DRAFT FINAL REPORT

on

FROG METAMORPHOSIS ASSAY DEMONSTRATION STUDY

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1.0 ABSTRACT

A previous work assignment in this contract (WA 2-20) was initiated to provide a detailed review of amphibian metamorphosis assays suitable for use in the Endocrine Disruptor Screening Program (EDSP). One of the candidate protocols described was a Prometamorphosis Assay with *Xenopus*. The recommended protocol further outlined a method beginning with Nieuwkoop and Faber [NF] stage 51 or 54 and continuing for 14 to 21 d with key endpoints based on changes in developmental stage and thyroid histology. This approach already has been utilized for several compounds at the U.S. Environmental Protection Agency (USEPA) Office of Research and Development/Mid-Continent Ecology Division (ORD/MED) Laboratory. The goal of this work assignment was to perform a multiple chemical demonstration and evaluation of the amphibian metamorphosis assay as developed by the USEPA ORD/MED. The primary experimental objective was to determine the differential sensitivity of beginning exposure with NF stage 51 and stage 54 larvae and to establish the necessary duration of exposure. The primary endpoints measured were mortality, developmental stage, growth, and histopathology status of the thyroid gland. To achieve this objective, two concurrent studies with organisms from the same cohort exposed from stage 51 for 21 d and from stage 54 for 14 d were conducted for each of the following chemicals: 6-n-propylthiouracil (PTU), methimazole, L-thyroxine (T4), dexamethasone, phenobarbital, and pregnenolone-16-α-carbonitrile (PCN). It is noted that a recognized synonym for PTU is 6-propyl-2-thiouracil.

Results from the present demonstration study of the frog metamorphosis assay indicated that PTU and methimazole inhibited development as marked by developmental stage and induced histopathological effects on the thyroid gland characteristic of the disruption observed. No effect on growth, or a slight effect in the case of methimazole, as measured by weight was observed. T4 accelerated development and reduced growth. Results with PTU, methimazole, and T4 were not unexpected as these test materials are known thyroid axis antagonists (PTU and methimazole) or agonists (T4) and have produced similar results in other amphibian-based assays. Thus, these three test materials served as reference substances. Less information regarding the thyroid axis altering potential of the latter three materials, dexamethasone, phenobarbital, and PCN, was available, however. Dexamethasone decreased the rate of development and growth (weight) regardless of exposure scenario. Phenobarbital and PCN did not markedly alter the rate of development or growth in either NF stage 51 organisms or in NF stage 54 larvae. PTU, methimazole, T4, phenobarbital, and PCN induced histological changes in the thyroid glands. Only minimal, relatively insignificant histological changes in thyroid gland histology were induced in NF stage 51 larvae exposed to dexamethasone for 21 d. No histological effects were observed in NF stage 54 larvae exposed to dexamethasone for 14 d.

Based on the results of the present study, we recommend that a pre- or prometamorphosis-based assay be strongly considered for use as an EDSP Tier 1S assay. The assay, regardless of exposure length or stage of test initiation, was straightforward to perform. Results generated from the demonstration testing were meaningful and provided insight into a thyroid disruption response in a representative vertebrate animal. Further, this study indicates that this assay can be successfully transferred to ecotoxicology testing laboratories with experience in amphibian testing methods. Although, in several cases, the 14-d exposure using

NF stage 54 larvae was found to be more sensitive, particularly with regards to histological evaluation, the 21-d protocol using NF stage 51 larvae may be more practicable with compounds with unknown activity and mechanisms of action, since it covers a broader range of metamorphosis. Regardless of which exposure scenario is utilized, it is imperative that thyroid histology be incorporated as a primary endpoint.

2.0 OBJECTIVES

A number of man-made environmental pollutants have the potential to interfere with endocrine function (1-6), and there is evidence of reproductive or endocrine dysfunction in wildlife species that have been exposed environmentally to such endocrine-disrupting contaminants (EDCs) (7-13). While there have been reports of alterations of the stress hormone axis in amphibians (14,15), there is little or no published evidence of reproductive dysfunction or developmental effects in this group as a result of exposure to EDCs. A special report on environmental endocrine disruption by the USEPA cited no reports of such effects in amphibians, although it concluded that this class of vertebrate represents a unique sentinel animal model for laboratory and field exposure studies (16).

In spite of increasing alarm over global population declines in amphibian species over the last 20 years, only recently has evidence of reproductive toxicity due to xenobiotic exposure been provided. Habitat destruction, fragmentation, or disturbance may be sufficient to explain some declines, but not all. Other potential causes include habitat acidification, predation, and competition by introduced species (17), increased ultraviolet (UV) radiation resulting from atmospheric ozone depletion (18), and exposure to toxic environmental contaminants (19,20). Amphibians may be particularly vulnerable to waterborne environmental contaminants due to their largely aquatic life histories and their highly permeable skin (21).

Metamorphosis is a period of substantial morphological change in which an organism alters its mode of living. Metamorphosis occurs in all major chordate groups with the exception of amniotes (15). In fact, metamorphosis is developmentally comparable to post-embryonic organogenesis in mammals (15). Three primary characteristics define metamorphosis: 1) change in non-reproductive structures between a post-hatch or larval state and sexual maturity; 2) form of the larvae enable it to occupy a unique ecological niche different from that used by the adult life stage; and 3) the morphological changes that occur at the conclusion of larval development depend on some environmental stimulus, either external (i.e., temperature or food supply) or internal (hormonal changes). Each of the three classes of amphibians (anurans, urodeles, and caecilians) undergoes metamorphosis, although not all species within each class metamorphose. For example, obligatory neotenic urodeles do not metamorphose, and reproduce as aquatic adult larvae.

Three primary morphological changes occur during metamorphosis: 1) resorption or regression of tissue or organ systems that have primary function only in the larval life stage; 2) the remodeling of larval organ systems to their adult form, which are suitable only for the adult; and 3) *de novo* development of tissues in the adult that are not required by the larvae. These changes are most marked in anuran species, and less obvious in urodeles and caecilians. In each of the three classes of amphibians, metamorphosis is controlled by thyroid hormone (TH),

although less is currently known about the role of TH in the metamorphosis of caecilian species. Amphibian metamorphosis has been most widely studied in anurans, primarily due to the dramatic nature of metamorphosis and the ease in use of anuran species in research. However, within the anurans, metamorphosis has been reasonably well studied in only three species, *Xenopus laevis* (South African clawed frog), *Rana catesbeiana* (bull frog), and *R. pipiens* (Northern Leopard frog).

Anuran metamorphosis is separated into three distinct periods, premetamorphosis, prometamorphosis, and metamorphic climax. Premetamorphosis refers to a period of embryonic and early larvae development that takes place without TH. Some advanced morphological developments occur during this stage, including hind limb bud development. More specific morphogenesis, such as differentiation of the toes and rapid growth (elongation) of the hind limbs, occurs during prometamorphosis. Biochemically, prometamorphosis is characterized by rising concentrations of endogenous TH. The final period is metamorphic climax, in which a surge of TH triggers the final processes associated with metamorphosis, including forelimb development and resorption of the tail. Drastic internal transformations at the organ system, tissue, and biochemical levels are also taking place during prometamorphosis and metamorphic climax.

From an evolutionary standpoint, amphibians are distinctively separated phylogenetically from other vertebrates. However, specific aspects of the thyroid axis are conserved amongst most chordates at both the morphological and molecular levels. Thus, the conserved nature of the thyroid axis enhances the ability to use an amphibian, particularly an anuran, as a general model for evaluating thyroid disruption that can be extrapolated to other vertebrate species.

The thyroid axis represents one potential target for environmental chemicals. Environmental agents, toxicants, natural products, and complex mixtures can alter metamorphosis by interacting with the thyroid axis. Further, the complexity of the thyroid axis yields many different possible mechanisms of inhibiting metamorphic processes in amphibians at differing biochemical and molecular levels. Thus, the use of amphibians as representatives of the chordates to screen for thyroid disrupting chemicals or chemical mixtures is not unreasonable.

To date, the debate on endocrine disruptors has mostly revolved around gonadal steroids, including estrogens and androgens, because of controversy regarding their possible link to infertility, breast cancer, and lower sperm counts. Thus, the thyroid has received comparatively little attention. The effects of synthetic chemicals in the environment on thyroid function have been surveyed (16). This review confirms the hypothesis of thyroid disruption by environmental chemicals in wildlife and supports the need for human population and laboratory animal studies on compounds already identified as thyroid disruptors. The effects of more than 40 pesticides and 45 industrial chemicals on the thyroid axis are described.

The USEPA is in the process of implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is an amphibian metamorphosis-screening assay designed to detect substances that interact with the thyroid systems of vertebrates. It is thought that the

inclusion of the amphibian metamorphosis assay in Tier 1 is important because amphibian metamorphosis provides a well-studied thyroid dependent process on which to build an assay. Additionally, the amphibian assay is considered essential in the overall screening battery to detect substances that may disrupt the vertebrate thyroid axis.

A previous work assignment in this contract (WA 2-20) was initiated to provide a detailed review of amphibian metamorphosis assays suitable for use in the EDSP. One of the candidate protocols described was a Prometamorphosis Assay with *Xenopus*. The recommended protocol further outlined a method beginning with Nieuwkoop and Faber [NF] (21) stage 51 or 54 and continuing for 14 to 21 d with key endpoints based on changes in developmental stage and thyroid histology. This approach already has been utilized for several compounds at the USEPA ORD/MED Laboratory (22). The goal of this work assignment was to perform a multiple chemical demonstration and evaluation of the amphibian metamorphosis assay as developed by the USEPA ORD/MED using the following test materials: 6-n-propylthiouracil (PTU), methimazole, L-thyroxine (T4), dexamethasone, phenobarbital, and pregnenolone-16-α-carbonitrile (PCN).

The primary experimental objective was to determine the differential sensitivity of beginning exposure with NF stage 51 and stage 54 larvae and to establish the necessary duration of exposure. The primary endpoints measured were mortality, developmental stage, growth, and histopathology status of the thyroid gland. To achieve this objective, two concurrent studies with organisms from the same cohort exposed from stage 51 for 21 d and from stage 54 for 14 d were conducted for each chemical.

3.0 MATERIALS AND METHODS

Adult Care and Breeding

Adult care and breeding were conducted in accordance with standard guidelines (23). The *X. laevis* larvae used for these studies were obtained exclusively from in-house cultures. To induce breeding, adult female and male breeding stock were injected with human chorionic gonadotropin (hCG). Essentially, 4 to 6 h prior to breeding, female and male specimens were injected with approximately 700 IU and 500 IU of hCG dissolved in amphibian saline, respectively.

Test Specimens

The embryos were cultured at 21˚ ± 1˚C for 4 days to allow for hatching, at which time they were divided into groups of 250 per 50 L dechlorinated tap water and maintained in a constant flow (50 mL/min.) at 21˚ ± 1˚C until they reached the desired developmental stages used in the studies. Larvae were fed a blended mixture of TetraFin® (Tetra Sales, Blacksburg, VA, USA), *Spirulina* algae discs (The Wardley Corporation, Secaucus, N.J., USA), and Silver Cup Trout Starter (Nelson & Sons Inc., Murray Utah, USA) along with live brine shrimp (Bio-Marine® Brand, Bio-Marine Inc., Hawthorne CA, USA) twice/d on weekdays and once/d on the weekends, regardless of treatment in accordance with feeding guidelines provided by USEPA ORD/MED.

Test Materials

The following test materials were obtained from the Battelle Chemical Repository following submittal of a chemical procurement request form: PTU, methimazole, T4, dexamethasone, phenobarbital, and PCN. Prior to the initiation of in-life studies, methods of analysis, including determination of method detection limits (MDLs); verification of chemical purity; and evaluation of stability (degradability) were performed by the Battelle Chemical Repository. Upon completion, test materials were shipped via commercial carrier to Fort Environmental Laboratories (FEL).

Test Initiation

Summaries of the experimental conditions used in the study for each of the six test materials are provided in Tables 1 through 6. After 16 to 17 days post-hatch, larvae were anesthetized using 100 mg/L of 3-aminobenoic acid ethyl ester methanesulfonate (MS-222) buffered with 200 mg/L of sodium bicarbonate, sorted by stage (21), and allowed to recover from anesthesia in dechlorinated tap water. After recovery, 200 stage 51 tadpoles were counted and randomly divided into tanks (20 tadpoles/tank). Remaining tadpoles were placed back in clean water until they reached stage 54 when they were used to start the stage 54 portion of each study (using similar methods used for the stage 51 tadpoles). Stage 51 and 54 larvae were exposed to four test concentrations and a control for PTU, methimazole, T4, and PCN, and to five test concentrations and a control for dexamethasone and phenobarbital. Each test concentration and control contained 20 organisms per replicate in duplicate. USEPA ORD/MED provided test concentrations for in-life studies with PTU, methimazole, T4, and dexamethasone. However, additional background work and range-finding studies were needed to assist in the ultimate selection of test concentrations for phenobarbital and PCN. Range-finding studies consisted of 4-d static-renewal exposures of NF stage 51 larvae. The objective was to determine lethal and malformation-inducing concentrations.

Test Termination

Once in the test system, mortality observations were made daily and any dead larvae were removed. On exposure days 21 and 14 for the stage 51 and stage 54 experiments, respectively, all organisms in the study were anesthetized in MS-222, evaluated for developmental stage, weighed, and fixed in Bouin's solution for possible histological analysis of the thyroid glands. Within 96 h, preserved specimens were rinsed three times in 70% v/v reagent alcohol and stored in 10% neutral buffered formalin.

