DRAFT FINAL REPORT

Inter-Laboratory Validation of the Fish Short-term Reproduction Assay, Run Simultaneously Across Three Independent Contract Laboratories,

Task Order #2
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Table of Contents

1.0 Introduction

The U.S. Environmental Protection Agency has implemented 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 fish short-term reproduction assay designed to detect substances that may affect the hypothalamic-pituitary-gonadal (HPG) axis, including estrogenic and androgenic pathways, of fish. It is thought that the inclusion of this fish 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. Further, given that endocrine-active chemicals in the environment appear to be impacting fish populations, this class of animals is a logical focal point for screening and testing.

An inter-laboratory study was undertaken that examined the reproducibility of a fish short-term reproduction assay designed to detect HPG-active chemicals. Three independent laboratories evaluated a standardized protocol of the assay using the same five chemicals. All of the work supporting these studies was conducted in accordance with Good Laboratory Practice (GLP) procedures. RTI International (RTI) is responsible for overall inter-laboratory study management, ensuring that the work of all three independent laboratories is high quality and delivered on schedule. The RTI Team includes Springborn Smithers Laboratories (Lead Laboratory, SSL, identified in this summary report as Lab B), Wildlife International, Ltd. (WLI, identified as Lab A), and Bayer CropScience, LP (BCS, identified as Lab C), and a single laboratory chosen to conduct the histopathological evaluation of the gonads, Integrated Laboratory Systems, Inc (ILS). RTI conducted analysis of the plasma hormones.

This report will summarize the results of each of the individual studies conducted at the three laboratories, and evaluate the robustness of specific endpoints in the assay. Additionally, difficulties and potential resolutions associated with conducting the assay will be discussed. Individual reports for each exposure have been prepared by each laboratory and are available

separately. These reports include full details on the exposures including methods, exposure analysis, and biochemical analysis, a complete description of the statistical analysis and discussion of the results of the non-histopathology data and a full description of the histopathology results and conclusions.

1.1 Protocol

This study was conducted according to the procedures outlined in the Quality Assurance Project Plan (QAPP) for EPA Contract Number EP-W-06-026. 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 and chemicals that affect sex steroid production. The test described in the protocol is an extension of existing standard practice for conducting a short-term reproduction test with fathead minnow (Ankley et al., 2001).

1.2 Test Substances

Five test substances were used to evaluate the fish short-term reproduction assay. Prochloraz, 4-t-octylphenol, ketoconazole and vinclozolin were tested at the same three exposure levels by each laboratory. Sodium dodecyl sulfate (SDS) was selected as a negative control with only one exposure level, which was different between the laboratories. The SDS exposure was run in conjunction with the vinclozolin exposure.

1.2.1 ID, mode of action

Prochloraz and ketoconazole are azole-based fungicides that inhibit the synthesis of ergosterol, a vital component of the fungal cell membrane. Prochloraz and ketoconazole both can inhibit sex steroid synthesis in vertebrates including the fathead minnow (Ankley et al. 2005; 2007). Prochloraz also may act as an androgen receptor antagonist.

Vinclozolin is a dicarboximide fungicide that affects DNA synthesis and lipid metabolism. Several metabolites of vinclozolin act as androgen receptor antagonists in rats and fish (Martinovic et al. in press).

4-t-Octylphenol is an alkylphenol often used as the backbone for alkylphneol ethoxylate industrial surfactants. Octylphenol is an estrogen receptor agonist and has been shown to influence the reproductive system in male fish.

SDS was chosen as a toxic "negative" chemical due to its well-described toxicity to fish due to its membrane disturbance activity.

1.3 Delivery

Laboratory B evaluated stock solution preparation techniques and exposure conditions for each test substance prior to Laboratories A and C beginning work with the same test substance. This practice was followed in an effort to resolve major issues regarding functional water solubility, stability and reliability of the delivery techniques before all of the laboratories began conducting the exposure. Although co-solvent free delivery of test substance is preferred, the many methods available to deliver co-solvent free test substance are test substance specific and require laboratory-to-laboratory adjustments. Three general co-solvent free techniques and one co-solvent technique were employed to deliver the test substances to the exposure systems in the inter-laboratory comparison. Even though these techniques were well described and were evaluated before the initiation of an exposure, the three laboratories experienced some difficulties in producing consistent stock solution concentrations and maintaining exposure levels with some of the test substance.

Laboratory B prepared the prochloraz stock solution using a chemical coating procedure. The prochloraz was first dissolved in acetone. The prochloraz solution was then added to a carboy, which had been placed on a rolling mill set at low speed. A constant, moderate flow of nitrogen was applied to evaporate the acetone leaving the prochloraz coated on the side walls of the

carboy. Dilution water was added to the carboy continuously at a rate of 10 mL/minute. Following the overnight equilibration period, the solution was engaged on the diluter system.

Laboratory B had difficulties maintaining exposure concentrations that were a result of variable stock solution concentrations. The procedure for stock solution preparation was changed prior to Laboratories A and C beginning their exposures. Laboratories A and C prepared the prochloraz stock solutions in Erlenmeyer flasks to achieve a desired stock concentration of 20 mg a.i./L. The use of Erlenmeyer flasks aided in mixing and allowed for sonication of the solutions. The solutions were covered with a black tarpaulin and allowed to stir on a stir plate overnight. Following stirring, the stock solutions were sonicated for approximately 20 minutes prior to use.

In all cases, the prochloraz stock solutions were prepared as needed throughout the exposures. The stock solutions were delivered to the diluter systems through calibrated pumps and mixed with dilution water to prepare the three exposure solutions

4-t-Octylphenol stock solutions were prepared by mixing a measured mass of the test substance with a known volume of triethylene glycol (TEG). The stock solutions contained no visible undissolved test substance. Stock solutions were prepared and engaged on the exposure system as needed. The stock solutions were delivered to the diluter systems through calibrated pumps and mixed with dilution water to prepare the three exposure solutions. Separate injections of TEG equalized the solvent load across the solvent control and all 4-t-octylphenol test levels via the flow dynamics of the diluter. The solvent load in each exposure vessel excluding the dilution water control was approximately 50 µL/L.

Ketoconazole stock solutions were prepared by mixing a measured mass of test substance with a known volume of dilution water using a laboratory mixer until solutions were observed to be clear and colorless with no visible undissolved test substance (typically overnight). The stock solutions were prepared as needed throughout the exposures. The stock solutions were delivered to the diluter systems through calibrated pumps and mixed with dilution water to prepare the three exposure solutions.

Vinclozolin stock solutions were prepared in acetone as needed throughout the exposures. This solution was added to stainless steel columns (2.5 x 30 cm) packed with glass wool. The acetone was evaporated by vacuum resulting in columns loaded with 5 grams of vinclozolin. Water was then slowly pumped over the wool through the column at a rate of 40 ml/minute. The flow from the columns was mixed with dilution water in the diluter systems to prepare the three exposure solutions.

Sodium dodecyl sulfate (SDS) stock solutions were prepared by mixing a measured mass of the test substance with a known volume of dilution water. The stock solutions were prepared as needed throughout the exposures. The stock solutions were delivered to the diluter systems through calibrated pumps and mixed with dilution water to prepare the three exposure solutions.

2.0 Methods

The methods that were followed for the fish short-term reproduction assay inter-laboratory comparison are summarized in Table 1 through Table 4, compared by compound for each of the three laboratories. The full protocol employed by each of the laboratories is included in the attachments.

3.0 Results – Comparison across laboratories by test substance

3.1.1 Prochloraz

3.1.1.1 Exposure levels

Nominal concentrations for the prochloraz exposures were 20, 100 and 300 µg/L. The table below summarizes the mean measured prochloraz concentrations from the three laboratories.

	Mean Measured Concentration (µg/L)		
Nominal Concentration	20	100	300
Laboratory A	16	77	220
Laboratory B	15	83	230
Laboratory C	23	90	270

The mean measured concentrations ranged from 75 to 112% of the nominal levels. Measured concentrations at Laboratories A and C remained relatively constant throughout the exposure period. Measured concentrations a Laboratory B exceeded nominal concentrations on day 0, dropped considerably (4 to 5 times lower than day 0 at their lowest levels) on days 7, 9 and 13, before recovering to day 0 levels on day 21.

3.1.1.2 Sensitive endpoints (Table 5)

Effects from prochloraz exposure were concentration-dependent and most extensive in the high treatment group. The only endpoints not affected from the prochloraz exposure in at least one laboratory were male and female survival, male vitellogenin concentration, male testosterone concentration, male body weight, male length and percent fertile embryos.

Tubercle score was the only male endpoint affected at the highest exposure level at all three laboratories. It was also affected in the mid treatment level at Laboratories A and C. Tubercle count and fatpad score were affected at the highest exposure level in Laboratories A and B and Laboratories B and C, respectively. Gonad weight and GSI were significantly increased at the highest exposure level in Laboratories B and C and at the mid exposure level at Laboratory C. The fatpad index was the most sensitive endpoint with significant reductions seen at all three treatment levels, however, this effect was only observed at Laboratory B.

Reductions in fecundity at the highest prochloraz test concentration were observed by all three laboratories. Laboratories B and C measured significant decreases in female vitellogenin concentrations at the two highest treatment levels, with Laboratory C also measuring significant reductions at the lowest treatment level. Decreases in female vitellogenin were not observed in Laboratory A even though fecundity was significantly reduced. The sex ratios throughout the exposure levels in Laboratory A were skewed toward male fish in a proportion greater than 2:4. This was due to miss-sexed females (males without prominent secondary sexcharacteristics) at initiation of the pre-exposure period that ended up being identified as male fish at the end of the exposure. Control fecundity was also lowest in the Laboratory A exposure, however neither the

skewed ratio nor the relatively low fecundity impacted the ability of the exposure to detect differences in fecundity at the high exposure level just as in Laboratories B and C.

Laboratories B and C measured significant decreases in female beta-estradiol concentrations at the highest treatment level, with Laboratory B measuring significant reductions at all three treatment levels. Laboratory A did not observe a significant dose trend in decreased beta-estradiol concentrations, but observed an overall significant difference in beta-estradiol concentrations with the lowest levels observed in the mid and high treatment levels. Female GSI was significantly larger at the mid and high treatment levels in Laboratory B, while significant increases in growth endpoints and gonadal weight were observed in the high treatment level. There was an empirical increased female GSI response in fish exposed at Laboratory A, which corroborates the GSI increases observed by Laboratory B. However, there was not a statistically significant dose response, only a global response.

3.1.1.3 Histopathology (Table 6)

The majority of male testes from all three laboratories were in stage 2 across all prochloraz treatment levels with the exception of the high treatment level from Laboratory C where the majority of the testes were at stage 3. The majority of female ovaries from all three laboratories were stage 3.

Significant treatment-related testicular degeneration was observed at the highest treatment level from Laboratories A and B, with possible treatment-related effects also observed from Laboratory C. These observations were also evident in the mid prochloraz treatment level from the male fish exposed at Laboratories B and C. In addition, male fish from Laboratory B had increased proportion of spermatogonia and increased proportion of interstitial cells when exposed to the high and mid concentrations of prochloraz.

Increased oocyte atresia was observed in the female fish at the mid and high exposures from Laboratory A and all three treatment levels from Laboratory B. In addition the female fish from Laboratory B had decreased post-ovulatory follicles at the mid and high prochloraz treatment

levels. No treatment related lesions were observed in the females exposed at Laboratory C. This may have been due the presence of microsporidian spores observed in some of these fish.

3.1.1.4 Conclusions

Prochloraz is an androgen antagonist that acts via two mechanisms: (a) inhibition of steroid (including testosterone) synthesis, and (b) direct antagonism of the androgen receptor. However, testosterone levels were not observed to decrease in male fish at any treatment level at any laboratory in this study. The effects on male fish were characterized as increased gonad weight and GSI, increased testicular degeneration and decreases in secondary sex characteristics, including both the tubercles and fatpads. The greatest incidence of effects was seen at the highest exposure concentration with incidence decreasing with decreasing exposure concentrations. Reduction in tubercle score appeared to be the most robust endpoint, being observed by all three laboratories at the highest treatment level and two of the three laboratories at the mid treatment level. Fatpad index was the most sensitive (effects seen at the lowest exposure concentrations) endpoint with significant decreases found at all three exposure levels, but in only one laboratory.

Female fecundity was reduced at the highest prochloraz treatment level. Increased oocyte atresia was present in the fish from two of the three laboratories at the mid and high treatment levels. Significant reductions in beta-estradiol concentration and significant reduction in female vitellogenin concentrations observed at all three treatment levels by one or two of the laboratories suggest that prochloraz exposure may impact fecundity via inhibition of egg yolk protein production.

The presence of microsporidian spores in the ovaries from fish exposed at Laboratory C did not impact vitellogenin production or fecundity of the female fish. Control vitellogenin concentrations were similar across all three laboratories and Laboratory C control fecundity was over 30 eggs/female/day or about six times higher than the other two laboratories.

3.1.2 4-t-Octylphenol

3.1.2.1 Exposure levels

Nominal concentrations for the 4-t-octylphenol exposures were 1.0, 50 and 150 µg/L. The table below summarizes the mean measured 4-t-octylphenol concentrations from the three laboratories.

	Mean Measured Concentration (µg/L)		
Nominal Concentration	1.0	50	150
Laboratory A	0.57	37	120
Laboratory B	0.58	31	98
Laboratory C	0.84	42	120

The mean measured concentrations ranged from 57 to 84% of the nominal levels. Measured concentrations from the three laboratories were generally highest on day 0 and generally greater than 70% of nominal concentrations. Concentrations generally decreased by day 7 and remained relatively constant with recoveries generally ranging between 50 and 70% of nominal concentrations at Laboratories A and B and generally greater than 70% of nominal concentrations at Laboratory C.

3.1.2.2 Sensitive endpoints (Table 7)

Male endpoints affected by 4-t-octlyphenol exposures based on statistical analysis included plasma vitellogenin, testosterone concentration, gonad weight, tubercle count and score, fatpad index, score and weight. Female endpoints significantly affected included plasma vitellogenin, beta-estradiol concentration, body weight, gonad weights, GSI, eggs/female, fertile eggs/female, percent fertile eggs/female and spawns/female. Laboratory A also saw a survival effect (decrease) at the high treatment level.

An increase in male plasma vitellogenin concentration was the most sensitive endpoint in all three laboratories, observed in both the mid and high test concentration, with Laboratory C also seeing significant increases in the low exposure. A decrease in testosterone concentration was observed in all three laboratories at the highest treatment level, by two of the three laboratories in the mid treatment level and one of the laboratories in the low treatment level. Reduced tubercle count and score were also observed by all three laboratories in the high exposure, with Laboratories A and C also seeing a reduced tubercle score in the mid exposure. Additionally,

Laboratory A found fatpad weight and index effects at the high exposure and significant effects at all three exposure levels for fatpad score.

Most of the effects seen in the female fish were confined to the high test concentration. A decreased number of spawns was the only endpoint affected in all three laboratories. The number of eggs, the number of fertile eggs and the percent of fertile eggs per female were also significantly reduced in the female fish exposed at laboratories A and B. The overall fecundity was noticeably greater in the Laboratory C exposure relative to both the Laboratories A and B exposures. Yet, the number of fecundity effects was greater in the Laboratories A and B exposures. Due to mortality and some miss–sexed female fish that ended up being male fish, the sex ratios in the Laboratory A and C exposures were skewed from the 2:4 ratio of the protocol. As seen in the prochloraz exposure, the screening assay experimental design is robust enough to compensate for some skewing of sex ratios and still detect effects in fecundity.

A decrease in gonad weight and GSI was observed by Laboratory A and a decrease in female body weight was observed by Laboratory C. Laboratory A saw an increase in vitellogenin concentration at both the mid and high concentrations while Laboratory C saw a decrease in beta-estradiol concentration at the high test concentration.

The co-solvent TEG delivered at 50 μL/L was used by all three laboratories for the 4-t-octylphenol exposure. Some unexplained differences between control and solvent control endpoints were observed in the sex steroid and vitellogenin concentrations. Laboratory A saw significantly higher male testosterone concentrations in the solvent control fish, while Laboratory C saw a significantly lower testosterone concentration in the solvent control male fish. Laboratory A also observed significantly higher beta-estradiol concentrations in the solvent control female fish. Laboratory B found female vitellogenin concentration was significantly lower in the solvent control females versus the control females.

3.1.2.3 Histopathology (Table 8)

The majority of male testes from all three laboratories were in stage 2 across all octylphenol test concentrations. The majority of female ovaries from all three laboratories were stage 3, except for the females in the high exposure concentration from Laboratory A where the staging ranged from 0 to 3.

Non-treatment related granulomatous inflammation in both the testicular and ovarian tissue of fish from Laboratory C confounded the interpretation of the potential effects of exposure to 4-t-ocytlphenol. Microsporidian spores were observed in one male fish. It is possible that the granulomatous inflammation was attributable to undetected Microsporidia.

Male histopathology results indicated an increased proportion of interstitial cells in the male testis and an altered proportion of spermatocytes or spermatids that was treatment related at all three 4-t-octylphenol treatment concentrations. No testis ova were observed in any of the male fish exposed at the three laboratories.

Female histopathology results indicated increased oocyte atresia in Laboratories A and B at the highest test concentration. This effect carried through all three treatment levels for the female fish exposed in Laboratory A.

3.1.2.4 Conclusions

4-t-Octylphenol is an estrogen receptor agonist. Consistent with other estrogen mimics, 4-t-octylphenol induced vitellogenin production in male fish. Octylphenol also significantly reduced testosterone levels in the male fish. Consistent with the feminization, increased testicular degeneration was observed and secondary sexual characteristics in males were reduced. Reductions in tubercle counts and scores were found at all three laboratories. Fatpad weight, score and index also were reduced in the male fish in one laboratory. Vitellogenin induction and testosterone decreases were observed at the mid and high treatment concentrations, while impacts on secondary sex characteristics were mostly evident at only the highest treatment level. Testicular degeneration was detected from all three laboratories at least at the highest treatment concentration. Testis ova have been observed in male fish exposed to estrogen agonists.

However, no testis ova were observed in any of the male fish exposed to 4-t-octylphenol. This may be a function of exposure time or potency of 4-t-octylphenol.

Fecundity and fertility effects were found at the highest 4-t-octylpheonol test concentration. The screening assay also demonstrated its ruggedness for detecting fecundity effects. Sex ratios were skewed from the 2:4 ratio in exposures A and C due to some mortality and some mis-sexed fish. In spite of the altered sex ratios, fecundity affects were still detected in both exposures at the highest test concentration. The screening assay is not designed to determine if the effects on fecundity and fertility are a result of chemical impacts on males or females. Regardless of the cause, however, effects on male secondary sex characteristics were correlated with increased ooctye atresia, decreases in fecundity and embryo fertility of female fish exposed under the same conditions.

The presence of granulomatous inflammation lesions in the testes from fish exposed at Laboratory C did not impact the effects on vitellogenin induction, decreased testosterone levels and decreased secondary sex characteristics. The male fish from all three laboratories had very similar responses to 4-t-ocytlyphenol exposure. As in the prochloraz exposure, the fecundity of the female control fish at Laboratory C was not impact by granulomatous inflammation lesions. Laboratory C control fecundity was nearly 30 eggs/female/day or about three to six times higher than the other two laboratories. Thus if the presence of Microsporidia was responsible for the granulomatous inflammation lesions, the screening assay was still robust enough to provide acceptable control performance and to detect effects in both male and female fish exposed to 4-t-octyphenol.

3.1.3 Ketoconazole

3.1.3.1 Exposure levels

Nominal concentrations for the ketoconazole exposures were 25, 100 and 400 µg/L. The table below summarizes the mean measured ketoconazole concentrations from the three laboratories.

	Mean Measured Concentration (µg/L)		
Nominal Concentration	25	100	400
Laboratory A	20	74	290
Laboratory B	18	81	320
Laboratory C	16	75	240

The mean measured concentrations ranged from 60 to 81% of the nominal levels. Measured concentrations at all three laboratories were generally near nominal concentrations on day 0. Measured concentrations dropped during the mid part of the exposure in all three laboratories and typically increased slightly by the end of the exposure.

3.1.3.2 Sensitive endpoints (Table 9)

Male endpoints impacted by ketoconazole exposures included gonad weight, GSI, and tubercle score. Female endpoints affected included vitellogenin concentration, gonad weight, GSI and spawns per day.

An increase in male GSI was detected in the high ketoconazole exposure in all three laboratories. Increased male GSI at the mid ketoconazole exposure were also detected at Laboratories B and C. Male gonad weight increases were detected in the high ketoconazole exposure at Laboratories B and C and in the mid exposure at Laboratory B.

Female GSI increased in the high ketoconazole exposure at Laboratories B and C and in the mid exposure at Laboratory B. Laboratory B also detected an increase in female gonad weight at the high ketoconazole exposure and a decrease in the number of spawns. Laboratory A measured decreases in vitellogenin concentration in the high and low exposure levels. The vitellogenin concentration in the mid exposure level also was less than the control concentration, but not statistically so.

