/12	9/21
Wildlife International Flutamide	0/15	3/15	2/15	0/20	0/20	0/20	4/15	5/20

[1]Highlighted results summarize comparisons between the parametric step down procedures (linear and Williams) and between the nonparametric step own procedures (linear and Jonkheere-Terpstra). When one procedure identifies more significant effects (p=0.05) than its comparison procedure it is highlighted. When both procedures identify the same number of significant effects they are both highlighted.

Results –Tables

1. Table 1. Outlier screen results. No screened values were deleted, based on direction from EPA (Springborn Smithers values) and because they looked physically reasonable.

2. Tables 2-7. Results of preliminary tests of normality and heterogeneity of variance. Significant results ($p \approx 0.05$) are highlighted in yellow. If either normality test or homogeneity test is significant then parametric tests are not used in principal tables.

3. Tables 8, 9. Contingency table test for homogeneity of 21-day survival in ABC Laboratories and Springborn Smithers.

   Table 8. Test of homogeneity among tanks within does groups. No evidence of departure from homogeneity.
   Table 9. Cochran Armitage step down test for trend in survival rate based on fish pooled across tanks within dose groups. Significant trend for high and middle dose groups in ABC Laboratories.

4. Tables 10-14. Results of four parametric and four nonparametric tests for each laboratory, chemical, and endpoint. For those endpoints with significant normality or heterogeneity results the parametric test endpoints are indicated very faintly and results are not highlighted. Linear trend tests (nonparametric and parametric), Williams test, and Jonckheere-Terpstra test are ordered alternatives tests. Other tests do not assume ordering among responses within dose groups.

   Significant results are highlighted in yellow. Results are given for overall tests (F-test, Kruskal-Wallis test) and for pairwise comparisons of low, medium, and high dose groups with the control group. Comparisons of amount of yellow across tables (e.g. Tables 10, 14) indicate relative number of significant effects found. Comparisons of relative amounts of yellow across columns within tables indicate which procedures were most sensitive.

   All analyses in Tables 10-14 were carried out on a per tank basis. For those endpoints determined on individual fish (e.g. VTG, male fatpad score, male or female GSI, etc.) an alternative analysis could be carried out on the individual fish data, incorporating tank as a blocking factor. If tank-to-tank variation is small, this would provide more sensitive estimates of variability. This needs to be examined in greater detail.

5. Tables 15-17 display summary statistics of untransformed data for each laboratory and chemical.

6. Tables 18-22 summarize the test results for all endpoints based on the recommended methods.

7. Some basic results.

    a. Springborn Smithers found far fewer significant results than ABC Laboratories or Wildlife International (Table 10 compared to Table 14, Table 12 compared to Table 13.)
    b. Step down test were more sensitive than general alternative s tests, e.g. linear trend tests versus Dunnett's test or Wilcoxon-Mann-Whitney test)
    c. Parametric and nonparametric tests had about the same sensitivity when parametric test were appropriate.
    d. Linear trend tests appear to be a bit more sensitive than Williams test or Jonckheere-Terpstra test, but the difference is slight. This would need to be studied in greater detail.

Table 1. Potential Outliers Identified by Grubb's Test.

Laboratories	Parameter	Chemical	Sex	Group	Replicate	Observed	Treated as Outlier
Springborn Smithers	VTG	Flutamide	Male	2_500ug/L	A	0.65272	No
	VTG	Potassium Permanganate	Male	1_225ug/L	C	0.23634	No
	VTG	Potassium Permanganate	Male	3_900ug/L	C	0.05628	No
	% of Infertile Eggs (logit)	Ketoconazole	Female	0_control	D	-4.2571	No
	GSI	Ketoconazole	Female	2_100ug/L	C	15.7562	No
ABC	# Counted Eggs	Potassium Permanganate	Female	2_450ug/L	C	350	No
	# Estimated Eggs	Potassium Permanganate	Female	2_450ug/L	C	350	No
	# Eggs per Female per Reproductive Day	Potassium Permanganate	Female	2_450ug/L	C	4.2	No
	# of Fertile eggs	Potassium Permanganate	Female	2_450ug/L	C	341.00	No
	# of Fertile eggs	Potassium Permanganate	Female	3_900ug/L	D	131.00	No
Wildlife International	VTG	Flutamide	Male	3_1000ug/L	C	18949.0	No
	# of Fertile eggs	Flutamide	Female	2_500ug/L L	B	1982.0	No

Table 2. ABC Laboratories Normality Test Results

Chemical	Parameter	Test	Stat	pValue	Sex
Potassium Permanganate	Survival Rate - Logit Transformed	Shapiro-Wilk	0.848996	0.0132	All
	log_VTG	Shapiro-Wilk	0.969574	0.8709	Females
	% of Infertile Eggs - Logit Transform	Shapiro-Wilk	0.921514	0.2987	Females
	# Estimated Eggs (log)	Shapiro-Wilk	0.899638	0.0793	Females
	# Counted Eggs (log)	Shapiro-Wilk	0.903744	0.0923	Females
	# Spawns (log)	Shapiro-Wilk	0.866719	0.0242	Females
	# Fertile Eggs (log)	Shapiro-Wilk	0.880186	0.0391	Females
	# Eggs/Female/Reproductive Day (log	Shapiro-Wilk	0.914862	0.1394	Females
	Length	Shapiro-Wilk	0.940047	0.3830	Females
	Weight	Shapiro-Wilk	0.943634	0.4303	Females
	Gonad Weight	Shapiro-Wilk	0.935006	0.3237	Females
	GSI	Shapiro-Wilk	0.948684	0.5040	Females
	log_VTG	Shapiro-Wilk	0.801635	0.0039	Males
	Length	Shapiro-Wilk	0.901692	0.1010	Males
	Weight	Shapiro-Wilk	0.950937	0.5393	Males
	Gonad Weight	Shapiro-Wilk	0.963975	0.7610	Males
	GSI	Shapiro-Wilk	0.960878	0.7077	Males
	Tubercle Score	Shapiro-Wilk	0.917347	0.1755	Males
	Fatpad Score	Shapiro-Wilk	0.922292	0.2088	Males
	Fatpad Weight	Shapiro-Wilk	0.976673	0.9417	Males
	FPI	Shapiro-Wilk	0.967558	0.8204	Males

Table 3. ABC Laboratories Heterogeneity of Variance Test Results

chemical	parameter	Method	DF	Sum of Squares	Mean Square	F Value	Pr > F	sex
Potassium Permanganate	Survival Rate - Logit Transformed	LV	3	2.6640	0.8880	1.65	0.2309	All
	log_VTG	LV	2	3.1053	1.5527	1.79	0.2210	Females
	% of Infertile Eggs - Logit Transform	LV	1	0.0495	0.0495	0.65	0.4513	Females
	# Estimated Eggs (log)	LV		4118.0	1372.7	15.39	0.0002	Females
	# Counted Eggs (log)	LV		3830.5	1276.8	13.29	0.0004	Females
	# Spawns (log)	LV	3	632.7	210.9	200.13	<.0001	Females
	# Fertile Eggs (log)	LV	3	3093.5	1031.2	2.55	0.1046	Females
	# Eggs/Female/Reproductive Day (log	LV		643.6	214.5	5.18	0.0159	Females
	Length	LV		21.8181	7.2727	3.52	0.0524	Females
	Weight	LV	3	0.00973	0.00324	2.14	0.1535	Females
	Gonad Weight	LV	3	0.000103	0.000034	3.35	0.0594	Females
	GSI	LV	3	308.8	102.9	3.80	0.0431	Females
	log_VTG	LV	3	342.3	114.1	1.77	0.2107	Males
	Length	LV	3	1.9382	0.6461	0.30	0.8245	Males
	Weight	LV	3	0.0798	0.0266	1.58	0.2510	Males
	Gonad Weight	LV	3	3.888E-7	1.296E-7	2.58	0.1066	Males
	GSI	LV	3	0.4364	0.1455	2.24	0.1413	Males
	Tubercle Score	LV	3	9276.7	3092.2	1.67	0.2309	Males
	Fatpad Score	LV	3	1.5612	0.5204	1.21	0.3508	Males
	Fatpad Weight	LV	3	0.000102	0.000034	1.12	0.3839	Males
	FPI	LV	3	24.1440	8.0480	0.91	0.4697	Males

Table 4. Wildlife International Laboratory Normality Test Results.

Chemical	Parameter	Test	Stat	pValue	Sex
Flutamide	VTG	Shapiro-Wilk	0.976396	0.9285	Females
	% of Infertile Eggs (logit)	Shapiro-Wilk	0.920185	0.2212	Females
	# Counted Eggs	Shapiro-Wilk	0.95734	0.6139	Females
	# Fertile Eggs (log)	Shapiro-Wilk	0.882884	0.0430	Females
	# Eggs/Female/Reproductive Day	Shapiro-Wilk	0.959462	0.6520	Females
	Length	Shapiro-Wilk	0.892163	0.0603	Females
	Weight	Shapiro-Wilk	0.913653	0.1333	Females
	Gonad Weight	Shapiro-Wilk	0.952214	0.5255	Females
	GSI	Shapiro-Wilk	0.952188	0.5251	Females
	VTG	Shapiro-Wilk	0.915011	0.1402	Males
	Length	Shapiro-Wilk	0.935387	0.2963	Males
	Weight	Shapiro-Wilk	0.9002	0.0810	Males
	Gonad Weight	Shapiro-Wilk	0.911304	0.1221	Males
	GSI	Shapiro-Wilk	0.94026	0.3522	Males
	Tubercle Score	Shapiro-Wilk	0.966216	0.7742	Males
	Tubercle Count	Shapiro-Wilk	0.943314	0.3916	Males
	Fatpad Score	Shapiro-Wilk	0.953566	0.5482	Males
	Fatpad Weight	Shapiro-Wilk	0.891543	0.0589	Males
	FPI	Shapiro-Wilk	0.887001	0.0500	Males

Table 5. Wildlife International Laboratory Heterogeneity of Variance (Levene's) Test Results

Chemical	Parameter	Method	DF	Sum of Squares	Mean Square	F Value	Pr > F	Sex
Flutamide	VTG	LV	3	4.809E28	1.603E28	1.67	0.2270	Females
	% of Infertile Eggs - Logit Transf	LV	2	76.4599	38.2299	2.03	0.1872	Females
	# Counted Eggs	LV	3	2.616E12	8.719E11	1.90	0.1828	Females
	# Fertile Eggs (log)	LV	3	2957.0	985.7	439.74	<.0001	Females
	# Eggs/Female/Reproductive Day	LV	3	199123	66374.4	2.36	0.1224	Females
	Length	LV	3	12.5064	4.1688	1.00	0.4257	Females
	Weight	LV	3	0.00312	0.00104	1.67	0.2264	Females
	Gonad Weight	LV	3	0.000013	4.252E-6	3.09	0.0676	Females
	GSI	LV	3	160.4	53.4687	5.91	0.0103	Females
	VTG	LV	3	6.911E15	2.304E15	1.29	0.3228	Males
	Length	LV	3	17.3604	5.7868	0.44	0.7265	Males
	Weight	LV	3	0.0511	0.0170	1.38	0.2955	Males
	Gonad Weight	LV	3	1.343E-8	4.477E-9	0.91	0.4636	Males
	GSI	LV	3	0.00412	0.00137	1.31	0.3158	Males
	Tubercle Score	LV	3	15477.8	5159.3	1.92	0.1799	Males
	Tubercle Count	LV	3	3654.5	1218.2	1.94	0.1778	Males
	Fatpad Score	LV	3	1.1394	0.3798	1.44	0.2793	Males
	Fatpad Weight	LV	3	0.000055	0.000018	1.04	0.4084	Males
	FPI	LV	3	44.2283	14.7428	1.65	0.2303	Males