Endpoints

The primary endpoints evaluated in the present study were mortality, developmental stage, growth (wet weight), and thyroid histology. These endpoints, including the measurement format and the frequency of measurement during the proposed studies, are described in Table 7. Gross morphology was evaluated as a secondary endpoint.

Exposure Systems

Benoit mini-diluter flow-through exposure systems were used for in-life studies with PTU, methimazole, T4, and dexamethasone. This system contained components constructed of glass and Teflon®, and was capable of generating five replicated exposure concentrations for each chemical with a dilution factor of 0.25 to 0.5 for test compounds, as well as replicated controls. Exposure tanks were equipped with standpipes, which provided an actual tank volume of 4.0 L. The flow rate to each tank was 25 mL/min. Fluorescent lamps within the laboratory provided a photoperiod of 12 hr light:12 hr dark at an average intensity that ranged from 781 to 1959 lux at the surface of the water. Limits of water solubility and requirements for high test concentrations relative to the limits of water solubility required that phenobarbital and PCN be tested using a static-renewal format. Five-gal tanks containing 8 L of test solution were used as summarized in Tables 5 and 6.

Chemical Analysis

Diluter system performance was monitored daily and exposure concentrations were analyzed based on the schedules outlined in Tables 1 through 6 for each test material. Analytical procedures for testing these chemicals, with the exception of PCN, were either obtained from USEPA ORD/MED, if available, or developed by the Battelle Chemical Repository. PCN analysis method development, chemical purity/stability analysis, and analysis of in-life samples were performed by the Battelle Chemistry Technical Center. The methods used to measure each test material are provided in Table 8.

Histological Procedures

Experimental Pathology Laboratories, Inc. performed histological preparation of thyroid tissue samples and pathological interpretation under the direction of pathologist Dr. Marilyn Wolfe. Five randomly selected larvae from each of the two replicates from each of the test concentrations for each of the six test substances and the control from both the 21-d and the 14-d exposures (620 specimens total) were allocated for thyroid gland histology.

The following procedures were followed for sectioning and evaluating the thyroid glands of *X. laevis* exposed to PTU under WA 3-11:

1. The head of the each frog was cut from the body in a transverse plane just caudal to the eyes.

2. The head was processed on an automatic tissue processor and then embedded in paraffin with the cut side down.

3. The microtomist rough-cut the frog head until the thyroid gland was observed bi-laterally in the section.

4. Upon reaching the thyroid glands, the microtomist made three step sections with approximately 32 microns between each step. These steps were mounted on one

slide, when possible.

5. The slides were then stained with hematoxylin and eosin (H&E).

6. An ACVP-certified veterinary pathologist reviewed the thyroid glands.

7. The histopathological evaluation of the thyroid glands included such changes in the glands as hypertrophy of follicular cells, hyperplasia of thyroid follicles, size of thyroid follicles and degree of colloid accumulation in the thyroid follicles, a qualitative assessment as to size of the thyroid gland, and any other relevant changes.

8. A Pathology Summary, which included the materials, methods, and the results comparing the thyroid tissue from control frogs with exposed frogs, was then prepared by EPL. Appropriate Individual Animal Tables and Summary Incidence Tables accompanied the Pathology Summary.

9. Digital photographs were taken to illustrate changes, if any, in the thyroid glands of exposed frogs as compared to thyroid glands of control frogs.

After histological examination was completed on PTU exposed *X. laevis*, the procedure for sectioning and evaluating thyroid gland tissue exposed to the remaining test materials was intensified under a new work assignment, WA 5-8. The following modified histological procedure was used on methimazole, T4, dexamethasone, phenobarbital, and PCN exposed specimens:

1. The head of the each frog was cut from the body in a transverse plane just caudal to the eyes.

2. The head was processed on an automatic tissue processor and then embedded in paraffin with appropriate orientation.

3. One representative section was cut from each embedded block.

4. The microtomist rough-cut the frog head until the thyroid gland was observed bi-laterally in the section. The exception to this process was sectioning of advanced NF stage 62 larvae, which required caudal cuts in order to locate the translocated thyroid glands, which typically reside rostral to the heart.

5. Upon reaching the thyroid glands, sectioning was in 5 µm sections, two serial sections at five steps, 30 µm apart, for a total of 10 sections, as appropriate. Both right and left lobes were evaluated, when possible.

6. All slides were stained with H&E and covered with glass cover slips. Periodic Acid Schiff was additionally used for phenobarbital specimens to better delineate colloid content.

7. An ACVP-certified veterinary pathologist reviewed the thyroid glands.

8. The histopathological evaluation of the thyroid glands included qualitative analysis such as thyroid gland size, follicle size and shape, colloid content and density, follicular cell shape and height, epithelium structure, follicular cell hyperplasia, and any other relevant changes.

9. A Pathology Summary, which included the materials, methods, and the results comparing the thyroid tissue from control frogs with exposed frogs, was then prepared by EPL. Appropriate Individual Animal Tables and Summary Incidence Tables accompanied the Pathology Summary.

10. Digital photographs were taken to illustrate changes, if any, in the thyroid glands of exposed frogs as compared to thyroid glands of control frogs.

Statistics

Developmental stage was analyzed based on the distribution of stages after 14 and 21 days of exposure for both tests conducted with stage 54 and 51 larvae, respectively. The non-parametric Kruskal-Wallis one-way analysis of variance (KW-ANOVA) was conducted on all developmental stage data sets. When warranted, pair-wise comparisons between treatments and control were conducted using Dunn's test ($p \leq 0.05$). Wet weight was analyzed by one-way ANOVA or KW-ANOVA followed by pair-wise comparisons between treatments and control using Dunn's test or Bonferroni t-test ($p \leq 0.05$ for both). Statistical analyses for each data set are provided in the Appendices for each test material, but are not presented in the body of the report due to uncertainty associated with analyzing data from only two replicates.

4.0 QUALITY ASSURANCE AND PROTOCOL DEVIATIONS

The quality assurance statement is presented as Appendix A. Quality assurance measures used in the present study are described in the Quality Assurance Project Plan (QAPP). The QAPP, Study Plan, and Study Protocol for WA 3-11 and WA 5-8 and any amendments and deviations to these guidance documents are included as Appendix B. After study initiation, it was determined that the experimental conditions outlined in the QAPP, Study Plan, and Study Protocol were not applicable to all of the test chemicals and numerous changes would be required. In lieu of generating a new amendment for each proposed change, one amendment was generated, requesting EPA to issue technical directives outlining any changes specific to the chemical being tested. These technical directives, along with pertinent teleconference records, are also included as part of Appendix B. The Quality Assurance Unit (QAU) at FEL reviewed each of the guidance documents, the data generated from the present study, and this report.

Study deviations and data errors regarding the in-life phase of study were found to be relatively minor in nature, generally inconsequential, and corrected in each case by the Study Director. Several deviations in test chemical analysis were noted by either the Battelle Chemical Repository or Battelle Chemistry Technical Center (see appropriate appendices for each test material). These deviations included sample holding time exceedances at the Battelle Chemistry

Technical Center during evaluation of T4. In addition, the measured concentration of PCN was substantially less (ca. 10-fold less) than originally intended. This may have been the result of physicochemical difficulties in solubilizing a relatively insoluble chemical in large quantities of test material (50 L). However, a 10-fold decrease of solubility would most likely have been noted by suspended or floating test material. Technical staff at FEL did not observe floating or submerged test material upon the termination of top stirring for 24 h. In turn, the less than anticipated stock and test concentrations may have been the result of sample degradation. Differences in analytical methodologies used, liquid chromatography-mass spectrometry (LC-MS)/MS (Battelle, Columbus, OH) and high performance liquid chromatography (HPLC)/photodiode array detector [USEPA ORD/MED] (22) may have also been a factor in the success of analysis. The final set of PCN samples analyzed from the in-life test, with the exception of the stock solution, which measured a concentration consistent with previous stock solution samples, was found to be not detectable. Since the high concentration and stock solution were theoretically the same nominal concentration, this discrepancy cannot be explained by the study team at FEL. Specific discussions of these deviations and technical issues are included in the Results section and in the Appendices for each respective test material. Each technical system audit (TSA) and audit of data quality (ADQ) for each test material evaluated in the present study are maintained at the lab where the work was eprformed.

5.0 RESULTS

Test Material Analytical Characterization

Results of analytical method development, determination of method detection limits (MDLs) and quantitation limits, purity, and stability for each test material are summarized in Table 8. Complete reports describing these characterization parameters are provided in Appendices C through H for each test material. A comparison of nominal and measured test solution concentrations from the in-life studies are provided in Table 9. Measured concentration data provided in Table 9 are based on an average measured concentration based on the analysis schemes provided in Tables 1 through 6. Complete reports describing the analyses of the in-life study samples are also provided in Appendices C through H.

Controls

Control mortality frequencies were ≤2.5% in each of the studies performed. Control performance based on the developmental and growth endpoints used in the present study are provided in Table 10. The mean stage of development obtained in controls ranged from 56.0 ± 0.21 (dexamethasone) to 57.7 ± 0.17 (phenobarbital) for NF stage 51 specimens cultured for 21 d. The mean stage of development obtained in controls ranged from 57.1 ± 0.17 (dexamethasone) to 58.4 ± 0.26 (methimazole) for NF stage 54 specimens cultured for 14 d. Mean wet weight at the conclusion of control culture of NF stage 51 organisms cultured for 21 d ranged from 0.48 ± 0.02 g (PCN) to 0.85 ± 0.03 g (phenobarbital). Mean wet weight at the conclusion of control culture of NF stage 54 organisms cultured for 14 d ranged from 0.59 ± 0.01 g (T4) to 0.90 ± 0.03 g (phenobarbital). Overall, the intra-study variability in control weight was relatively low; however, the inter-study variability in control weight was somewhat greater. In addition, control weight was more variable than developmental rate from both an intra- and

inter-study standpoint. In each case, thyroid glands from control specimens appeared normal based on histological examination. As a point of reference, the rate of normal *X. laevis* development from NF stage 51 for 21-d and NF stage 54 for 14-d should yield NF stage 56 to 57 and 57 to 58, respectively, in accordance with Nieuwkoop and Faber (21).

Test Materials

6-n-propylthiouracil (PTU, also known as 6-propyl-2-thiouracil)

The stage distribution profiles for larvae exposed to PTU at NF stage 51 for 21 d and at NF stage 54 for 14 d are presented in Tables 11a and 11b, respectively. The concentration-related effects of PTU exposure on the rate of development and weight are illustrated in Figures 1a through 1d. PTU exposure inhibited the rate of development in a concentration-dependent manner, but had no apparent effect on weight. The histological effects of PTU exposure are enumerated in Tables 12a and 12b and illustrated in Appendix C. In NF stage 51 specimens (21-d exposure), increased frequency of enlarged, diffuse follicles was noted at 5.0 mg/L PTU. An increase in the frequency of follicular hyperplasia was noted at 10.0 mg/L PTU. A decrease in follicular colloid was noted in only two specimens exposed to 20 mg/L PTU. In NF stage 54 specimens (14-d exposure), increased frequency of enlarged, diffuse follicles; follicular distension; and follicular hyperplasia were noted at 5.0 mg/L PTU. Changes in follicular colloid were not noted. Overall, histological effects were slightly more dramatic in NF stage 54 specimens exposed to PTU for 14-d compared to NF stage 51 specimens exposed to PTU for 21-d. Raw data for in-life tests with PTU are provided in Appendix C.

Methimazole

The stage distribution profiles for larvae exposed to methimazole at NF stage 51 for 21 d and at NF stage 54 for 14 d are presented in Tables 13a and 13b, respectively. The concentration-related effects of methimazole exposure on the rate of development and weight are illustrated in Figures 2a through 2d. Methimazole exposure inhibited the rate of development in a concentration-dependent manner for organisms exposed for 21 d (NF stage 51) and 14 d (NF stage 54). There was no apparent effect on weight for organisms exposed for 21 d (NF stage 51), but a slight increase in growth (weight) in larvae exposed at NF stage 54 for 14 d. The histological effects of methimazole exposure are enumerated in Tables 14a and 14b and illustrated in Appendix D. In NF stage 51 specimens (21-d exposure), an increase in follicular distension; enlarged, diffuse follicles; and follicular hyperplasia were noted at 6.25 mg/L methimazole. A decrease in follicular colloid was noted in several specimens exposed to 12.5 mg/L methimazole. In NF stage 54 specimens (14-d exposure), increased frequency of enlarged, diffuse follicles; follicular distension; follicular hyperplasia; and a reduction in follicular colloid were noted at 12.5 mg/L methimazole. Raw data for in-life tests with methimazole are provided in Appendix D.

L-Thyroxine (T4)

The stage distribution profiles for larvae exposed to T4 at NF stage 51 for 21 d and at NF stage 54 for 14 d are presented in Tables 15a and 15b, respectively. The concentration-related

effects of T4 exposure on the rate of development and weight are illustrated in Figures 3a through 3d. T4 exposure accelerated the rate of development and reduced larval weight in a concentration-dependent manner in both the 21-d and 14-d assays. Increased larval mortality was observed at 4.0 µg/L possibly due to greatly enhanced rates of development and smaller body size. The histological effects of T4 exposure are enumerated in Tables 16a and 16b and illustrated in Appendix E. Due to increased mortality in the NF stage 51 larvae exposed to 4.0 µg/L T4 for 21 d, no histology was performed on this set. In NF stage 51 specimens (21-d exposure), decreased follicular size and colloid content were noted at 0.5 µg/L T4. An increase in the foamy nature of colloid was noted with increasing concentration. In NF stage 54 specimens (14-d exposure), no thyroid gland tissue could be found in larvae exposed to 4.0 µg/L T4. Decreased colloid was observed at 0.5 µg/L T4. In general, the histological effects were more dramatic in the NF stage 51 larvae exposed to T4 for 21 d. Raw data for in-life tests with T4 are provided in Appendix E.