3.1.3.3 Histopathology (Table 10)

The majority of male testes from Laboratories B and C were in stage 2 across all ketoconazole treatment levels. The majority of male testes from Laboratory A were in either stage 2 or 3. The majority of female ovaries from all three laboratories were stage 3.

Male histopathology results indicated an increased proportion of interstitial cells in the male testis that was treatment related at all three ketoconazole test concentrations in fish from all three laboratories. In addition, increased testicular degeneration was observed in the male fish exposed at Laboratory A.

Female histopathology results indicated an increased incidence of oocyte atresia with increasing exposure concentration from Laboratories A and B, in spite of the presence of microsporidian spores. No treatment-related lesions were observed in the female fish from Laboratory C. Incidence levels of oocyte atresia were greater in control fish.

3.1.3.4 Conclusions

Ketoconazole has been found to inhibit gonadal testosterone synthesis in males. Increased male GSI, increased testes weight and increased proportion of interstitial cells in the testis appear to be consistent with a compensatory response to reduced testosterone synthesis (Ankley et al. 2007). However, there were no significant decreases observed in the testosterone levels from the male plasma at any treatment level. Increased male GSI and increased proportion of interstitial cells in the male testis were the most robust endpoints, being observed at all three laboratories. Increased male GSI was the most sensitive quantitative endpoint being observed by two of the three laboratories at the mid ketoconazole exposure level. However, the mode of action of ketoconazole was defined by the testicular tissue degeneration.

Ketoconazole exposure increased oocyte atresia, increased female GSI, increased gonad weight and reduced the number of spawns in fathead minnows. However these effects were only observed by one or two laboratories. Microsporidian spores were observed in ovaries of fish from Laboratories A and B. Oocyte atresia related to ketoconazole exposure was able to be distinguished from any impact due to the presence of the Microsporidia. Although control

fecundity was less than 15 eggs/female/day in all three laboratories, there was no correlation between the presence of Microsporidia and fecundity. Somewhat low fecundity in the control exposures also did not impact the evaluation of the fecundity endpoint, since most of the treatments had fecundity levels greater than the control levels.

3.1.4 Vinclozolin

3.1.4.1 Exposure levels

Nominal concentrations for the vinclozolin exposures were 100, 300 and 900 µg/L. The table below summarizes the mean measured vinclozolin concentrations from the three laboratories.

	Mean Measured Concentration (µg/L)		
Nominal Concentration	100	300	900
Laboratory A	75	280	830
Laboratory B	150	370	1200
Laboratory C	84	270	760

The mean measured concentrations ranged from 75 to 150% of the nominal levels. Measured concentrations at Laboratory A ranged between 73 and 83% of nominal concentrations and increased through day 14 where they remained near nominal concentrations for the remainder of the exposure. Some intermediate measurements at the low and mid concentrations between days 11 and 21 suggest that the measured concentrations could vary day to day but the variability between replicates was negligible. Measured concentrations at Laboratory B ranged between 80 and 89% of the nominal concentrations and increased through day 14 to 160 to 190% of the nominal concentrations before decreasing slightly by day 21. Measured concentrations at Laboratory C started near nominal concentrations (86 to 93% of nominal concentrations) on day 0, increasing slightly on day 7 and dropping slightly below the day 0 measurements throughout the rest of the exposure.

3.1.4.2 Sensitive endpoints (Table 11)

All three laboratories saw a number of significant effects at the high vinclozolin test concentration in both the male and female fish. In addition, Laboratory B saw these effects carry through the mid exposure for the females and through the low exposure for the males.

Tubercle score was significantly reduced in male fish at the highest treatment level in all three laboratories. Tubercle count was significantly reduced in male fish at this same treatment level in Laboratories A and B and empirically reduced in the fish exposed at Laboratory C. The male fish exposed at Laboratory B had reduced tubercle counts and scores at all three vinclozolin treatment levels. Fadpad weight and fatpad index were also significantly reduced in all three treatment levels from fish exposed by Laboratory B. Fatpad score was significantly decreased in the high treatment level in Laboratory B. Male gonad weight and GSI were significantly increased in the highest treatment level from Laboratory C and all three treatment levels from Laboratory B. The male fish from Laboratory B had significantly reduced testosterone levels in the highest treatment level, which may be due to the higher (~1.5 times higher) mean measured concentration of vinclozolin. The male fish exposed at the mid and high vinclozolin treatment levels from Laboratory C also had significantly increased vitellogenin levels.

Fecundity was significantly reduced in the high vinclozolin concentration in all three laboratories and continued into the mid treatment level in Laboratory B. The effects seen in the mid treatment level at Laboratory B may be partially explained by the fact that the mean measured exposure concentrations were noticeably higher than those measured in Laboratories A and C. Decreased levels of beta-estradiol were also observed from the female fish exposed to the mid and high treatment levels in Laboratory B. Significant increases in gonad weight were observed in the female fish exposed at Laboratories B and C, with an increase in GSI also observed at laboratory C. Increased length and body weight were observed in the female fish exposed to the mid and high treatment levels at Laboratory B. Increased vitellogenin levels were measured from the mid and high treatment levels in the female fish exposed at Laboratory C.

3.1.4.3 Histopathology (Table 12)

The majority of male testes from all three laboratories were in stage 2 across all vinclozolin treatment levels, with the exception of the high treatment level from Laboratory A, where the majority of the testes were at stage 3. The majority of female ovaries from all three laboratories were stage 3 with the exception of the low treatment level from Laboratory C where the majority of the female ovaries were either stage 2 or 3.

Granulomatous inflammation in the testicular and ovarian tissues from Laboratory C confounded the evaluation of vinclozolin exposure to the fish exposed by Laboratory C. The evaluation of oocyte atresia in the fish exposed by Laboratory A was also confounded by the presence of microsporidian spores and the associated granulomatous inflammation.

Male histopathology results from Laboratories A and B indicated an increased proportion of spermatogonia, increased testicular degeneration and an increased proportion of interstitial cells in the male testis. There were also an altered proportion of spermatocytes or spermatids that was treatment related at all three vinclozolin test concentrations.

Potential increased oocyte atresia was observed in female fish exposed to vinclozolin from all three laboratories. However, the female fish from Laboratory B were the only ones that could be correlated with vinclozolin exposure due to the presence of microsporidian spores in the fish from the other two laboratories.

3.1.4.4 Conclusions

Studies in rats and fish indicate that metabolites of vinclozolin are androgen receptor antagonists. In the present analysis, results support this MOA. Male secondary sex characteristics, testicular degeneration and male gonad weight and GSI were the most robust and sensitive endpoints. At higher exposures, testosterone levels in male fish may have also been a robust endpoint. Tubercle score was significantly reduced in males exposed to the highest vinclozolin treatment at all three laboratories. Tubercle count was reduced in two of the three laboratories in the high exposure and empirically reduced in Laboratory C. Male gonad weight and GSI were significantly

increased and increased incidence of testicular degeneration was observed in the male fish exposed to the high treatment level at two of the three laboratories. The presence of granulomatous inflammation in the testicular tissues of the fish from Laboratory C did not impact the effects of vinclozolin exposure on other endpoints (e.g., GSI, gonad weight and tubercle score). Only Laboratory C measured significant increases in male vitellogenin concentrations at the mid and high vinclozolin treatment levels.

Fecundity effects were found at the highest vinclozolin exposure in all three laboratories and the mid exposure level at Laboratory B. The screening assay is not designed to determine if the effects in fecundity were a result of chemical effects on males or females. Regardless of the cause, effects in male secondary sex characteristics were correlated with decreases in fecundity of female fish exposed under the same conditions. Female gonad weight was significantly increased in fish exposed at the high treatment level from two of the three laboratories and at the mid treatment level in one of the laboratories. GSI was also increased in the high exposure at one of the laboratories. Similar to the male exposure, female vitellogenin concentrations were significantly greater in the mid and high treatment levels from fish exposed at Laboratory C. The presence of Microsporidia and the associated granulomatous inflammation confounded the evaluation of oocyte atresia in two of the three laboratory exposures (Laboratories A and C). However, the presence of microspridian spores in the ovarian tissue did not impact control performance fecundity as Laboratories' A and C control fecundity exceeded Laboratory B. The presence of microsporidian spores also did not impact the ability of the assay to detect significant differences between control and treatment level fecundity performance.

3.1.5 SDS

3.1.5.1 Exposure level

The nominal concentration for the SDS exposures ranged from 2 to 14 mg/L at the three laboratories. SDS toxicity could in part be a function of water hardness, thus each laboratory conducted acute testing with SDS to assist in choosing a nominal exposure concentration. Laboratory B conducted the first exposure using 14 mg/L as a nominal exposure concentration. Even though Laboratory B could not maintain nominal concentrations, they still saw significant

reductions in reproduction. Laboratories A and C selected much lower nominal concentrations recognizing that measured exposure levels would be lower than the selected nominal concentration. The table below summarizes the mean measured SDS concentrations from the three laboratories.

	Laboratory A	Laboratory B	Laboratory C
Nominal Concentration (mg/L)	2	14	2
Mean Measured Concentration (mg/L)	0.27	<0.44-13	0.62

Rapid microbial degradation of SDS resulted in relatively low and often variable recoveries from the three laboratories. The nominal SDS concentration at Laboratory A was 2.0 mg/L. The mean measured concentration was 0.27 mg/L. The SDS exposure on Day 0 and through Day 7 was approximately 0.4 mg/L and then decreased to 0.1 mg/L on day 14 and decreased further to 0.05 mg/L by day 21. The nominal SDS concentration at Laboratory B was 14 mg/L. SDS recoveries were variable at Laboratory B and dropped below the limit of quantitation on day 7, thus a mean measured concentration was not calculated. However, an estimate of the mean exposure concentration is about 4.5 mg/L. The SDS exposure was 13 mg/L on day 0, dropping to <0.44 mg/L on day 7, increasing slightly to 0.79 mg/L on day 14 and increasing further to 3.6 mg/L on day 21. The nominal SDS concentration at Laboratory C was 2.0 mg/L. The mean measured concentration was 0.62 mg/L or 31% of the nominal level. At Laboratory C the SDS concentrations again started relatively high at approximately 0.76 mg/L, dropped to 0.39 mg/L on day 7, increased to 0.85 mg/L on day 14 and then dropping on day 21 to 0.46 mg/L.

3.1.5.2 Sensitive endpoints (Table 13)

The results from the SDS exposure varied according to the exposure levels maintained by each laboratory. Laboratory A had the lowest mean measured concentrations, with testicular degeneration and oocyte atresia being the only effects observed in the exposure. Laboratory B had the highest exposure levels and saw an increase in basal endpoints (length, body weight, organ weight) in both the males and females, increases in testicular degeneration and oocyte atresia, and a decrease in fecundity endpoints. Laboratory C had a mean exposure about twice as

high as Laboratory A and about seven times lower than Laboratory B. They reported effects on the male fatpad and increased vitellogenin concentration in both the male and female fish. Granulomatous inflammation in the testicular and ovarian tissues from Laboratory C confounded the evaluation of SDS exposure to the fish exposed by Laboratory C.

3.1.5.3 Histopathology (Table 14)

The majority of male testes from two of the three laboratories were in stage 2 while the male testes from the third laboratory were at stage 1. The majority of female ovaries from two of the three laboratories were stage 3 while the female ovaries from the third laboratory were either stage 2 or 3.

Histopathology results from Laboratories A and B showed increased proportion of spermatogonia, increased testicular degeneration, increased proportion of interstitial cells, altered proportions of spermatocytes or spermatids in males, and increased oocyte atresia in females.

3.1.5.4 Conclusions

SDS proved to be a difficult substance and may have been a poor choice for a reference compound due to its instability in water even under flow-through exposure conditions. The results from the SDS exposure were additionally complicated by two significant factors: a single dose (thus no evaluation of a dose response was possible) and the single exposure levels were not consistent among the three laboratories. The effects seen in the SDS exposure were correlated with the mean exposure concentration tested, therefore drawing conclusions regarding the reproducibility of the assay and the suitability of SDS as a positive control is not possible. Laboratory B had the highest exposure concentration and saw the most effects centering around gonad degeneration (testicular degeneration and oocyte atresia), gonad weight and GSI, as well as reductions in fecundity. Laboratory A had the lowest exposure and only saw effects centering on gonadal degeneration; both testicular degeneration and increased oocyte atresia. Laboratory C's exposure fell between those of Laboratories A and B. They found fatpad effects, and increased vitellogenin concentrations in both male and female fish. Although comparisons among laboratories can not be made in the SDS exposure, effects such as testicular degeneration,

oocyte atresia, increased GSI (biological compensation for endocrine disruption, Ankley et al, 2007), decreases in fatpad endpoints and vitellogenin induction in males (although relative small induction) suggest that SDS may affect the endocrine system in fathead minnows.

3.2 Comparison by sensitive endpoints

3.2.1 Basal endpoints

Basal endpoints include fish length and weight, gonad weight and GSI. On several occasions throughout this study, one of the laboratories observed an effect on body length or whole body weight. However, these endpoints were never robust or sensitive. Increases in male gonad weight and GSI were observed in each of the prochloraz, ketoconazole, and vinclozolin exposures. Increased male GSI was the defining effect in the ketoconazole exposure. An increase in female gonad weight and GSI was also observed in each of the three fungicide tests, although the effects were not as commonly observed in males. A significant decrease in male and female gonad weight and/or GSI was detected by at least one laboratory in the octylphenol exposure. All of the significant effects observed at each laboratory for the basal endpoints were in agreement with each other. Male and female GSI comparison figures are presented in Figure 1 and Figure 2.

3.2.2 Fecundity endpoints

Fecundity endpoint figures are presented in Figure 3 through Figure 5. Fecundity endpoints (number of eggs, percent of fertile embryos, and number of spawns) were significantly reduced at the high treatment level of two of the three fungicide exposures, but generally not seen with ketoconazole. The fecundity endpoints were also significantly reduced in the highest treatment level of the octylphenol exposure. In addition the percent of fertile embryos was significantly reduced by 4-tert-octylphenol. The number of spawns per female per day endpoint had more significant effects than the number of eggs per female endpoint and the percentage of fertile embryos end point.

Fecundity endpoints are typically variable. The actual measurements of the fecundity endpoints may have differed between laboratories but the measurements within a laboratory and within an

exposure were consistent enough to see significant dose responses rather than just presence or absence of spawning. The cumulative number of eggs collected over the pre-exposure and exposure periods are presented in Figure 6 through Figure 10. This is best demonstrated in the Laboratory A prochloraz exposure. The control performance for all three fecundity endpoints (number of eggs, number of embryos and number of spawns) was low in two replicates and completely shut down in a third replicate. Nevertheless, a trend of statistical differences was detected at the high exposure level. This improved consistency and reduced variability is likely due to selecting spawning cohorts with demonstrated spawning performance during the pre-exposure period.

This assay is not designed to determine if the fecundity endpoint effects are a result of male or female performance. However, the following observations were made. The only decrease in percent fertile embryos occurred in the octyphenol exposure, which is an estrogen agonist, suggesting that the potential feminization of the males could have affected fertility rate. The one fungicide exposure (ketoconazole) that did not affect secondary male sex characteristics also resulted in almost no significant reductions in fecundity endpoints.

3.2.3 Secondary male sex characteristics

Tubercle and fatpad endpoints were significantly decreased when an effect was observed. Effects were observed in two of the three anti-androgenic exposures (prochloraz, ketoconazole, and vinclozalin) and the one estrogenic exposure (octylphenol). Tubercle count and/or score were the more robust effect, observed most often. Reductions in these endpoints were observed by each laboratory and when the reductions were not significant, there was still evidence of a dose-responsive relationship among treatment levels. The fatpad weights, scores and indices were not as robust, but often more were more sensitive with effects observed by at least one laboratory at all three exposure levels. Significant effects observed for the fatpad endpoints were typically observed by only one laboratory when they were present. They also were typically affected at all three treatment levels. Even though male fish with visible fatpads were selected to initiate the pre-exposure period, the fatpad endpoints may be more difficult to measure with the relatively young male fathead minnows used in these exposures. However, it appears that if there is

adequate fatpad development in a population of male fish, these endpoints are less variable and can be very sensitive.

3.2.4 Biochemical endpoints

Male blood plasma vitellogenin concentration (Figure 13) was a sensitive and robust endpoint in the estrogen mimic exposure (octylphenol). Significant increases were seen by all three laboratories at the mid and high treatment levels and by one laboratory at the lowest exposure level. There was considerable variability in the measurements between the laboratories at the detection limit levels of the procedure. However, the presence of vitellogenin induction in male fish can increase blood plasma vitellogenin levels by orders of magnitude making the variability at the detection limit irrelevant.

Significant decreases in female blood plasma vitellogenin (Figure 14) were observed by two of the three laboratories in the prochloraz exposure. The third laboratory recorded the empirically highest vitellogenin concentrations at the highest treatment level which is contrary to the observations by the other two laboratories. One laboratory measured significant decreases in vitellogenin concentration in the ketoconazole exposure, but this may be due to the relatively high levels of vitellogenin in the control fish rather than an effect of the ketoconazole exposure. One laboratory (Laboratory A) measured a significant increase in vitellogenin concentration during the 4-tert-octyphenol exposure. Laboratory B also measured increased vitellogenin production from females in the high exposure. Relative to the control fish the increase was also in excess of an order of magnitude; however, the increase was not statistically significant and was not as empirically as high as those measured by Laboratory A. It is apparent that female blood plasma vitellogenin concentrations are also quite variable and may not be a robust endpoint.

Laboratory C observed increased vitellogenin effects in both males and females exposed to the mid and high vinclozolin treatment levels and the SDS treatment level. Since both treatments were compared against the same controls, it is possible that the effects were relative to low control concentrations of blood plasma vitellogenin. For example, the male vitellogenin

concentrations at the affected treatment levels are only about one order of magnitude greater than the control values. This is compared to three or four orders of magnitude increases in vitellogenin levels in the 4-tert-octylphenol exposure. Thus, it is not clear if this could be a procedural issue or just coincidence.

Only one significant decrease in male testosterone concentration was observed by one laboratory in the three fungicide exposures (Figure 15). This is contrasted by a significant decrease in male testosterone measured by all three laboratories at the high treatment level, two of the three laboratories at the mid treatment level and one laboratory at the low treatment level in the 4-tert-octylphenol exposure.

Beta-estradiol (Figure 16) was significantly reduced in the prochloraz exposure. This effect was observed by two of the laboratories, with one of the laboratories measuring the effect through the lowest treatment level. During the octylphenol exposure, beta-estradiol was significantly reduced at the high treatment level at one laboratory. There was also an empirical dose-response reduction among the octylphenol treatment levels observed by the other two laboratories. One laboratory also observed significant reductions in beta-estradiol concentration in the vinclozolin exposure.

Beta-estradiol may be a more sensitive sex steroid endpoint than testosterone. However, both sex steroid endpoints appear to be effectively sensitive when there is a clear inhibition mechanism.

3.2.5 Histopathology

Differences in the degeneration of testicular tissue could be distinguished between control and treatment fish. In addition, the differences were often observed to be dose dependent. Testicular degeneration was characterized by increased proportion of spermatogonia, altered proportion of spermatocytes or spermatids and increased proportion of interstitial cells. When these observations were made, they were typically present throughout the treatment range. Testis ova have been reported in male fish exposed to estrogen-mimicking compounds. No testis ova were

observed in any of the male fish from the octylphenol exposure. Testis ova were observed in only two male fish from the entire inter-laboratory comparison.

Testes of fish from two of the exposures (Laboratory C, octylphenol and vinclozolin) exhibited granulomatous inflammation at a high enough frequency to confound the evaluation of treatment-related effects. In the octylphenol exposure, mirosporidian spores were observed in one of the male fish. Granulomatous infiltrates can be caused by a variety of factors such as environmental contaminants in the water, foreign materials, or aftermath of a necrotic process. In other exposures from the inter-laboratory comparison, granulaomatous inflammation was attributed to basophilic foreign material or testicular degeneration. Granulaomatous infiltrates were more of a confounding factor in the ovarian tissue.

Increased oocyte atresia was the major dose-related observation made from the female gonads. Granulomatous inflammation was also commonly observed; however, it was often not dose dependent, and confounded interpretation of potential dose related effects in the ovarian tissues in five exposures (33% of the exposures). As in the testes, granulomatous infiltrates can be caused by a variety of factors such as environmental contaminants in the water, foreign materials, or aftermath of a necrotic process. In some cases granulomatous inflammation appeared to be in response to egg debris in the oviduct. In three of the five exposures were confounded by the presence of granulomatous inflammation, microsporidian spores were observed in the population. Thus in some cases microsporidian spores could be present in the tissues, but not observed and still cause effects. It was also noted that in some females, microsporidian spores were observed and they were not associated with gramulomatous inflammation or oocyte atresia.

Half of the fish populations (6 out of 12) used in the inter-laboratory comparison were found to have microsporidian spores present in the gonadal tissues. The incidence rate was typically confined to female fish and ovarian tissues. In one of the six populations, the Microsporidan spores were only observed in one male fish. Microsporidian spores were present in at least one population of fish from each laboratory. The morphology of the microspordian spores was consistent with *Pleistophora sp*. At no point did the presence of microsporidian spores appear to

affect fecundity or vitellogenin endpoints, which is consistent with the findings of Ruehl-Fehlert et al (2005).