Table 6. Springborn Smithers Laboratory Normality Test Results

Chemical	Parameter	Test	Stat	pValue	Sex
Flutamide	VTG	Shapiro-Wilk	0.935986	0.3027	Females
	% of Infertile Eggs (logit)	Shapiro-Wilk	0.931902	0.2913	Females
	# Estimated Eggs	Shapiro-Wilk	0.830692	0.0072	Females
	# Counted Eggs	Shapiro-Wilk	0.838703	0.0093	Females
	# Spawns	Shapiro-Wilk	0.906165	0.1009	Females
	# Fertile Eggs	Shapiro-Wilk	0.84859	0.0130	Females
	# Eggs/Female/Reproductive Day	Shapiro-Wilk	0.860905	0.0198	Females
	Length	Shapiro-Wilk	0.971808	0.8670	Females
	Weight	Shapiro-Wilk	0.92685	0.2172	Females
	Gonad Weight	Shapiro-Wilk	0.944313	0.4052	Females
	GSI	Shapiro-Wilk	0.967669	0.7996	Females
	VTG	Shapiro-Wilk	0.598004	0.0001	Males
	Length	Shapiro-Wilk	0.925727	0.2084	Males
	WeightG	Shapiro-Wilk	0.965028	0.7531	Males
	Gonad Weight	Shapiro-Wilk	0.961477	0.6887	Males
	GSI	Shapiro-Wilk	0.945538	0.4225	Males
	Tubercle Score	Shapiro-Wilk	0.929908	0.2430	Males
	Fatpad Weight	Shapiro-Wilk	0.898055	0.0748	Males
	FPI	Shapiro-Wilk	0.888199	0.0522	Males
Ketoconazole	VTG	Shapiro-Wilk	0.924752	0.2011	Females
	% of Infertile Eggs (logit)	Shapiro-Wilk	0.950583	0.5337	Females
	# Estimated Eggs	Shapiro-Wilk	0.896866	0.0716	Females
	# Counted Eggs	Shapiro-Wilk	0.914675	0.1384	Females
	# Spawns	Shapiro-Wilk	0.925153	0.2041	Females
	# Fertile Eggs	Shapiro-Wilk	0.90299	0.0897	Females
	# Eggs/Female/Reproductive Day	Shapiro-Wilk	0.916934	0.1505	Females
	Length	Shapiro-Wilk	0.9459	0.4276	Females
	Weight	Shapiro-Wilk	0.967162	0.7908	Females
	Gonad Weight	Shapiro-Wilk	0.980835	0.9699	Females
	GSI	Shapiro-Wilk	0.841591	0.0103	Females
	log_VTG	Shapiro-Wilk	0.816631	0.0046	Males
	Length	Shapiro-Wilk	0.986317	0.9946	Males

Table 6. Springborn Smithers Laboratory Normality Test Results

Chemical	Parameter	Test	Stat	pValue	Sex
	Weight	Shapiro-Wilk	0.928836	0.2336	Males
	Gonad Weight	Shapiro-Wilk	0.926755	0.2165	Males
	GSI	Shapiro-Wilk	0.918746	0.1610	Males
	Tubercle Score	Shapiro-Wilk	0.892412	0.0608	Males
	Fatpad Weight	Shapiro-Wilk	0.713621	0.0002	Males
	FPI	Shapiro-Wilk	0.732901	0.0004	Males
Potassium Permanganate	VTG	Shapiro-Wilk	0.939478	0.4913	Females
	% of Infertile Eggs (logit)	Shapiro-Wilk	0.946118	0.5022	Females
	# Estimated Eggs	Shapiro-Wilk	0.87733	0.0353	Females
	# Counted Eggs	Shapiro-Wilk	0.8784	0.0366	Females
	# Spawns	Shapiro-Wilk	0.904481	0.0948	Females
	# Fertile Eggs	Shapiro-Wilk	0.879481	0.0381	Females
	# Eggs/Female/Reproductive Day	Shapiro-Wilk	0.861237	0.0200	Females
	Length	Shapiro-Wilk	0.948307	0.4634	Females
	Weight	Shapiro-Wilk	0.911982	0.1253	Females
	Gonad Weight	Shapiro-Wilk	0.934987	0.2921	Females
	GSI	Shapiro-Wilk	0.930913	0.2520	Females
	log_VTG	Shapiro-Wilk	0.923877	0.3197	Males
	Length	Shapiro-Wilk	0.960263	0.6666	Males
	Weight	Shapiro-Wilk	0.960794	0.6762	Males
	Gonad Weight	Shapiro-Wilk	0.953873	0.5534	Males
	GSI	Shapiro-Wilk	0.919825	0.1676	Males
	Tubercle Score	Shapiro-Wilk	0.939258	0.3400	Males
	Fatpad Weight	Shapiro-Wilk	0.762368	0.0009	Males
	FPI	Shapiro-Wilk	0.782767	0.0016	Males

Table 7. Springborn Smithers Laboratory Heterogeneity of Variance (Levene's) Test Results

Chemical	Parameter	Method	DF	Sum of Squares	Mean Square	F Value	Pr > F	Sex
Flutamide	VTG	LV	3	13414155	4471385	1.92	0.1799	Females
	% of Infertile Eggs (logit)	LV	3	1.3792	0.4597	3.64	0.0483	Females
	# Estimated Eggs	LV	3	4.457E11	1.486E11	2.08	0.1560	Females
	# Counted Eggs	LV	3	8.755E11	2.918E11	2.04	0.1619	Females
	# Spawns	LV	3	387.5	129.2	1.26	0.3310	Females
	# Fertile Eggs	LV	3	5.987E11	1.996E11	2.00	0.1681	Females
	# Eggs/Female/Reproductive Day	LV	3	16515.9	5505.3	1.90	0.1830	Females
	Length	LV	3	21.3014	7.1005	1.83	0.1958	Females
	Weight	LV	3	0.000262	0.000087	0.56	0.6515	Females
	Gonad Weight	LV	3	5.742E-7	1.914E-7	1.96	0.1742	Females
	GSI	LV	3	34.7985	11.5995	3.28	0.0587	Females
	VTG	LV	3	0.0183	0.00610	2.25	0.1351	Males
	Length	LV	3	33.4875	11.1625	0.98	0.4363	Males
	WeightG	LV	3	0.0233	0.00778	1.31	0.3176	Males
	Gonad Weight	LV	3	5.909E-9	1.97E-9	0.90	0.4697	Males
	GSI	LV	3	0.000900	0.000300	0.24	0.8654	Males
	Tubercle Score	LV	3	4020.9	1340.3	1.85	0.1927	Males
	Fatpad Weight	LV	3	1.819E-8	6.063E-9	1.30	0.3205	Males
	FPI	LV	3	0.0282	0.00940	1.06	0.4012	Males
Ketoconazole	VTG	LV	3	22700.8	7566.9	1.68	0.2232	Females
	% of Infertile Eggs (logit)	LV	3	16.6327	5.5442	2.43	0.1206	Females
	# Estimated Eggs	LV	3	1.8559E9	6.1863E8	0.55	0.6563	Females
	# Counted Eggs	LV	3	1.8745E9	6.2483E8	0.52	0.6777	Females
	# Spawns	LV	3	267.3	89.0872	0.95	0.4459	Females
	# Fertile Eggs	LV	3	1.9704E9	6.5681E8	0.60	0.6250	Females
	# Eggs/Female/Reproductive Day	LV	3	114.2	38.0531	1.19	0.3548	Females
	Length	LV	3	46.4670	15.4890	1.70	0.2194	Females
	Weight	LV	3	0.00195	0.000649	3.73	0.0420	Females
	Gonad Weight	LV	3	8.793E-6	2.931E-6	4.44	0.0256	Females
	GSI	LV	3	14.7491	4.9164	0.67	0.5858	Females
	log_VTG	LV	3	256.9	85.6235	2.12	0.1510	Males
	Length	LV	3	293.3	97.7780	8.81	0.0023	Males
	Weight	LV	3	0.1686	0.0562	14.02	0.0003	Males

Table 7. Springborn Smithers Laboratory Heterogeneity of Variance (Levene's) Test Results

Chemical	Parameter	Method	DF	Sum of Squares	Mean Square	F Value	Pr > F	Sex
	Gonad Weight	LV	3	5.926E-9	1.975E-9	0.34	0.8000	Males
	GSI	LV	3	0.00211	0.000703	0.87	0.4836	Males
	Tubercle Score	LV	3	33904.5	11301.5	1.79	0.2019	Males
	Fatpad Weight	LV	3	7.899E-6	2.633E-6	1.57	0.2473	Males
	FPI	LV	3	12.5678	4.1893	1.76	0.2090	Males
Potassium Permanganate	VTG	LV	1	596696	596696	3.12	0.1275	Females
	% of Infertile Eggs (logit)	LV	3	0.0529	0.0176	0.36	0.7836	Females
	# Estimated Eggs	LV	3	6.231E10	2.077E10	0.78	0.5270	Females
	# Counted Eggs	LV	3	5.891E11	1.964E11	1.42	0.2864	Females
	# Spawns	LV	3	1063.7	354.6	0.99	0.4308	Females
	# Fertile Eggs	LV	3	5.293E11	1.764E11	1.39	0.2921	Females
	# Eggs/Female/Reproductive Day	LV	3	34873.4	11624.5	1.60	0.2409	Females
	Length	LV	3	71.2230	23.7410	0.76	0.5375	Females
	Weight	LV	3	0.00466	0.00155	4.51	0.0245	Females
	Gonad Weight	LV	3	0.000014	4.58E-6	1.19	0.3559	Females
	GSI	LV	3	680.4	226.8	2.14	0.1479	Females
	log_VTG	LV	1	0.1310	0.1310	0.02	0.8886	Males
	Length	LV	3	577.0	192.3	1.36	0.3027	Males
	Weight	LV	3	0.4287	0.1429	1.98	0.1715	Males
	Gonad Weight	LV	3	5.938E-9	1.979E-9	0.62	0.6178	Males
	GSI	LV	3	0.00131	0.000438	0.97	0.4385	Males
	Tubercle Score	LV	3	1198.4	399.5	1.34	0.3076	Males
	Fatpad Weight	LV	3	0.000015	5.095E-6	1.15	0.3697	Males
	FPI	LV	3	4.8226	1.6075	1.39	0.2940	Males

Table 8. Cochran-Mantel-Haenszel Test for Heterogenity Among Tanks Within Dose Groups for Survival. By Laboratory and Chemical.

Laboratory	Chemical	Cochran-Mantel-Haenszel Statistic	P-Value (2-sided)
ABC	Potassium Permanganate	6.5714	0.0869
Springborn Smithers	Flutamide	6.0000	0.1116
Springborn Smithers	Ketoconazole	3.0000	0.3916
Springborn Smithers	Potassium Permanganate	6.4186	0.0929

Table 9. Exact Cochran-Armitage Test for Trend for Survival. By Laboratory and Chemical. Male and Female Fish Combined

Laboratory	Chemical	Dose Group	P-Value (2-sided)
ABC	Potassium Permanganate	High	<0.0001
		Mid	0.0049
		Low	1.0000
Springborn Smithers	Flutamide	High	0.7474
		Mid	1.0000
		Low	1.0000
Springborn Smithers	Ketoconazole	High	1.0000
		Mid	0.6667
		Low	--
Springborn Smithers	Potassium Permanganate	High	0.1108
		Mid	1.0000
		Low	1.0000

Table 10. Summary Results of Statistical Analyses - Test Article: Flutamide, Participating Laboratory: Springborn Smithers[1]