Dexamethasone

The stage distribution profiles for larvae exposed to dexamethasone at NF stage 51 for 21 d and at NF stage 54 for 14 d are presented in Tables 17a and 17b, respectively. The concentration-related effects of dexamethasone exposure on the rate of development and weight are illustrated in Figures 4a through 4d. Dexamethasone exposure inhibited the rate of development and reduced growth (weight) in a concentration-dependent manner. The histological effects of dexamethasone exposure are enumerated in Table 18 and illustrated in Appendix F. In NF stage 51 specimens (21-d exposure), minimal decreases in follicular size and colloid were noted at 15.6 µg/L and minimal to mild decreases were noted at 62.5 µg/L dexamethasone. In NF stage 54 specimens (14-d exposure), no histological effects were noted. Raw data for in-life tests with dexamethasone are provided in Appendix F.

Phenobarbital

The stage distribution profiles for larvae exposed to phenobarbital at NF stage 51 for 21 d and at NF stage 54 for 14 d are presented in Tables 19a and 19b, respectively. The concentration-related effects of phenobarbital exposure on the rate of development and weight are illustrated in Figures 5a through 5d. Phenobarbital exposure nominally increased the rate of development and growth (weight) in NF stage 51 larvae exposed to phenobarbital for 21 d. This effect, however, was very subtle. Phenobarbital exposure had no appreciable effect on the rate of development and growth in NF stage 54 organisms exposed to phenobarbital for 14 d. The histological effects of phenobarbital exposure are enumerated in Tables 20a and 20b and illustrated in Appendix G. In NF stage 51 specimens (21-d exposure), an increase in enlarged, diffuse follicles, and follicular hyperplasia were noted at 1,000 mg/L phenobarbital. In NF stage 54 specimens (14-d exposure), increased frequency of follicular hyperplasia was noted at 1,500 mg/L phenobarbital. Raw data for in-life tests with phenobarbital are provided in Appendix G.

Pregnenolone-16-α-Carbonitrile (PCN)

The stage distribution profiles for larvae exposed to PCN at NF stage 51 for 21 d and at NF stage 54 for 14 d are presented in Tables 21a and 21b, respectively. The concentration-

related effects of PCN exposure on the rate of development and weight are illustrated in Figures 6a through 6d. PCN exposure had no appreciable effect on the rate of development or growth (weight) in either the NF stage 51 organisms exposed for 21 d or in NF stage 54 organisms exposed to PCN for 14 d. It should be noted, however, that the potential effect of PCN on larvae growth (weight) is not completely clear since the control weight for the study with NF stage 51 larvae was low. The histological effects of PCN exposure are enumerated in Tables 22a and 22b and illustrated in Appendix H. In NF stage 51 specimens (21-d exposure), an increase in follicular hyperplasia was noted at 22.2 µg/L PCN. Increased incidence of follicular karyomegaly was noted in several specimens exposed to 66.7 µg/L PCN. In NF stage 54 specimens (14-d exposure), increased frequency of enlarged, diffuse follicles and follicular hyperplasia were noted at 22.2 µg/L PCN. Follicular karyomegaly was found in only one specimen (22.2 µg/L treatment) in the NF stage 54 larvae exposed to PCN for 14 d. Interestingly, the histological effects noted were also observed by USEPA ORD/MED (24), but at a markedly greater concentration (ca. 300 mg/L). These effects were not reported at lower test concentrations. These results, in addition to the evidence described in Section 4.0, Quality Assurance, suggest that the test solutions from the present study may have been reasonably close to the nominal concentrations. Raw data for in-life tests with PCN are provided in Appendix H.

6.0 DISCUSSION

Summary of Results

Results from the present demonstration study of the frog metamorphosis assay indicated that PTU and methimazole inhibited development as marked by developmental stage and induced histopathological effects on the thyroid gland characteristic of the disruption observed. No effect on growth, or a slight effect in the case of methimazole, as measured by weight was observed. T4 accelerated development and reduced growth. Results with PTU, methimazole, and T4 were not unexpected as these test materials are known thyroid axis antagonists (PTU and methimazole) or agonists (T4) and have produced similar results in other amphibian-based assays. Thus, these three test materials served as reference substances. Less information regarding the thyroid axis altering potential of the latter three materials, dexamethasone, phenobarbital, and PCN, was available, however. Dexamethasone decreased the rate of development and growth (weight) regardless of exposure scenario. Phenobarbital did not markedly alter the rate of development or growth in NF stage 51 organisms exposed to phenobarbital for 21 d or in NF stage 54 larvae exposed to phenobarbital for 14 d. PCN had no appreciable effect on the rate of development and growth (weight) in either NF stage 51 organisms exposed to PCN for 21 d or in NF stage 54 larvae exposed to PCN for 14 d. PTU, methimazole, T4, phenobarbital, and PCN induced histological changes in the thyroid glands. Only minimal, relatively insignificant histological changes in thyroid gland histology were induced in NF stage 51 larvae exposed to dexamethasone for 21 d. No histological effects were observed in NF stage 54 larvae exposed to dexamethasone for 14 d.

Endpoint Sensitivity

Of the primary endpoints measured in the present study, changes in thyroid gland histology were noted with each of the six test materials. The severity of histological changes

varied with each test material:

methimazole>PTU>T4>>PCN≥phenobarbital>>>dexamethasone.

Alteration of developmental stage was also found in studies with each of the six test materials. T4 was the only test material found to accelerate development, whereas PTU, methimazole, dexamethasone, and to a lesser extent phenobarbital and PCN were found to inhibit developmental rate. Alteration in growth (weight) was found in specimens exposed to five of the six test materials. T4 and dexamethasone both inhibited growth (weight), whereas phenobarbital (NF stage 51 at 21-d exposure only), PCN (NF stage 51 at 21-d exposure only), and methimazole (NF stage 54 at 14-d exposure only) increased growth (weight). Histological changes were found at test concentrations below those inducing effects on developmental rate or growth (weight) in organisms exposed to PTU, methimazole, T4, phenobarbital, and PCN. Based on these findings, thyroid gland histology appeared to be the most consistent and sensitive indicator of thyroid axis impairment. Alteration of developmental rate was somewhat less sensitive than histology, but was more sensitive than growth. This relationship was not necessarily surprising since thyroid histology is the most direct link to the thyroid axis of the endpoints measured. Effects on growth, and, to a lesser extent, developmental stage may or may not be directly indicative of effects induced at the thyroid axis level. Further, effects on growth alone may be the result of indiscriminant toxicological insult. Without histological data, understanding the effects of phenobarbital and PCN on the thyroid axis would be difficult.

Stage Sensitivity

Results from the present study suggested that the sensitivity of the stages used for exposure and the importance of the duration of exposure were test material-dependent. Measurement endpoint sensitivity was also reflected in the sensitivity of the two different exposure periods. For example, in terms of developmental rate and growth, PTU was nearly equipotent in both exposure profiles. However, somewhat more dramatic histological effects were observed in organisms exposed at NF stage 54 for 14 d than NF stage 51 organisms exposed for 21 d. In this case, both NF stage 51 (21-d) and NF stage 54 (14-d) exposures were similarly sensitive to the development of diffuse enlargement of the thyroid gland. However, the presence of follicular cell hyperplasia and pale, foamy colloid were greater among the NF stage 54 (14-d exposure) specimens than the NF stage 51 specimens (21-d exposure).

A similar relationship between the two exposure profiles was observed with methimazole. Similar to PTU, both NF stage 51 (21-d) and NF stage 54 (14-d) exposures were sensitive to the development of diffuse enlargement of the thyroid gland, follicular hyperplasia, and the presence of pale and foamy colloid. However, the NF stage 54 (14-d) exposure resulted in an increased sensitivity to decreased colloid compared to the NF stage 51 (21-d) exposure. T4 was equipotent in terms of effects on developmental rates and growth in both exposure profiles in organisms exposed at NF stages 51 and 54 for 21 and 14 d, respectively. Like T4, dexamethasone was equipotent in terms of effects on developmental rates and growth in both exposure profiles in organisms exposed at NF stages 51 and 54 for 21 and 14 d, respectively. Marked stage sensitivity differences were not observed with either phenobarbital or PCN. A stimulatory effect on growth in NF stage 54 organisms exposed to methimazole for 14 d was noted, but not in NF stage 51 organisms exposed to methimazole for 21 d.

In terms of the total number of significant responses for effects on developmental stage and growth for both exposure scenarios evaluated in the present study, a greater number of positive or negative responses was observed with the NF stage 51 organisms exposed to test material for 21 d. This response was not necessarily surprising because of the longer and more inclusive exposure period. Without consideration of thyroid histology, the 21-d exposure profile would appear to be more sensitive. However, when thyroid histology was considered in the endpoint battery, the 14-d exposure using NF stage 54 organisms appeared to be somewhat more sensitive for PTU. The slightly decreased sensitivity of the NF stage 51 organisms exposed to PTU, and to a lesser extent methimazole, for 21 d may be the result of increased compensatory response stemming from prometamorphic exposure. However, more work will be needed to delineate this hypothesis. On the contrary, the 21-d exposure using NF stage 51 organisms appeared to be somewhat more sensitive for T4, dexamethasone, phenobarbital, and PCN. Overall, the present study demonstrated the importance of histology endpoints to more clearly delineate thyroid axis impairment. In the case of dexamethasone, which induced only slight histological changes in thyroid tissue in NF stage 51 tadpoles exposed for 21 d, but not in specimens subjected to dexamethasone at NF stage 54 for 14 d, alteration in development and growth was most likely the result of a non-thyroidal mechanism of action.

Response in Relation to Mechanism of Action

PTU and methimazole block the synthesis of TH by inhibiting thyroid peroxidase (TPO), preventing oxidation of inorganic iodide (I) to reactive iodine (I_2). This process represents a critical step in the synthesis of TH. Ultimately, decreased circulating levels of TH occur, which provide positive feedback to the pituitary to release thyrotropin (thyroid stimulating hormone [TSH]). Increased TSH stimulates the thyroid gland, resulting in hypertrophy and hyperplasia of thyroid follicular cells. T4, which belongs to the family of active THs in *Xenopus*, along with the active triidothyronine (T3), serves as a TH agonist inducing rapid metamorphosis and histological effects on the thyroid gland, including decreased follicular size and colloid.

Dexamethasone, a corticosteroid, consistently inhibited both developmental rate and growth (weight), but did not induce histological changes in thyroid tissue consistent with TH antagonists, such as PTU or methimazole. Today, evidence suggests that TH-induced metamorphosis can be altered by other hormones, including prolactin (PRL) and corticosteroids.

For example, PRL has been shown to repress and corticosterone to accelerate metamorphosis and various, but not all, aspects of TH-induced metamorphosis. The mechanisms by which these other hormones alter TH-induced metamorphosis at a molecular level are still not entirely clear. Regardless of the mechanism, it appears that the effects of glucocorticoids on TH-induced metamorphosis are tissue-specific. For example, Kaltenbach (25) and Kikuyama et al. (26) found that exogenous administration of corticoids via the culture media enhanced tail resorption of premetamorphic tadpoles. Similar responses in cultured anuran tails have also been noted as the result of exogenous corticoid (15, 26, 27, 28, 29). Several different tissues in the metamorphosing anuran appear to be responsive to the impact of corticoids on TH action, including the limbs (15, 30, 31) and skin (32). Further, corticoid receptor sites have been identified in the metamorphosing anuran tail and determined to be important in the control of metamorphosis (33, 34).

A study by Hayes et al. (15) suggested that corticoids might operate under a dual mode of action based on the stage of anuran metamorphosis. Based on this study (15), corticoids appeared to slow development during early embryogenesis. Prior to and during the early stages of prometamorphosis, endogenous TH levels are low. As TH levels begin to rise with the onset of metamorphosis, corticoids enhance the capacity of TH to induce metamorphosis, although a clear mechanism is not yet known. Based on results from the present study, the timeframe in which corticosteriods inhibit metamorphosis could potentially extend later into prometamorphosis. However, much of the evidence supporting the role of corticoids in amphibian metamorphosis is based on *in vitro* studies involving cell and organ cultures, and not on whole organisms. Many investigators suggest that corticoids exert negative feedback at the pituitary and hypothalamic levels in anurans (15, 30, 32, 35, 36, 37, 38, 39, 40).

The influence of corticoids on TH-induced metamorphic events has also been observed at the cell and molecular level (15, 30, 31). For example, maturation of the skin, which occurs during the metamorphic transition of the larvae to an adult, involves the expression of adult keratin genes in the epidermis of *X. laevis*. Under normal physiological conditions, TH controls up-regulation. However, corticoids have also been shown to potentiate the response of these genes to TH.

As previously indicated, the nature of this effect is highly tissue-specific. Current research suggests that corticoids act through a nuclear receptor, the glucocorticoid receptor (GR). The GR appears to be similar to classical nuclear-based steroid receptors, which essentially belong to the same super family of receptors that includes TH receptors (41, 42, 43). Thus, as with most steroid hormones, corticoid effects are induced at the transcriptional level. Results from the present study indicate that dexamethasone had only subtle effects on the thyroid gland itself based on histological status resulting from 21-d exposure starting at NF stage 51. No histological effects were found in NF stage 54 larvae exposed to dexamethasone for 14 d. These results appear to be generally consistent with the lines of evidence developed by Schneider and Galton (39) that corticosteriods act by reducing thyroid receptor (TR) expression, inhibiting metamorphosis, and ultimately acting as a modulator of TH-induced metamorphosis. If TR expression is repressed and circulating levels of TH are at relatively normal physiological levels, no direct effect on the thyroid gland would be expected, which is consistent with our finding.