4.0 Discussion and Conclusions

The measured concentrations from the high treatment level in four of the exposures ranged within 20 to 35% among the three laboratories. This variation in exposure levels did not impact the interpretation of the results among the three laboratories. Thus the fish short-term reproduction assay has some built-in robustness regarding moderate differences in exposure concentrations. However, when exposure concentrations varied by greater than 40% (SDS exposure) the fish short-term reproduction assay, like any other aquatic toxicity test, can provide highly variable results.

The age of the fish used during an exposure typically ranged from 23 to 26 weeks between the three laboratories. However, during the ketoconaozle exposure the age of the fish ranged from 25 to 35 weeks, with no significant differences in response. Thus, the assay is robust enough to compensate for differences in ages of fish of up to 10 weeks. The fish short-term reproduction assay can also compensate for the typical differences in water quality that exists between laboratories.

In all cases where statistically significant effects were observed by two or more of the laboratories in a specific exposure, the results always corroborated each other and never contradicted each other. In one case (female vitellogenin concentration) a statistically significant decrease observed by two laboratories appeared to be contradicted by an empirical increase in the endpoint observed in the third laboratory.

The inter-laboratory comparison demonstrated the strengths of many of the fish short-term reproduction assay endpoints. Since there is no single definitive endpoint for endocrine disruption, it is important to include a number of relevant endpoints that will assist in the interpretation of the results for identifying chemicals with the potential to affect the endocrine system when looking at a complex whole organism model. GSI was a sensitive endpoint in the

anti-androgen exposures and significant increases in male GSI in these exposures demonstrates possible biological compensation first reported by Ankley et al (2007) with ketoconazole.

Fecundity and fertility endpoints were robust endpoints even when the fecundity criterion of 15 eggs/female/day was not met. It appears that control fecundity rates of approximately 5 eggs/female/day consistently detected effects in the exposures. The robustness of the fecundity endpoint is likely a result of the 2 male: 4 female sex ratio per replicate and use of proven spawners as determined by the pre-exposure monitoring period used to qualify replicates for inclusion in the exposure phase of the study. The 2 male: 4 female sex ratio also provides a buffer for some expected mortality and/or mis-sexed fish. Generally, the number of spawns per female per day was slightly more robust than the number of eggs per female per day and the number of fertile embryos per female per day across the laboratories in this study.

The presence of nuptial tubercles was the most robust male secondary sex characteristic endpoint. Fatpad endpoints, when present, were typically affected at all treatment levels and thus potentially more sensitive than the tubercle endpoints. The fatpad endpoints may be more robust, if slightly older fish are used in the exposures. However using only older males would require the use of two fish populations, one for the males and one for the females. Using an older single fish population may confound the fecundity endpoints in an effort to enhance fatpad endpoints. It is important at a minimum to only select male fish with developed fatpads for the pre-exposure spawning period.

Fathead minnow blood plasma is very limited particularly from the female fish. Thus biochemical endpoints derived from blood plasma analysis have to be prioritized. Vitellogenin is relatively easy to analyze with commercially available kits. However, there is considerable variability in the vitellogenin measurement and large differences between controls and treatments are needed to see significant differences as occurs in induced males, but not so readily in females. Sex steroid analysis may be more definitive analyses for mode of action determination and a more sensitive endpoint when there are effects. However, commercially reliable assays for measuring testosterone and estradiol in fish plasma are not currently available. Thus more skill is required in measuring the sex steroids.

Testicular degeneration was a sensitive and pertinent histological endpoint for male fish. It was also the defining endpoint in the ketoconazole exposure. Testicular degeneration was characterized by increased proportion of spermatogonia, altered proportion of spermatocytes or spermatids and increased proportion of interstitial cells. Increased oocyte atresia was the major dose related observation made from the female gonads and was a sensitive endpoint when present.

Granulomatous inflammation was also a commonly observed lesion in both the testicular and ovarian tissues. However, granulomatous infiltrates can be caused by a variety of factors such as environmental contaminants in the water, foreign materials or aftermath of a necrotic process. Because granulomatous inflammation can suggest more diffuse systemic processes, it can confound the potential affects of dose related exposures. Granulomatous inflammation confounded observations on testes in three exposures (Laboratory C, octlyphenol, vinclozolin and SDS) and on ovaries in five exposures (Laboratory A, vinclozolin, Laboraotry C, octlyhpenol, ketoconazole, vincolozolin and SDS). In four of the exposures confounded by the presence of granulomatous inflammation, microsporidian spores were observed in the population (Laboratory A, vinclozolin, Laboratory C, octylphenol males and females, vinclozolin). Half of the fish populations (6 out of 12) used in the inter-laboratory comparison were found to have microsporidian spores present in the gonadal tissues. The incidence rate was typically confined to female fish and ovarian tissue. Microsporidian spores were present in at least one population of fish from each laboratory. The morphology of the microspordian spores was consistent with *Pleistophora sp*. At no point did the presence of microsporidian spores appear to affect fecundity or vitellogenin endpoints.

The use of a co-solvent (TEG) in the octylphenol exposure resulted in sporadic differences between control and solvent control endpoints. Interestingly, all of the differences centered on biochemical endpoints. There was no correlation between increases or decreases of these endpoints relative to the presence or absence of the co-solvent. Thus it is more likely that the differences observed between control and solvent control endpoints are due to chance. There were also three global effects (prochloraz Laboratory A male length, ketoconazole Laboratory C

male length, vinclozolin Laboratory C tubercle count) that were not a result of exposure based on the absence of an apparent dose response. However, if co-solvents can be avoided it would be recommended not to use co-solvents as they complicate the experimental design.

The effects seen in the SDS exposure were correlated with the mean exposure concentration tested, therefore drawing conclusions regarding the reproducibility of the assay and the suitability of SDS as a negative control is not possible. Although comparisons between laboratories can not be made in the SDS exposure, effects such as testicular degeneration, oocyte atresia, increased GSI (biological compensation for endocrine disruption), decreases in fatpad endpoints and vitellogenin induction in males (although relatively small induction) suggest that SDS may affect the endocrine system in fathead minnows.

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Table 1. Experimental design for the assay method in all laboratories for prochloraz.

Parameter	Assay Protocol			
	Protocol Recommendation	Lab A	Lab B	Lab C
Test species: Fathead minnow age (months)	4.5-6 months old	23 weeks (5.4 months)	25 weeks old (5.8 months)	26 weeks 6 months
Fish husbandry conditions: Temperature Range (ºC) D.O. Range (mg/L) Photo period (h light:h dark) Light Intensity (lux)	 25ºC ± 1 >5.0 16 h light: 8 h dark 540 to 1080	 24.1 – 25.1 6.6 – 8.1 16 h light: 8 h dark --	 23 to 25 5.12 to 7.17 16 h light: 8 h dark 917	 23.9 to 25.7 >5.0 16 h light: 8 h dark 560 - 926
Pre-exposure evaluation Duration (days)	minimum of 14 days	22 days	14 days	14
Dilution water Total Alkalinity (mg/L as $CaCO_3$) Total organic carbon (mg/L) Unionized Ammonia (mg/L)	Clean, surface, well or reconstituted water >20 ≤5 ≤ 35	Laboratory well water 180 – 182 <1.0 0.0 as total ammonia	Laboratory well water 22 to 25 0.30 to 0.34 0.178 mg/L (measured as total ammonia)	Laboratory processed (mixed spring /R.O) 29 to 47 <5.0 <0.001 mg/L (measured as unionized ammonia)
Test chamber size Volume (L) Dimensions (cm)	 18 to 20 L 40 x 20 x 20 cm	 19L 39x20x25 cm.	 20 L 39 x 20 x 25 cm	 22 L 34.3 x 21.6 x 30.5 cm
Test volume (L)	10 to 12 L	~12 L	11 L	12 L (34.3 x 21.6 x 16 cm)
# Exchanges/day (tank volume exchanges)	6 tank volume exchanges	6	6.1 tank volume exchanges	6 tank volume exchanges
Flow rate (l/h)	2.7 L / hr	~3.0 L/hr	2.8 L / hr	3.0 L / hr
# Concentration / chemical	3	3	3	3
# Replicates:	4	4	4	4
Weight of each fish	NS	NS	NS	NS
# Fish/vessel (females/males)	4 females and 2 males	4 females and 2 males	4 females and 2 males	4 females and 2 males
Total # fish/concentration	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)
Feeding regime	Frozen brine shrimp, twice a day	Frozen brine shrimp, twice a day		Frozen brine shrimp twice daily first 7 days of pre-exposure, rest of study live brine shrimp twice daily
# Controls Dilution water control Solvent control	1 Dilution water control or a solvent control added if a solvent used	 1 0	 1 0	 1 0
# Fish/control vessel (females/males)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)
Photo period (h light:h dark)	16 h light : 8 h dark	16 h ligt: 8 h dark	16 h light : 8 h dark	16 h light : 8 h dark
Temperature range (ºC)	25ºC ± 1	24.8 – 25.8	24 to 25 ºC	24.1 to 25.5
Light intensity (lux)	540 to 1080	552 - 714	618 to 981	596 - 773
Aeration: (yes or no)	None unless D.O. <4.9 mg/L	None	yes (initiated on test day 10)	Yes (added on day 8)
pH range	6.5 - 9.0	8.0 – 8.2	6.8 to 7.6	7.5 – 8.1

Table 2. Experimental design for the assay method in all laboratories for 4-octylphenol.

Parameter	Assay Protocol			
	Protocol Recommendation	Lab A	Lab B	Lab C
Test species: Fathead minnow age (months)	 4.5-6 months old	~24 weeks (~5.6 months)	25 weeks old (5.8 months)	24 weeks 5 months
Fish husbandry conditions: Temperature Range (ºC) D.O. Range (mg/L) Photo period (h light:h dark) Light Intensity (lux)	 25ºC ± 1 >5.0 16 h light: 8 h dark 540 to 1080	 24.0 – 25.1 6.8 – 8.1 16 h light: 8 h dark --	 23 to 25 5.61 to 6.60 16 h light: 8 h dark 619	 23.9 to 25.6 >5.0 16 h light: 8 h dark 549 - 893
Pre-exposure evaluation Duration (days)	minimum of 14 days	15 days	22 days	14
Dilution water Total Alkalinity (mg/L as $CaCO_3$) Total organic carbon (mg/L) Unionized Ammonia (mg/L)	Clean, surface, well or reconstituted water >20 ≤5 ≤ 35	Laboratory well water 182 – 184 <1.0 <0.25	Laboratory well water 20 to 23 0.30 to 0.58 0.178 mg/L (measured as total ammonia)	Laboratory processed (mixed spring /R.O) 44 to 49 <5.0 <0.001 mg/L (measured as unionized ammonia)
Test chamber size Volume (L) Dimensions (cm)	 18 to 20 L 40 x 20 x 20 cm	 19 L 39x20x25 cm	 20 L 39 x 20 x 25 cm	 22 L 34.3 x 21.6 x 30.5 cm
Test volume (L)	10 to 12 L	~10 L	11 L	12 L (34.3 x 21.6 x 16 cm)
# Exchanges/day (tank volume exchanges)	6 tank volume exchanges	6	6 tank volume exchanges	6 tank volume exchanges
Flow rate (l/h)	2.7 L / hr	~2.5 L / hr	2.75 L / hr	3.0 L / hr
# Concentration / chemical	3	3	3	3
# Replicates:	4	4	4	4
Weight of each fish	NS	NS	NS	NS
# Fish/vessel (females/males)	4 females and 2 males	4 females and 2 males	4 females and 2 males	4 females and 2 males
Total # fish/concentration	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (120 fish per test chemical)
Feeding regime	Frozen brine shrimp, twice a day	Frozen brine shrimp, twice a day		Live brine shrimp, twice a day
# Controls Dilution water control Solvent control	1 Dilution water control or a solvent control added if a solvent used	 1 1	 1 1	 1 1
# Fish/control vessel (females/males)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (120 fish per test chemical)
Photo period (h light:h dark)	16 h light : 8 h dark	16 h light:8 h dark	16 h light : 8 h dark	16 h light : 8 h dark
Temperature range (ºC)	25ºC ± 1	24.5 – 25.4	24 to 25 ºC	22.7 to 26.0
Light intensity (lux)	540 to 1080	632 – 776	551 to 962	568 - 844
Aeration: (yes or no)	None unless D.O. <4.9 mg/L	None	yes (initiated on test day 11)	Yes (added on day 7)
pH range	6.5 - 9.0	8.0 – 8.2	6.9 to 7.6	7.7 – 8.2

Table 3. Experimental design for the assay method in all laboratories for ketoconazole.

Parameter	Assay Protocol			
	Protocol Recommendation	Lab A	Lab B	Lab C
Test species: Fathead minnow age (months)	4.5-6 months old	~25 weeks (~5.8 months)	35 weeks old (8.2 months)	26 weeks 6 months
Fish husbandry conditions: Temperature Range (ºC) D.O. Range (mg/L) Photo period (h light:h dark) Light Intensity (lux)	 25ºC ± 1 >5.0 16 h light: 8 h dark 540 to 1080	 24.4 – 25.6 6.2 – 8.2 16 h light: 8 h dark --	 22 to 24 4.42 to 8.93 16 h light: 8 h dark 556	 24.0 to 26.0 >5.0 16 h light: 8 h dark 581 - 926
Pre-exposure evaluation Duration (days)	minimum of 14 days	16 days	22 days	14
Dilution water Total Alkalinity (mg/L as $CaCO_3$) Total organic carbon (mg/L) Unionized Ammonia (mg/L)	Clean, surface, well or reconstituted water >20 ≤5 ≤ 35	Laboratory well water 182 – 184 <1.0 <0.25	Laboratory well water 21 to 23 0.48 to 0.58 0.178 mg/L (measured as total ammonia)	Laboratory processed (mixed spring /R.O) 32 to 49 <5.0 <0.001 mg/L (measured as unionized ammonia)
Test chamber size Volume (L) Dimensions (cm)	 18 to 20 L 40 x 20 x 20 cm	 19 L 39x20x25 cm	 20 L 39 x 20 x 25 cm	 22 L 34.3 x 21.6 x 30.5 cm
Test volume (L)	10 to 12 L	~11 L	11 L	12 L (34.3 x 21.6 x 16 cm)
# Exchanges/day (tank volume exchanges)	6 tank volume exchanges	6	6 tank volume exchanges	6 tank volume exchanges
Flow rate (l/h)	2.7 L / hr	~2.75 L/hr	2.8 L / hr	3.0 L / hr
# Concentration / chemical	3	3	3	3
# Replicates:	4	4	4	4
Weight of each fish	NS	NS	NS	NS
# Fish/vessel (females/males)	4 females and 2 males	4 females, 2 males	4 females and 2 males	4 females and 2 males
Total # fish/concentration	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)
Feeding regime	Frozen brine shrimp, twice a day	Frozen brine shrimp, twice a day		Live brine shrimp twice a day, one day of exposure fed Tetramin flakes twice
# Controls Dilution water control Solvent control	1 Dilution water control or a solvent control added if a solvent used	1 0	 1 0	 1 0
# Fish/control vessel (females/males)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)
Photo period (h light:h dark)	16 h light : 8 h dark	16 h light: 8 h dark	16 h light : 8 h dark	16 h light : 8 h dark
Temperature range (ºC)	25ºC ± 1	24.3 – 25.8	25 ºC	24.1 to 25.9
Light intensity (lux)	540 to 1080	383 - 658	576 to 963	630 - 775
Aeration: (yes or no)	None unless D.O. <4.9 mg/L	None	yes (initiated on test day 10)	Yes (added on day 6)
pH range	6.5 - 9.0	8.0 – 8.3	6.8 to 7.5	7.7 – 8.1

Table 4. Experimental design for the assay method in all laboratories for vinclozolin & sodium dodecyl sulfate (SDS).

Parameter	Assay Protocol			
	Protocol Recommendation	Lab A	Lab B	Lab C
Test species: Fathead minnow age (months)	4.5-6 months old	23 weeks (5.4 months)	25 weeks old (5.8 months)	26 weeks 6 months
Fish husbandry conditions: Temperature Range (ºC) D.O. Range (mg/L) Photo period (h light:h dark) Light Intensity (lux)	 25ºC ± 1 >5.0 16 h light: 8 h dark 540 to 1080	 24.1 – 25.1 6.6 – 8.1 16 h light: 8 h dark --	 23 to 25 5.12 to 7.17 16 h light: 8 h dark 917	 23.9 to 25.7 >5.0 16 h light: 8 h dark 560 - 926
Pre-exposure evaluation Duration (days)	minimum of 14 days	22 days	14 days	14
Dilution water Total Alkalinity (mg/L as $CaCO_3$) Total organic carbon (mg/L) Unionized Ammonia (mg/L)	Clean, surface, well or reconstituted water >20 ≤5 ≤ 35	Laboratory well water 180 – 182 <1.0 0.0 as total ammonia	Laboratory well water 22 to 25 0.30 to 0.34 0.178 mg/L (measured as total ammonia)	Laboratory processed (mixed spring /R.O) 29 to 47 <5.0 <0.001 mg/L (measured as unionized ammonia)
Test chamber size Volume (L) Dimensions (cm)	 18 to 20 L 40 x 20 x 20 cm	 19L 39x20x25 cm.	 20 L 39 x 20 x 25 cm	 22 L 34.3 x 21.6 x 30.5 cm
Test volume (L)	10 to 12 L	~12 L	11 L	12 L (34.3 x 21.6 x 16 cm)
# Exchanges/day (tank volume exchanges)	6 tank volume exchanges	6	6.1 tank volume exchanges	6 tank volume exchanges
Flow rate (l/h)	2.7 L / hr	~3.0 L/hr	2.8 L / hr	3.0 L / hr
# Concentration / chemical	3	3	3	3
# Replicates:	4	4	4	4
Weight of each fish	NS	NS	NS	NS
# Fish/vessel (females/males)	4 females and 2 males	4 females and 2 males	4 females and 2 males	4 females and 2 males
Total # fish/concentration	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)
Feeding regime	Frozen brine shrimp, twice a day	Frozen brine shrimp, twice a day		Frozen brine shrimp twice daily first 7 days of pre-exposure, rest of study live brine shrimp twice daily
# Controls Dilution water control Solvent control	1 Dilution water control or a solvent control added if a solvent used	 1 0	 1 0	 1 0
# Fish/control vessel (females/males)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)
Photo period (h light:h dark)	16 h light : 8 h dark	16 h ligt: 8 h dark	16 h light : 8 h dark	16 h light : 8 h dark
Temperature range (ºC)	25ºC ± 1	24.8 – 25.8	24 to 25 ºC	24.1 to 25.5
Light intensity (lux)	540 to 1080	552 - 714	618 to 981	596 - 773
Aeration: (yes or no)	None unless D.O. <4.9 mg/L	None	yes (initiated on test day 10)	Yes (added on day 8)
pH range	6.5 - 9.0	8.0 – 8.2	6.8 to 7.6	7.5 – 8.1

Table 5. Summary of prochloraz quantitative results.

Endpoint	Exposure Level (measured range in µg/L)								
	Low (20)			Mid (100)			High (300)		
	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
	Males								
Male histopathology[a]			↑		↑	↑	↑	↑	↑
VTG									
Testosterone									
Length									
Weight									
Gonad Weight						↑		↑	↑
GSI						↑		↑	↑
Tubercle Score				↓		↓	↓	↓	↓
Tubercle Count							↓	↓	
Fatpad Weight					↓			↓	
Fatpad Score					↓			↓	↓
Fatpad Index		↓			↓			↓	
Survival (% M&F)									
	Females								
Female histopathology[a]		↑		↑	↑		↑	↑	
VTG			↓		↓	↓		↓	↓
Beta-Estradiol		↓			↓		Global[b]	↓	↓
Length								↑	
Weight								↑	
Gonad Weight					↑			↑	
GSI							Global[c]	↑	
Eggs/Female/Day							↓	↓	↓
# Fertile Eggs/Female/Day							↓	↓	↓
Spawns/Female/Day							↓	↓	↓
% of Fertile Eggs									

[a] Greater histopathology detail is presented in Table 6

[b] Beta Estradiol Global effect – greatest difference between control and mid

[c] GSI Global effect – greatest difference between low and control

Legend
↑ significant increase
↓ significant decrease

Table 6. Summary of prochloraz histopathology results.

Endpoint	Exposure Level (measured range in µg/L)								
	Low (20)			Mid (100)			High (300)		
	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
	Males								
Increased proportion of spermatogonia			↑		▲	↑		▲	
Increased testicular degeneration			↑		▲	↑	▲	▲	↑
Increased proportion of interstitial cells			↑		▲	↑		▲	
Presence of testis-ova									
Decreased proportion of spermatogonia									↑
Increased interstitial or vascular proteinaceous fluid									
Asynchronous gonad development									
Altered proportions of spermatocytes or spermatids			↑						
Granulomatous inflammation									
Gonadal staging	2	2	2	2	2	2	2	2	3
	Female								
Increased oocyte atresia		▲		▲	▲		▲	▲	
Perifollicular cell hyperplasia/hypertrophy									
Decreased yolk formation									
Interstitial fibrosis									
Egg debris in the oviduct									
Granulomatous inflammation									
Decreased post-ovulatory follicles				▲			▲		
Gonadal staging	3	3	3	3	3	3	3	3	3

Lab C microsporidian spheres in female

Legend
↑ increase
↓ decrease
▲ greater incidence

Table 7. Summary of 4-t-octylphenol quantitative results.