Endpoint	Parametric Procedure[2]										Non-Parametric Procedure									
	Linear Trend[3]			Williams' Test[4]			F TEST	Dunnett's Test[5]			Linear Trend – Ranked Data[6]			Jonckheere-Terpstra Test[7]			Krukal-Wallis Test	Wilcoxon-Mann-Whitney Test[8]		
	Low	Med	High	Low	Med	High		Low	Med	High	Low	Med	High	Low	Med	High		Low	Med	High
Male Length	0.0507	0.5073	0.1644	0.1790	0.2119	0.0759	0.1345	0.1215	0.8350	0.1468	0.0728	0.5769	0.1588	0.1489	0.6605	0.1911	0.1618	0.5818	1.0000	0.3371
Female Length	0.5896	0.1257	0.3386	0.5896	0.3169	0.3379	0.4533	0.9005	0.2798	0.8332	0.7105	0.0930	0.2351	1.0000	0.1877	0.3504	0.3141	1.0000	0.3371	0.9370
Male Weight	0.3406	0.5439	0.3844	0.4345	0.5192	0.3868	0.7030	0.6427	0.8665	0.5898	0.5496	0.6406	0.4761	0.3865	0.5582	0.4006	0.8350	1.0000	1.0000	1.0000
Female Weight	0.4313	0.3020	0.8396	0.5569	0.6645	0.7103	0.6596	0.7573	0.5869	0.9984	0.5293	0.1755	0.3363	0.7728	0.3055	0.4550	0.5265	1.0000	0.5818	0.9370
# of Eggs - Estimated	0.8387	0.6567	0.5568	1.0000	0.7975	0.8514	0.8881	0.9934	0.9400	0.9530	0.8238	0.3804	0.1097	0.7728	0.2416	0.0618	0.3198	1.0000	0.9370	0.9370
# of Eggs_- Counted	0.9574	0.4631	0.4537	0.9574	0.6157	0.6583	0.8342	0.9999	0.7918	0.8878	0.8804	0.3031	0.0896	0.7728	0.1877	0.0499	0.2846	1.0000	0.5818	0.9370
# of Spawns	0.8147	0.7255	0.0825	1.0000	0.8608	0.1206	0.2271	0.9901	0.9685	0.2225	0.5989	0.9397	0.1500	0.5590	1.0000	0.1863	0.3429	1.0000	1.0000	0.5818
# of Fertile Eggs	0.9523	0.4336	0.3561	0.9523	0.5342	0.5712	0.7675	0.9998	0.7598	0.7895	0.8804	0.3031	0.0896	0.7728	0.1877	0.0499	0.2846	1.0000	0.5818	0.9370
% of infertile eggs[9]	0.9754	0.8810	0.0053	1.0000	1.0000	0.0078	0.0124	1.0000	0.9974	0.0117	0.9232	0.7009	0.0158	1.0000	0.6605	0.0309	0.0743	1.0000	1.0000	0.1555
# of Eggs per Female per Reproductive Day	0.8758	0.4360	0.3424	0.8758	0.5210	0.5423	0.7759	0.9970	0.7625	0.7495	0.7631	0.2146	0.0688	0.7728	0.1073	0.0317	0.2770	1.0000	0.3371	0.9370
Male Tubercle Score	0.5143	0.9417	0.8806	0.7703	0.9119	0.8733	0.8613	0.8414	0.9997	0.9546	0.7880	0.6391	0.9322	0.7674	0.6050	0.8879	0.8164	1.0000	1.0000	1.0000
Male Fatpad Weight	0.1498	0.2302	0.0736	0.1864	0.2208	0.0631	0.2310	0.3269	0.4723	0.1242	0.1420	0.2835	0.1012	0.1663	0.2196	0.0461	0.1726	0.6558	1.0000	0.2068
Male FPI	0.1838	0.2873	0.0887	0.2309	0.2745	0.0766	0.2721	0.3906	0.5646	0.1481	0.3056	0.4087	0.1455	0.2817	0.2874	0.0600	0.2152	1.0000	1.0000	0.2068
Male Gonad Weight	0.4564	0.4872	0.3722	0.4716	0.5635	0.4329	0.7753	0.7848	0.8161	0.6412	0.5471	0.5043	0.4009	0.5637	0.5582	0.4006	0.7924	1.0000	1.0000	0.9370
Female Gonad Weight	0.8727	0.5611	0.4605	1.0000	0.6878	0.7351	0.8274	0.9967	0.8800	0.9025	0.5913	0.8396	0.5812	0.7728	0.7697	0.5133	0.7817	1.0000	1.0000	1.0000
Male GSI	0.8619	0.7372	0.5942	0.8619	0.8742	0.7784	0.9586	0.9958	0.9723	0.9147	0.7931	0.7432	0.6492	0.7728	0.7697	0.6406	0.9625	1.0000	1.0000	1.0000
Female GSI	0.7696	0.2085	0.2016	0.7696	0.2991	0.3189	0.5209	0.9811	0.4347	0.5854	0.7210	0.2127	0.1646	0.7728	0.3055	0.1911	0.4531	1.0000	0.3371	0.5818
Male VTG	0.9710	0.1964	0.6768	1.0000	0.6121	0.6544	0.4425	1.0000	0.4134	1.0000	0.7926	0.7926	0.6192	1.0000	0.8836	0.6406	0.9498	1.0000	1.0000	1.0000
Female VTG	0.3631	0.5566	0.3339	0.4533	0.5417	0.3337	0.6751	0.6734	0.8766	0.5262	0.2529	0.6298	0.5721	0.0833	0.3798	0.4006	0.6275	0.3371	1.0000	1.0000

1. All analyses are applied to the total count (or %) data, or mean data over each tank (replicate) within each dose group.
2. Parametric procedures assume common variance across dose groups.
3. Stepdown linear dose trend tests assume equal spacing among the dose groups, starting from the linear trend among all four dose levels until finding the highest dose level for which a linear trend incorporating responses from all dose levels (including vehicle) up to and including the given dose level, is not statistically significant at the 0.05 level.
4. Williams' trend tests include a series of increasing trend tests and a series of decreasing trend tests. Each series is conducted in the stepdown fashion as described in the above item 3. The p-values are defined as 2*min(p_decreasing, p_increasing). If the p-values are larger than 1 then 1 was reported.

5. Dunnett's tests are applied when a significant dose effect at the 0.05 level is observed.
6. Stepdown linear dose trend tests are applied to the ranked scores.
7. Jonckheere-Terpstra tests are conducted in a stepdown fashion at the 0.05 level (2-sided).
8. When a dose effect by Kruskal-Wallis test is observed, pairwise Wilcoxon-Mann-Whitney test are applied to each dose and control pair at the 0.05 level, adjusted for simultaneity by Bonferroni's method.
9. A logit-transformation is applied to the % of infertile egg data.

Table 11. Summary Results of Statistical Analyses
Test Article: Ketoconazole
Participating Laboratory: Springborn Smithers[1]

Endpoint	Parametric Procedure[2]										Non-Parametric Procedure								
	Linear Trend[3]			Williams' Test[4]			F TEST	Dunnett's Test[5]			Linear Trend – Ranked Data[6]			Jonckheere-Terpstra Test[7]			Krukal-Wallis Test	Wilcoxon-Mann-Whitney Test[8]		
	Low	Med	High	Low	Med	High		Low	Med	High	Low	Med	High	Low	Med	High		Low	Med	High
Male Length	0.3483	0.0595	0.0146	0.3483	0.0690	0.0258	0.0884	0.6533	0.1410	0.0542	0.2101	0.0937	0.0273	0.5637	0.1432	0.0317	0.1486	1.0000	0.1818	0.0911
Female Length	0.8096	0.1982	0.4573	0.8096	0.4610	0.4927	0.5659	0.9892	0.4165	0.9461	0.7247	0.1958	0.5643	0.5637	0.1073	0.4550	0.5179	1.0000	0.5818	1.0000
Male Weight	0.4097	0.2800	0.1890	0.4097	0.3336	0.2446	0.5598	0.7321	0.5533	0.4091	0.2942	0.1693	0.2197	0.5637	0.3055	0.3043	0.4531	1.0000	0.5818	0.3371
Female Weight	0.9656	0.1759	0.4988	0.9656	0.4894	0.5232	0.4618	0.9999	0.3760	0.9859	0.8865	0.1914	0.5602	1.0000	0.1877	0.5753	0.4687	1.0000	0.5818	1.0000
# of Eggs - Estimated	0.5821	0.6463	0.8985	0.6949	0.8256	0.8810	0.9354	0.8953	0.9346	0.9964	0.7405	0.7909	0.9331	0.5637	0.6077	0.8881	0.9802	1.0000	1.0000	1.0000
# of Eggs_ - Counted	0.5454	0.8696	0.9177	0.7317	0.8679	0.9256	0.9352	0.8677	0.9965	0.9870	0.5085	1.0000	0.9327	0.5637	1.0000	0.9256	0.8614	1.0000	1.0000	1.0000
# of Spawns	0.5076	0.2343	0.3171	0.5076	0.3586	0.3828	0.6519	0.8353	0.4793	0.6959	0.5794	0.1917	0.3236	0.5439	0.2595	0.4458	0.7103	1.0000	1.0000	1.0000
# of Fertile Eggs	0.5464	0.8218	0.8784	0.7092	0.8420	0.8983	0.9395	0.8685	0.9912	0.9819	0.5108	0.8423	0.9665	0.5637	0.8836	1.0000	0.8974	1.0000	1.0000	1.0000
% of infertile eggs[9]	0.3264	0.1494	0.3809	0.3264	0.3764	0.3171	0.4978	0.6265	0.3290	0.7982	0.3009	0.1462	0.2514	0.1663	0.0962	0.2542	0.4604	0.6558	0.7257	1.0000
# of Eggs per Female per Reproductive Day	0.5323	0.9608	0.8399	0.7720	0.9138	0.8403	0.8732	0.8569	0.9999	0.9424	0.4656	1.0000	0.9324	0.5637	1.0000	0.9256	0.8244	1.0000	1.0000	1.0000
Male Tubercle Score	0.5326	0.8574	0.2488	1.0000	1.0000	0.3595	0.3774	0.8572	0.9954	0.5576	0.3782	0.7656	0.2338	0.6631	0.5566	0.1595	0.3244	1.0000	1.0000	1.0000
Male Fatpad Weight	0.8241	0.3962	0.2590	1.0000	0.4733	0.5059	0.5836	0.9915	0.7157	0.7157	0.5390	0.5390	0.3370	0.8501	0.4289	0.2209	0.5419	1.0000	1.0000	1.0000
Male FPI	0.6493	0.4740	0.3003	1.0000	0.5663	0.6055	0.5683	0.9362	0.8030	0.8030	0.5390	0.5390	0.3370	0.8501	0.4289	0.2209	0.5419	1.0000	1.0000	1.0000
Male Gonad Weight	0.6325	0.0474	0.0006	0.6325	0.0547	0.0012	0.0043	0.9271	0.1140	0.0029	0.6829	0.0583	0.0015	0.7728	0.0790	0.0021	0.0299	1.0000	0.3371	0.0911
Female Gonad Weight	0.9922	0.1848	0.0488	1.0000	0.2188	0.1122	0.1957	1.0000	0.3924	0.2087	0.8777	0.1247	0.0946	1.0000	0.1073	0.0838	0.2744	1.0000	0.1818	1.0000
Male GSI	0.9442	0.0283	0.0003	0.9442	0.0324	0.0008	0.0018	0.9997	0.0700	0.0019	0.5488	0.0119	0.0002	0.5637	0.0281	0.0005	0.0137	1.0000	0.1818	0.0911
Female GSI	0.8966	0.3567	0.0388	1.0000	0.4260	0.0725	0.1531	0.9982	0.6648	0.1410	0.9358	0.3832	0.0372	0.5637	0.4642	0.0399	0.1558	1.0000	0.5818	0.3371
Male VTG[10]	0.8465	0.6816	0.0002	1.0000	1.0000	0.0001	0.0001	0.9943	0.9516	0.0003	0.3799	0.2339	0.0235	0.4678	0.2704	0.1227	0.0260	1.0000	0.9283	0.0911
Female VTG	0.3123	0.9477	0.9242	0.6821	0.8107	0.8654	0.6705	0.6022	0.9998	0.9857	0.8370	0.4169	0.4405	0.7728	0.3798	0.4006	0.6772	1.0000	1.0000	1.0000

1. All analyses are applied to the total count (or %) data, or mean data over each tank (replicate) within each dose group.
2. Parametric procedures assume common variance across dose groups.
3. Stepdown linear dose trend tests assume equal spacing among the dose groups, starting from the linear trend among all four dose levels until finding the highest dose level for which a linear trend incorporating responses from all dose levels (including vehicle) up to and including the given dose level, is not statistically significant at the 0.05 level.