Kurata et al. (44) and Tsuchiya et al. (45) found that phenobarbital induced both uridine diphosphate glucuronosyl transferase (UDPGT) and thyroid hypertrophy in marmosets and rats, respectively. Induction of UDPGT was somewhat insignificant in comparison to microsomal cytochrome P-450 enzymes. Kurata et al. (44) administered 50 mg/kg/d of phenobarbital to marmosets for 4 weeks. The histological effects of phenobarbital administration on the thyroid gland were similar to that induced by methimazole, but generally less severe. Hypertrophied follicular cells and decreased colloid were noted. However, no consistent decrease in circulating T4 levels were detected following phenobarbital exposure, as opposed to methimazole exposure, which dramatically reduced T4 levels. Interestingly, O'Connor et al. (46) evaluated the effect of phenobarbital as a thyroid toxicant in Sprague-Dawley rats. Phenobarbital was administered at doses from 5 to 100 mg/kg/d for 28 d. In the female battery, only serum T3 and T4 levels were decreased, with no effects on serum TSH levels and histological changes in the thyroid gland noted. However, in the male battery, phenobarbital exposure increased TSH levels, decreased serum T3 and T4 levels, and reduced thyroid colloid. UDPGT was induced by phenobarbital regardless of gender.

Cole and Little (47) evaluated the role of bile pigments and bilirubin UDPGTs during the metamorphosis of *R. catesbeiana* tadpoles. These investigators found that the major bile pigments in this species were bilirubin Ixα (bilverdin was also measured in the bile), which increased in the bile and the plasma during metamorphosis. UDPGT activity was measured in the livers of premetamorphic larvae; however, naturally metamorphosing tadpoles showed slight increases in activity. T3-stimulated specimens demonstrated at least two-fold increases in UDPGT activity. In the present study, no outstanding effects were noted on development and growth with phenobarbital or PCN exposure, although histological effects were detected. Since phenobarbital and PCN are not marked inducers of UDPGT and *Xenopus* have a generally less developed phase II conjugation capacity than mammals, induction may not be sufficient to clear TH to a level that would alter development. Hood and Klaassen (48) found that UDPGT inducers, including phenobarbital and PCN, were more effective in reducing T4 levels than reducing the T3 concentration in rats. In the case of phenobarbital and PCN, as opposed to other UDPGT inducers (3-methylcholanthrene and polychlorinated biphenyls[PCBs]), greater levels of serum T3 could be maintained by increased serum TSH. As an alternative hypothesis, increased peripheral deiodinase activity could account for the greater impact on T4 levels than T3 in UDPGT-induced rats. However, decreased deiodinase activities in a suite of peripheral tissues was found following administration with the various microsomal enzyme inducers, and cannot be directly attributed to either preferential reductions in T4 compared to T3, or the increases in serum TSH.

Overall, the mechanisms by which dexamethasone, phenobarbital, and PCN alter thyroid axis function are highly complex and require more detailed examination of changes in TSH and TH titers, thyroid histology, deiodinase activities, TH excretion (phenobarbital and PCN), and TR expression. Regardless, the results of the present study clearly underscore the importance of histological endpoints in the amphibian metamorphosis assay and demonstrate the transferability of this assay into the ecotoxicology laboratory community.

7.0 SUMMARY OF IN-LIFE AND ENDPOINT DATA

Results from the present study indicated that PTU, methimazole, and dexamethasone

were capable of inhibiting the rate of development as marked by developmental stage, whereas T4 was capable of accelerating development. Phenobarbital and PCN had little effect on the rate of development. T4 and dexamethasone inhibited growth (weight), while methimazole slightly increased growth (weight). Phenobarbital exposure did not markedly alter growth (weight). The effect of PCN on larvae growth (weight) is not completely clear since the control weight for the study with NF stage 51 larvae was low. PTU, methimazole, T4, phenobarbital, and PCN each induced histological change in thyroid gland tissue characteristic of thyroid axis disturbance. Although minimal histological effects on the thyroid glands were detected in NF stage 51 organisms exposed to dexamethasone for 21 d, no substantive histological changes were noted in the 21-d exposure studies, indicating that the developmental and growth effects may not be the result of effects on the thyroid gland, or the thyroid axis. Both exposure scenarios were effective in detecting developmental and growth changes with the appropriate test substances. However, organisms exposed to PTU, and to a lesser extent methimazole, at NF stage 54 for 14 d demonstrated a slightly increased sensitivity to histological changes of the thyroid gland, whereas specimens exposed to T4 and phenobarbital at NF stage 51 for 21 d demonstrated a slightly greater sensitivity to histological changes. No difference in thyroid gland histological sensitivity was noted with PCN. Overall, regardless of the exposure period, the importance of using thyroid histology to delineate thyroidal from non-thyroidal mechanisms of changes in development or growth patterns during metamorphosis was strongly emphasized in the present study.

8.0 CONCLUSIONS

Based on the results of the present study, we recommend that a pre- or prometamorphosis-based assay be strongly considered for use as an EDSP Tier 1S assay. The assay, regardless of exposure length or stage of test initiation, was straightforward to perform. Results generated from the demonstration testing were meaningful and provided insight into a thyroid disruption response in a representative vertebrate animal. Further, this study indicates that this assay can be successfully transferred to ecotoxicology testing laboratories with experience in amphibian testing methods. However, it should be noted that due to technical difficulties encountered during the PCN study, this test material should be re-evaluated. Although, in several cases, the 14-d exposure using NF stage 54 larvae was found to be more sensitive, particularly with regards to histological evaluation, the 21-d protocol using NF stage 51 larvae may be more practicable with compounds with unknown activity and mechanisms of action, since it covers a broader range of metamorphosis.

Regardless of which exposure scenario is utilized, it is imperative that thyroid histology be incorporated as a primary endpoint. However, in order to fully evaluate the effectiveness and robustness of this model system, additional assay demonstration is recommended. We recommend that several additional test materials be considered that increase the breadth of mechanism of action covered in this initial demonstration study. In the present study, TH synthesis inhibitors, a thyroid hormone agonist, a corticosteroid with controversial activity on the thyroid axis, and potential inducers of TH metabolism were evaluated. To complete the spectrum of potential mechanisms by which the thyroid axis could be disrupted, test materials, that affect TH synthesis via iodine uptake, TH transport (TTR), elimination of TH via deiodination, and TR antagonism should be evaluated to determine the broader efficacy of this

method. Additional demonstration compounds could include perchlorate (I uptake), iopanoic acid (IOP, deiodinase inhibitor), and amiodarone (TR antagonist).

9.0 REFERENCES

1. Tulner, W.W. Uterotrophic action of the insecticide methoxychlor. Science 133:647-648, 1961.

2. Welch, R.M., Levin, W. and Conney, A.H. Estrogenic action of DDT and its analogues. Toxicol. Appl. Pharmacol. 14:358-367, 1969.

3. Jobling, S. and Sumpter, J.P. Detergent components in sewage effluent are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquat. Toxicol. 27:361-372, 1993.

4. Palmiter, R.D. and Mulvihill, E.R. Estrogenic activity of the insecticide kepone on the chicken oviduct. Science 201:356-358, 1978.

5. Gray, L.E.J., Ostby, J.S. and Kelce, W.R. Developmental effects of an environmental antiandrogen: the fungicide vinclosolin alters sex differentiation of the male rat. Toxicol. Appl. Pharmacol. 129:46-52, 1994.

6. Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen J.A. and Wilson, E.M. Persistent DDT metabolite p,p=-DEE is a potent androgen receptor antagonist. Nature 375:581-585, 1995.

7. Guillette, L.J. Jr., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F. and Woodward, A.R. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 102:680-688, 1994.

8. Guillette, L.J. Jr., Pickford, D.B., Crain, D.A., Rooney, A.A. and Percival, H.F. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. Gen. Comp. Endocrinol. 101:32-42, 1996.

9. Crain, D.A., Guillette, L.J. Jr., Rooney, A.A. and Pickford, D.B. Alterations in steroidogenesis in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. Environ. Health Perspect. 105:528-533, 1997.

10. Crain, D.A., Guillette, L.J. Jr., Pickford, D.B., Percival, H.F. and Woodward, A.R. Sex steroid and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida, USA. Environ. Toxicol. Chem. 17:446-452, 1998.

11. Folmar, L.C., Denslow, N.D., Rao, V., Chow, M., Crain, D.A., Enblom, J., Marcino, J. and

Guillette, L.J. Jr. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environ. Health Perspect. 104:1096-1101, 1996.

12. Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Taylor, C.R. and Sumpter, J.P. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8:275-285, 1994.

13. Van der Kraak, G.J., Munkittrick, K.R., McMaster, M.E., Portt, C.B. and Chang, J.P. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. Toxicol. Appl. Pharmacol. 115:224-233, 1992.

14. Gendron, A.D., Bishop, C.A., Fortin, R. and Hontela, A. *In vivo* testing of the functional integrity of the corticosterone-producing axis in mudpuppy (Amphibia) exposed to chlorinated hydrocarbons in the wild. Environ. Toxicol. Chem. 16:1694-1706, 1997.

15. Hayes, T.B., Wu, T.H. and Gill, T.N. DDT-like effects as a result of corticosterone treatment in an anuran amphibian: is DDT a corticoid mimic or a stressor? Environ. Toxicol. Chem. 16:1948-1953, 1997.

16. U.S. EPA. Special report on environmental endocrine disruption: an effects assessment and analysis. EPA/630/R-96/012. Washington, DC: U.S. Environmental Protection Agency, 1997.

17. Wake, D.B. Declining amphibian populations. Science 253:860, 1991.

18. Blaustein, A.R., Hoffman, P.D., Hokit, K.G., Kiesecker, J.M., Walls, S.C. and Hays, J.B. UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines. Proc. Natl. Acad. Sci. USA 91:1791-1795, 1994.

19. Hall, R.J. and Henry, P.F.P. Assessing effects of pesticides on amphibians and reptiles: status and needs. Herpetol. J. 2:65-71, 1992.

20. Carey, C. and Bryant, C.J. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environ. Health Perspect. 103(suppl 4):13-17, 1995.

21. Nieuwkoop, P.D. and Faber, J. *Normal Tables of Xenopus laevis (Daudin)*. Garland Publishing, London, 1994.

22. USEPA Office of Research and Development/Mid-Continent Ecology Division (ORD/MED) Laboratory staff, personal communication, 2003.

23. Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX). American Society for Testing and Materials (ASTM), E 1439-98, 1998.

24. Chowdhury, M.J., Holcombe, G.W., Kosian, P.A., Tietge, J.E., Degitz, S.J. Phenobarbital affects thyroid histology and larval development in the African clawed frog, *Xenopus Laevis*. Abstract No. 1756. Prepared for U.S. EPA ORD/MED. New Orleans, LA: Society of Toxicology, 2005.

25. Kaltenbach, J.C. Amphibian metamorphosis: influence of thyroid and steroid hormones, in *Current Trends in Comparative Endocrinology*, B. Lofts and W.N. Holmes (eds), Hong Kong University Press, Hong Kong, p. 533-534, 1985.

26. Kikuyama, S., Niki, K., Mayumi, M., Shibayama, R., Nishikawa, M., and Shitake, N. Studies on corticoid action on the toad tadpoles *in vitro*. Gen. Comp. Endocrinol. 52: 395-399, 1983.

27. Hayes, T.B., Chan, R., and Licht, P. Interaction of temperature and steroids on larval growth, development and metamorphosis in a toad (*Bufo boreas*). J. Exp. Zool. 266: 206-215, 1993.

28. Hayes, T.B. and WU, T.H. Interdependence of corticosterone and thyroid hormones in larval growth and development in the western toad (*Bufo boreas*). II. Regulation of corticosterone and thyroid hormones. J. Exp. Zool. 271: 103-111, 1995a.

29. Hayes, T.B. and WU, T.H. The role of corticosterone in anuran metamorphosis and its potential role in stress-induced metamorphosis, in *Proceedings of the 17th Conference of the European Society of Comparative Endocrinology: Netherlands*. J. Exp. Zool. 45: 107-109, 1995b.

30. Galton, V.A. Mechanisms underlying the acceleration of thyroid hormone-induced tadpole metamorphosis by corticosterone. Endocrinology (Baltimore) 127: 2997-3002, 1990.

31. Kikuyama, S., Kawamura, K., Tanaka, S., and Yamamoto, K. Aspects of amphibian metamorphosis: hormonal control. Internatl. Rev. Cytol.145: 105-148, 1993.

32. Shimizu-Nishikawa, K. and Miller, L. Hormonal regulation of adult type keratin gene expression in larval epidermal cells of the frog *Xenopus laevis*. Differentiation 49: 77-83, 1992.

33. Woody, C.J. and Jaffe, R.C. Binding of dexamethasone by hepatic, intestine and tail fin cytosol in *Rana catesbeiana* tadpoles during spontaneous and induced metamorphosis. Gen. Comp. Endocrinol. 54: 194-202, 1984.

34. Yamamoto, K. and Kikuyama, S. Binding of aldosterone by epidermal cytosol in the tail of bullfrog larvae. Gen. Comp. Endocrinol. 89: 283-290, 1993.

35. Denver, R.J. and Licht, P. Neuropeptide stimulation of thyrotropin secretion in the larval bullfrog: evidence for a common neuroregulator of thyroid and interregnal activity during metamorphosis. J. Exp. Zool. 252: 101-104, 1989.