Endpoint	Exposure Level (measured range in µg/L)								
	Low (1.0)			Mid (50)			High (150)		
	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
	Males								
Male histopathology[a]	↑	↑		↑	↑		↑	↑	↑
VTG			↑	↑	↑	↑	↑	↑	↑
Testosterone	↓			↓		↓	↓	↓	↓
Length									
Weight									
Gonad Weight							↓		
GSI									
Tubercle Score				↓		↓	↓	↓	↓
Tubercle Count							↓	↓	↓
Fatpad Weight							↓		
Fatpad Score	↓			↓			↓		
Fatpad Index							↓		
Survival (% M&F)							↓		
	Females								
Female histopathology[a]	↑			↑			↑	↑	
VTG				↑			↑		
Beta-Estradiol									↓
Length									
Weight									↓
Gonad Weight							↓		
GSI							↓		
Eggs/Female/Day							↓	↓	
# Fertile Eggs/Female/Day							↓	↓	
Spawns/Female/Day							↓	↓	↓
% of Fertile Eggs							↓	↓	

[a] Greater histopathology detail is presented in Table 8

Lab A – male testosterone and female beta estradiol, solvent control was greater than control
Lab B – female vitellogenin, solvent control was less than control
Lab C – male testosterone, solvent control was less than control

Legend
↑ significant increase
↓ significant decrease

Table 8. Summary of 4-t-octylphenol histopathology results.

Endpoint	Exposure Level (measured range in µg/L)								
	Low (1.0)			Mid (50)			High (150)		
	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
	Males								
Increased proportion of spermatogonia	▲	▲		▲	▲		▲	▲	▲
Increased testicular degeneration				↑			↑		
Increased proportion of interstitial cells									
Presence of testis-ova									
Decreased proportion of spermatogonia									↑
Increased interstitial or vascular proteinaceous fluid									
Asynchronous gonad development									
Altered proportions of spermatocytes or spermatids	▲			▲	▲		▲	▲	
Granulomatous inflammation				↑			↑		
Gonadal staging	2	2	2	2	2	2	2	2	2
	Females								
Increased oocyte atresia	▲			▲			▲	▲	
Perifollicular cell hyperplasia/hypertrophy									
Decreased yolk formation									
Interstitial fibrosis									
Egg debris in the oviduct									
Granulomatous inflammation									
Decreased post-ovulatory follicles									
Gonadal staging	3	3	3	3	3	3	0-3	3	3

Lab C microsporidian spheres in female
Lab C inconclusive

Legend
↑ increase
↓ decrease
▲ greater incidence

Table 9. Summary of ketoconazole quantitative results.

Endpoint	Exposure Level (measured range in µg/L)								
	Low (25)			Mid (100)			High (400)		
	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
	Males								
Male histopathology[a]	↑	↑	↑	↑	↑	↑	↑	↑	↑
VTG									
Testosterone									
Length									Global[b]
Weight									
Gonad Weight					↑			↑	↑
GSI					↑	↑	↑	↑	↑
Tubercle Score								↓	
Tubercle Count									
Fatpad Weight									
Fatpad Score									
Fatpad Index									
Survival (% M&F)									
	Females								
Female histopathology[a]	↑							↑	
VTG	↓						↓		
Beta-Estradiol									
Length									
Weight									
Gonad Weight								↑	
GSI						↑		↑	↑
Eggs/Female/Day									
# Fertile Eggs/Female/Day									
Spawns/Female/Day								↓	
% of Fertile Eggs									

[a] Greater histopathology detail is presented in Table 10

[b] Global effect – greatest difference between mid and control.

Legend
↑ significant increase
↓ significant decrease

Table 10. Summary of ketoconazole histopathology results.

Endpoint	Exposure Level (measured range in µg/L)								
	Low (25)			Mid (100)			High (400)		
	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
	Males								
Increased proportion of spermatogonia									
Increased testicular degeneration	▲			▲			▲		
Increased proportion of interstitial cells	▲	▲	▲	▲	▲	▲	▲	▲	▲
Presence of testis-ova									
Decreased proportion of spermatogonia									
Increased interstitial or vascular proteinaceous fluid									
Asynchronous gonad development									
Altered proportions of spermatocytes or spermatids									
Granulomatous inflammation									
Gonadal staging	2-3	2	2	2-3	2	2	2-3	2	2
	Female								
Increased oocyte atresia	▲	▲		▲	▲		▲	▲	
Perifollicular cell hyperplasia/hypertrophy									
Decreased yolk formation									
Interstitial fibrosis									
Egg debris in the oviduct									
Granulomatous inflammation									
Decreased post-ovulatory follicles									
Gonadal staging	3	3	3	3	3	3	3	3	3

Legend
↑ increase
↓ decrease
▲ greater incidence

Table 11. Summary of vinclozolin quantitative results.

Endpoint	Exposure Level (measured range in µg/L)								
	Low (100)			Mid (300)			High (900)		
	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
	Males								
Male histopathology[a]	↑	↑		↑	↑		↑	↑	
VTG						↑			↑
Testosterone								↓	
Length									
Weight									
Gonad Weight		↑			↑			↑	↑
GSI		↑			↑			↑	↑
Tubercle Score		↓			↓		↓	↓	↓
Tubercle Count					↓		↓	↓	Global[b]
Fatpad Weight		↓			↓			↓	
Fatpad Score								↓	
Fatpad Index		↓			↓			↓	
Survival (% M&F)									
	Females								
Female histopathology[a]								↑	
VTG						↑			↑
Beta-Estradiol					↓			↓	
Length					↑			↑	
Weight					↑			↑	
Gonad Weight					↑			↑	↑
GSI									↑
Eggs/Female/Day					↓		↓	↓	↓
# Fertile Eggs/Female/Day					↓		↓	↓	↓
Spawns/Female/Day					↓		↓	↓	↓
% of Fertile Eggs									

[a] Greater histopathology detail is presented in Table 12

[b] Global effect – greatest difference between mid and high. High treatment level is empirically less than the control

Legend
↑ significant increase
↓ significant decrease

Table 12. Summary of vinclozolin histopathology results.

Endpoint	Exposure Level (measured range in µg/L)								
	Low (100)			Mid (300)			High (900)		
	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
	Males								
Increased proportion of spermatogonia	▲	▲		▲	▲		▲	▲	
Increased testicular degeneration	▲	▲		▲			▲	▲	
Increased proportion of interstitial cells	▲	▲		▲	▲		▲	▲	
Presence of testis-ova									
Decreased proportion of spermatogonia									
Increased interstitial or vascular proteinaceous fluid									
Asynchronous gonad development									
Altered proportions of spermatocytes or spermatids	▲	▲		▲	▲		▲	▲	
Granulomatous inflammation									
Gonadal staging	2	2	2	2	2	2	3	2	2
	Female								
Increased oocyte atresia								▲	
Perifollicular cell hyperplasia/hypertrophy								↑	
Decreased yolk formation									
Interstitial fibrosis								↑	
Egg debris in the oviduct									
Granulomatous inflammation	↑						↑		
Decreased post-ovulatory follicles									
Gonadal staging	3	3	2-3	3	3	3	3	3	3

Lab A microsporidian spheres in female
Lab C microsporidian spheres in female
Lab C inconclusive

Legend
↑ increase
↓ decrease
▲ greater incidence

Table 13. Summary of SDS quantitative results.

Endpoint	Exposure Level (measured range in mg/L)		
	2 - 14 mg/L		
	Lab A	Lab B	Lab C
	Males		
Male histopathology[a]	↑	↑	
VTG			↑
Testosterone			
Length		↑	
Weight			
Gonad Weight		↑	
GSI		↑	
Tubercle Score			
Tubercle Count			
Fatpad Weight			↓
Fatpad Score			↓
Fatpad Index			↓
Survival (% M&F)			
	Females		
Female histopathology[a]	↑	↑	
VTG			↑
Beta-Estradiol			
Length			
Weight		↑	
Gonad Weight		↑	
GSI		↑	
Eggs/Female/Day		↓	
# Fertile Eggs/Female/Day		↓	
Spawns/Female/Day		↓	
% of Fertile Eggs			

[a] Greater histopathology detail is presented in Table 14

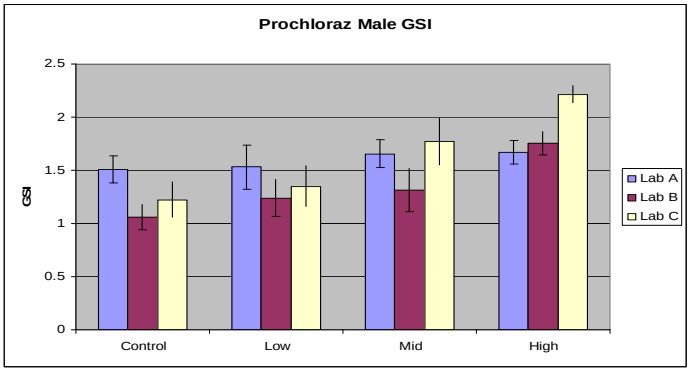
Legend
↑ significant increase
↓ significant decrease

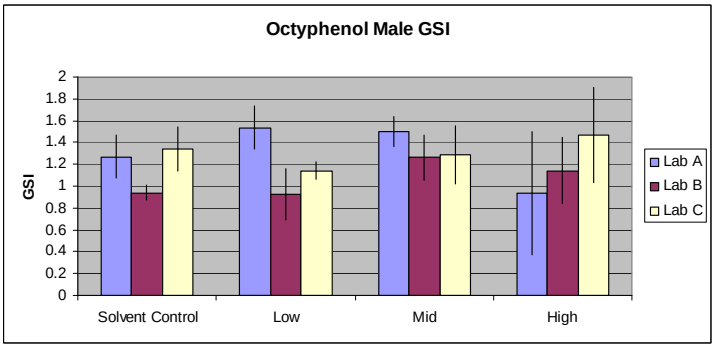
Table 14. Summary of SDS histopathology results.

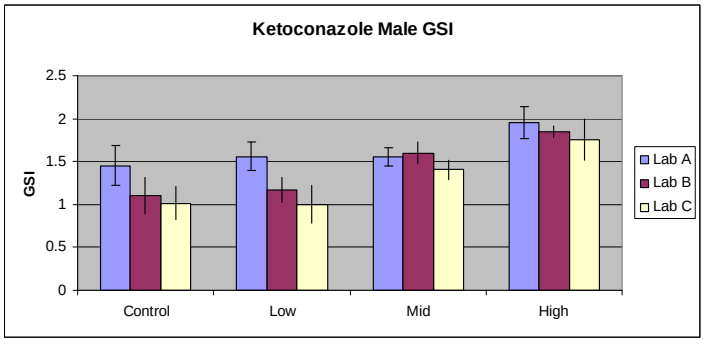
Endpoint	Exposure Level (measured range in mg/L)		
	2 - 14 mg/L		
	Lab A	Lab B	Lab C
	Males		
Increased proportion of spermatogonia	▲	▲	
Increased testicular degeneration	▲	▲	
Increased proportion of interstitial cells	▲	↑	
Presence of testis-ova			
Decreased proportion of spermatogonia			
Increased interstitial or vascular proteinaceous fluid			
Asynchronous gonad development			
Altered proportions of spermatocytes or spermatids	▲	▲	
Granulomatous inflammation			
Gonadal staging	2	2	1
	Female		
Increased oocyte atresia	▲	▲	
Perifollicular cell hyperplasia/hypertrophy			
Decreased yolk formation			
Interstitial fibrosis			
Egg debris in the oviduct			
Granulomatous inflammation			
Decreased post-ovulatory follicles			
Gonadal staging	3	3	2-3

Legend
↑ increase
↓ decrease
▲ greater incidence

Figure 1. Male GSI comparisons (error bars represent treatment level CV)







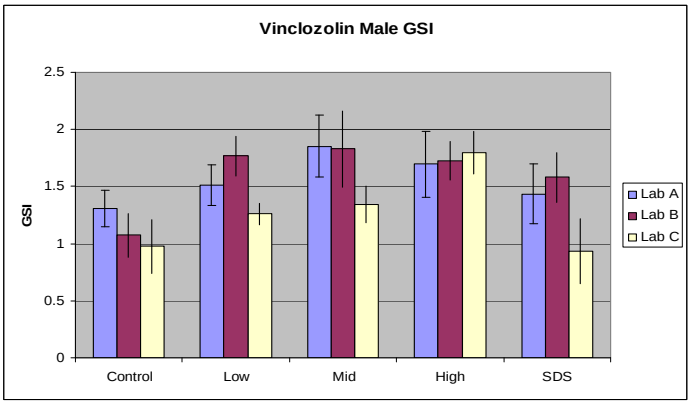
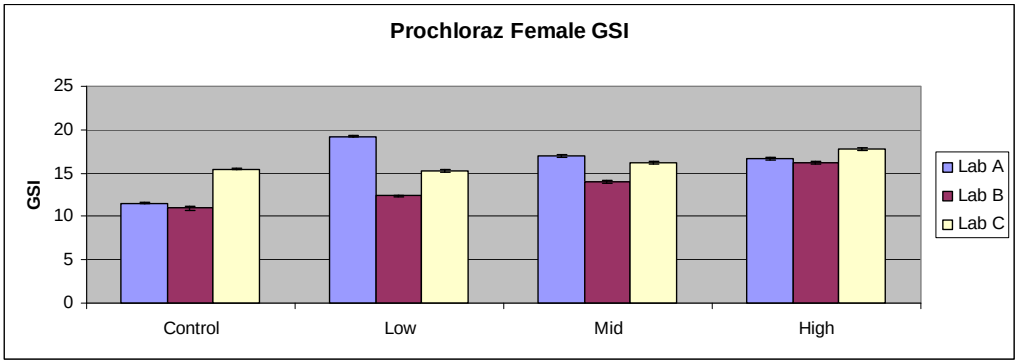
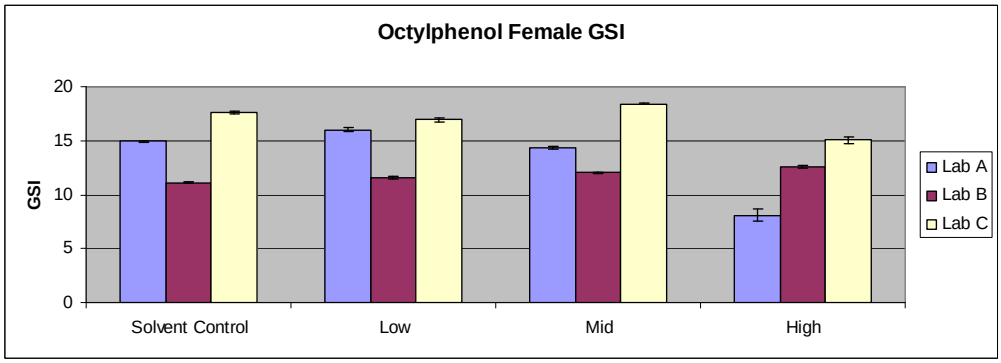
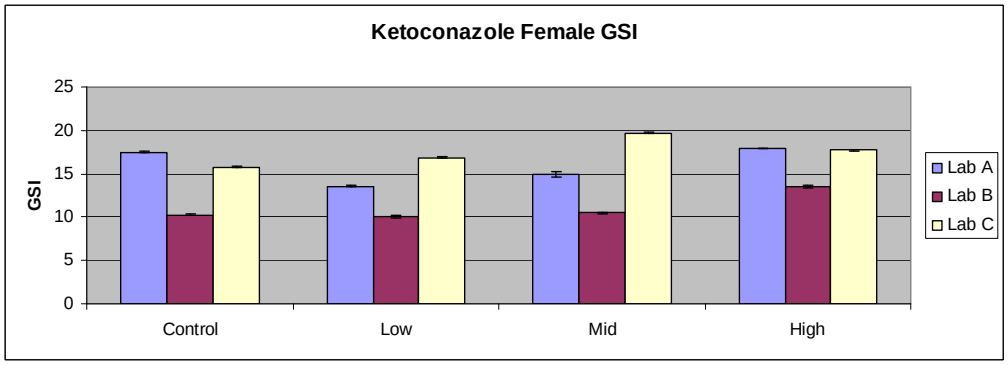


Figure 2. Female GSI comparisons (error bars represent treatment level CV)







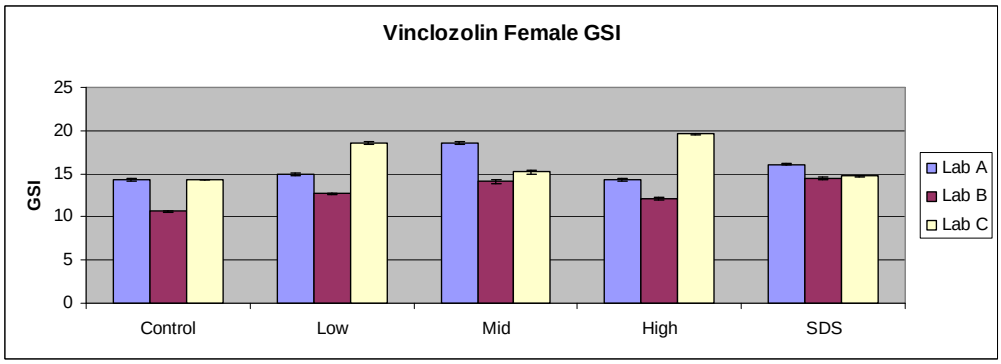
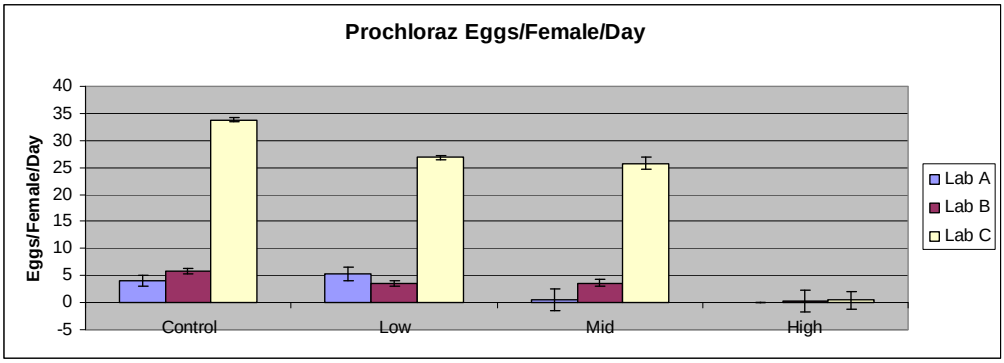
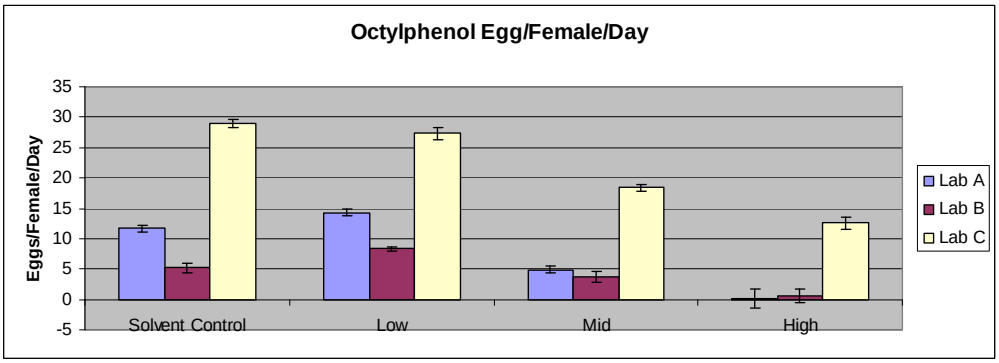
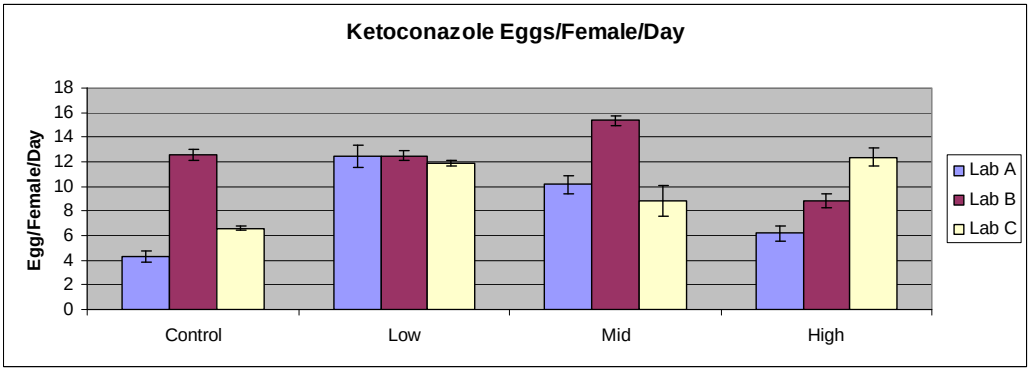