4. Williams' trend tests include a series of increasing trend tests and a series of decreasing trend tests. Each series is conducted in the stepdown fashion as described in the above item 3. The p-values are defined as 2*min(p_decreasing, p_increasing). If the p-values are larger than 1 then 1 was reported.
5. Dunnett's tests are applied when a significant dose effect at the 0.05 level is observed.
6. Stepdown linear dose trend tests are applied to the ranked scores.
7. Jonckheere-Terpstra tests are conducted in a stepdown fashion at the 0.05 level (2-sided).
8. When a dose effect by Kruskal-Wallis test is observed, pairwise Wilcoxon-Mann-Whitney test are applied to each dose and control pair at the 0.05 level, adjusted for simultaneity by Bonferroni's method.
9. A logit-transformation is applied to the % of infertile egg data.
10. A log-transformation is applied to the male VTG data.

Table 12. Summary Results of Statistical Analyses
Test Article: Potassium Permanganate
Participating Laboratory: Springborn Smithers[1]

Endpoint	Parametric Procedure[2]										Non-Parametric Procedure									
	Linear Trend[3]			Williams' Test[4]			F TEST	Dunnett's Test[5]			Linear Trend – Ranked Data[6]			Jonckheere-Terpstra Test[7]			Krukal-Wallis Test	Wilcoxon-Mann-Whitney Test[8]		
	Low	Med	High	Low	Med	High		Low	Med	High	Low	Med	High	Low	Med	High		Low	Med	High
Male Length	0.5963	0.2046	0.4287	0.5963	0.4196	0.4482	0.6227	0.9050	0.4279	0.8801	0.5341	0.2246	0.5992	0.5637	0.2416	0.6406	0.5894	1.0000	0.9370	1.0000
Female Length	0.4780	0.6833	0.7715	0.6107	0.7276	0.7774	0.9075	0.8070	0.9524	0.9524	0.5532	0.9471	0.8339	0.3865	0.7697	0.7087	0.8974	1.0000	1.0000	1.0000
Male Weight	0.6965	0.4171	0.9642	0.7526	0.8917	0.9506	0.7415	0.9579	0.7409	0.9945	0.8357	0.3765	0.9651	1.0000	0.3798	0.9256	0.6275	1.0000	0.9370	1.0000
Female Weight	0.4414	0.9107	0.9201	0.7373	0.8743	0.9323	0.8500	0.7686	0.9989	0.9987	0.3877	0.7362	0.9659	0.3865	0.6605	1.0000	0.7817	1.0000	1.0000	1.0000
# of Eggs - Estimated	0.5676	0.6346	0.8496	0.9609	1.0000	1.0000	0.7597	0.8849	0.9283	0.9973	0.5291	0.5752	0.8064	0.5637	0.6605	0.8885	0.6306	1.0000	1.0000	1.0000
# of Eggs_- Counted	0.6835	0.8007	0.5714	1.0000	0.9464	0.8930	0.8581	0.9524	0.9877	0.9611	0.6257	0.5319	0.7738	0.7728	0.5582	0.8152	0.6729	1.0000	1.0000	1.0000
# of Spawns	0.4705	0.8712	0.5250	0.7769	0.9194	0.4335	0.6070	0.7994	0.9966	0.6419	0.2202	0.8125	0.6184	0.3749	0.8216	0.5286	0.4298	1.0000	1.0000	1.0000
# of Fertile Eggs	0.6786	0.7958	0.5786	1.0000	0.9408	0.9071	0.8587	0.9503	0.9868	0.9654	0.6257	0.5319	0.7738	0.7728	0.5582	0.8152	0.6729	1.0000	1.0000	1.0000
% of infertile eggs[9]	0.5149	0.8224	0.1967	1.0000	1.0000	0.3178	0.3343	0.8465	0.9916	0.4637	0.7758	0.9263	0.2891	0.7137	0.9319	0.3000	0.4806	1.0000	1.0000	1.0000
# of Eggs per Female per Reproductive Day	0.8237	0.8342	0.5062	1.0000	0.9841	0.7341	0.8716	0.9914	0.9928	0.8909	0.6257	0.5319	0.7738	0.7728	0.5582	0.8152	0.6729	1.0000	1.0000	1.0000
Male Tubercle Score	0.5753	0.3523	0.0802	0.5753	0.4207	0.1036	0.3342	0.8905	0.6589	0.1944	0.6050	0.3804	0.1287	0.6631	0.3377	0.1109	0.4743	1.0000	1.0000	0.5818
Male Fatpad Weight	0.0760	0.6149	0.6517	0.5689	0.6787	0.7254	0.1348	0.1770	0.9168	0.9749	0.1687	0.4780	0.6118	0.1391	0.4132	0.4193	0.0692	0.5495	1.0000	1.0000
Male FPI	0.0700	0.5604	0.7520	0.5360	0.6398	0.6840	0.1140	0.1642	0.8795	0.9146	0.2384	0.4919	0.7841	0.1391	0.4132	0.4840	0.0890	0.5495	1.0000	1.0000
Male Gonad Weight	0.8238	0.0092	0.0219	1.0000	0.0397	0.0416	0.0194	0.9914	0.0237	0.2718	0.8461	0.0096	0.0154	0.7728	0.0570	0.0618	0.0407	1.0000	0.1818	0.3371
Female Gonad Weight	0.4675	0.6867	0.4046	1.0000	0.8160	0.7774	0.5891	0.7964	0.9538	0.9142	0.4070	0.6252	0.6273	0.3865	0.5582	0.5753	0.5528	1.0000	1.0000	1.0000
Male GSI	0.5408	0.0034	0.0011	1.0000	0.0066	0.0068	0.0020	0.8640	0.0089	0.0261	0.7828	0.0003	0.0002	0.7728	0.0281	0.0117	0.0086	1.0000	0.0911	0.0911
Female GSI	0.7052	0.4958	0.2395	1.0000	0.5922	0.4659	0.5395	0.9613	0.8243	0.6760	0.5656	0.3215	0.3686	0.5637	0.2416	0.3043	0.4125	1.0000	0.9370	1.0000
Male VTG[10]	0.5360	0.7757	0.6742	0.8379	0.9145	0.9048	0.7448	0.8358	0.9779	0.9748	0.5977	0.7592	0.5962	1.0000	0.7868	0.3478	0.5836	1.0000	1.0000	1.0000
Female VTG	0.5147	0.9674	0.3111	1.0000	1.0000	0.3904	0.4269	0.8157	0.9999	0.6856	0.3101	0.8796	0.5382	0.4386	0.7868	0.3140	0.4548	1.0000	1.0000	1.0000

1. All analyses are applied to the total count (or %) data, or mean data over each tank (replicate) within each dose group.
2. Parametric procedures assume common variance across dose groups.

3. Stepdown linear dose trend tests assume equal spacing among the dose groups, starting from the linear trend among all four dose levels until finding the highest dose level for which a linear trend incorporating responses from all dose levels (including vehicle) up to and including the given dose level, is not statistically significant at the 0.05 level.
4. Williams' trend tests include a series of increasing trend tests and a series of decreasing trend tests. Each series is conducted in the stepdown fashion as described in the above item 3. The p-values are defined as 2*min(p_decreasing, p_increasing). If the p-values are larger than 1 then 1 was reported.
5. Dunnett's tests are applied when a significant dose effect at the 0.05 level is observed.
6. Stepdown linear dose trend tests are applied to the ranked scores.
7. Jonckheere-Terpstra tests are conducted in a stepdown fashion at the 0.05 level (2-sided).
8. When a dose effect by Kruskal-Wallis test is observed, pairwise Wilcoxon-Mann-Whitney test are applied to each dose and control pair at the 0.05 level, adjusted for simultaneity by Bonferroni's method.
9. A logit-transformation is applied to the % of infertile egg data.
10. A log-transformation is applied to the male VTG data.

Table 13. Summary Results of Statistical Analyses
Test Article: Potassium Permanganate
Participating Laboratory: ABC Laboratories[1]

Endpoint	Parametric Procedure[2]										Non-Parametric Procedure									
	Linear Trend[3]			Williams' Test[4]			F TEST	Dunnett's Test[5]			Linear Trend – Ranked Data[6]			Jonckheere-Terpstra Test[7]			Krukal-Wallis Test	Wilcoxon-Mann-Whitney Test[8]		
	Low	Med	High	Low	Med	High		Low	Med	High	Low	Med	High	Low	Med	High		Low	Med	High
Survival Rate[9]	0.5603	0.0112	<.0001	0.5603	0.0126	0.0001	0.0004	0.8794	0.0286	0.0003	0.3827	0.0011	<.0001	0.3173	0.0057	0.0002	0.0064	1.0000	0.0531	0.0606
Male Length	0.4132	0.7224	0.7798	0.8536	1.0000	1.0000	0.6767	0.7398	0.9682	0.9997	0.5074	0.6046	0.6938	0.3719	0.7663	0.7950	0.7122	1.0000	1.0000	1.0000
Female Length	0.7968	0.1531	0.0724	1.0000	0.1808	0.2286	0.1955	0.9872	0.3361	0.3410	0.8640	0.0592	0.0460	0.7674	0.0389	0.0263	0.1249	1.0000	0.0911	1.0000
Male Weight	0.6559	0.0293	0.0164	1.0000	0.0437	0.0807	0.0303	0.9408	0.0728	0.1550	0.8367	0.0336	0.0071	0.7728	0.1073	0.0181	0.0498	1.0000	0.3371	0.1555
Female Weight	0.7977	0.0420	0.0177	1.0000	0.0486	0.0883	0.0463	0.9874	0.1026	0.1364	0.7862	0.0663	0.0424	0.7728	0.0570	0.0309	0.1120	1.0000	0.1818	1.0000
# of Eggs - Estimated[10]	0.9352	0.0150	0.0050	0.9352	0.0195	0.0203	0.0183	0.9996	0.0380	0.0492	0.3413	0.0002	<.0001	0.3865	0.0053	0.0010	0.0086	1.0000	0.0882	0.0882
# of Eggs_- Counted[10]	0.8834	0.0120	0.0036	0.8834	0.0147	0.0153	0.0140	0.9975	0.0306	0.0358	0.0993	<.0001	<.0001	0.1489	0.0021	0.0005	0.0073	0.5818	0.0882	0.0882
# of Spawns[10]	0.9851	0.0130	0.0037	0.9851	0.0159	0.0166	0.0134	1.0000	0.0331	0.0385	0.8710	0.0003	<.0001	0.7674	0.0118	0.0024	0.0087	1.0000	0.0853	0.0882
# of Fertile Eggs[10]	0.8887	0.0086	0.0006	0.8887	0.0097	0.0026	0.0033	0.9978	0.0222	0.0059	0.1610	0.0001	<.0001	0.2482	0.0034	0.0003	0.0071	0.9370	0.0882	0.0796
% of Infertile eggs[9]	0.7094	0.7969	0.0580	1.0000	0.9721	0.1259	0.1712	0.9655	0.9880	0.1577	0.3239	0.9337	0.1809	0.2482	0.5613	0.4720	0.2573	0.9370	1.0000	0.7415
# of Eggs per Female per Reproductive Day[10]	0.8199	0.0040	0.0013	0.8199	0.0056	0.0058	0.0047	0.9909	0.0105	0.0163	0.2140	0.0002	<.0001	0.2482	0.0034	0.0008	0.0079	0.9370	0.0882	0.0882
Male Tubercle Score	0.6723	0.0957	0.0997	0.6723	0.1387	0.2135	0.2751	0.9485	0.2207	0.3758	0.5802	0.1154	0.1033	0.5637	0.1235	0.0800	0.2895	1.0000	0.5818	0.4607
Male Fatpad Score	0.5759	0.0284	0.0474	0.5759	0.0582	0.1029	0.1039	0.8928	0.0706	0.2518	0.6494	0.0321	0.0468	0.5566	0.0515	0.0166	0.0829	1.0000	0.1726	0.1248
Male Fatpad Weight	0.5151	0.0276	0.0131	0.5151	0.0316	0.0460	0.0574	0.8446	0.0688	0.0711	0.5701	0.0329	0.0116	1.0000	0.0570	0.0136	0.0793	1.0000	0.0911	0.1555
Male FPI	0.4634	0.0495	0.0264	0.4634	0.0573	0.0740	0.1148	0.7952	0.1197	0.1143	0.2444	0.0153	0.0064	0.3865	0.0192	0.0055	0.0587	1.0000	0.0911	0.1555
Male Gonad Weight	0.1297	0.7314	0.2619	0.8419	0.8675	0.7286	0.1740	0.2902	0.9711	0.8702	0.0607	0.9260	0.1658	0.0433	0.7697	0.3550	0.0946	0.1818	1.0000	1.0000
Female Gonad Weight	0.1718	0.0008	0.0010	0.1718	0.0015	0.0041	0.0029	0.3715	0.0021	0.0101	0.0190	0.0002	0.0003	0.0209	0.0004	0.0007	0.0134	0.0911	0.0911	0.1555
Male GSI	0.2238	0.6816	0.8469	0.5658	0.6749	0.7868	0.5836	0.4649	0.9526	0.9998	0.1622	0.8174	0.7509	0.1489	0.6605	0.9181	0.4007	0.5818	1.0000	1.0000
Female GSI	0.1010	0.0003	0.0005	0.1010	0.0006	0.0017	0.0011	0.2318	0.0007	0.0050	0.0179	0.0001	0.0001	0.0209	0.0004	0.0005	0.0111	0.0911	0.0911	0.1555
Male VTG[10]	0.5448	0.3361	0.9959	1.0000	0.9724	0.8180	0.3956	0.8695	0.6403	0.9220	0.6988	0.9227	0.1016	0.3173	0.5127	0.5855	0.3530	1.0000	1.0000	1.0000
Female VTG[10]	0.7335	0.0072	0.0180	0.7335	0.0159	0.0758	0.0217	0.9725	0.0189	0.1479	0.8278	0.0108	0.0475	0.7728	0.0128	0.0224	0.0637	1.0000	0.0911	1.0000