36. Nishikawa, A., Shimizu-Nishikawa, K., and Miller, L. Spatial, temporal and hormonal regulation of epidermal keratin expression during development of the frog, *Xenopus laevis*. Develop. Biol. 151: 145-153, 1992.

37. Gancedo, B., Corpas, I., Alonso-Gomez, A.L., Delgado, M.J., Morreale de Escobar, G., and Alonso-Bedate, M. Corticotropin-releasing factor stimulates metamorphosis and increases thyroid hormone concentration in prometamorphic *Rana catesbeiana*. Endocrinology 87: 6-13, 1992.

38. Denver, R.J. Acceleration of anuran amphibian metamorphosis by corticotropin releasing hormone-like peptides. Gen. Comp. Endocrinol. 91: 38-51, 1993.

39. Schneider, M.J. and Galton, V.A. Effect of glucocorticoids on thyroid hormone action in cultured red blood cells from *Rana catesbeiana* tadpoles. Endocrinology 136: 1435-1440, 1995.

40. Tata, J.R. How hormones regulate programmed cell death during amphibian-metamorphosis, in *Programmed Cell Death*, Shi, Y-B., Shi, Y., Xu, Y., and Scott, D.W. (eds), Plenum Press, New York, p. 1-11, 1997.

41. Evans, R.M. The steroid and thyroid hormone receptor super family. Science 240: 889-895, 1988.

42. Green, S. and Chambon, P. Nuclear receptors enhance our understanding of transcription regulation. Trends Genet. 4: 309-313, 1988.

43. Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. The nuclear receptor super family: the second decade. Cell 83: 835-839, 1995.

44. Kurata, Y., Wako, Y., Tanaka, K., Inoue, Y., and Makinodan, F. Thyroid hyperactivity induced by methimazole, spironolactone and phenobarbital in marmosets (*Callithrix jacchus*): histopathology, plasma thyroid hormone levels and hepatic T4 metabolism. J. Vet. Med. Sci. 62(6): 607-614, 2000.

45. Tsuchiya, Y., Ohno, H., Satoh, H., Sugawara, T., Kajimura, T., and Nomura, M. Thyroid hypertrophic effect of semotiadil fumarate, a new calcium antagonist, in rats. J. Toxicol. Sci. 25(2): 121-130, 2000.

46. O'Connor, J.C., Frame, S.R., Davis, L.G., and Cook, J.C. Detection of thyroid toxicants in a tier I screening battery and alterations in thyroid endpoints over 28 days of exposure. Toxicol. Sci. 51: 54-70, 1999.

47. Cole, K.D. and Little, G.H. Bile pigments and bilirubin UDP-glucuronyl transferase during metamorphosis of *Rana catesbeiana* tadpoles. Comp. Biochem. Physiol. B. 76: 503-506, 1983.

48. Hood, A. and Klaassen, C.D. Effects of microsomal enzyme inducers on outer-ring deiodinase activity toward thyroid hormones in various rat tissues. Toxicol. Appl. Pharmacol. 163: 240-248, 2000.

Table 1. Experimental Design and Methodological Conditions for In-Life Exposure Studies 6-n-propylthiouracil [PTU] (CAS # 51-52-5)

Test Start Date	3/5/2004 (study d 0 for stage 51); 3/12/2004 (study d 0 for stage 54)
Test End Date	3/26/2004 (study d 21 for stage 51 and study d 14 for stage 54)
Test Type	Prometamorphosis Assay
Test Animal	*X. laevis* larvae
Initial Larval Stages	Stage 51 and Stage 54
Test Duration	21 d for Stage 51 and 14 d for Stage 54
Exposure Method	Flow-through
Exposure System	Benoit Mini-Diluter
Dilution Media	Dechlorinated tap water
Test Solution Volume/Tank	4.0 L
Flow Rate	25 mL/min
Tank volume Replacement Frequency	2.7 h
Replicates/Test Concentration and Control	2
Larvae/Tank	20
Larvae/Concentration	40
Photoperiod	12 h dark and 12 h light
Light Intensity at Water Surface	61 to 139 lumens
Water Temperature	21±1°C
Feed Type	TetraFin®, *Spirulina* algae, Silver Cup Trout Starter®, live brine shrimp
Feed Volume/Frequency	Twice daily on weekdays and once daily on weekends
Stock Concentration	700 mg/L
Nominal Test Concentrations	2.5, 5.0, 10.0, 20.0 mg/L
Laboratory Control	Dechlorinated tap water
Analytical Sampling Schedule	3/5/04, 3/12/04, 3/19/04, and 3/26/04
Analytical Sampling Location	Mini-Diluter Flow Booster Cell for each concentration

Table 2. Experimental Design and Methodological Conditions for In-Life Exposure Studies Methimazole (CAS # 60-56-0)

Test Start Date	4/8/2004 (study d 0 for stage 51); 4/16/2004 (study d 0 for stage 54)
Test End Date	4/29/2004 (study d 21 for stage 51); 4/30/2004 (study d 14 for stage 54)
Test Type	Prometamorphosis Assay
Test Animal	*X. laevis* larvae
Initial Larval Stages	Stage 51 and Stage 54
Test Duration	21 d for Stage 51 and 14 d for Stage 54
Exposure Method	Flow-through
Exposure System	Benoit Mini-Diluter
Dilution Media	Dechlorinated tap water
Test Solution Volume/Tank	4.0 L
Flow Rate	25 mL/min
Tank volume Replacement Frequency	2.7 h
Replicates/Test Concentration and Control	2
Larvae/Tank	20
Larvae/Concentration	40
Photoperiod	12 h dark and 12 h light
Light Intensity at Water Surface	61 to 139 lumens
Water Temperature	21±1°C
Feed Type	TetraFin®, *Spirulina* algae, Silver Cup Trout Starter®, live brine shrimp
Feed Volume/Frequency	Twice daily on weekdays and once daily on weekends
Stock Concentration	2,500 mg/L
Test Concentrations	6.25, 12.5, 25.0, 50.0 mg/L
Laboratory Control	Dechlorinated tap water
Analytical Sampling Schedule	4/8/04, 4/16/04, 4/22/04, and 4/29/04
Analytical Sampling Location	Mini-Diluter Flow Booster Cell for each concentration

Table 3. Experimental Design and Methodological Conditions for In-Life Exposure Studies L-Thyroxine [T4] (CAS # 51-48-9)

Test Start Date	6/10/2004 (study d 0 for stage 51); 6/17/2004 (study d 0 for stage 54)
Test End Date	7/1/2004 (study d 21 for stage 51 and study d 14 for stage 54)
Test Type	Prometamorphosis Assay
Test Animal	*X. laevis* larvae
Initial Larval Stages	Stage 51 and Stage 54
Test Duration	21 d for Stage 51 and 14 d for Stage 54
Exposure Method	Flow-through
Exposure System	Benoit Mini-Diluter
Dilution Media	Dechlorinated tap water
Test Solution Volume/Tank	4.0 L
Flow Rate	25 mL/min
Tank volume Replacement Frequency	2.7 h
Replicates/Test Concentration and Control	2
Larvae/Tank	20
Larvae/Concentration	40
Photoperiod	12 h dark and 12 h light
Light Intensity at Water Surface	61 to 139 lumens
Water Temperature	21±1°C
Feed Type	TetraFin®, *Spirulina* algae, Silver Cup Trout Starter®, live brine shrimp
Feed Volume/Frequency	Twice daily on weekdays and once daily on weekends
Stock Concentration	4.04 mg/L
Nominal Test Concentrations	0.5, 1.0, 2.0, 4.0 µg/L
Laboratory Control	Dechlorinated tap water
Analytical Sampling Schedule	6/10/04, 6/17/04, 6/24/04, and 7/1/04. Only 2 highest concentrations analyzed.
Analytical Sampling Location	From the aquarium, alternating between replicates A, B, C, and D per sampling event

Table 4. Experimental Design and Methodological Conditions for In-Life Exposure Studies Dexamethasone (CAS # 50-02-2)

Test Start Date	7/15/2004 (study d 0 for stage 51); 7/21/2004 (study d 0 for stage 54)
Test End Date	8/5/2004 (study d 21 for stage 51); 8/4/2004 (study d 14 for stage 54)
Test Type	Prometamorphosis Assay
Test Animal	*X. laevis* larvae
Initial Larval Stages	Stage 51 and Stage 54
Test Duration	21 d for Stage 51 and 14 d for Stage 54
Exposure Method	Flow-through
Exposure System	Benoit Mini-Diluter
Dilution Media	Dechlorinated tap water
Test Solution Volume/Tank	4.0 L
Flow Rate	25 mL/min
Tank volume Replacement Frequency	2.7 h
Replicates/Test Concentration and Control	2
Larvae/Tank	20
Larvae/Concentration	40
Photoperiod	12 h dark and 12 h light
Light Intensity at Water Surface	61 to 139 lumens
Water Temperature	21±1°C
Feed Type	TetraFin®, *Spirulina* algae, Silver Cup Trout Starter®, live brine shrimp
Feed Volume/Frequency	Twice daily on weekdays and once daily on weekends
Stock Concentration	20 mg/L
Nominal Test Concentrations	1.0, 3.9, 15.6, 62.5, 250 µg/L
Laboratory Control	Dechlorinated tap water
Analytical Sampling Schedule	7/15/04, 7/21/04, 7/29/04, and 8/4/04. Only 2 highest concentrations analyzed.
Analytical Sampling Location	From the aquarium, alternating between replicates A, B, C, and D per sampling event

Table 5. Experimental Design and Methodological Conditions for In-Life Exposure Studies Phenobarbital (CAS # 50-06-6)

Test Start Date	8/26/2004 (study d 0 for stage 51); 9/1/2004 (study d 0 for stage 54)
Test End Date	9/16/2004 (study d 21 for stage 51); 9/15/2004 (study d 14 for stage 54)
Test Type	Prometamorphosis Assay
Test Animal	*X. laevis* larvae
Initial Larval Stages	Stage 51 and Stage 54
Test Duration	21 d for Stage 51 and 14 d for Stage 54
Exposure Method	Static Renewal
Exposure System	Glass Aquaria
Dilution Media	Dechlorinated tap water
Test Solution Volume/Tank	8.0 L
Flow Rate	6.0 L/d
Tank volume Replacement Frequency	24 h
Replicates/Test Concentration and Control	2
Larvae/Tank	20
Larvae/Concentration	40
Photoperiod	12 h dark and 12 h light
Light Intensity at Water Surface	61 to 139 lumens
Water Temperature	21±1°C
Feed Type	TetraFin®, *Spirulina* algae, Silver Cup Trout Starter®, live brine shrimp
Feed Volume/Frequency	Twice daily on weekdays and once daily on weekends
Stock Concentration	8,000 mg/L
Nominal Test Concentrations	125, 250, 500, 1,000 mg/L for Stage 51; 250, 500, 1,000, 1,500 mg/L for Stage 54
Laboratory Control	Dechlorinated tap water
Analytical Sampling Schedule	Stage 51 – 8/26/04 and 9/16/04 with only 1 conc. sampled on 9/9/04. Stage 54 – 9/1/04 and 9/15/04 with only 1 conc. sampled on 9/9/04
Analytical Sampling Location	From the aquarium

Table 6. Experimental Design and Methodological Conditions for In-Life Exposure Studies Pregnenolone-16-α-Carbonitrile [PCN] (CAS # 1434-54-4)

Test Start Date	10/14/2004 (study d 0 for stage 51); 10/20/2004 (study d 0 for stage 54)
Test End Date	11/4/2004 (study d 21 for stage 51); 11/3/2004 (study d 14 for stage 54)
Test Type	Prometamorphosis Assay
Test Animal	*X. laevis* larvae
Initial Larval Stages	Stage 51 and Stage 54
Test Duration	21 d for Stage 51 and 14 d for Stage 54
Exposure Method	Static Renewal
Exposure System	Glass Aquaria
Dilution Media	Dechlorinated tap water
Test Solution Volume/Tank	8.0 L
Flow Rate	6.0 L/d
Tank volume Replacement Frequency	24 h
Replicates/Test Concentration and Control	2
Larvae/Tank	20
Larvae/Concentration	40
Photoperiod	12 h dark and 12 h light
Light Intensity at Water Surface	61 to 139 lumens
Water Temperature	21±1°C
Feed Type	TetraFin®, *Spirulina* algae, Silver Cup Trout Starter®, live brine shrimp
Feed Volume/Frequency	Twice daily on weekdays and once daily on weekends
Stock Concentration	200 µg/L
Nominal Test Concentrations	7.4, 22.2, 66.7, 200 µg/L
Laboratory Control	Dechlorinated tap water
Analytical Sampling Schedule	10/14/04, 10/20/04, 10/28/04, and 11/3/04
Analytical Sampling Location	From the aquarium, alternating between replicates A, B, C, and D per sampling event

Table 7. Primary and Secondary Endpoints

Primary Endpoint	Frequency of Measurement	Measurement Format
Mortality	Daily	% at Test Conclusion
Developmental Stage	At Test Conclusion	Individual Stage Distribution
Growth	At Test Conclusion	Weight
Thyroid Histology	At Test Conclusion	Pathology Summary
Secondary Endpoint		
Gross Morphology	At Test Conclusion	% External Abnormality

Table 8. Physicochemical Properties of Test Materials

Test Material	CAS #	Method of Analysis	LOD[1] (µg/L)	LOQ[2] (µg/L)	Purity[3] (%)	Stability[4]
6-n-propylthiouracil (PTU)	51-52-5	HPLC with UV/Vis Detector	18.9	NA[5]	100/99.9	>90% @ 818 mg/L/24 d >90% @ 1.57 mg/L/24 d
Methimazole	60-56-0	HPLC with UV/Vis Detector	34	NA[5]	99.9/100	>90% @ 2630 mg/L/25 d >90% @ 26.0 mg/L/60 d
L-Thyroxine (T4)	51-48-9	IC-ICP/MS	0.09	NA[5]	99.4/93.01	>90% @ 3.9 mg/L/7 d 28% @ 1.0 µg/L/8 d
Dexamethasone	50-02-2	HPLC with UV/Vis Detector	1.36	NA[5]	99/99.54	89.5% @ 9.9 mg/L/7 d <90% @ 15.8 µg/L/3 d
Phenobarbital	50-06-6	HPLC with UV/Vis Detector	111	NA[5]	99/99.96	>90% @ 7767 mg/L/21 d >90% @ 124.3 mg/L/21 d
Pregnenolone-16-∝-Carbonitrile (PCN)	1434-54-4	LC-MS/MS	1.018	3.392	99/92.4	>100% @ 7.4 µg/L/7 d

[1] Limit of detection or method detection limit.
[2] Limit of quantitation defined as 10 times the SD of lowest standard.
[3] Manufacturer's Certificate of Analysis reported value / Battelle's measured reported value.
[4] Stability of the stock concentration and 1 diluter concentration over a designated time expressed in days.
[5] Not available.