Figure 3. Eggs/Female/Day comparisons (error bars represent treatment level CV)







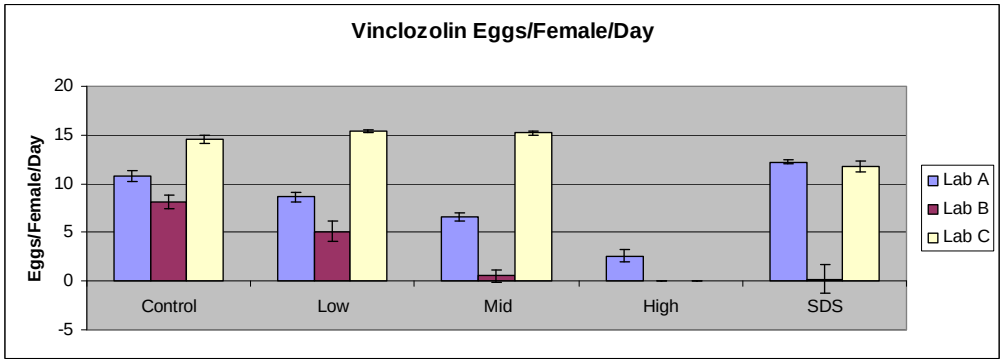
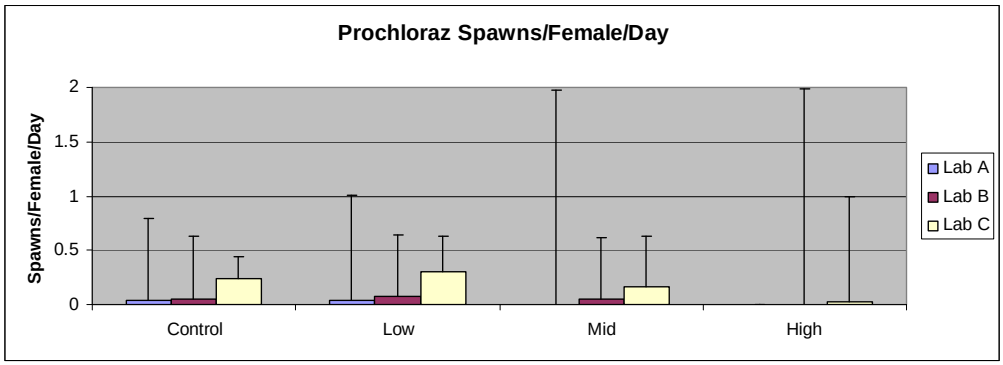
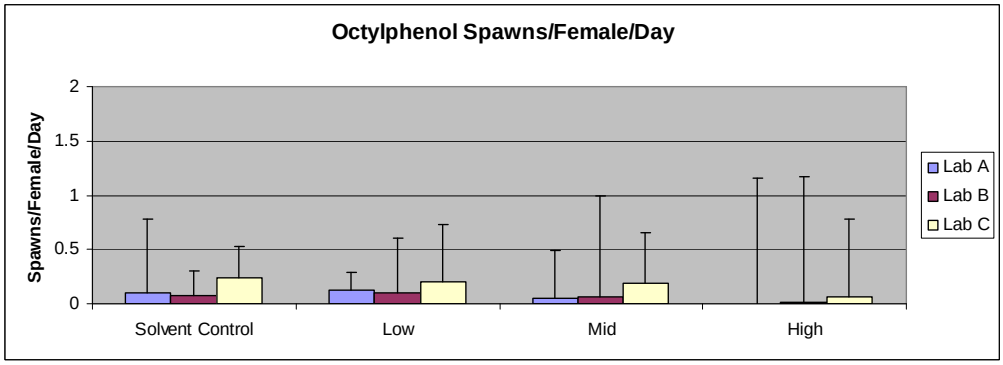
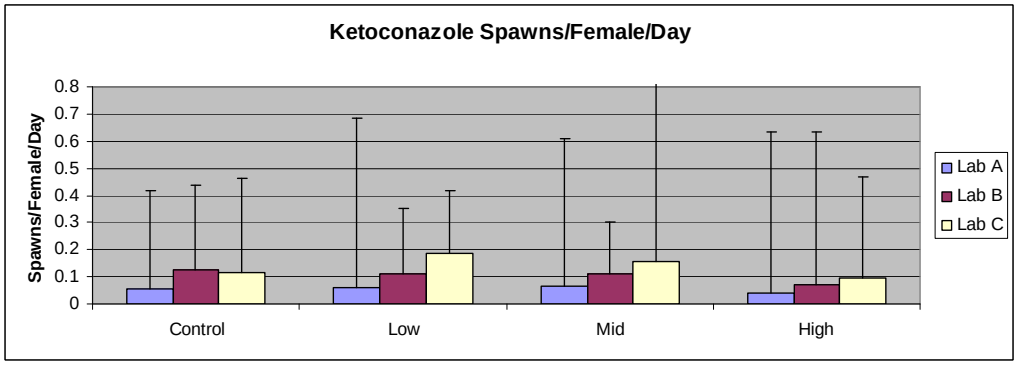


Figure 4. Spawns/Female/Day comparisons (error bars represent treatment level CV)







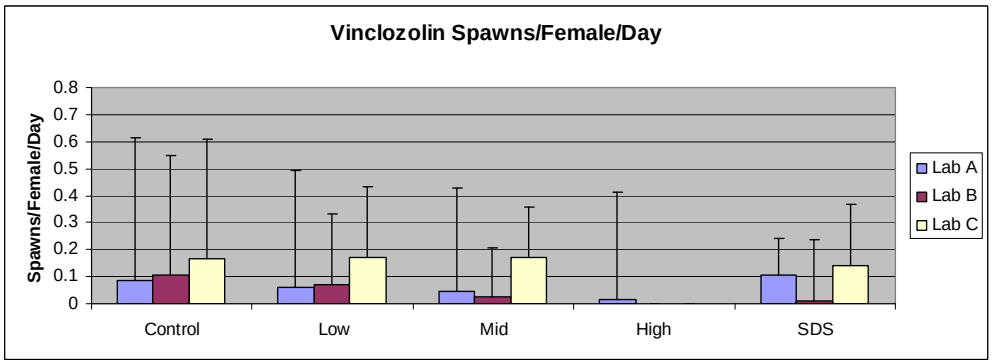
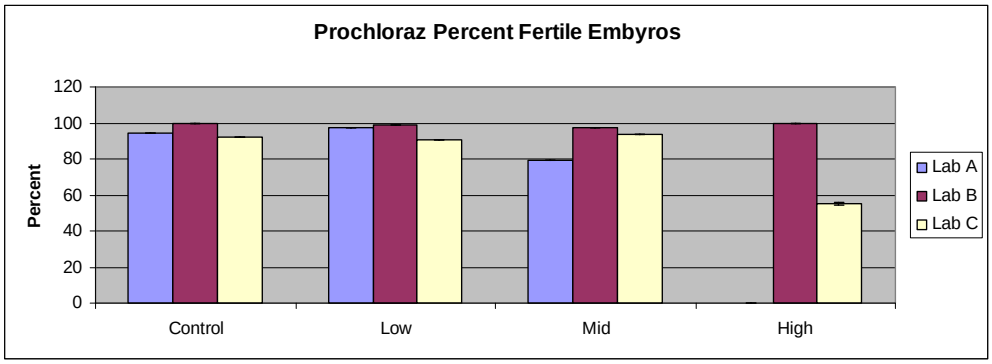
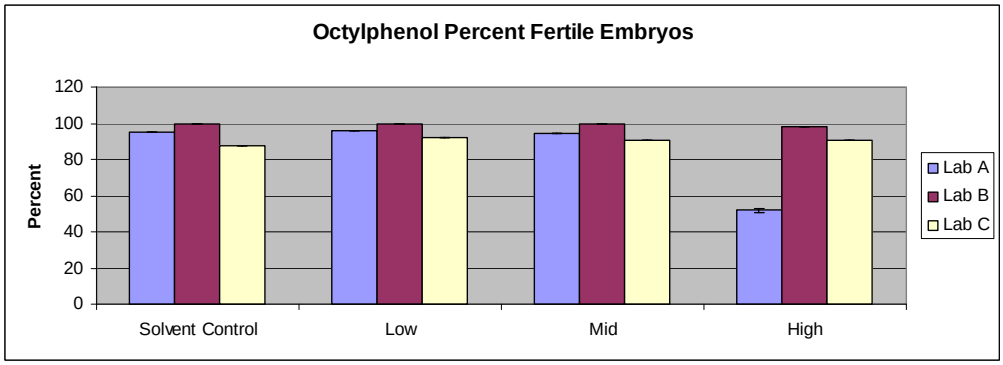
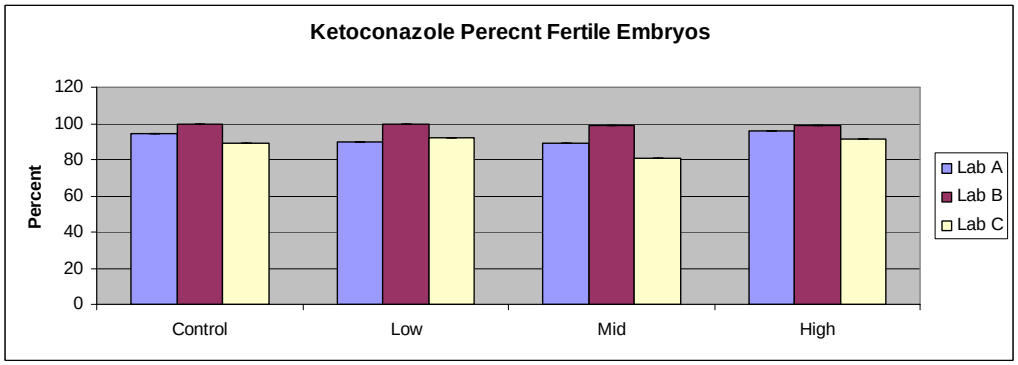


Figure 5. **Percent fertile embryo comparisons (error bars represent treatment level CV)**







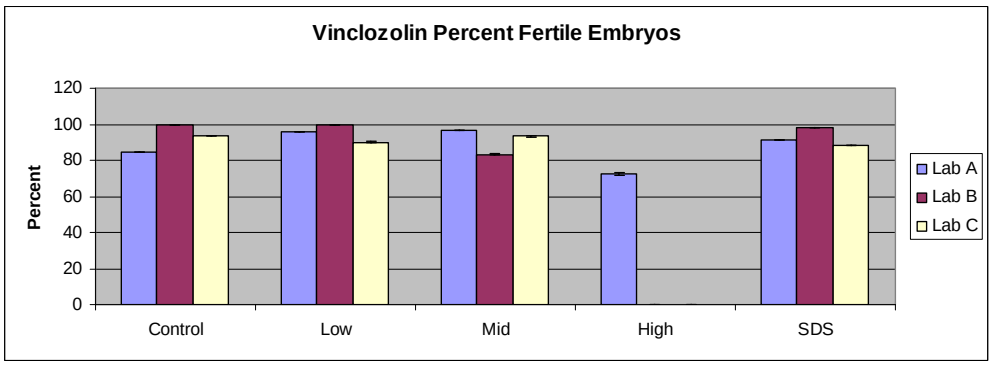
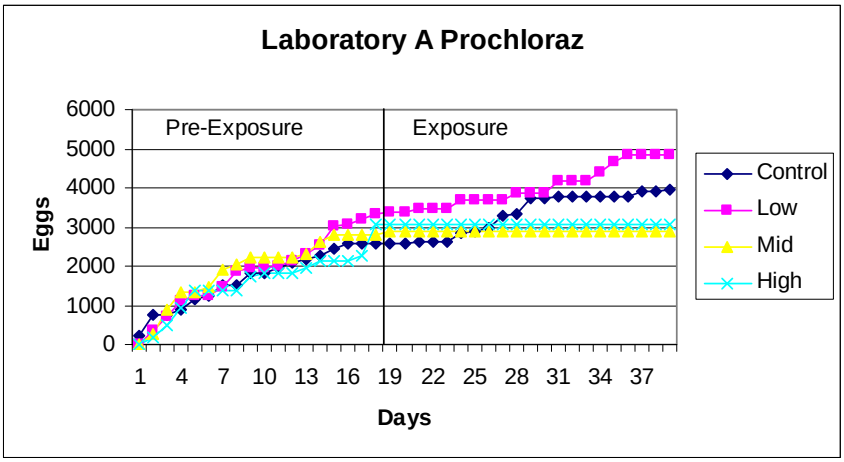
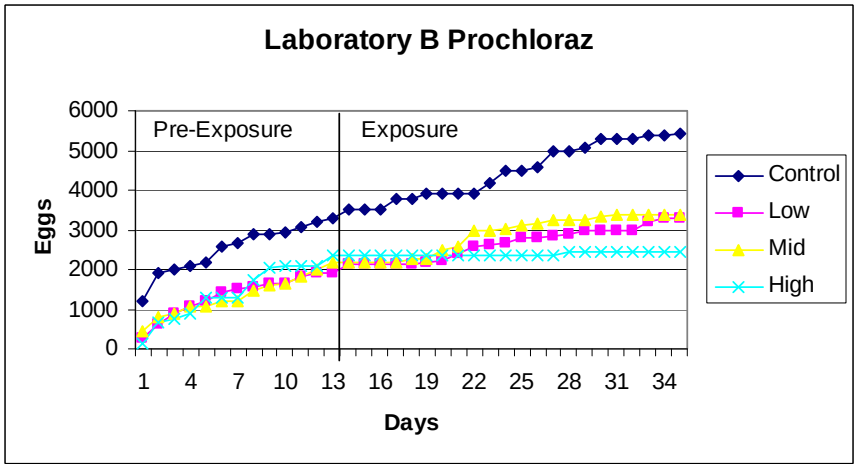


Figure 6. **The cumulative number of eggs collected over the pre-exposure and exposure periods for the prochloraz exposure.**





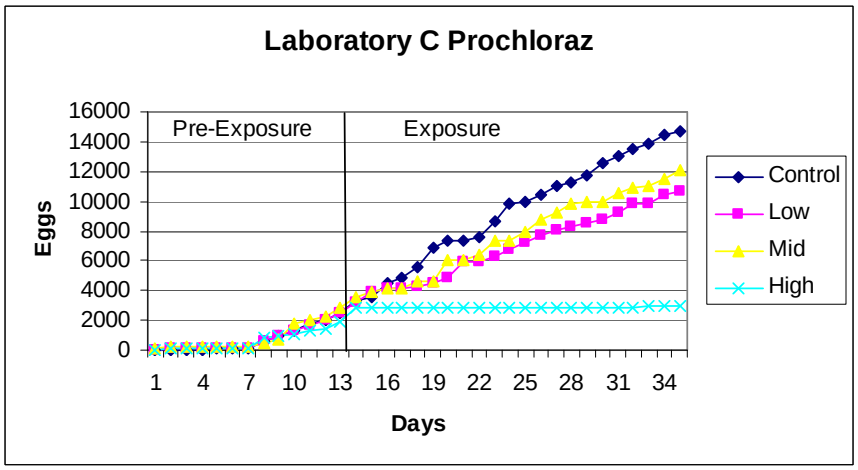
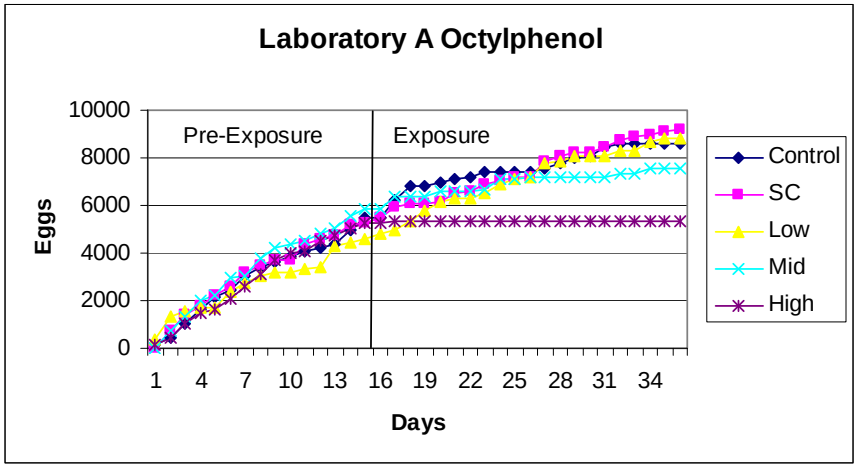
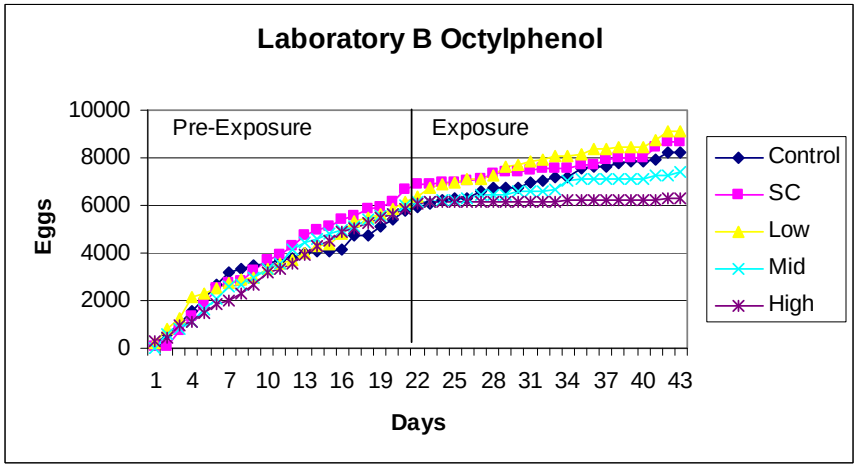


Figure 7. **The cumulative number of eggs collected over the pre-exposure and exposure periods for the octylphenol exposure.**





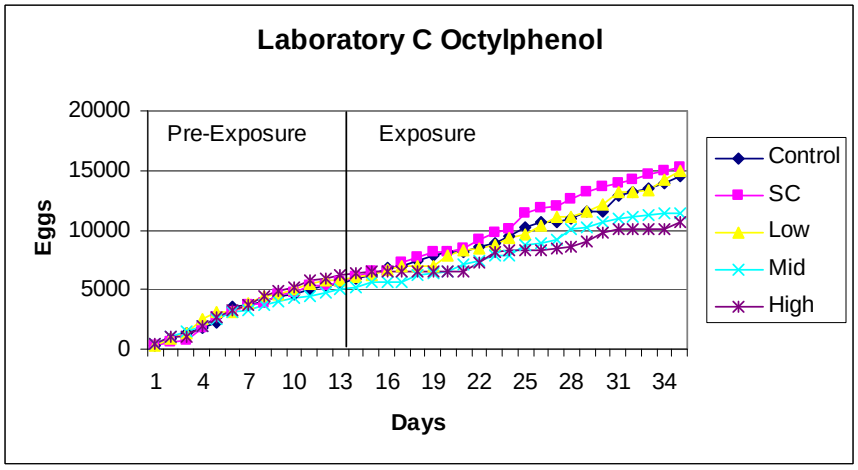
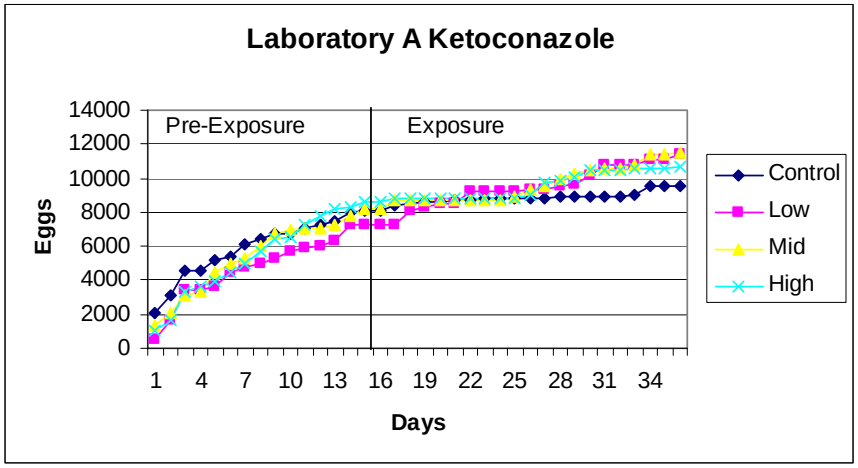
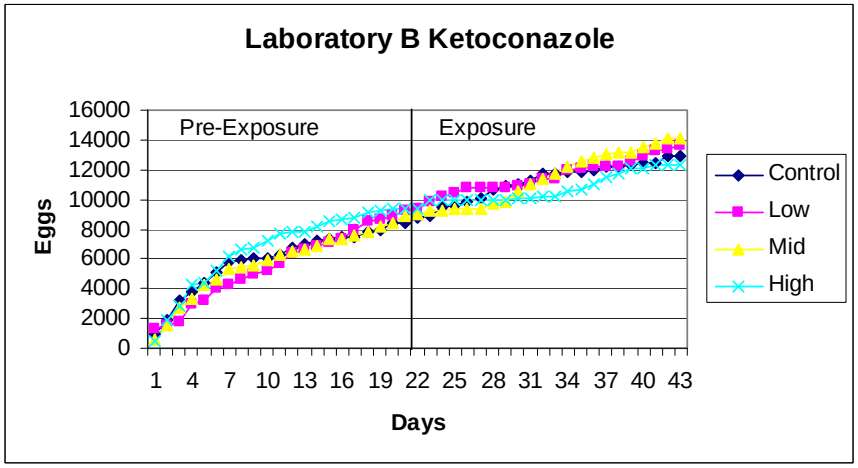