1. All analyses are applied to the total count (or %) data, or mean data over each tank (replicate) within each dose group.
2. Parametric procedures assume common variance across dose groups.
3. Stepdown linear dose trend tests assume equal spacing among the dose groups, starting from the linear trend among all four dose levels until finding the highest dose level for which a linear trend incorporating responses from all dose levels (including vehicle) up to and including the given dose level, is not statistically significant at the 0.05 level.
4. Williams' trend tests include a series of increasing trend tests and a series of decreasing trend tests. Each series is conducted in the stepdown fashion as described in the above item 3. The p-values are defined as 2*min(p_decreasing, p_increasing). If the p-values are larger than 1 then | was reported.
5. Dunnett's tests are applied when a significant dose effect at the 0.05 level is observed.
6. Stepdown linear dose trend tests are applied to the ranked scores.
7. Jonckheere-Terpstra tests are conducted in a stepdown fashion at the 0.05 level (2-sided).
8. When a dose effect by Kruskal-Wallis test is observed, pairwise Wilcoxon-Mann-Whitney test are applied to each dose and control pair at the 0.05 level, adjusted for simultaneity by Bonferroni's method.
9. A logit-transformation is applied to the % of infertile egg data.
10. A log-transformation is applied.

Table 14. Summary Results of Statistical Analyses
Test Article: Flutamide
Participating Laboratory: Wildlife International Laboratories[1]

Endpoint	Parametric Procedure[2]										Non-Parametric Procedure									
	Linear Trend[3]			Williams' Test[4]			F TEST	Dunnett's Test[5]			Linear Trend – Ranked Data[6]			Jonckheere-Terpstra Test[7]			Krukal-Wallis Test	Wilcoxon-Mann-Whitney Test[8]		
	Low	Med	High	Low	Med	High		Low	Med	High	Low	Med	High	Low	Med	High		Low	Med	High
Male Length	0.5209	0.0815	0.1990	1.0000	0.2838	0.3024	0.1214	0.8472	0.1889	0.8892	0.4692	0.1226	0.2137	0.5614	0.1409	0.2403	0.1786	1.0000	0.4395	1.0000
Female Length	0.8340	0.8788	0.6525	0.8564	1.0000	0.7914	0.9644	0.9928	0.9972	0.9210	0.6366	0.5897	0.3649	0.6631	0.4189	0.2612	0.7725	1.0000	1.0000	1.0000
Male Weight	0.2121	0.0036	0.0053	1.0000	0.0192	0.0200	0.0018	0.4410	0.0095	0.1795	0.3021	0.0037	0.0034	0.3865	0.0570	0.0399	0.0155	1.0000	0.0911	0.5818
Female Weight	0.9303	0.7388	0.4690	1.0000	0.8760	0.6781	0.8765	0.9995	0.9727	0.8561	0.9443	0.7274	0.2464	1.0000	0.6605	0.1911	0.5709	1.0000	1.0000	0.9370
# of Eggs_-Counted	0.6789	0.1984	0.0111	1.0000	0.2352	0.0295	0.0429	0.9504	0.4169	0.0615	0.4905	0.1803	0.0055	0.5637	0.2416	0.0089	0.0381	1.0000	0.9370	0.1772
# of Fertile Eggs[10]	0.8357	0.4908	0.0044	1.0000	0.5863	0.0063	0.0132	0.9930	0.8196	0.0143	0.4021	0.1544	0.0028	0.3865	0.3055	0.0089	0.0263	1.0000	0.9370	0.0882
% of infertile eggs[9]	0.7860	0.7094	0.3759	1.0000	0.8421	0.6568	0.7631	0.9855	0.9646	0.7886	0.9423	0.9423	0.9697	0.7728	1.0000	1.0000	0.9987	1.0000	1.0000	1.0000
# of Eggs per Female per Reproductive Day	0.9821	0.3421	0.0269	0.9821	0.4084	0.0455	0.1168	1.0000	0.6448	0.0920	0.6625	0.6011	0.0270	0.5637	0.7697	0.0398	0.0873	1.0000	1.0000	0.1772
Male Tubercle Score	0.0907	0.0058	0.0006	0.0907	0.0064	0.0010	0.0049	0.2082	0.0150	0.0024	0.1007	0.0017	0.0003	0.0833	0.0026	0.0006	0.0157	0.3371	0.0911	0.0911
Male Tubercle Count	0.4458	0.0797	0.0020	0.4458	0.0930	0.0032	0.0147	0.7734	0.1850	0.0073	0.7602	0.0232	0.0015	0.3836	0.0153	0.0012	0.0310	1.0000	0.3371	0.1818
Male Fatpad Score	0.7995	0.3195	0.1103	0.7995	0.3812	0.1817	0.4171	0.9875	0.6127	0.3182	0.8761	0.2007	0.0560	0.8770	0.2860	0.0663	0.2768	1.0000	1.0000	0.4025
Male Fatpad Weight	0.6615	0.5081	0.0976	1.0000	0.6068	0.1946	0.2727	0.9423	0.8357	0.3374	0.8736	0.2773	0.0430	1.0000	0.2416	0.0355	0.1729	1.0000	1.0000	0.3371
Male FPI	0.8007	0.9217	0.1866	1.0000	1.0000	0.2520	0.4104	0.9877	0.9992	0.4193	1.0000	0.2722	0.0340	1.0000	0.3055	0.0317	0.1573	1.0000	1.0000	0.1818
Male Gonad Weight	0.4803	0.5180	0.6184	0.5271	0.6293	0.6728	0.8794	0.8094	0.8446	0.8980	0.4669	0.5075	0.6734	0.5637	0.5582	0.7087	0.8456	1.0000	1.0000	1.0000
Female Gonad Weight	0.1728	0.4883	0.1796	0.7674	0.9087	0.2946	0.0958	0.3703	0.8172	0.4763	0.3163	0.9320	0.0641	0.2482	0.7697	0.1124	0.0985	0.9370	1.0000	0.3371
Male GSI	0.7952	0.0207	0.0495	0.7952	0.0688	0.0723	0.0723	0.9867	0.0518	0.3438	0.9320	0.0311	0.0391	0.7728	0.0281	0.0399	0.0985	1.0000	0.3371	0.5818

Female GSI	0.1355	0.3839	0.1232	0.7476	0.8860	0.1949	0.0454	0.2993	0.7004	0.3378	0.2181	1.0000	0.0368	0.0833	0.5582	0.1351	0.0623	0.3371	1.0000	0.0911
Male VTG	0.4369	0.7497	0.8184	0.8430	0.9940	1.0000	0.7206	0.7636	0.9759	0.9984	0.3017	0.7784	0.5800	0.1489	0.7139	0.5435	0.6503	0.5818	1.0000	1.0000
Female VTG	0.5621	0.6258	0.7265	0.6247	0.7441	0.7949	0.9356	0.8808	0.9233	0.9550	0.7443	0.8446	0.8363	0.7728	0.7697	0.7794	0.9846	1.0000	1.0000	1.0000

1. All analyses are applied to the total count (or %) data, or mean data over each tank (replicate) within each dose group.
2. Parametric procedures assume common variance across dose groups.
3. Stepdown linear dose trend tests assume equal spacing among the dose groups, starting from the linear trend among all four dose levels until finding the highest dose level for which a linear trend incorporating responses from all dose levels (including vehicle) up to and including the given dose level, is not statistically significant at the 0.05 level.
4. Williams' trend tests include a series of increasing trend tests and a series of decreasing trend tests. Each series is conducted in the stepdown fashion as described in the above item 3. The p-values are defined as 2*min(p_decreasing, p_increasing). If the p-values are larger than 1 then 1 was reported.
5. Dunnett's tests are applied when a significant dose effect at the 0.05 level is observed.
6. Stepdown linear dose trend tests are applied to the ranked scores.
7. Jonckheere-Terpstra tests are conducted in a stepdown fashion at the 0.05 level (2-sided).
8. When a dose effect by Kruskal-Wallis test is observed, pairwise Wilcoxon-Mann-Whitney test are applied to each dose and control pair at the 0.05 level, adjusted for simultaneity by Bonferroni's method.
9. A logit-transformation is applied to the % of infertile egg data.
10. A log-transformation is applied to the # of fertile egg data

Table 14a. Summary Results of Statistical Analyses
Test Article: Flutamide
Participating Laboratory: Wildlife International Laboratories[1]
Estimated Number of Eggs[9]

Endpoint	Parametric Procedure[2]										Non-Parametric Procedure									
	Linear Trend[3]			Williams' Test[4]			F TEST	Dunnett's Test[5]			Linear Trend – Ranked Data[6]			Jonckheere-Terpstra Test[7]			Kruskal-Wallis Test	Wilcoxon-Mann-Whitney Test[8]		
	Low	Med	High	Low	Med	High		Low	Med	High	Low	Med	High	Low	Med	High		Low	Med	High
Estimated Number of Eggs	0.9260	0.7260	0.0737	1.0000	0.8614	0.0980	0.2112	0.9994	0.9687	0.1850	0.9433	0.8869	0.2387	0.7728	0.8836	0.1750	0.4301	1.0000	1.0000	0.5738

1. All analyses are applied to the total count (or %) data, or mean data over each tank (replicate) within each dose group.
2. Parametric procedures assume common variance across dose groups.
3. Step down linear dose trend tests assume equal spacing among the dose groups, starting from the linear trend among all four dose levels until finding the highest dose level for which a linear trend incorporating responses from all dose levels (including vehicle) up to and including the given dose level, is not statistically significant at the 0.05 level.
4. Williams' trend tests include a series of increasing trend tests and a series of decreasing trend tests. Each series is conducted in the step down fashion as described in the above item 3. The p-values are defined as 2*min(p_decreasing, p_increasing). If the p-values are larger than 1 then 1 was reported.
5. Dunnett's test is applied when a significant dose effect at the 0.05 level is observed.
6. Step down linear dose trend tests are applied to the ranked scores.
7. Jonckheere-Terpstra tests are conducted in a step down fashion at the 0.05 level (2-sided).
8. When a dose effect by Kruskal-Wallis test is observed, pairwise Wilcoxon-Mann-Whitney test are applied to each dose and control pair at the 0.05 level, adjusted for simultaneity by Bonferroni's method.
9. Counted numbers of eggs were 0 for concentration 1000ug/L, replicates B and C. Estimated number of eggs were not reported for these replicates, but were set to 0 for analysis.

Shapiro-Wilk W Test of Normality	W = 0.964185		Pr < W = 0.7379
Levene's Heterogeneity Test	SS=28349565 D df=3	MS=9449855 F=0.90	Pr > F = 0.4711

Parametric procedures are okay for this variable.