Table 9. Comparison of Nominal Versus Measured Test Concentrations for Thyroid Disruption Assays

	NF Stage 51 (21-d)			NF Stage 54 (14-d)		
Test Material	Nominal Concentration[1] (mg/L)	Measured Concentration[2] (mg/L)	SEM[3]	Nominal Concentration[1] (mg/L)	Measured Concentration[2] (mg/L)	SEM[3]
6-n-propylthiouracil (PTU)	2.5	2.6	0.22	2.5	2.5	0.18
	5.0	6.1	0.56	5.0	5.6	0.39
	10.0	12.0	0.28	10.0	11.9	0.38
	20.0	20.5	0.11	20.0	20.4	0.16
Methimazole	6.25	7.3	0.94	6.25	7.5	1.31
	12.5	13.9	1.18	12.5	14.6	1.34
	25.0	26.2	0.68	25.0	26.1	0.96
	50.0	52.8	1.13	50.0	52.7	1.60
L-Thyroxine[4]	0.0005	-	-	0.0005	-	-
(T4)	0.001	-	-	0.001	-	-
	0.002	0.0017	0.12	0.002	0.0017	0.16
	0.004	0.0034	0.21	0.004	0.0035	0.27
Dexamethasone[4]	0.001	-	-	0.001	-	-
	0.0039	-	-	0.0039	-	-
	0.0156	-	-	0.0156	-	-
	0.0625	0.0729	3.13	0.0625	0.075	3.20
	0.25	0.255	10.24	0.25	0.265	5.64
Phenobarbital	125.0	115.7	11.15	250.0	214.8	14.18
	250.0	217.6	9.70	500.0	436.4	30.24
	500.0	436.3	19.69	1000.0	930.6	116.5
	1000.0	908.6	56.02	1500.0	1380.8	138.1
Pregnenolone-16-∝-Carbonitrile (PCN)	0.0074	0.00051	0.00	0.0074	0.00051	0.00
	0.0222	0.0006	0.09	0.0222	0.00051	0.00
	0.0667	0.0042	1.04	0.0667	0.0035	1.27
	0.2	0.0144	0.68	0.2	0.0142	1.10

[1] Target test concentrations initially proposed for assay.
[2] Mean of actual measured concentrations corresponding to the nominal concentrations.
[3] Standard error of the mean for the measured concentration.
[4] Following instructions imposed by USEPA, only the 2 highest nominal test concentrations were measured.

Table 10. Control Mean Developmental Stage and Weight Data from Thyroid Disruption Assays

Test Material	Test Exposure System	Initial Test Stage[1] (Exposure Duration)	Mean Stage[1] (SEM[2])	Mean Weight (g) (SEM[2])
6-n-propylthiouracil (PTU)	Flow-Thru	51 (21-d)	57.4 (0.167)	0.736 (0.022)
		54 (14-d)	58.0 (0.182)	0.825 (0.030)
Methimazole	Flow-Thru	51 (21-d)	56.6 (0.142)	0.539 (0.020)
		54 (14-d)	58.4 (0.264)	0.636 (0.026)
L-Thyroxine (T4)	Flow-Thru	51 (21-d)	56.7 (0.177)	0.538 (0.016)
		54 (14-d)	58.2 (0.176)	0.587 (0.014)
Dexamethasone	Flow-Thru	51 (21-d)	56.0 (0.207)	0.695 (0.041)
		54 (14-d)	57.1 (0.167)	0.790 (0.026)
Phenobarbital	Static Renewal	51 (21-d)	57.7 (0.169)	0.852 (0.033)
		54 (14-d)	58.0 (0.188)	0.897 (0.031)
Pregnenolone-16-∝-Carbonitrile (PCN)	Static Renewal	51 (21-d)	56.3 (0.162)	0.481 (0.023)
		54 (14-d)	58.0 (0.158)	0.827 (0.025)

[1] Developmental stages are based on Nieuwkoop and Faber (1994).
[2] Standard error of the mean.

Table 11a. Developmental Stage Distribution of 21-d 6-n-propylthiouracil (PTU) Exposure on *X. laevis* Larvae – T_0 = Stage 51

Nominal Test Concentration (mg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0					5	24	1	8	1					39
2.5					11	21	1	6	1					40
5.0				1	9	18	1	11						40
10.0			5	8	10	12	5							40
20.0		4	17	16	2			1						40

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 11b. Developmental Stage Distribution of 14-d 6-n-propylthiouracil (PTU) Exposure on *X. laevis* Larvae – T_0 = Stage 54

Nominal Test Concentration (mg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0					6	7	12	13	2					40
2.5						4	22	13	1					40
5.0					4	10	14	10	2					40
10.0					8	19	8	5						40
20.0					1	28	8	3						40

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 12a. Effect of 21-d 6-n-propylthiouracil (PTU) Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 51

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (mg/L)				
Types of Effects	**0.0**	**2.5**	**5.0**	**10.0**	**20.0**
Colloid, Decreased	0/10	0/10	0/10	0/10	2/10 (Mild-Mod)
Enlargement, Diffuse	0/10	1/10 (Mild)	7/10 (Min-Mild)	7/10 (Min-High)	6/10 (Mild-High)
Hyperplasia, Follicular Cell	0/10	1/10 (Mild)	1/10 (Min)	6/10 (Min-High)	10/10 (Mild-High)
Interfollicular Pigment	7/10 (Mild-Mod)	7/10 (Mild)	9/10 (Mild)	6/10 (Mild -Mod)	8/10 (Min -Mild)

Table 12b. Effect of 14-d 6-n-propylthiouracil (PTU) Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 54

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (mg/L)				
Types of Effects	**0.0**	**2.5**	**5.0**	**10.0**	**20.0**
Distension, Follicles	0/10	2/10 (Min-Mild)	6/10 (Min-Mild)	8/10 (Min-Mod)	6/10 (Min-Mild)
Enlargement, Diffuse	0/10	0/10	4/10 (Min-Mild)	9/10 (Min-Mod)	5/10 (Mild-Mod)
Hyperplasia, Follicular Cell	0/10	1/10 (Min)	9/10 (Min-Mild)	10/10 (Mild-Mod)	10/10 (Min-Mod)
Interfollicular Pigment	9/10 (Mild-Mod)	9/10 (Mild)	8/10 (Mild-Mod)	7/10 (Mild-Mod)	7/10 (Min-Mild)

[1] Results presented in these tables were taken from EPL Pathology Report 237-008, June 10, 2004.

Table 13a. Developmental Stage Distribution of 21-d Methimazole Exposure on *X. laevis* Larvae – T_0 = Stage 51

Nominal Test Concentration (mg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0				2	17	18		3						40
6.25			2	5	16	15		2						40
12.5	2	4	5	10	14	5								40
25.0		3	20	17										40
50.0		4	19	13	1									37

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 13b. Developmental Stage Distribution of 14-d Methimazole Exposure on *X. laevis* Larvae – T_0 = Stage 54

Nominal Test Concentration (mg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0					2	17	1	13	3		4			40
6.25					3	16		16	4	1				40
12.5					7	32		1						40
25.0				3	14	22	1							40
50.0				9	15	14	1	1						40

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 14a. Effect of 21-d Methimazole Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 51

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (mg/L)				
Types of Effects	**0.0**	**6.25**	**12.5**	**25.0**	**50.0**
Colloid, Decreased	0/9	1/10 (Min)	3/10 (Mild-Mod)	7/10 (Min-Mod)	10/10 (Mild-Mod)
Colloid, Foamy & Pale	0/9	0/10	6/10	3/10	7/10
Distension, Follicles	0/9	4/10 (Min-Mild)	3/10 (Min-Mod)	1/10 (Min)	0/10
Enlargement, Diffuse	0/9	2/10 (Min)	6/10 (Min-Mild)	6/10 (Min-High)	9/10 (Mild-Mod)
Eosinophilic Droplets, Follicular Cell	0/9	0/10	0/10	0/10	3/10 (Min-Mild)
Hyperplasia, Follicular Cell	0/9	2/10 (Min)	8/10 (Min-Mod)	10/10 (Min-Mod)	10/10 (Mild-Mod)

Table 14b. Effect of 14-d Methimazole Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 54

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (mg/L)				
Types of Effects	**0.0**	**6.25**	**12.5**	**25.0**	**50.0**
Colloid, Decreased	0/10	0/9	9/10 (Mild-Mod)	10/10 (Min-Mod)	9/9 (Min-High)
Colloid, Foamy & Pale	0/10	0/9	9/10	7/10	6/9
Distension, Follicles	0/10	0/9	6/10 (Min-Mod)	3/10 (Min-Mild)	0/9
Enlargement, Diffuse	0/10	0/9	8/10 (Min-Mod)	8/10 (Min-Mod)	7/9 (Min-Mod)
Eosinophilic Droplets, Follicular Cell	0/10	0/9	0/10	3/10 (Min-Mod)	2/9 (Min-Mild)
Hyperplasia, Follicular Cell	0/10	0/9	10/10 (Min-Mod)	10/10 (Min-Mod)	9/9 (Min-Mod)

[1] Results presented in these tables were taken from EPL Pathology Report 237-009, February 2, 2005.

Table 15a. Developmental Stage Distribution of 21-d L-Thyroxine (T4) Exposure on *X. laevis* Larvae – T_0 = Stage 51

Nominal Test Concentration (µg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0				4	16	14	1	5						40
0.5					6	26	1	7						40
1.0								34	1	1	2	1		39
2.0								19	7	9	4	1		40
4.0												8	3	11

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 15b. Developmental Stage Distribution of 14-d L-Thyroxine (T4) Exposure on *X. laevis* Larvae – T_0 = Stage 54

Nominal Test Concentration (µg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0						17	1	20	1	1				40
0.5						19	2	14	1	4				40
1.0						6	9	24		1				40
2.0								25	3	12				40
4.0										3	30	4	2	39

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 16a. Effect of 21-d L-Thyroxine (T4) Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 51

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (µg/L)				
Types of Effects	**0.0**	**0.5**	**1.0**	**2.0**	**4.0[2]**
Colloid, Decreased	0/10	7/10 (Min-Mod)	7/10 (Min-Mod)	6/7 (Min-Mod)	-
Colloid, Light Eosinophilic Foamy	0/10	1/10 (Min)	2/10 (Min)	5/7 (Min)	-
Thyroid Follicles, Decreased Size	0/10	8/10 (Min-Mod)	7/10 (Min-Mod)	6/7 (Mild-Mod)	-
Interfollicular Pigment	7/10 (Min-Mod)	7/10 (Min-Mod)	10/10 (Min-Mild)	5/7 (Min-Mild)	-

Table 16b. Effect of 14-d L-Thyroxine (T4) Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 54

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (µg/L)				
Types of Effects	**0.0**	**0.5**	**1.0**	**2.0**	**4.0[3]**
Colloid, Decreased	1/10 (Min)	4/10 (Min-Mild)	3/10 (Min)	8/10 (Min-Mod)	-
Colloid, Light Eosinophilic Foamy	0/10	0/10	2/10 (Min)	2/10 (Min)	-
Interfollicular Pigment	8/10 (Mild-Mod)	7/10 (Mild-Mod)	10/10 (Min-Mod)	6/10 (Mild-Mod)	-

[1] Results presented in these tables were taken from EPL Pathology Report 237-013, March 4, 2005.
[2] Due to high lethality at the 4.0 µg/L concentration, thyroid histology was not requested.
[3] No thyroid gland tissue could be found to analyze in the 10 specimens exposed to 4.0 µg/L T4.

Table 17a. Developmental Stage Distribution of 21-d Dexamethasone Exposure on *X. laevis* Larvae – T_0 = Stage 51

Nominal Test Concentration (µg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0			3	12	14	7		4						40
1.0			3	10	16	9		2						40
3.9				13	18	6		3						40
15.6		1	3	11	20	4		1						40
62.5		2	5	20	12	1								40
250		1	6	21	9	3								40

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 17b. Developmental Stage Distribution of 14-d Dexamethasone Exposure on *X. laevis* Larvae – T_0 = Stage 54

Nominal Test Concentration (µg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0				1	11	17	5	6						40
1.0				3	10	16	5	4	2					40
3.9				2	9	11	6	9	3					40
15.6				3	11	24	1	1						40
62.5				6	23	11								40
250				6	26	8								40

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 18. Effect of 21-d Dexamethasone Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 51

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1, 2]					
	Nominal Exposure Concentration (µg/L)					
Types of Effects	**0.0**	**1.0**	**3.9**	**15.6**	**62.5**	**250**
Colloid, Decreased	0/10	0/10	0/10	2/10 (Min)	7/10 (Min-Mild)	10/10 (Min-Mod)
Thyroid Follicles, Decreased Size	0/10	0/10	0/10	2/10 (Min)	7/10 (Min-Mild)	10/10 (Min-Mild)
Interfollicular Pigment	0/10	0/10	0/10	1/10 (Min)	1/10 (Mild)	1/10 (Min)

[1] Results presented in these tables were taken from EPL Pathology Report 237-014, March 4, 2005.
[2] No effect was found during histological examination of 14-d Dexamethasone exposure on *X. laevis* thyroid glands.