Figure 8. **The cumulative number of eggs collected over the pre-exposure and exposure periods for the ketoconazole exposure.**





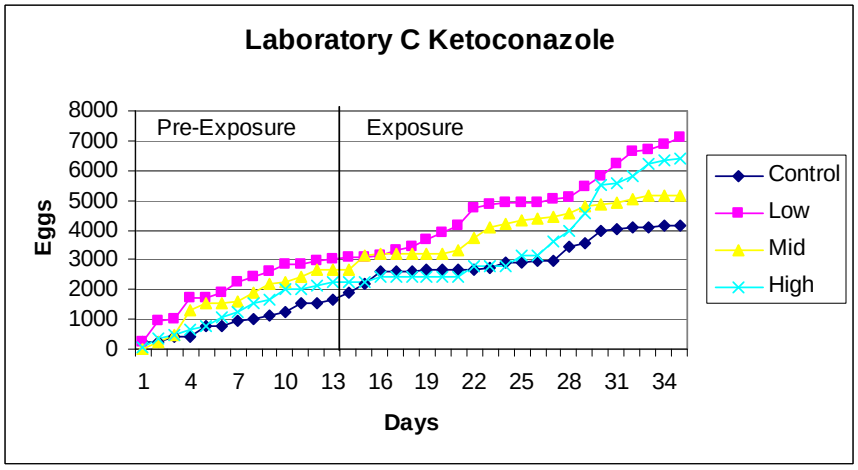
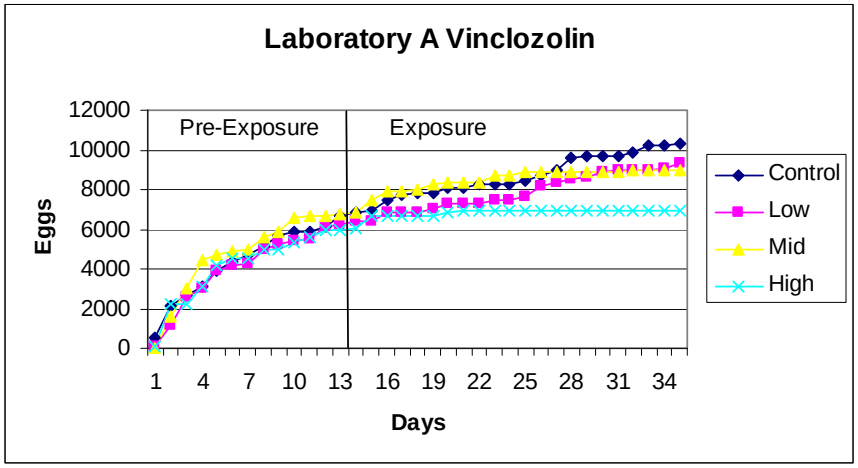
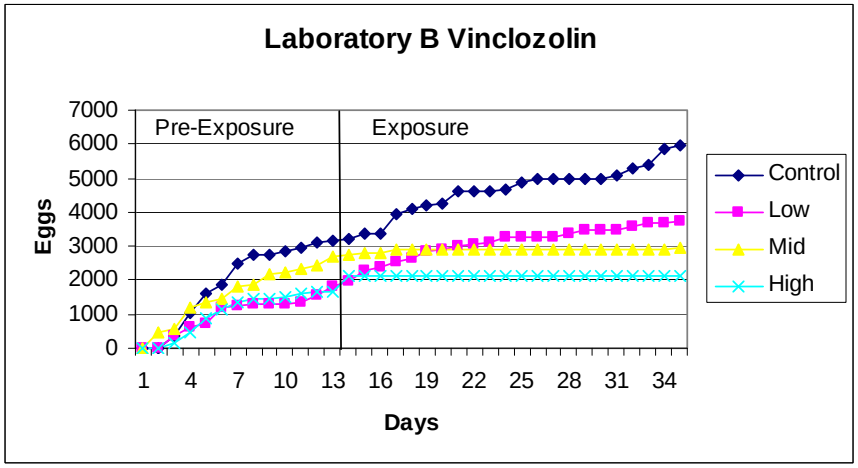


Figure 9. **The cumulative number of eggs collected over the pre-exposure and exposure periods for the vinclozolin exposure.**





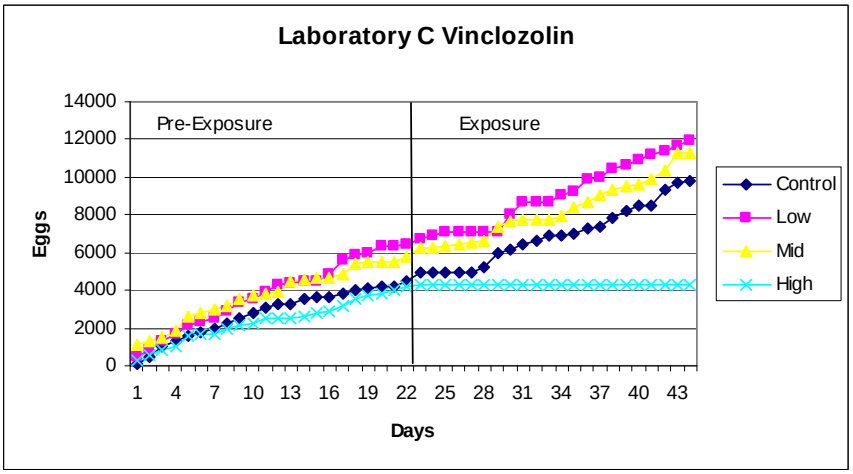


Figure 10. **The cumulative number of eggs collected over the pre-exposure and exposure periods for the SDS exposure.**

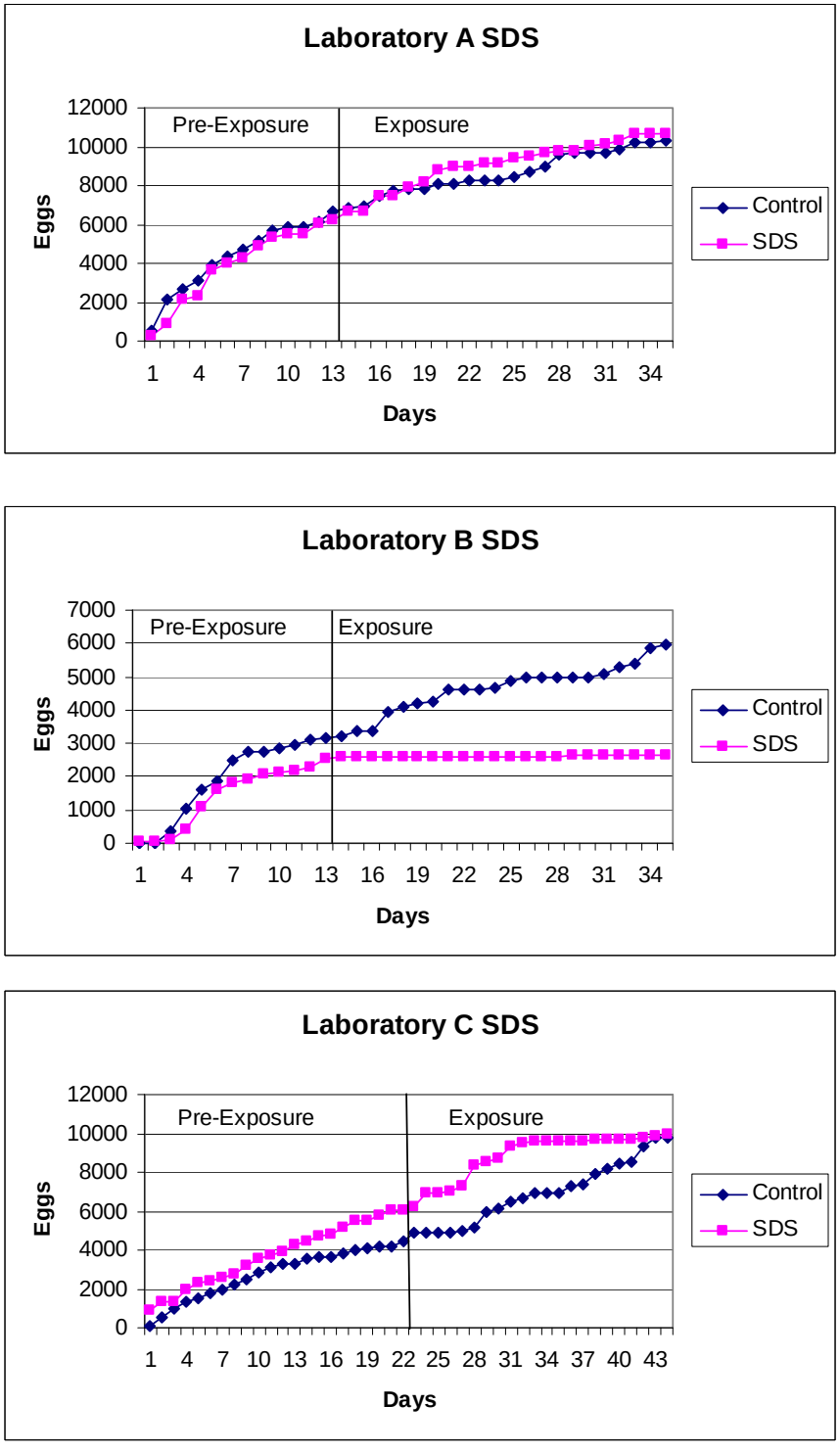
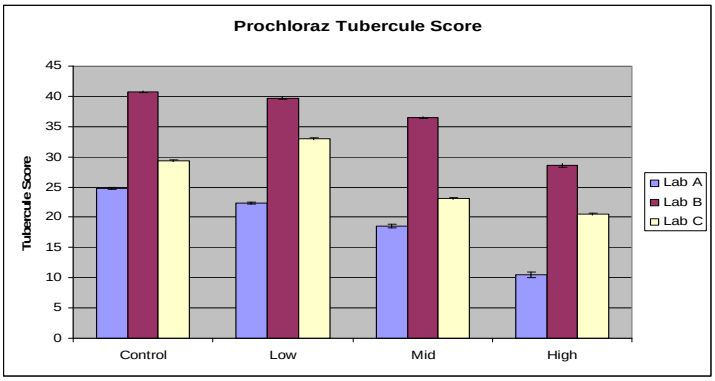
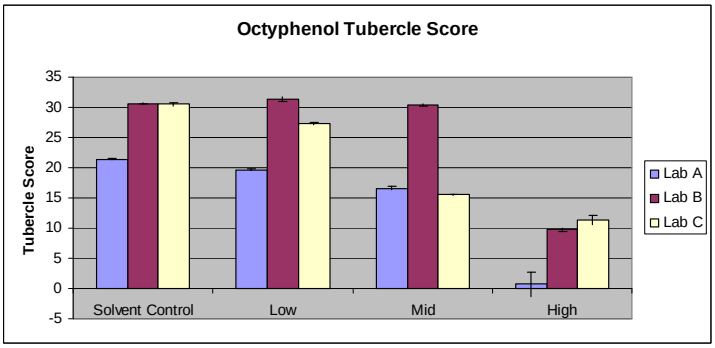
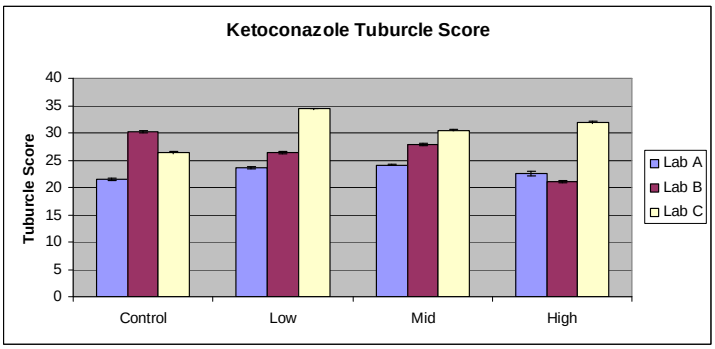


Figure 11. Tubercle score comparisons (error bars represent treatment level CV)







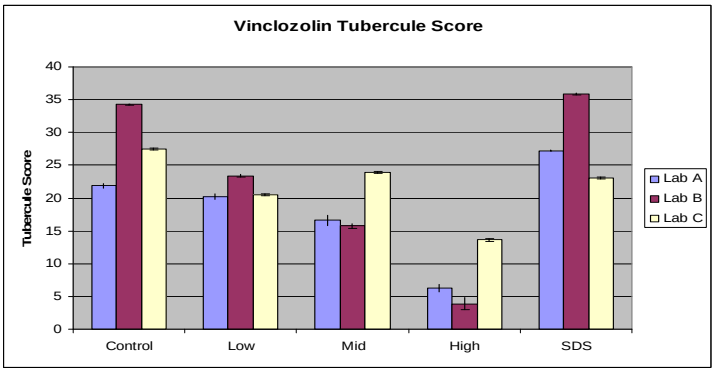


Figure 12. Fatpad index comparisons (error bars represent treatment level CV)

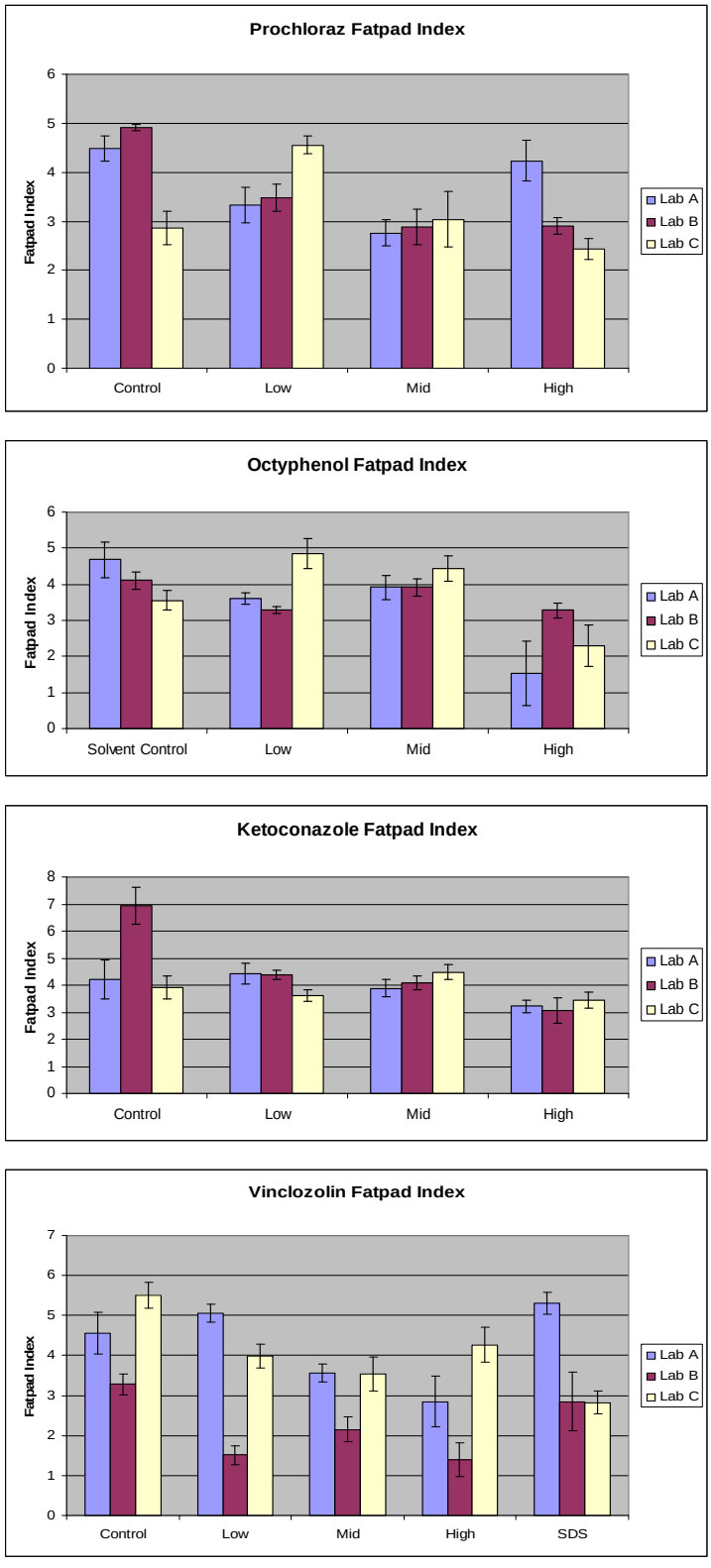
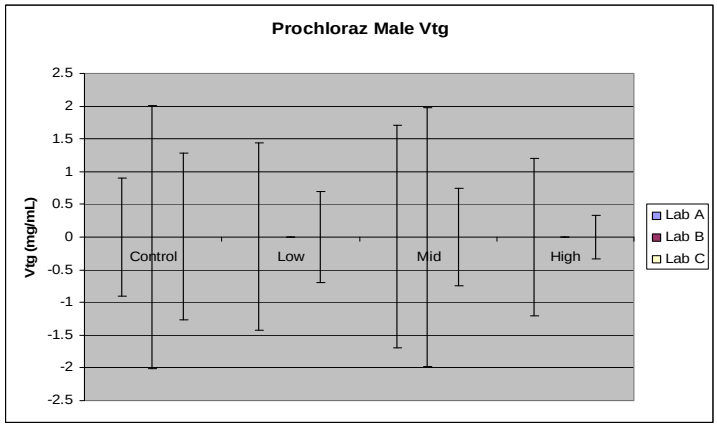
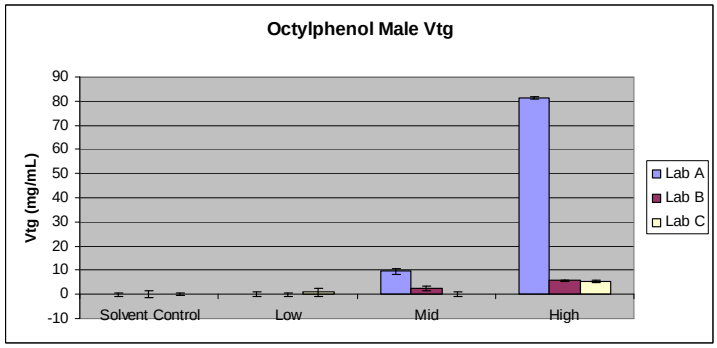
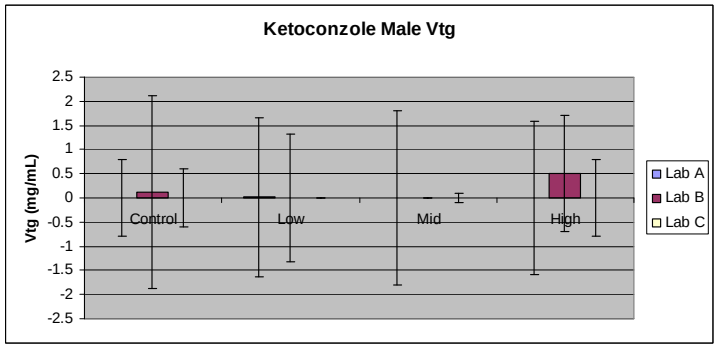


Figure 13. Male vitellogenin comparisons (error bars represent treatment level CV)