Summary Statistics for Estimated Number of Eggs

Dose Group	N	Mean	Standard Deviation	CV (%)	Sex
0_Control	4	119.15	60.56	50.832	F
1_100 ug/L	4	122.88	16.46	13.394	F
2_500 ug/L	4	105.02	57.55	54.802	F
3_1000 ug/L	4	43.75	71.81	164.130	F

Table 15. Springborn Smithers Summary Statistics

Chemical	Parameter	N	Dose Group	Mean	Stand Deviation	CV (%)	Sex
Flutamide	**# Counted Eggs**	4	0_Control	800.250	275.024	34.367	Females
		4	1_100ug/L	781.750	179.673	22.983	Females
		4	2_500ug/L	543.250	205.879	37.898	Females
		4	3_1000ug/L	603.000	877.166	145.467	Females
Flutamide	**# Eggs/Female/Reproductive Day**	4	0_Control	10.646	3.521	33.076	Females
		4	1_100ug/L	9.976	2.025	20.296	Females
		4	2_500ug/L	7.266	3.898	53.648	Females
		4	3_1000ug/L	7.179	10.442	145.467	Females
Flutamide	**# Estimated Eggs**	4	0_Control	625.000	168.770	27.003	Females
		4	1_100ug/L	683.750	245.845	35.955	Females
		4	2_500ug/L	496.250	76.852	15.487	Females
		4	3_1000ug/L	507.500	737.264	145.274	Females
Flutamide	**# Fertile Eggs**	4	0_Control	793.500	276.857	34.891	Females
		4	1_100ug/L	774.250	174.731	22.568	Females
		4	2_500ug/L	538.250	206.046	38.281	Females
		4	3_1000ug/L	553.500	802.636	145.011	Females
Flutamide	**# Spawns**	4	0_Control	7.500	3.109	41.455	Females
		4	1_100ug/L	8.000	2.160	27.003	Females
		4	2_500ug/L	6.750	1.258	18.642	Females
		4	3_1000ug/L	3.750	4.349	115.982	Females
Flutamide	**% of Infertile Eggs**	4	0_Control	0.954	0.598	62.659	Females
		4	1_100ug/L	0.898	0.511	56.939	Females
		4	2_500ug/L	1.004	0.501	49.907	Females
		3	3_1000ug/L	8.826	7.422	84.098	Females
Flutamide	**FPI**	4	0_Control	0.420	0.344	82.012	Males
		4	1_100ug/L	0.131	0.263	200.000	Males
		4	2_500ug/L	0.192	0.384	200.000	Males
		4	3_1000ug/L	0.000	0.000	.	Males
Flutamide	**Fatpad Weight**	4	0_Control	0.012	0.010	83.821	Males
		4	1_100ug/L	0.004	0.007	200.000	Males
		4	2_500ug/L	0.005	0.010	200.000	Males
		4	3_1000ug/L	0.000	0.000	.	Males
Flutamide	**GSI**	4	0_Control	8.594	1.014	11.801	Females
		4	1_100ug/L	8.895	2.329	26.177	Females
		4	2_500ug/L	9.931	0.983	9.898	Females
		4	3_1000ug/L	9.682	0.824	8.515	Females
		4	0_Control	1.290	0.248	19.254	Males

Table 15. Springborn Smithers Summary Statistics

Chemical	Parameter	N	Dose Group	Mean	Stand Deviation	CV (%)	Sex
		4	1_100ug/L	1.318	0.227	17.235	Males
		4	2_500ug/L	1.343	0.215	15.983	Males
		4	3_1000ug/L	1.371	0.184	13.445	Males
Flutamide	Gonad Weight	4	0_Control	0.109	0.017	15.904	Females
		4	1_100ug/L	0.107	0.026	24.530	Females
		4	2_500ug/L	0.116	0.009	7.442	Females
		4	3_1000ug/L	0.115	0.008	6.654	Females
		4	0_Control	0.038	0.006	15.182	Males
		4	1_100ug/L	0.042	0.009	21.504	Males
		4	2_500ug/L	0.042	0.009	22.031	Males
		4	3_1000ug/L	0.044	0.006	13.244	Males
Flutamide	Length	4	0_Control	50.636	1.025	2.024	Females
		4	1_100ug/L	50.117	2.088	4.166	Females
		4	2_500ug/L	49.096	1.103	2.246	Females
		4	3_1000ug/L	49.993	0.614	1.228	Females
		4	0_Control	62.112	2.511	4.043	Males
		4	1_100ug/L	65.206	1.901	2.916	Males
		4	2_500ug/L	63.086	2.274	3.604	Males
		4	3_1000ug/L	65.044	1.077	1.655	Males
Flutamide	Tubercle Score	4	0_Control	25.125	1.931	7.686	Males
		4	1_100ug/L	27.375	7.620	27.835	Males
		4	2_500ug/L	24.875	4.171	16.767	Males
		4	3_1000ug/L	26.500	3.240	12.228	Males
Flutamide	VTG	4	0_Control	47.729	11.794	24.711	Females
		4	1_100ug/L	72.787	19.039	26.157	Females
		4	2_500ug/L	63.758	44.826	70.306	Females
		4	3_1000ug/L	78.869	55.768	70.710	Females
		4	0_Control	0.012	0.009	70.004	Males
		4	1_100ug/L	0.008	0.001	16.658	Males
		4	2_500ug/L	0.169	0.323	191.536	Males
		4	3_1000ug/L	0.010	0.009	88.878	Males
Flutamide	Weight	4	0_Control	1.255	0.086	6.869	Females
		4	1_100ug/L	1.193	0.093	7.765	Females
		4	2_500ug/L	1.173	0.098	8.338	Females
		4	3_1000ug/L	1.245	0.144	11.602	Females
		4	0_Control	2.990	0.385	12.866	Males
		4	1_100ug/L	3.206	0.169	5.282	Males
		4	2_500ug/L	3.126	0.375	11.999	Males

Table 15. Springborn Smithers Summary Statistics

Chemical	Parameter	N	Dose Group	Mean	Stand Deviation	CV (%)	Sex
		4	3_1000ug/L	3.224	0.249	7.733	Males
Ketoconazole	# Counted Eggs	4	0_Control	288.500	262.301	90.919	Females
		4	1_25ug/L	192.000	179.618	93.551	Females
		4	2_100ug/L	262.500	232.507	88.574	Females
		4	3_400ug/L	247.750	192.945	77.879	Females
Ketoconazole	# Eggs/Female/Reproductive Day	4	0_Control	3.519	3.039	86.359	Females
		4	1_25ug/L	2.286	2.138	93.551	Females
		4	2_100ug/L	3.615	3.496	96.714	Females
		4	3_400ug/L	2.658	1.840	69.217	Females
Ketoconazole	# Estimated Eggs	4	0_Control	274.500	255.821	93.195	Females
		4	1_25ug/L	192.500	188.392	97.866	Females
		4	2_100ug/L	206.250	163.777	79.407	Females
		4	3_400ug/L	250.000	201.039	80.416	Females
Ketoconazole	# Fertile Eggs	4	0_Control	287.000	259.478	90.410	Females
		4	1_25ug/L	191.250	179.836	94.032	Females
		4	2_100ug/L	251.500	237.406	94.396	Females
		4	3_400ug/L	241.500	185.200	76.687	Females
Ketoconazole	# Spawns	4	0_Control	5.000	4.243	84.853	Females
		4	1_25ug/L	3.500	3.317	94.761	Females
		4	2_100ug/L	2.250	2.217	98.549	Females
		4	3_400ug/L	3.000	2.160	72.008	Females
Ketoconazole	% of Infertile Eggs	4	0_Control	0.228	0.455	200.000	Females
		4	1_25ug/L	3.196	5.312	166.219	Females
		3	2_100ug/L	9.410	15.087	160.340	Females
		4	3_400ug/L	1.528	1.773	116.048	Females
Ketoconazole	FPI	4	0_Control	0.521	1.043	200.000	Males
		4	1_25ug/L	0.850	1.700	200.000	Males
		4	2_100ug/L	0.000	0.000	.	Males
		4	3_400ug/L	0.000	0.000	.	Males
Ketoconazole	Fatpad Weight	4	0_Control	0.019	0.037	200.000	Males
		4	1_25ug/L	0.023	0.047	200.000	Males
		4	2_100ug/L	0.000	0.000	.	Males
		4	3_400ug/L	0.000	0.000	.	Males
Ketoconazole	GSI	4	0_Control	11.360	1.156	10.175	Females
		4	1_25ug/L	11.203	1.433	12.793	Females
		4	2_100ug/L	12.493	2.179	17.446	Females
		4	3_400ug/L	13.820	1.741	12.601	Females
		4	0_Control	1.014	0.205	20.208	Males

Table 15. Springborn Smithers Summary Statistics

Chemical	Parameter	N	Dose Group	Mean	Stand Deviation	CV (%)	Sex
		4	1_25ug/L	1.024	0.194	18.958	Males
		4	2_100ug/L	1.382	0.164	11.838	Males
		4	3_400ug/L	1.682	0.262	15.549	Males
Ketoconazole	Gonad Weight	4	0_Control	0.166	0.008	4.890	Females
		4	1_25ug/L	0.165	0.029	17.235	Females
		4	2_100ug/L	0.197	0.021	10.545	Females
		4	3_400ug/L	0.207	0.051	24.862	Females
		4	0_Control	0.035	0.010	28.545	Males
		4	1_25ug/L	0.039	0.012	30.731	Males
		4	2_100ug/L	0.051	0.009	16.881	Males
		4	3_400ug/L	0.066	0.009	14.121	Males
Ketoconazole	Length	4	0_Control	52.737	1.543	2.925	Females
		4	1_25ug/L	53.051	0.444	0.838	Females
		4	2_100ug/L	54.473	2.000	3.672	Females
		4	3_400ug/L	53.295	2.533	4.752	Females
		4	0_Control	66.438	2.372	3.570	Males
		4	1_25ug/L	68.100	3.894	5.718	Males
		4	2_100ug/L	69.983	1.318	1.884	Males
		4	3_400ug/L	70.930	0.825	1.163	Males
Ketoconazole	Tubercle Score	4	0_Control	32.250	8.261	25.617	Males
		4	1_25ug/L	28.750	2.533	8.811	Males
		4	2_100ug/L	31.250	2.533	8.106	Males
		4	3_400ug/L	38.375	12.499	32.571	Males
Ketoconazole	VTG	4	0_Control	14.134	11.899	84.185	Females
		4	1_25ug/L	8.618	5.351	62.093	Females
		4	2_100ug/L	14.484	4.676	32.281	Females
		4	3_400ug/L	12.714	5.169	40.651	Females
		4	0_Control	0.004	0.002	56.298	Males
		4	1_25ug/L	0.003	0.002	69.893	Males
		4	2_100ug/L	0.003	0.003	94.344	Males
		4	3_400ug/L	33.249	32.418	97.500	Males
Ketoconazole	Weight	4	0_Control	1.448	0.073	5.037	Females
		4	1_25ug/L	1.452	0.042	2.904	Females
		4	2_100ug/L	1.582	0.156	9.890	Females
		4	3_400ug/L	1.473	0.195	13.236	Females
		4	0_Control	3.493	0.369	10.561	Males
		4	1_25ug/L	3.735	0.622	16.648	Males
		4	2_100ug/L	3.814	0.319	8.352	Males