Table 19a. Developmental Stage Distribution of 21-d Phenobarbital Exposure on *X. laevis* Larvae – T_0 = Stage 51

Nominal Test Concentration (mg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0					3	21	1	15						40
125						16	2	21						39
250						11	3	26						40
500						11	2	26	1					40
1000					1	11		25						37

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 19b. Developmental Stage Distribution of 14-d Phenobarbital Exposure on *X. laevis* Larvae – T_0 = Stage 54

Nominal Test Concentration (mg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0					4	13	3	18	2					40
250						10	2	28						40
500						20	6	14						40
1000						12	1	27						40
1500					1	16	4	19						40

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 20a. Effect of 21-d Phenobarbital Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 51

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (mg/L)				
Types of Effects	**0.0**	**125**	**250**	**500**	**1000**
Colloid, Pale	0/10	2/10 (Min)	0/10	0/10	0/10
Enlargement, Diffuse	0/10	0/10	0/10	0/10	2/10 (Min-Mild)
Hyperplasia, Follicular Cell	0/10	0/10	0/10	0/10	6/10 (Min)
Interfollicular Pigment	3/10 (Mild-Mod)	4/10 (Min-Mod)	2/10 (Mild)	2/10 (Min-Mod)	3/10 (Min-Mild)

Table 20b. Effect of 14-d Phenobarbital Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 54

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (mg/L)				
Types of Effects	**0.0**	**250**	**500**	**1000**	**1500**
Hyperplasia, Follicular Cell	0/9	0/10	0/10	0/10	2/10 (Min)
Interfollicular Pigment	2/9 (Min-Mild)	4/10 (Min-Mild)	3/10 (Mild-Mod)	2/10 (Min)	4/10 (Min-Mild)

[1] Results presented in these tables were taken from EPL Pathology Report 237-012, March 3, 2005.

Table 21a. Developmental Stage Distribution of 21-d Pregnenolone-16-∝-Carbonitrile Exposure on *X. laevis* Larvae – T_0 = Stage 51

Nominal Test Concentration (µg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0				7	19	11		3						40
7.4					3	20	2	14						39
22.2				2	4	22	2	10						40
66.7						33	2	5						40
200					4	34	1	1						40

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 21b. Developmental Stage Distribution of 14-d Pregnenolone-16-∝-Carbonitrile Exposure on *X. laevis* Larvae – T_0 = Stage 54

Nominal Test Concentration (µg/L)	Developmental Stage[1] of Surviving Larvae													Total Larvae Surviving
	52	53	54	55	56	57	58	59	60	61	62	63	64	
0.0						20	1	19						40
7.4					1	17	3	19						40
22.2						15	1	23	1					40
66.7						13	1	24	2					40
200						22	2	16						40

[1] Developmental stages are based on Nieuwkoop and Faber (1994).

Table 22a. Effect of 21-d Pregnenolone-16-α-Carbonitrile (PCN) Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 51

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (µg/L)				
Types of Effects	**0.0**	**7.4**	**22.2**	**66.7**	**200**
Enlargement, Diffuse	0/10	1/10 (Min)	0/10	0/10	2/10 (Min)
Karyomegaly, Follicular Cell	0/10	0/10	1/10 (Min)	7/10 (Min)	4/10 (Min)
Hyperplasia, Follicular Cell	2/10 (Min)	4/10 (Min)	7/10 (Min-Mild)	7/10 (Min-Mild)	8/10 (Min)

Table 22b. Effect of 14-d Pregnenolone-16-α-Carbonitrile (PCN) Exposure on *X. laevis* Thyroid Gland Histology – T_0 = Stage 54

	Response (#Responding/#Evaluated) (Severity Rating – Minimal/Mild/Moderate/High)[1]				
	Nominal Exposure Concentration (µg/L)				
Types of Effects	**0.0**	**7.4**	**22.2**	**66.7**	**200**
Colloid, Decreased	1/10 (Min)	1/10 (Min)	0/10	0/10	1/10 (Min)
Enlargement, Diffuse	0/10	0/10	2/10 (Min)	4/10 (Min)	1/10 (Min)
Karyomegaly, Follicular Cell	0/10	0/10	1/10 (Min)	0/10	0/10
Hyperplasia, Follicular Cell	5/10 (Min)	3/10 (Min)	7/10 (Min)	7/10 (Min)	7/10 (Min)

[1] Results presented in these tables were taken from EPL Pathology Report 237-011, March 4, 2005.

Figure 1a. Effect of 21-d 6-n-Propyl-2-thiouracil (PTU) Exposure on *X. laevis* Developmental Stage - T_0 = Stage 51

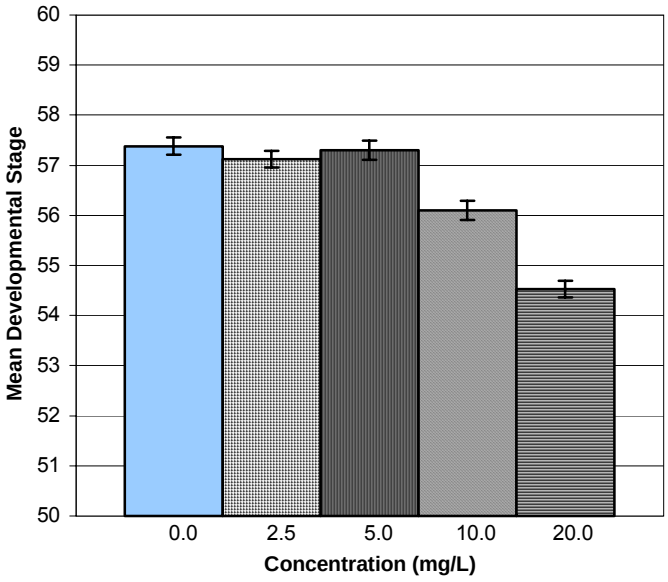

Figure 1b. Effect of 21-d 6-n-Propyl-2-thiouracil (PTU) Exposure on *X. laevis* Larval Weight - T_0 = Stage 51

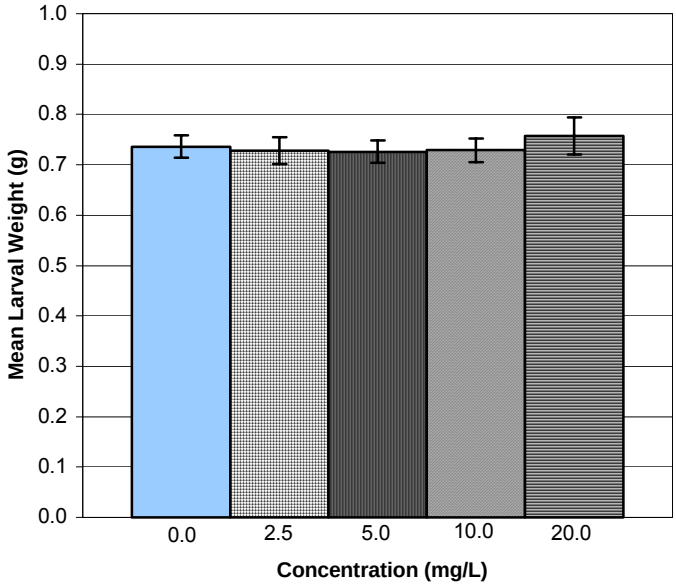

Figure 1c. Effect of 14-d 6-n-Propyl-2-thiouracil (PTU) Exposure on *X. laevis* Developmental Stage - T_0 = Stage 54

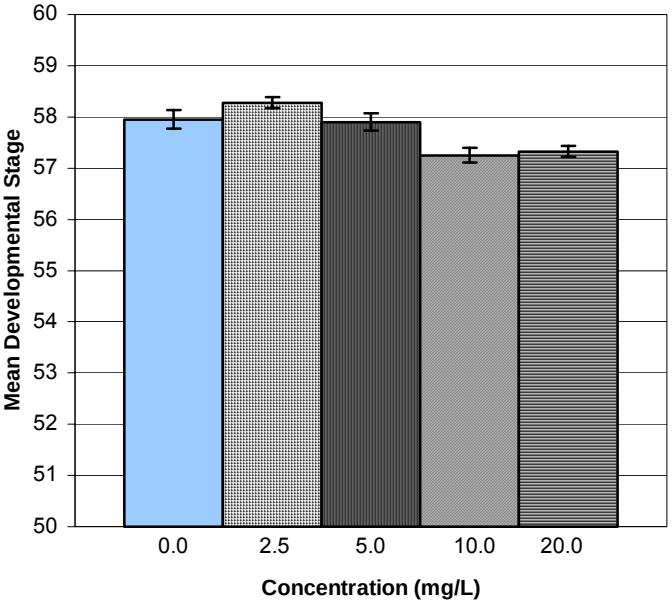

Figure 1d. Effect of 14-d 6-n-Propyl-2-thiouracil (PTU) Exposure on *X. laevis* Larval Weight - T_0 = Stage 54

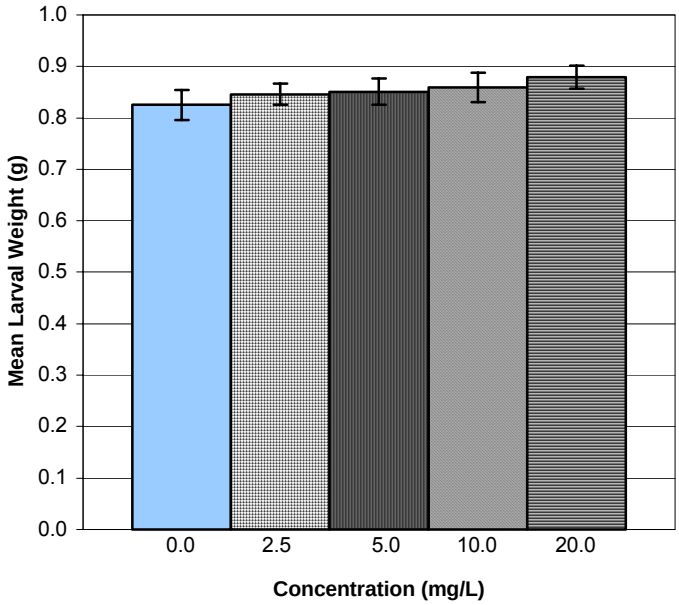

Figure 2a. Effect of 21-d Methimazole Exposure on *X. laevis* Developmental Stage - T_0 = Stage 51

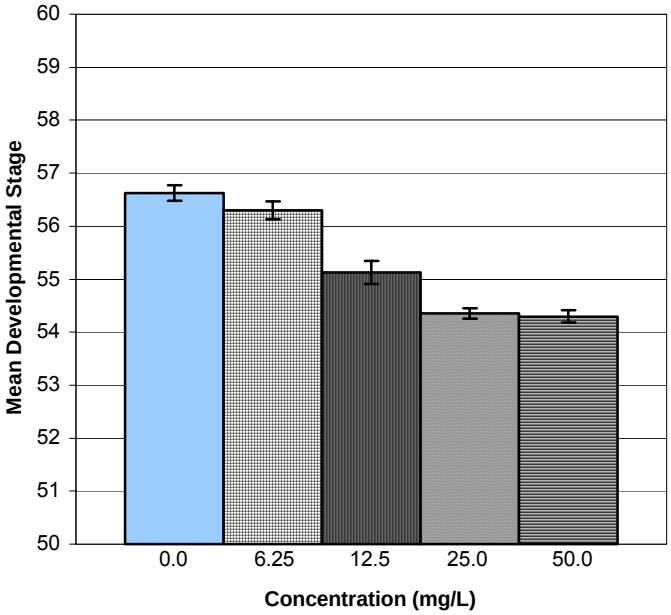

Figure 2b. Effect of 21-d Methimazole Exposure on *X. laevis* Larval Weight - T_0 = Stage 51

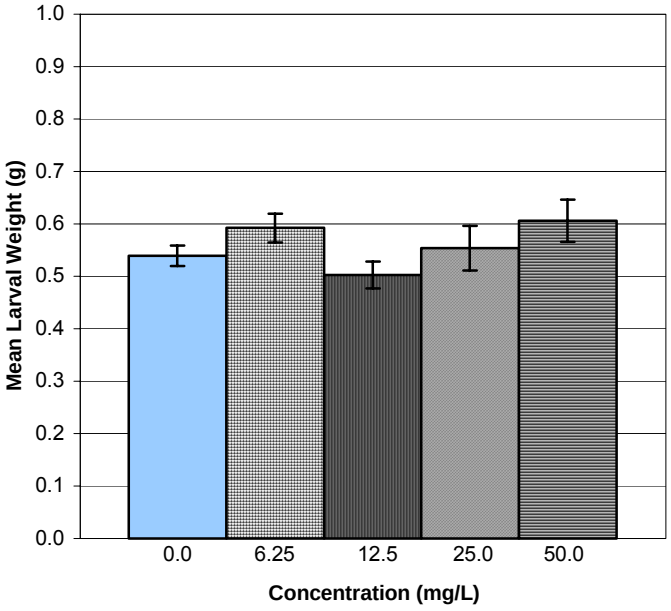

Figure 2c. Effect of 14-d Methimazole Exposure on *X. laevis* Developmental Stage - T_0 = Stage 54