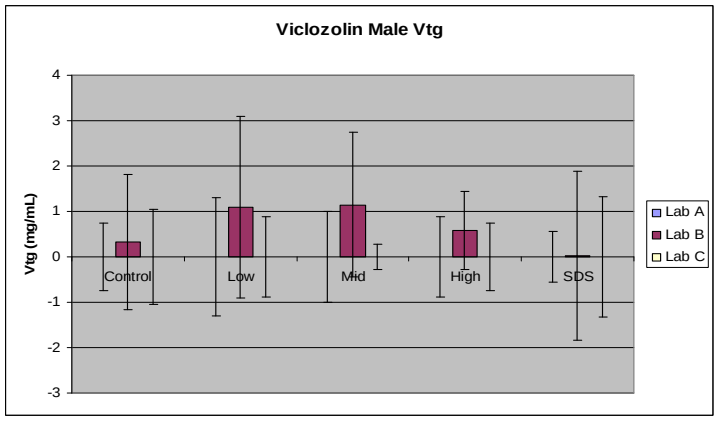
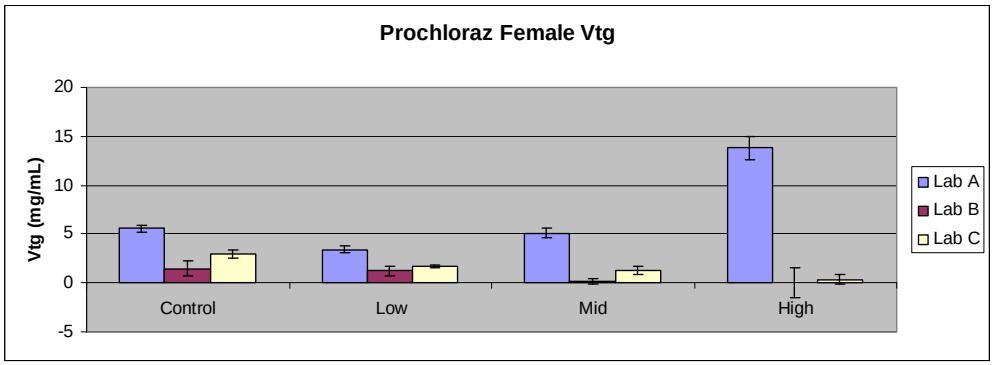
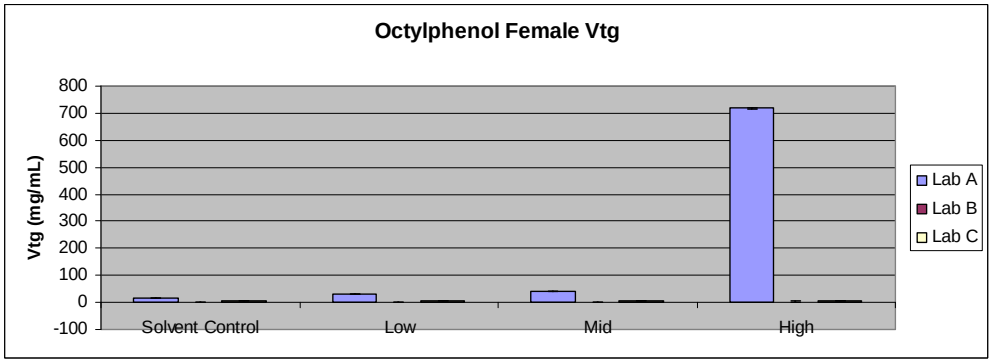
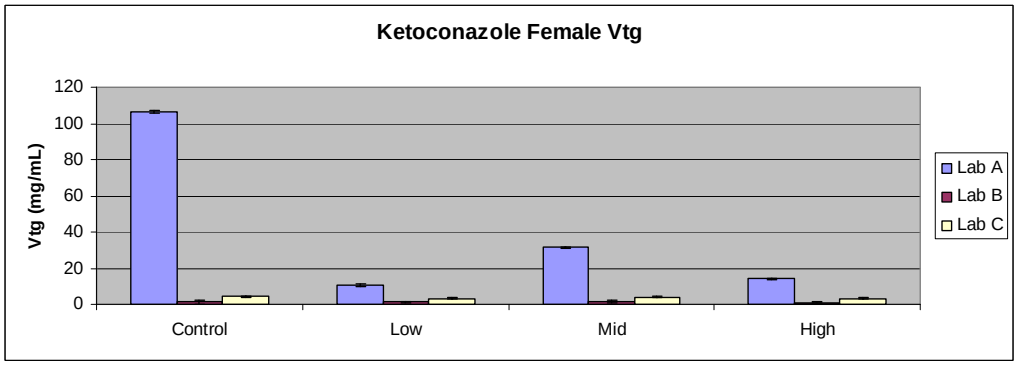


Figure 14. Female vitellogenin comparisons (error bars represent treatment level CV)







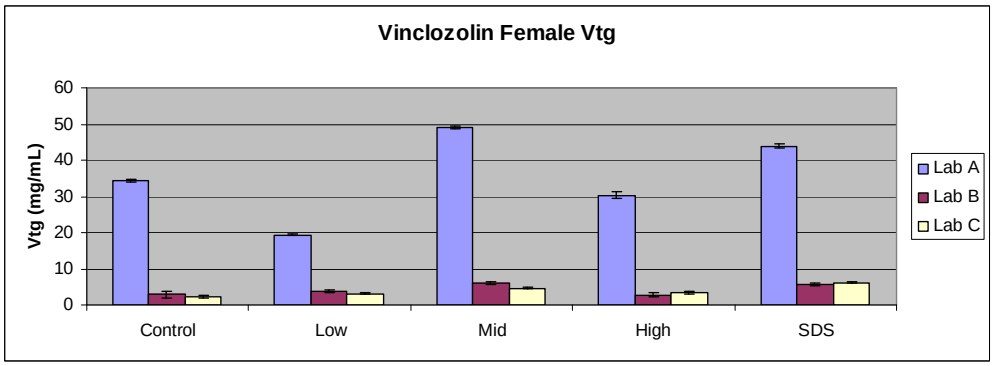
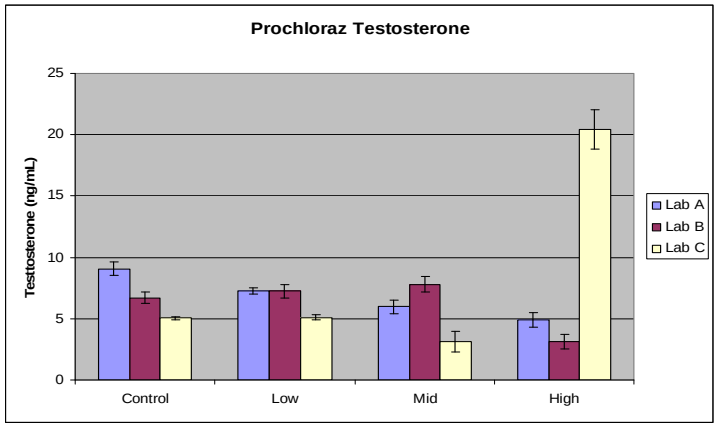
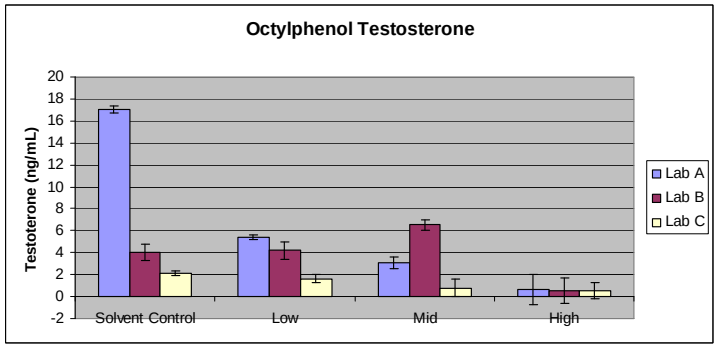
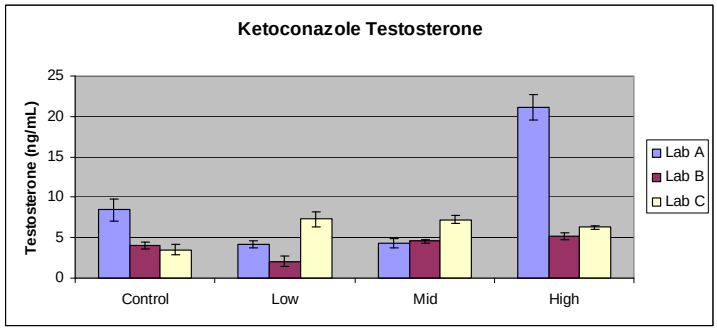


Figure 15. Testosterone comparisons (error bars represent treatment level CV)







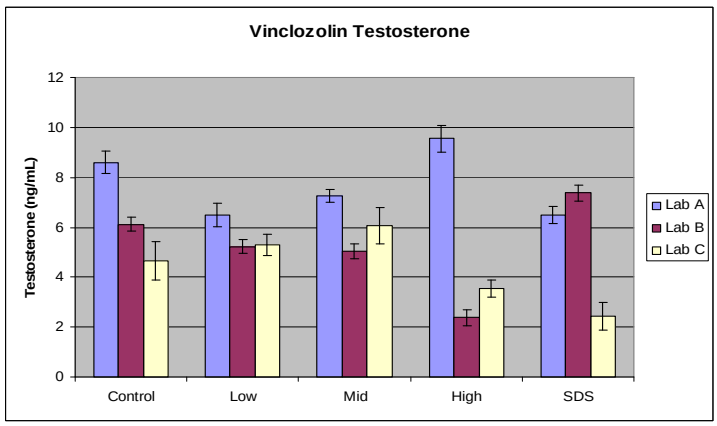
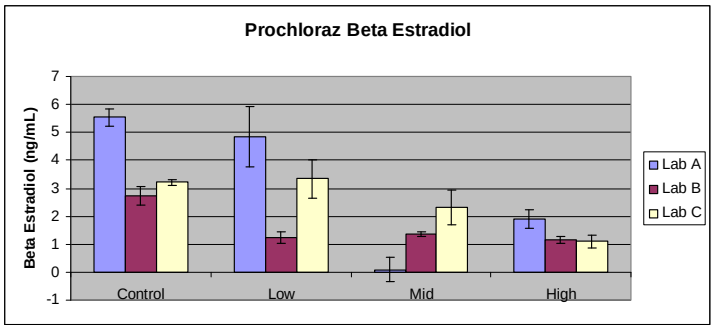
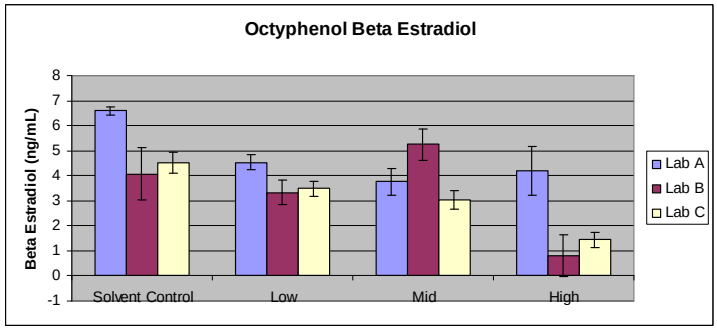
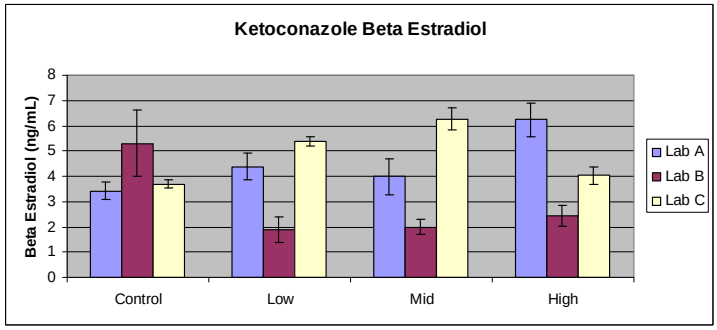
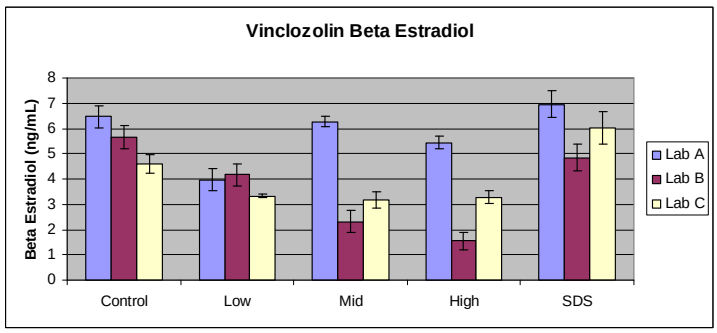


Figure 16. Beta Estradiol comparisons (error bars represent treatment level CV)









APPENDIX 1 - STUDY PLAN

November 21, 2006

STUDY PLAN

on

FISH SCREENING ASSAY INTER-LABORATORY COMPARISON

TASK ORDER #2

PREPARED FOR

Les Touart, Ph.D.

TASK ORDER PROJECT OFFICER

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Springborn Smithers Study No. 13888.6105

TABLE OF CONTENTS

1.0 INTRODUCTION

The U.S. Environmental Protection Agency (US-EPA 2002) has described a short-term reproduction assay 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 assay (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 steroids.

1.1 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 ensure that potential toxicological and endocrine mechanisms of concern are detected for the test chemical.

The assays will be initiated with mature male and female fish. 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, 17β-estradiol and testosterone.

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

The pre-exposure observations will occur in a similar 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 (4.5-6 months old)
Fish husbandry conditions:	Temp: 25°C ± 1°C; D.O. >5.0 mg/L; Light: 16 h light: 8 h dark with 540 to 1080 lux; Feed: frozen brine shrimp twice daily.
Pre-exposure evaluation	Duration: minimum of 14 days; Data Collected: fecundity (estimated daily)
Dilution water	Clean, surface, well or reconstituted water Total Alkalinity = >20 mg/L (as $CaCO_3$) Total organic carbon = ≤5 mg/L Unionized Ammonia = ≤ 35 mg/L
Test material	NS
Test chamber size	18 to 20 L ~ (40 x 20 x 20 cm)
Test volume:	10 to 12L
# Exchanges/day	6 tank volume exchanges
Flow rate:	2.7 L / hr
# Concentration / chemical	3
# Replicates:	4
Weight of each fish	NS
# Fish/vessel	4 females and 2 males
Total # fish/concentration	4 adult females and 2 adult males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)
Feeding regime	Frozen brine shrimp, twice a day
# Controls	1 Dilution water control or a solvent control added if a solvent used
# Fish/control	4 adult females and 2 adult males per replicate = 24 fish total per control. (96 fish per test chemical)
Photo period:	16 h light : 8 h dark
Temperature:	25°C ± 1°C
Light intensity	540 - 1080 lux
Aeration:	None unless D.O. <4.9 mg/L
PH	6.5 - 9.0
Biological endpoints:	Adult survival, reproductive behavior, secondary sexual characteristics, GSI, gonadal histology, VTG, fecundity, fertility and plasma sex steroids
Test validity criteria:	D.O. = >4.9 mg/L (≥60% saturation); mean temp. 25°C ± 1°C; 90% survival in the controls and successful egg production in controls. Spawning occurs at least every 4 days in each control replicate, or approximately 15 eggs/female/day/replicate. Fertility > 80%. Measured exposure concentration CV <20% for all replicates.

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. Based on the technical judgment of experienced laboratory personnel, fish will be reproductively mature (namely, with clear secondary sexual characteristics visible) and capable of 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 should be 4.5 to 6 months of age, assuming they have been cultured at 25°C ± 2 °C

Adult fathead minnows will be obtained from brood stock maintained at Springborn Smithers Laboratories or received from a commercial supplier. Purchased fish will be between 30 and 60 days old. Fish will be acclimated for at least thirty days 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). Whenever possible, test fish be drawn from an in-house culture as shipping of adult fish is stressful and will interfere with reliable spawning. Fish will be fed twice per day throughout the holding period and during the exposure phase.

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.

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 table below shows the typical water quality parameters for the three laboratories:

Table 2. Water Quality Parameters

	Springborn Smithers Laboratories	Wildlife International	Bayer CropScience
Hardness ($mgCaCO_3/L$)	30 to 60	120 to 150	40 to 60
Alkalinity ($mgCaCO_3/L$)	20 to 45	170 to 190	30 to 50
pH	6.9 to 7.7	7.8 to 8.8	7.5 to 8.2
TOC (mg/L)	<2.0	<2.0	<2.0
Ammonia (mg/L)	<0.5	<0.25	<0.001
Conductivity (µmohs/cm)	140 to 250	270 to 330	140 to 170

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

Eighteen liter 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 to 12 L of test solution, which is renewed once every 4 hours (6 volume exchanges per day). 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.

Each test vessel will be constructed of glass and silicone adhesive and will measure 39 x 20 x 25 centimeters (L x W x H). The test chambers will be chemically cleaned before the test is started. The aquaria will be washed with hot water and a detergent, rinsed with acetone, and then rinsed extensively with water. In addition, all test chambers will be brushed and siphoned to remove detritus and uneaten food as needed (weekly at a minimum) during the test. Water depth will be maintained by a constant level overflow drain 14.5 cm from the bottom of each test aquarium. The total test solution volume in each aquarium will be thus maintained at 11 L. Aquaria will be labeled to identify the treatment/control and the replicate designation

A diluter (e.g., Mount and Brungs, 1967) will be employed to deliver three test substance concentrations and a negative control or solvent control to the test aquaria. The exposure system will be constructed entirely of glass, silicone, and nylon. The diluter will be labeled with the study number. A flow-splitting chamber will be used between the diluter cells and the aquaria to promote mixing of the test substance solution and diluent water. In each chamber, four separate standpipes will be employed to split the test solution equally between the four replicate test vessels. Flow rates will be 6 test chamber volume additions per 24 hours.

Treatment levels will be randomly assigned in the exposure system and spawning groups will be assigned to treatment groups using a rank-order approach. Based on data collected during the

pre-spawning phase, the top 16 performing spawning groups will be selected and ranked accordingly. The top 4 performers will be randomly assigned to one replicate of each treatment level for a given chemical exposure. The next 4 performers will be randomly assigned to a second replicate exposure chamber for each treatment level and so on. 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. 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 Preparation and Testing of Chemical Exposure Water

Test chemicals will possess some varied physicochemical properties that will likely require different approaches for preparation of chemical exposure water. When possible, direct addition to water will be utilized if the test chemical has sufficiently high water solubility or stability in water. For test chemicals with reduced water solubility, the use of a saturator column will be required to prepare the concentrated stock solution (US-EPA 2002). For difficult to test substances, a solvent may be employed as a last resort (OECD GD on difficult substances). All aqueous stock solutions will be encased in black tarpaulin during agitation and subsequent storage to prevent photo-oxidation.

Table 3. Test Chemicals and Exposure Concentrations

Test Chemical	Lot Number	Purity	Exposure Concentration (µg/L)		
			Low	Med	High
Vinclozolin	372-39A	99.5%	100	300	900
Prochloraz	372-42B	99.4%	20	100	300
4-t-octylphenol	O76O4CD	99.3%	1	50	150
Sodium dodecyl sulfate	125K0181	99.8%			7
Ketoconazole	VF0320	Tested as 100%	25	100	400

2.5 Analytical Determinations

After preparation of the stock solution, determinations of the concentration will be made using appropriate methods. 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 treatment level (High, Med, Low, and Control, in alternating replicates). This sampling scheme is appropriate only for test systems employing a common splitting chamber on the diluter system, effectively splitting a prepared solution into replicate test vessels. If a splitter chamber is not employed, samples from all replicate test solutions must be analyzed. Further, if a diluter malfunction has occurred, sampling will be initiated for all replicate test solutions.

All test chemicals will be directly measured using spectrophotometry, gas chromatography - electron capture detection (GC-ECD), gas chromatography-mass selective detection (GC-MS), HPLC, (high performance liquid chromatography) or ion-chromatography with conductivity detection as appropriate.

96 Hour Range-finding: A range-finding test may be necessary to establish test chemical concentrations.

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. The 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 test using 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). 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 or 1/3 the derived 96-hr LC50 will serve as the highest exposure concentration in the 21-day test.

2.6 Biochemical Determinations

Vitellogenin (VTG): Enzyme-linked immunosorbent assay (ELISA) tests will be conducted using commercially available test kits from either EnBioTec Laboratories Co. Ltd, (Japan) orEnzyme ImmunoAssay (EIA) kit (Biosense Laboratories AS, Bergen, Norway). The methods used for the bioanalytical measurements of VTG will follow manufacturer's specifications. Samples of blood plasma can be frozen at the time of collection, thawed and divided for vitellogen and sex steroid determination. The portion of plasma designated for sex steroid determination can be refrozen until analysis.

Sex Steroids: Plasma concentrations of 17β-estradioland testosterone should be determined using radioimmunoassay (RIA) techniques optimized for the relatively small sample volumes obtained from the fathead minnow (Jensen et al. 2001). For females, after vitellogenin determinations, remaining plasma will be used to measure (in order of priority) 17β-estradiol and testosterone. For males, after vitellogenin determinations, remaining plasma will be used to measure (in order of priority) testosterone and 17β-estradiol. In males the presence of 11-ketotestosterone is closely linked with the presence of testosterone and thus will not be measured in this assay since plasma amounts are expected to be limited.

2.7 21-day Assay Initiation and Conduct

2.7.1 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 by sex at the start of the test will be kept, if possible within ±20% of the arithmetic mean weight of the same sex. This range

can be expanded to ±30%, only as needed, to minimize the number of fish to be replaced. A subsample of fish will be weighed before the test in order to estimate the mean weight.

2.7.2 Conditions of exposure

The test duration is 21 days.

2.7.3 Feeding

The fish will be fed twice per day with brine shrimp at a sufficient rate to promote active reproduction and 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.

2.7.4 Pre-exposure

The pre-exposure phase will last a minimum of 14 days. The assay will use fish that are generally within the age range of 4.5 – 6 months, 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 should 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 sex cannot be identified will be excluded from the assay. It has been reported that, at 4.5 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. Fecundity data will be collected (estimated) daily. For each assay, successful pre-exposure (suitability for testing) is established when regular spawning occurs in each replicate test chamber at least once in the immediate preceding 7 days prior to test initiation.. It is recommend that 20-24 groups are established during the pre-spawning phase.