Table 15. Springborn Smithers Summary Statistics

Chemical	Parameter	N	Dose Group	Mean	Stand Deviation	CV (%)	Sex
		4	3_400ug/L	3.883	0.142	3.658	Males
Potassium Permanganate	# Counted Eggs	4	0_Control	452.250	454.380	100.471	Females
		4	1_225ug/L	290.750	480.294	165.191	Females
		4	2_450ug/L	552.000	188.960	34.232	Females
		4	3_900ug/L	602.250	849.953	141.130	Females
Potassium Permanganate	# Eggs/Female/Reproductive Day	4	0_Control	5.384	5.409	100.471	Females
		4	1_225ug/L	4.120	6.758	164.036	Females
		4	2_450ug/L	6.571	2.250	34.232	Females
		4	3_900ug/L	8.576	12.903	150.458	Females
Potassium Permanganate	# Estimated Eggs	4	0_Control	455.000	467.547	102.758	Females
		4	1_225ug/L	278.750	446.885	160.318	Females
		4	2_450ug/L	601.250	202.088	33.611	Females
		4	3_900ug/L	408.750	510.202	124.820	Females
Potassium Permanganate	# Fertile Eggs	4	0_Control	449.250	451.175	100.429	Females
		4	1_225ug/L	288.000	474.819	164.868	Females
		4	2_450ug/L	549.750	189.257	34.426	Females
		4	3_900ug/L	590.500	829.925	140.546	Females
Potassium Permanganate	# Spawns	4	0_Control	6.500	4.359	67.060	Females
		4	1_225ug/L	4.250	5.965	140.357	Females
		4	2_450ug/L	7.000	2.708	38.686	Females
		4	3_900ug/L	3.500	3.317	94.761	Females
Potassium Permanganate	% of Infertile Eggs	4	0_Control	0.573	0.465	81.170	Females
		3	1_225ug/L	0.364	0.630	173.205	Females
		4	2_450ug/L	0.470	0.430	91.456	Females
		3	3_900ug/L	1.446	1.013	70.054	Females
Potassium Permanganate	FPI	4	0_Control	0.377	0.754	200.000	Males
		4	1_225ug/L	1.628	0.782	48.001	Males
		4	2_450ug/L	0.000	0.000		Males
		4	3_900ug/L	0.705	1.410	200.000	Males
Potassium Permanganate	Fatpad Weight	4	0_Control	0.015	0.030	200.000	Males
		4	1_225ug/L	0.071	0.058	81.928	Males
		4	2_450ug/L	0.000	0.000		Males
		4	3_900ug/L	0.024	0.049	200.000	Males
Potassium Permanganate	GSI	4	0_Control	11.806	1.615	13.675	Females
		4	1_225ug/L	12.575	2.009	15.980	Females
		4	2_450ug/L	10.413	1.393	13.380	Females
		4	3_900ug/L	9.940	4.782	48.107	Females
		4	0_Control	1.105	0.055	5.012	Males

Table 15. Springborn Smithers Summary Statistics

Chemical	Parameter	N	Dose Group	Mean	Stand Deviation	CV (%)	Sex
		4	1_225ug/L	1.036	0.176	16.985	Males
		4	2_450ug/L	1.504	0.176	11.704	Males
		4	3_900ug/L	1.439	0.176	12.231	Males
Potassium Permanganate	Gonad Weight	4	0_Control	0.138	0.028	19.979	Females
		4	1_225ug/L	0.160	0.039	24.479	Females
		4	2_450ug/L	0.126	0.029	23.104	Females
		4	3_900ug/L	0.123	0.062	50.345	Females
		4	0_Control	0.033	0.006	19.372	Males
		4	1_225ug/L	0.032	0.010	32.940	Males
		4	2_450ug/L	0.051	0.008	16.243	Males
		4	3_900ug/L	0.043	0.007	16.817	Males
Potassium Permanganate	Length	4	0_Control	48.681	2.746	5.641	Females
		4	1_225ug/L	50.050	2.985	5.963	Females
		4	2_450ug/L	49.463	1.502	3.036	Females
		4	3_900ug/L	49.462	3.046	6.159	Females
		4	0_Control	61.364	4.772	7.777	Males
		4	1_225ug/L	62.773	4.438	7.070	Males
		4	2_450ug/L	64.839	1.920	2.961	Males
		4	3_900ug/L	62.912	2.742	4.358	Males
Potassium Permanganate	Tubercle Score	4	0_Control	29.625	5.692	19.213	Males
		4	1_225ug/L	27.542	3.351	12.167	Males
		4	2_450ug/L	26.125	4.442	17.002	Males
		4	3_900ug/L	22.813	6.427	28.173	Males
Potassium Permanganate	VTG	2	0_Control	52.681	17.892	33.963	Females
		2	1_225ug/L	66.235	9.535	14.396	Females
		4	2_450ug/L	53.407	9.723	18.205	Females
		4	3_900ug/L	37.332	28.685	76.837	Females
		2	0_Control	0.009	0.004	42.881	Males
		2	1_225ug/L	0.120	0.165	137.556	Males
		4	2_450ug/L	0.033	0.044	135.535	Males
		4	3_900ug/L	0.016	0.027	167.554	Males
Potassium Permanganate	Weight	4	0_Control	1.166	0.179	15.379	Females
		4	1_225ug/L	1.282	0.253	19.694	Females
		4	2_450ug/L	1.182	0.105	8.900	Females
		4	3_900ug/L	1.183	0.256	21.617	Females
		4	0_Control	3.025	0.552	18.244	Males
		4	1_225ug/L	3.199	0.865	27.056	Males

Table 15. Springborn Smithers Summary Statistics

Chemical	Parameter	N	Dose Group	Mean	Stand Deviation	CV (%)	Sex
		4	2_450ug/L	3.391	0.429	12.658	Males
		4	3_900ug/L	2.940	0.528	17.945	Males

Table 16. ABC Laboratories Summary Statistics

Chemical	Parameter	Dose Group	n	Mean	Standard Deviation	CV(%)	Sex
Potassium Permanganate	**# Counted Eggs**	0_Control	4	2957.75	856.69	28.964	Females
		1_225ug/L	4	2007.00	1195.03	59.543	Females
		2_450ug/L	4	88.75	174.18	196.262	Females
		3_900ug/L	4	45.25	67.58	149.357	Females
Potassium Permanganate	**# Eggs/Female/Reproductive Day**	0_Control	4	38.63	14.19	36.750	Females
		1_225ug/L	4	25.90	14.86	57.357	Females
		2_450ug/L	4	1.07	2.09	196.262	Females
		3_900ug/L	4	1.17	2.03	174.671	Females
Potassium Permanganate	**# Estimated Eggs**	0_Control	4	2266.25	402.64	17.767	Females
		1_225ug/L	4	1883.50	960.72	51.007	Females
		2_450ug/L	4	88.75	174.18	196.262	Females
		3_900ug/L	4	57.25	72.18	126.082	Females
Potassium Permanganate	**# Fertile Eggs**	0_Control	4	2725.00	896.70	32.907	Females
		1_225ug/L	4	1923.75	1155.51	60.066	Females
		2_450ug/L	4	86.00	170.01	197.681	Females
		3_900ug/L	4	32.75	65.50	200.000	Females
Potassium Permanganate	**# Spawns**	0_Control	4	12.75	3.30	25.914	Females
		1_225ug/L	4	12.00	1.41	11.785	Females
		2_450ug/L	4	0.50	0.58	115.470	Females
		3_900ug/L	4	0.75	0.96	127.657	Females
Potassium Permanganate	**% of Infertile Eggs**	0_Control	4	8.77	5.98	68.184	Females
		1_225ug/L	4	4.59	1.92	41.766	Females
		2_450ug/L	2	21.29	26.47	124.337	Females
		3_900ug/L	2	54.20	64.78	119.524	Females
Potassium Permanganate	**FPI**	0_Control	4	4.84	1.82	37.642	Males
		1_225ug/L	4	3.90	2.31	59.321	Males
		2_450ug/L	4	2.10	1.51	72.015	Males
		3_900ug/L	3	1.84	0.77	41.749	Males
Potassium Permanganate	**Fatpad Score**	0_Control	4	3.08	0.69	22.287	Males
		1_225ug/L	4	2.75	0.87	31.492	Males
		2_450ug/L	4	1.63	1.11	68.226	Males
		3_900ug/L	3	2.00	0.00	0.000	Males
Potassium Permanganate	**Fatpad Weight**	0_Control	4	0.21	0.08	39.773	Males
		1_225ug/L	4	0.17	0.10	58.178	Males
		2_450ug/L	4	0.07	0.06	76.750	Males
		3_900ug/L	3	0.06	0.03	43.628	Males

Table 16. ABC Laboratories Summary Statistics

Chemical	Parameter	Dose Group	n	Mean	Standard Deviation	CV(%)	Sex
Potassium Permanganate	**GSI**	0_Control	4	13.75	0.70	5.074	Females
		1_225ug/L	4	10.80	1.43	13.217	Females
		2_450ug/L	4	5.06	2.15	42.431	Females
		3_900ug/L	3	6.53	4.38	67.033	Females
		0_Control	4	1.11	0.23	20.655	Males
		1_225ug/L	4	1.54	0.37	24.056	Males
		2_450ug/L	4	1.25	0.77	61.535	Males
		3_900ug/L	3	1.13	0.22	19.848	Males
Potassium Permanganate	**Gonad Weight**	0_Control	4	0.28	0.02	7.106	Females
		1_225ug/L	4	0.22	0.03	14.298	Females
		2_450ug/L	4	0.09	0.06	62.135	Females
		3_900ug/L	3	0.12	0.11	86.734	Females
		0_Control	4	0.05	0.01	24.636	Males
		1_225ug/L	4	0.06	0.01	14.821	Males
		2_450ug/L	4	0.04	0.02	56.784	Males
		3_900ug/L	3	0.04	0.01	14.280	Males
Potassium Permanganate	**Length**	0_Control	4	44.21	0.81	1.837	Females
		1_225ug/L	4	44.44	1.14	2.573	Females
		2_450ug/L	4	42.88	0.37	0.862	Females
		3_900ug/L	3	42.78	2.27	5.304	Females
		0_Control	4	53.25	1.19	2.235	Males
		1_225ug/L	4	54.13	1.44	2.653	Males
		2_450ug/L	4	52.88	1.65	3.124	Males
		3_900ug/L	3	53.33	1.53	2.864	Males
Potassium Permanganate	**Survival Rate (%)**	0_Control	4	100.00	0.00	0.000	All
		1_225 ug/L	4	95.75	8.50	8.877	All
		2_450 ug/L	4	74.75	16.50	22.074	All
		3_900 ug/L	4	37.50	31.26	83.352	All
Potassium Permanganate	**Tubercle Score**	0_Control	4	25.33	6.94	27.396	Males
		1_225ug/L	4	22.75	7.96	35.004	Males
		2_450ug/L	4	14.50	11.03	76.071	Males
		3_900ug/L	3	16.00	6.24	39.031	Males
Potassium Permanganate	**VTG**	0_Control	4	182749929.25	249465378.53	136.506	Females
		1_225ug/L	4	73424084.69	35542273.45	48.407	Females
		2_450ug/L	4	7127426.79	5551990.50	77.896	Females
		3_900ug/L	2	27316248.67	35616697.98	130.386	Females

Table 16. ABC Laboratories Summary Statistics

Chemical	Parameter	Dose Group	n	Mean	Standard Deviation	CV(%)	Sex
		0_Control	4	11828.08	22156.17	187.318	Males
		1_225ug/L	4	750.00	0.00	0.000	Males
		2_450ug/L	4	876610.00	1748927.46	199.510	Males
		3_900ug/L	3	882.33	315.81	35.793	Males
Potassium Permanganate	**Weight**	0_Control	4	2.05	0.18	8.675	Females
		1_225ug/L	4	2.09	0.13	6.423	Females
		2_450ug/L	4	1.70	0.19	11.064	Females
		3_900ug/L	3	1.70	0.35	20.852	Females
		0_Control	4	4.09	0.42	10.288	Males
		1_225ug/L	4	4.23	0.51	12.159	Males
		2_450ug/L	4	3.30	0.53	16.063	Males
		3_900ug/L	3	3.39	0.15	4.422	Males