2.7.5 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, as well as a diluent water control and solvent control (if a solvent is necessary), with four experimental units (replicates) per treatment. Each replicate tank will contain four female and two male fish. In the absence if a visible dorsal nape pad, males will be identified by the presence of other secondary sex characteristics such as body shape/color and presence of nuptial tubercles. The test chemical will be delivered to the exposure chamber using a proportional diluter or other appropriate delivery systems. 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 and sex steroids. Alternatively, hepatopancreas samples can be collected and analyzed for VTG to conserve all blood plasma for sex steroid analysis. The gonads will also be removed for GSI determination and later histological analyses.

2.7.6 Frequency of analytical determinations and measurements

Prior to initiation of the exposure period, proper function of the chemical delivery system will be ensured. Additionally, all analytical methods necessary 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. The type of toxicant delivery system and diluent delivery system for each diluter will dictate the diluter monitoring. Flow rates will be appropriate checks in some cases and cycle rate will be appropriate checks in other cases. Specific procedures will be described in laboratory specific protocols. Actual test chemical concentrations will be measured in a single replicate vessel at the start of the test and in alternating replicates at weekly intervals thereafter, provided the diluter system employs a common splitting chamber.

- 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.

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

Body Weights: Recorded at test termination.

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 reproductive 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. It is expected that one spawn typically will be composed of 50 to 250 eggs (EPA, 2002). If no embryos are present, the substrate is either left in the tank or replaced with a clean substrate. Clean 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 and as cumulative eggs laid over the test

Fertilization Success: After the spawning substrate has been removed from the tank, the embryos will either be carefully rolled off with a gentle circular motion of an index finger and visually inspected under appropriate magnification or left on the substrate and transferred to clean water for incubation to the eyed stage. 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 and androgen receptor antagonists 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 Appendix C. In determining the size of the dorsal nape pad, no dissection of females is necessary unless a fatpad is visible. In males (that have been confirmed to be males by morphological observations such as tubercles and body shape) where no fatpad is visible, confirmation (via dissection) should be done to confirm the absence of a fatpad. It may be

possible to excise fatty adipose tissue without the outward visible presence of a fatpad. If none is present (confirmed by dissection), then a score of 0 is appropriate.

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 and sex steroids.

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 anesthetization of an individual fish, length and weight measurements will be taken, collection of fresh tissues (e.g., blood, hepatopancreas, fatpad) will also be performed, and gonads will be perfused with fixative (Appendix B).

The following steps will be followed for gonad fixation:

1. 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 the following manner:
    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.
2. 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. The volume of fixative in the container will be at least 20 times the approximated volume of the tissues. The fixative container will be gently agitated for 5 seconds to dislodge air bubbles from the cassette.
3. After at least 24 hours of fixation, the gonads will be rinsed with 70% ethanol and placed in neutral-buffered formalin for shipment to ILS for histopatology

After (1) sampling the blood and (2) fatpad scoring and removal for FPI determination (FPI=100 x fatpad wt/body wt), the gonads will be fixed in situ (3) and "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.

Using the carcass, the secondary sex characteristics will be assessed (e.g., dorsal nape pad, nuptial tubercles - Appendix C). Routine histological procedures will be used to assess the

condition of testes and ovaries from the fish using procedures provided in Histopathology Guidelines for Fathead Minnow (*Pimephales promelas*) 21-Day Reproduction Assay (6 July 2006). The Histopathology Guidelines for Fathead Minnow (*Pimephales promelas*) 21-Day Reproduction Assay details the post mortem and histotechnical procedures that will be used. The three participating laboratories will be providing fixed gonad tissue to Integrated Laboratory Systems (ILS), Research Triangle Park, NC. All tissues will be shipped within 7-10 days of collection. Details of the histopathology procedures are presented in "Histopathology guidelines for the Fathead Minnow (*Pimephales promelas*) 21-day reproduction assay".

Vitellogenin (VTG): The measurement of VTG in plasma or hepatopancreas 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.

Sex Steroids: Plasma concentrations of β-estradiol, testosterone, and 11-ketotestosterone should be determined using radioimmunoassay (RIA) techniques optimized for the relatively small sample volumes obtained from the fathead minnow. Detailed instructions for the measurement of fathead minnow plasma sex steroids using RIA are provided in US-EPA (2002).

Table 4. 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 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 behavior, particularly loss of territorial aggressiveness by males. Qualitative anecdotal observations.
Fecundity: Egg production will be determined daily, but only during the morning.	Fecundity will be expressed either on the basis of average number of eggs laid per surviving female per reproductive (test) day per replicate.	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 or replaced with a new substrate; new substrates should 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 will be opaque or clear with a white dot where the yolk has precipitated. Control fertilization ≥ 95% is ideal, but 80-90% is acceptable.
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.	Not Applicable	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, number of nuptial tubercles in males; ovipositor in females).
Body Weights	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 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, polyclonal Fathead minnow (FHM) (*Pimephales promelas*) VTG antibody and purified VTG protein, also from the FHM, will be utilized.

Parameter	Units	Expected Results
Gonad Size: After sampling the blood, the fixed gonads 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 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 placed in a fixative (Davidson's fixative). A toxicologic pathologist with experience with small fish will perform histology procedures and will refer to the Histopathology Guidelines for Fathead Minnow (*Pimephales promelas*) 21-Day Reproduction assay (6 July 2006).	Not Applicable	Refer to the Histopathology Guidelines for Fathead Minnow (*Pimephales promelas*) 21-Day reproduction Assay (6 July 2006).

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

3.2 Statistical Methods

Statistical analysis will evaluate for within laboratory variability and between laboratory variability. All statistical analysis will be coordinated through the Data Coordination Center (DCC). 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 end point 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 non-parametric 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 of 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 75^{th} and 25^{th} 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°C between test vessels at any one

time during the exposure period and was maintained within a range of 2°C within the temperature ranges specified for the test species)

- There will 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 4 days. Typically, there will be 15 eggs/female/day/test chamber.

- There will be greater than 80% fertility of eggs from the control animals during the exposure.

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(s) 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, photo period, light intensity, chamber size, numbers of male and female fish per replicate, number and composition of spawning substrates, 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 (using data template provided) on biological observations of fecundity, fertility, gross morphology (including secondary sex characteristics), GSI, vitellogenin and sex steroids; detailed report on gonadal histology (using template provided); 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.

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, the experiment should be terminated and the cause investigated.

- 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 concentrations fall outside of the range of 70 to 120% of the nominal concentrations, adjustments will be made to the diluter to correct the problem.

5.0 LITERATURE

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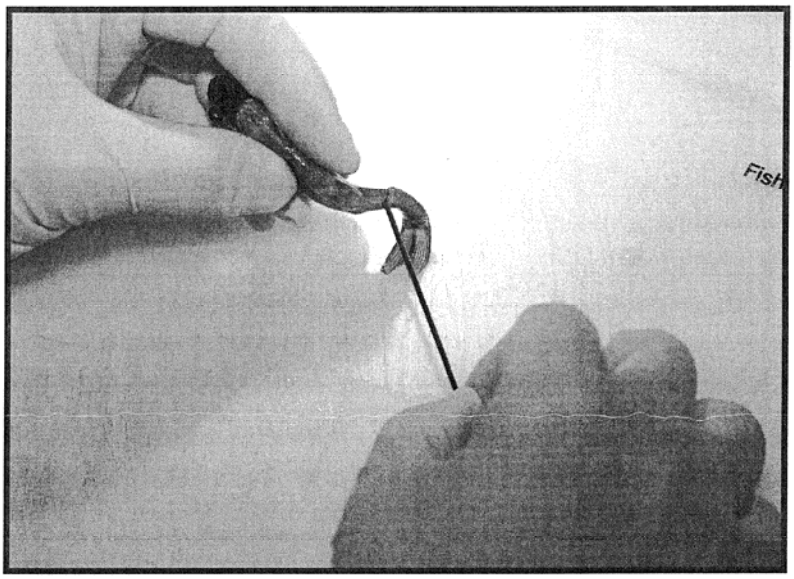
APPENDIX A

SAMPLE COLLECTION PROCEDURES FOR VITELLOGENIN ANALYSIS

Procedure 1A: Fathead Minnow, Blood Collection from the Caudal Vein/Artery



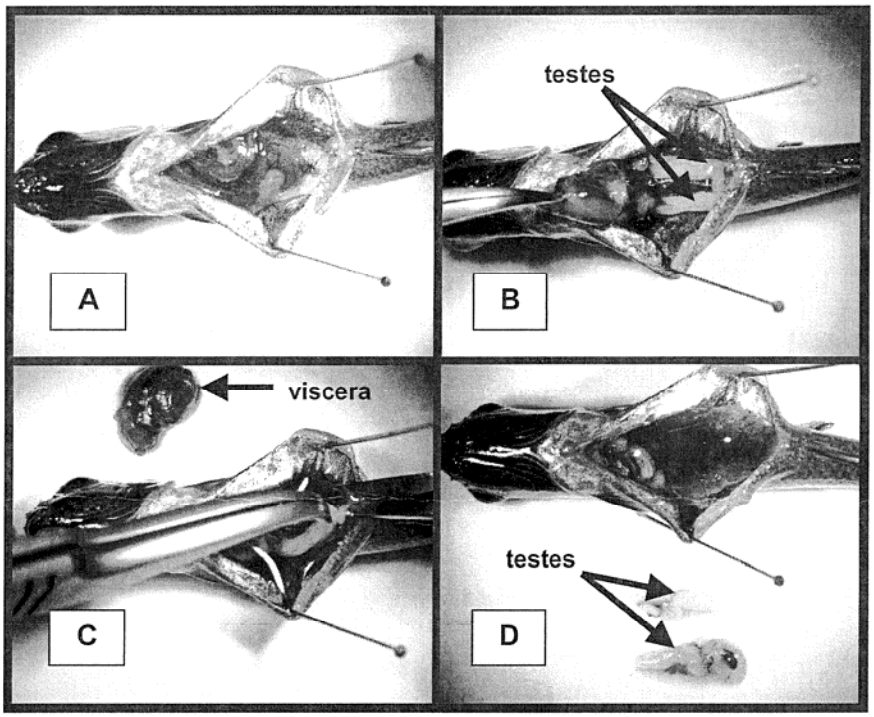
After anaesthetization, the caudal peduncle is partially severed with a scalpel blade and blood is collected from the caudal vein/artery with a heparinized microhematocrit capillary tube. After the blood has been collected, the plasma is quickly isolated by centrifugation for 3 min at 15,000 *g*. If 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 and sex steroid concentrations 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

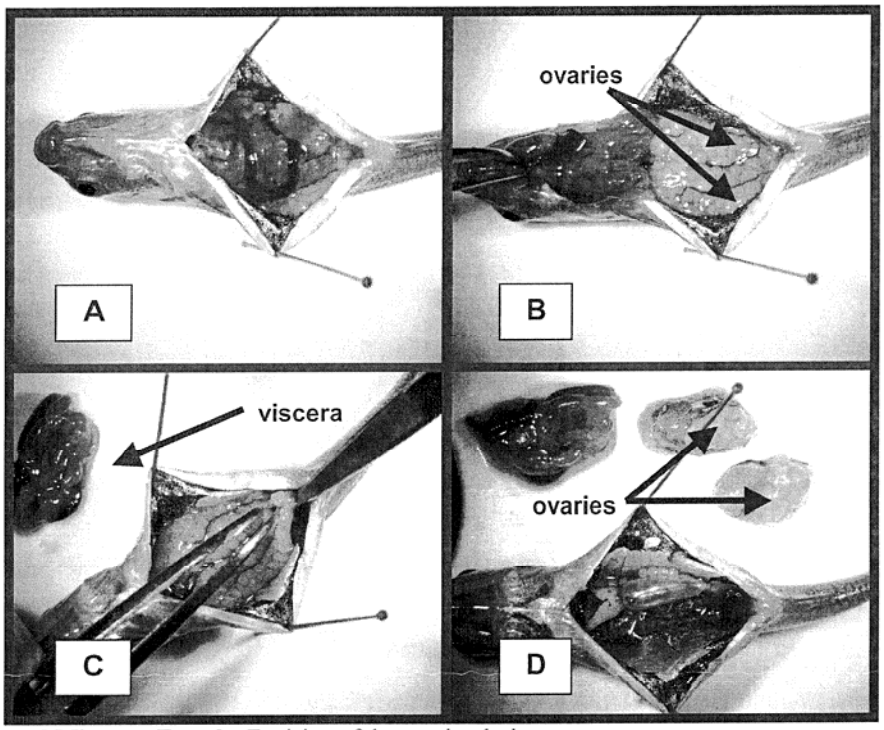
Alternatively, blood may also be collected by cardiac puncture using a heparinized syringe (1000 units of heparin per ml). The blood is transferred into Eppendorf tubes (held on ice) and then centrifuged (5 min, 7,000 g, room temperature). The plasma should be transferred into clean Eppendorf tubes (in 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

Assessment of Secondary Sex Characteristics (Nuptial Tubercle and Fat Pad Scores) in EDC Tests with Fathead Minnows

Michael Kahl and Gerald Ankley

Nuptial Tubercle Scoring

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
   a. Allocate 100 mg of MS-222 and 200 mg of sodium bicarbonate to weigh pan
   b. Add weighed chemicals to control water and stir (ca., 1 minute)
2. Transfer dissolved chemical solution to stainless steel bowl for easy fish handling
   c. 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.
   a. If handling toxicant-exposed fish, start with control fish and work up with increasing EDC concentrations.
2. Place organisms in MS-222 solution.
   a. 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.
   a. 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.
   a. 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.
    a. Keep fish moist during procedure.
    b. Limit the amount of time used to score tubercles.
    c. 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 (see below). 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. 1C).

Figure1C. The actual number of tubercles in some fish may be greater than the template boxes (see template below) 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; 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_____

Date_____

Total Score_____

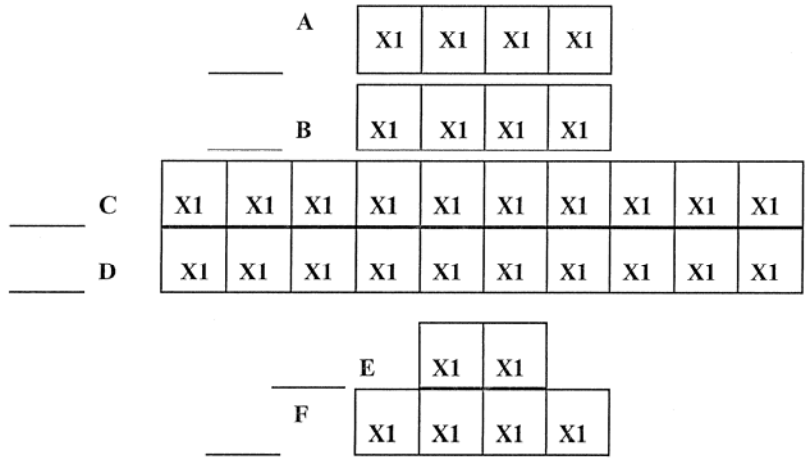
Tubercle Template

Numerical Rating

1-present

2-enlarged

3-pronounced



Fatpad Scoring

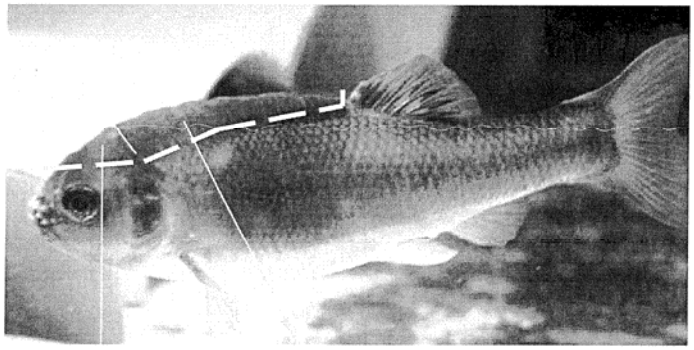
For evaluating the fatpads, use a very similar system as to that used in tubercle assessment:

1. No fatpad visible
2. Small fatpad evident
3. Fatpad is clearly visible and is just above the 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 (see fatpad index described below).

Fatpad Index (based on personal communication from Karen Thorpe

The fatpad index is expressed as a percentage of the body weight, i.e. Fatpad index = fatpad weight/total wet body weight.



Head pad Dorsal pad

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

SIGNATURE PAGE

Springborn Smithers Laboratories Study Protocol

Fish Screening Assay Inter-Laboratory Comparison
EPA Contract Number EP-W-06-026
Concurrences and Approvals

Carrie Ingalls, B.S. Contractor Quality Assurance Officer RTI International Research Triangle Park, NC	Carrie Ingalls 12/07/2006 *Signature* *Date*
Julia D. George, Ph.D. Contractor Task Order Leader RTI International Research Triangle Park, NC	Julia D George 12/05/06 *Signature* *Date*
Ronald C. Biever Lead Laboratory Project Leader Springborn Smithers Laboratories Wareham, MA	11.29.06 *Signature* *Date*
J. Thomas McClintock, Ph.D. Quality Assurance Manager U.S. EPA Washington, DC	Thomas McClintock 12/4/06 *Signature* *Date*
Les Touart, Ph.D. EPA Task Order Project Officer U.S. EPA Washington, DC	Linda Phillips for Les Touart 12/4/06 *Signature* *Date*
Mark Cafarella Study Director Springborn Smithers Laboratories Wareham, MA	11/29/06 *Signature* *Date*
Jean D'Alessandro Quality Assurance Officer Springborn Smithers Laboratories Wareham, MA	Jean D'Alessandro 11-29-06 *Signature* *Date*

STUDY PLAN AMENDMENT

Amendment No.: 1

Effective Date: 8 November 2006

Study Plan Title: Fish Screening Assay Inter-laboratory Comparison

Species: *Pimephales promelas*

Study Sponsor: RTI International

Test Substance: See below

Study No.: 13888.6105

Amendment:

The following sections are being added to the study plan

Test Materials

Test Material	Purity	Lot Number
4-t-octylphenol	99.3%	07604CD
Prochloraz	99.5%	2226X
Vinclozolin	99.5%	329-72B
Ketoconazole		
Sodium dodecyl sulfate	99.8%	125K0181

Test Organisms

Origin and Acclimation
Adult fathead minnows will be obtained from brood stock maintained at Springborn Smithers Laboratories or received from a commercial supplier. Purchased fish will be between 30 and 60 days old and will be acclimated according to the criteria outlined in the Study Plan.

Diluter
A diluter (e.g., Mount and Brungs, 1967) will be employed to deliver three test substance concentrations and a negative control or solvent control to the test aquaria. The exposure system will be constructed entirely of glass, silicone, and nylon. The diluter will be labeled with the study number. A flow-splitting chamber will be used between the diluter cells and the aquaria to promote mixing of the test substance solution and diluent water. In each chamber, four separate standpipes will be employed to split the test solution equally between the four replicate test vessels. Flow rates will be 6 test chamber volume additions per 24 hours.

Test Chambers
Each test vessel will be constructed of glass and silicone adhesive and will measure 39 x 20 x 25 centimeters (L x W x H). The test chambers will be chemically cleaned before the test is started. The aquaria will be washed with hot water and a detergent, rinsed with acetone, and then rinsed extensively with water. In addition, all test chambers will be brushed and siphoned to remove detritus and uneaten food as needed (weekly at a minimum) during the test. Water depth will be maintained by a constant level overflow drain 14.5 cm from the bottom of each test aquarium. The total test solution volume in each aquarium will be thus maintained at 11 L. Aquaria will be labeled to identify the treatment/control and the replicate designation.

None of the above changes will have a negative impact on the study.

Approval Signatures:

Mark A. Cafarella
Springborn Smithers Study Director

10/05/07
Date

Study Sponsor Representative

9/21/07
Date

STUDY PLAN AMENDMENT

Amendment No.: 2

Effective Date: 8 November 2006

Study Plan Title: Fish Screening Assay Inter-laboratory Comparison

Species: *Pimephales promelas*

Study Sponsor: RTI International

Test Substance: See below

Study No.: 13888.6105

Amendment:

The following sections are being added to the study plan

Test Materials

Test Material	Purity	Lot Number
4-t-octylphenol	99.3%	07604CD
Prochloraz	99.5%	2226X
Vinclozolin	99.5%	329-72B
Ketoconazole	100%	VF0320
Sodium dodecyl sulfate	99.8%	125K0181

Table 1, Page 4

The assay protocol states that one dilution water control or a solvent control will be added if a solvent is used. The protocol is amended to read that a dilution water control will be used in all cases and a solvent control will be added along with the dilution water control when a solvent is used. In addition, the solvent control will be used to statistically determine effects from treatment.

Endpoints, Vitellogenin (VTG)

The assay protocol states that the measurement of VTG in plasma or hepatopancreas samples will be performed using an enzyme-linked immunoabsorbant assay (ELISA). During this study, measurement of VTG will be performed using plasma samples.

None of the above changes will have a negative impact on the study.

Approval Signatures:

Mark A. Cafarella
Springborn Smithers Study Director

10/05/07
Date

Study Sponsor Representative

9/21/07
Date