Table 17. Wildlife International Summary Statistics

Chemical	Parameter	Dose Group	n	Mean	Standard Deviation	CV(%)	Sex
Flutamide	**# Counted Eggs**	0_Control	4	1687.50	1169.58	69.308	Females
		1_100ug/L	4	1931.50	368.44	19.076	Females
		2_500ug/L	4	904.50	1000.93	110.661	Females
		3_1000ug/L	4	212.00	375.05	176.910	Females
Flutamide	**# Eggs/Female/Reproductive Day**	0_Control	4	23.20	19.37	83.494	Females
		1_100ug/L	4	22.99	4.39	19.076	Females
		2_500ug/L	4	14.44	14.60	101.127	Females
		3_1000ug/L	4	2.52	4.46	176.910	Females
Flutamide	**# Fertile Eggs**	0_Control	4	1289.50	702.11	54.448	Females
		1_100ug/L	4	1761.25	369.75	20.994	Females
		2_500ug/L	4	573.00	941.61	164.330	Females
		3_1000ug/L	4	43.50	51.51	118.422	Females
Flutamide	**% of Infertile Eggs**	0_Control	4	15.78	17.01	107.779	Females
		1_100ug/L	4	9.02	4.78	53.014	Females
		2_500ug/L	4	28.53	43.59	152.782	Females
		3_1000ug/L	2	45.43	58.67	129.134	Females
Flutamide	**FPI**	0_Control	4	2.97	0.77	25.854	Males
		1_100ug/L	4	3.24	1.20	37.116	Males
		2_500ug/L	4	2.87	2.44	85.218	Males
		3_1000ug/L	4	1.56	0.83	53.542	Males
Flutamide	**Fatpad Score**	0_Control	4	3.13	0.63	20.133	Males
		1_100ug/L	4	3.00	0.41	13.608	Males
		2_500ug/L	4	2.63	1.03	39.268	Males
		3_1000ug/L	4	2.38	0.48	20.156	Males
Flutamide	**Fatpad Weight**	0_Control	4	0.13	0.04	28.615	Males
		1_100ug/L	4	0.15	0.06	42.083	Males
		2_500ug/L	4	0.10	0.09	85.280	Males
		3_1000ug/L	4	0.07	0.04	61.987	Males
Flutamide	**GSI**	0_Control	4	9.86	1.15	11.676	Females
		1_100ug/L	4	12.14	1.50	12.395	Females
		2_500ug/L	4	11.15	3.37	30.191	Females
		3_1000ug/L	4	7.71	1.13	14.606	Females
		0_Control	4	1.26	0.24	19.324	Males
		1_100ug/L	4	1.30	0.11	8.368	Males
		2_500ug/L	4	1.67	0.24	14.113	Males
		3_1000ug/L	4	1.49	0.26	17.228	Males

Table 17. Wildlife International Summary Statistics

Chemical	Parameter	Dose Group	n	Mean	Standard Deviation	CV(%)	Sex
Flutamide	Gonad Weight	0_Control	4	0.16	0.03	15.945	Females
		1_100ug/L	4	0.20	0.04	18.053	Females
		2_500ug/L	4	0.18	0.06	33.176	Females
		3_1000ug/L	4	0.13	0.03	21.541	Females
		0_Control	4	0.05	0.01	23.402	Males
		1_100ug/L	4	0.06	0.01	13.980	Males
		2_500ug/L	4	0.06	0.01	14.708	Males
		3_1000ug/L	4	0.06	0.01	19.788	Males
Flutamide	Length	0_Control	4	54.42	0.79	1.453	Females
		1_100ug/L	4	54.19	1.39	2.565	Females
		2_500ug/L	4	54.25	1.66	3.057	Females
		3_1000ug/L	4	53.88	1.96	3.644	Females
		0_Control	4	70.00	2.48	3.548	Males
		1_100ug/L	4	71.00	2.12	2.988	Males
		2_500ug/L	4	67.13	1.55	2.306	Males
		3_1000ug/L	4	69.13	2.29	3.308	Males
Flutamide	Tubercle Count	0_Control	4	24.63	5.51	22.389	Males
		1_100ug/L	4	22.00	2.12	9.642	Males
		2_500ug/L	4	18.25	1.55	8.518	Males
		3_1000ug/L	4	12.13	7.17	59.127	Males
Flutamide	Tubercle Score	0_Control	4	39.38	6.22	15.805	Males
		1_100ug/L	4	30.25	4.77	15.768	Males
		2_500ug/L	4	22.75	3.97	17.445	Males
		3_1000ug/L	4	17.63	10.93	62.040	Males
Flutamide	VTG	0_Control	4	14600092.50	3574413.39	24.482	Females
		1_100ug/L	4	18880036.88	10260636.47	54.346	Females
		2_500ug/L	4	18193395.00	14678157.37	80.678	Females
		3_1000ug/L	4	17539200.00	8878258.84	50.620	Females
		0_Control	4	6260.60	5025.36	80.270	Males
		1_100ug/L	4	2650.09	2231.24	84.195	Males
		2_500ug/L	4	7726.25	7259.50	93.959	Males
		3_1000ug/L	4	5679.30	8848.50	155.803	Males
Flutamide	Weight	0_Control	4	1.61	0.09	5.705	Females
		1_100ug/L	4	1.62	0.08	5.012	Females
		2_500ug/L	4	1.58	0.10	6.603	Females
		3_1000ug/L	4	1.55	0.23	14.660	Females

Table 17. Wildlife International Summary Statistics

Chemical	Parameter	Dose Group	n	Mean	Standard Deviation	CV(%)	Sex
		0_Control	4	4.31	0.25	5.746	Males
		1_100ug/L	4	4.59	0.29	6.377	Males
		2_500ug/L	4	3.52	0.13	3.569	Males
		3_1000ug/L	4	3.88	0.47	12.031	Males

Table 18. Springborn Smithers/Flutamide

Endpoint	Significant difference from the Control				Normality	Heterogeneity
	Low	Mid	High	Procedure Applied		
Male Length				Parametric linear trend, and F-test		
Female Length				Parametric linear trend, and F-test		
Male Weight				Parametric linear trend, and F-test		
Female Weight				Parametric linear trend, and F-test		
# of Eggs - Estimated				Nonparametric linear, and K-W	√	
# of Eggs_- Counted			(√)	Nonparametric linear, and K-W	√	
# of Spawns				Parametric linear trend, and F-test		
# of Fertile Eggs			(√)	Nonparametric linear	√	
% of infertile eggs[9]			√	Nonparametric linear		√
# of Eggs per Female per Reproductive Day			(√)	Nonparametric linear	√	
Male Tubercle Score				Parametric linear trend, and F-test		
Male Fatpad Weight				Parametric linear trend, and F-test		
Male FPI				Nonparametric linear, and K-W	√	
Male Gonad Weight				Parametric linear trend and F-test		
Female Gonad Weight				Parametric linear trend, and F-test		
Male GSI				Parametric linear trend, and F-test		
Female GSI				Nonparametric linear and K-W		√
Male VTG				Nonparametric linear and K-W	√	
Female VTG				Parametric linear trend and F-test		

√ means p 20.05 (√) means 0.05 <p<0.1

Table 19. Springborn Smithers/Ketoconazole[1]

Endpoint	Significant difference from the Control				Normality	Heterogeneity
	Low	Mid	High	Procedure Applied		
Male Length			√	Nonparametric linear trend		√
Female Length				Parametric linear trend, and F-test		
Male Weight				Nonparametric linear trend, and K-W		√
Female Weight				Nonparametric linear trend, and K-W		√
# of Eggs - Estimated				Parametric linear trend, and F-test		
# of Eggs_- Counted				Parametric linear trend, and F-test		
# of Spawns				Parametric linear trend, and F-test		
# of Fertile Eggs				Parametric linear trend, and F-test		
% of infertile eggs[9]				Parametric linear trend, and F-test		
# of Eggs per Female per Reproductive Day				Parametric linear trend, and F-test		
Male Tubercle Score				Nonparametric linear trend, and K-W	√	
Male Fatpad Weight				Nonparametric linear trend, and K-W	√	
Male FPI				Nonparametric linear trend, and K-W	√	
Male Gonad Weight		√	√	Parametric linear trend		
Female Gonad Weight				Nonparametric linear trend, and K-W		√
Male GSI		√	√	Parametric linear trend		
Female GSI			√	Nonparametric linear trend	√	
Male VTG			√	Parametric linear trend	√	
Female VTG				Parametric linear trend, and F-test		

[1] √ means $p < 0.05$

Table 20. Springborn Smithers/Potassium Permanganate[1]

Endpoint	Significant difference from the Control				Normality	Heterogeneity
	Low	Mid	High	Procedure Applied		
Male Length				Parametric linear trend and F-test		
Female Length				Parametric linear trend and F-test		
Male Weight				Parametric linear trend and F-test		
Female Weight				Nonparametric linear trend and K-W		√
# of Eggs - Estimated				Nonparametric linear trend and K-W	√	
# of Eggs_- Counted				Nonparametric linear trend and K-W	√	
# of Spawns				Parametric linear trend and F-test		
# of Fertile Eggs				Nonparametric linear trend and K-W	√	
% of infertile eggs[9]				Parametric linear trend and F-test		
# of Eggs per Female per Reproductive Day				Nonparametric linear trend and K-W	√	
Male Tubercle Score				Parametric linear trend and F-test		
Male Fatpad Weight				Nonparametric linear trend and K-W	√	
Male FPI				Nonparametric linear trend and K-W	√	
Male Gonad Weight		√	√	Parametric linear trend		
Female Gonad Weight				Parametric linear trend and F-test		
Male GSI		√	√	Parametric linear trend		
Female GSI				Parametric linear trend and F-test		
Male VTG				Parametric linear trend and F-test		
Female VTG				Parametric linear trend and F-test		

[1] √ means $p < 0.05$

Table 21. ABC Laboratories/Potassium Permanganate[1]

Endpoint	Significant difference from the Control				Normality	Heterogeneity
	Low	Mid	High	Procedure Applied		
Male Length				Parametric linear trend and F-test		
Female Length		(√)	√	Nonparametric linear trend		√
Male Weight		√	√	Parametric linear trend		
Female Weight		√	√	Parametric linear trend		
# of Eggs - Estimated		√	√	Nonparametric linear trend		√
# of Eggs_- Counted	(√)	√	√	Nonparametric linear trend		√
# of Spawns		√	√	Nonparametric linear trend	√	√
# of Fertile Eggs		√	√	Nonparametric linear trend	√	
% of infertile eggs[9]				Parametric linear trend and F-test		
# of Eggs per Female per Reproductive Day		√	√	Nonparametric linear trend		√
Male Tubercle Score				Parametric linear trend and F-test		
Male Fatpad Score		√	√	Parametric linear trend		
Male Fatpad Weight		√	√	Parametric linear trend		
Male FPI		√	√	Parametric linear trend		
Male Gonad Weight				Parametric linear trend and F-test		
Female Gonad Weight	√	√	√	Nonparametric linear trend		√
Male GSI				Parametric linear trend and F-test		
Female GSI	√	√	√	Nonparametric linear trend		√
Male VTG				Nonparametric linear trend, then K-W test	√	
Female VTG		√	√	Parametric linear trend		

[1] √ means $p <= 0.05$ (√) means $0.05<p<0.10$

Table 22. Wildlife International/Flutamide[1]

Endpoint	Significant difference from the Control				Normality	Heterogeneity
	Low	Mid	High	Procedure Applied		
Male Length				Parametric linear trend and F-test		
Female Length				Nonparametric linear trend and K-W	√	
Male Weight		√	√	Parametric linear trend		
Female Weight				Parametric linear trend and F-test		
# of Eggs - Estimated				Parametric linear trend and F-test		
# of Eggs_ - Counted			√	Parametric linear trend and F-test		
# of Spawns	(Data were not available)					
# of Fertile Eggs			√	Nonparametric linear trend	√	√
% of infertile eggs[9]				Parametric linear trend and F-test		
# of Eggs per Female per Reproductive Day			√	Parametric linear trend		
Male Tubercle Score	(√)	√	√	Parametric linear trend		
Male Tubercle Count		(√)	√	Parametric linear trend		
Male Fatpad Score				Parametric linear trend and F-test		
Male Fatpad Weight			√	Nonparametric linear trend	√	
Male FPI			√	Nonparametric linear trend	√	
Male Gonad Weight				Parametric linear trend and F-test		
Female Gonad Weight				Parametric linear trend and F-test		
Male GSI		√	√	Parametric linear trend		
Female GSI			√	Nonparametric linear trend		√
Male VTG				Parametric linear trend and F-test		
Female VTG				Parametric linear trend and F-test		

[1] √ means $p <= 0.05$ (√) means $0.05<p<0.10$