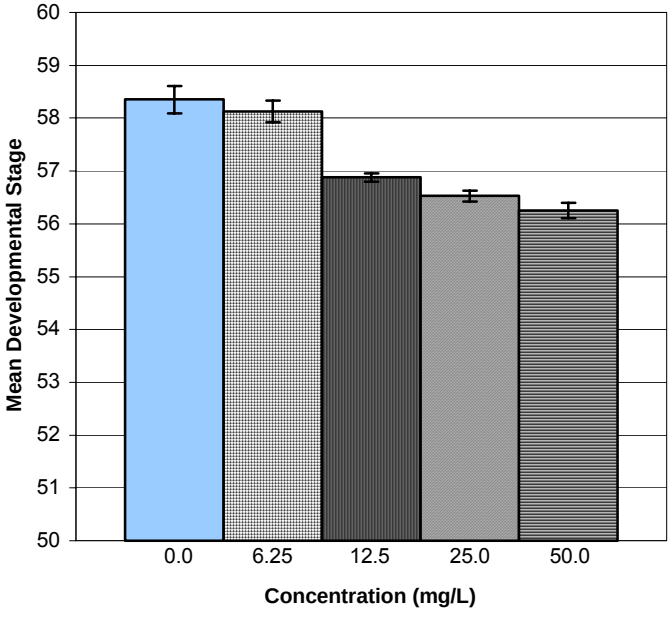

Figure 2d. Effect of 14-d Methimazole Exposure on *X. laevis* Larval Weight - T_0 = Stage 54

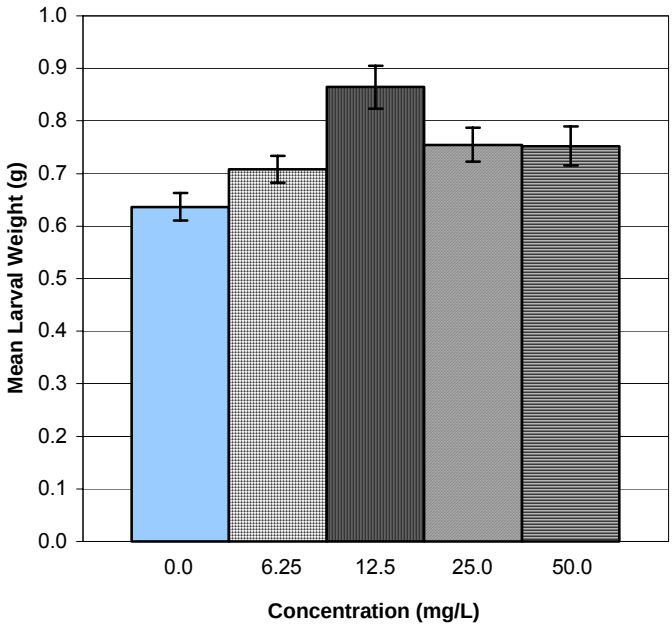

Figure 3a. Effect of 21-d L-Thyroxine (T4) Exposure on *X. laevis* Developmental Stage - T_0 = Stage 51

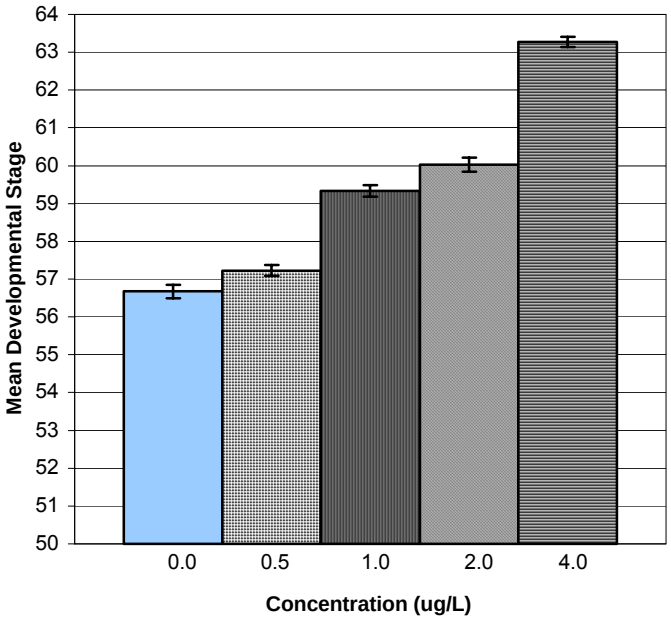

Figure 3b. Effect of 21-d L-Thyroxine (T4) Exposure on *X. laevis* Larval Weight - T_0 = Stage 51

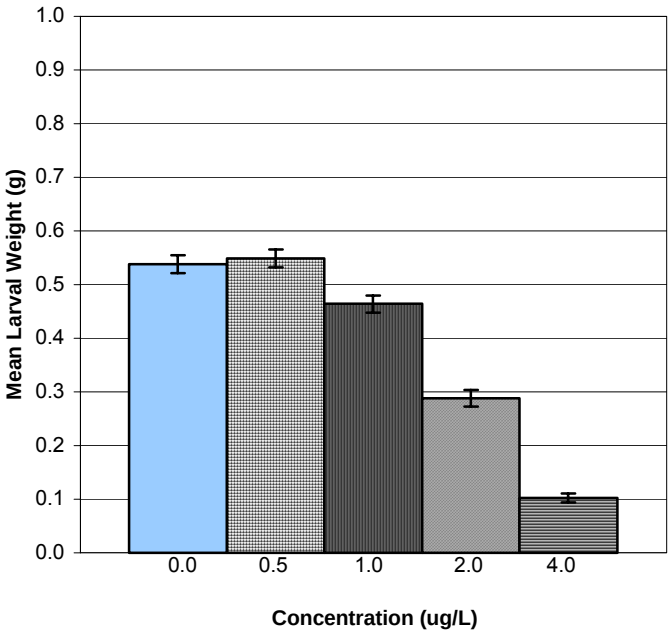

Figure 3c. Effect of 14-d L-Thyroxine (T4) Exposure on *X. laevis* Developmental Stage - T_0 = Stage 54

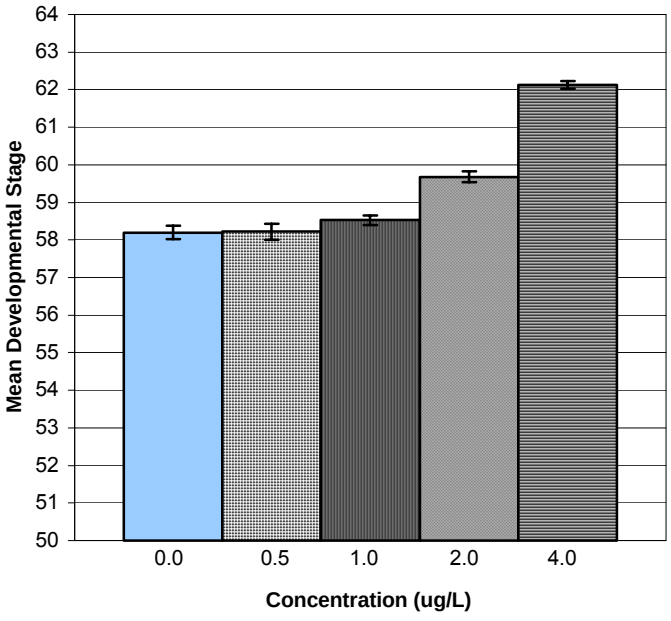

Figure 3d. Effect of 14-d L-Thyroxine (T4) Exposure on *X. laevis* Larval Weight - T_0 = Stage 54

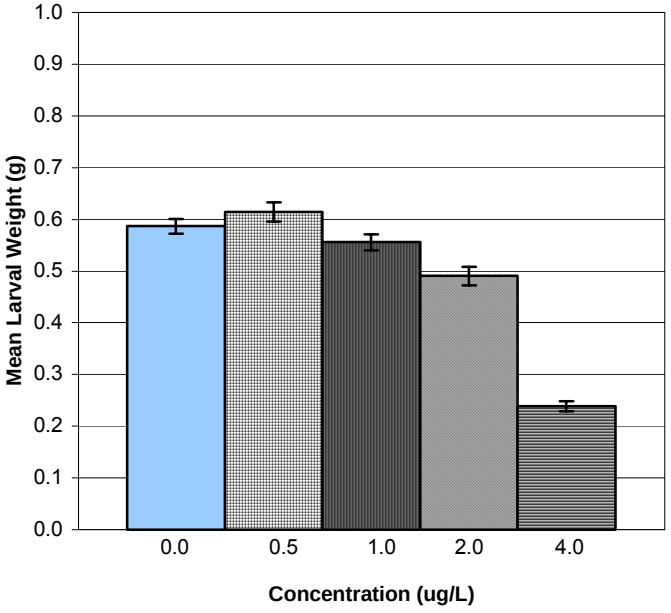

Figure 4a. Effect of 21-d Dexamethasone Exposure on *X. laevis* Developmental Stage - T_0 = Stage 51

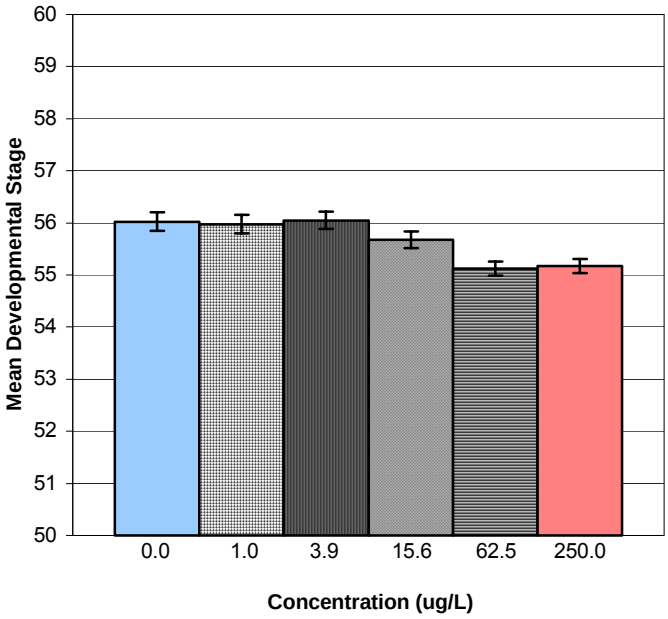

Figure 4b. Effect of 21-d Dexamethasone Exposure on *X. laevis* Larval Weight - T_0 = Stage 51

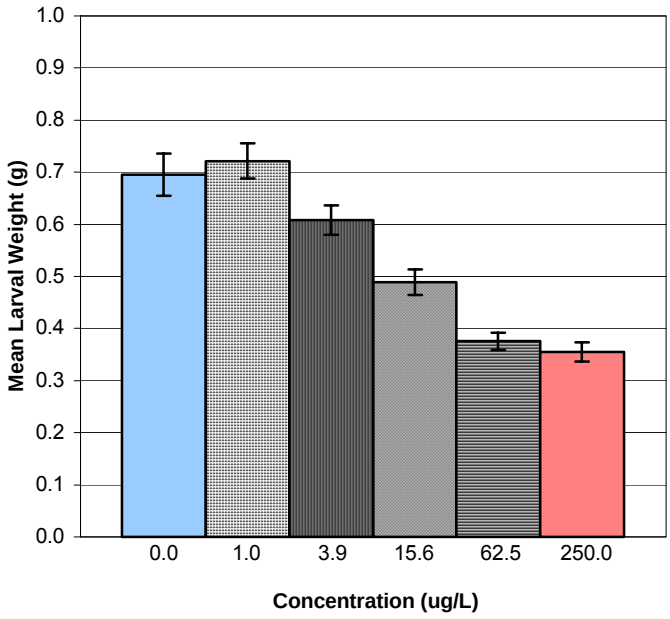

Figure 4c. Effect of 14-d Dexamethasone Exposure on *X. laevis* Developmental Stage - T_0 = Stage 54

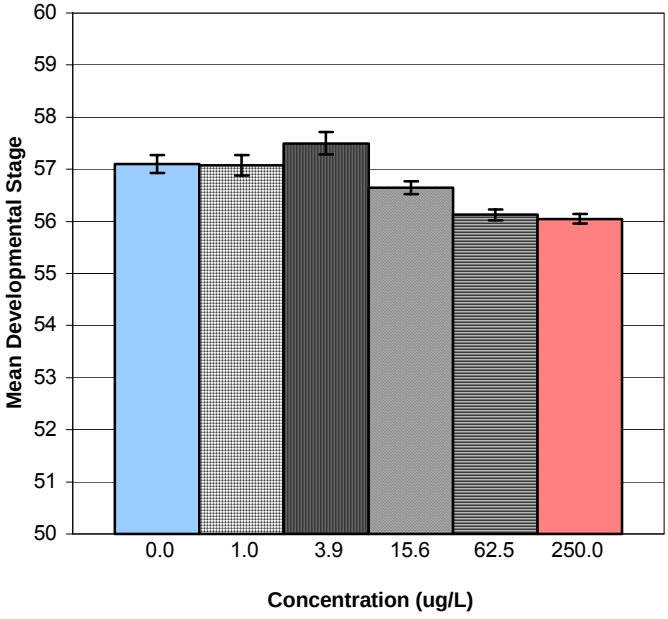

Figure 4d. Effect of 14-d Dexamethasone Exposure on *X. laevis* Larval Weight - T_0 = Stage 54

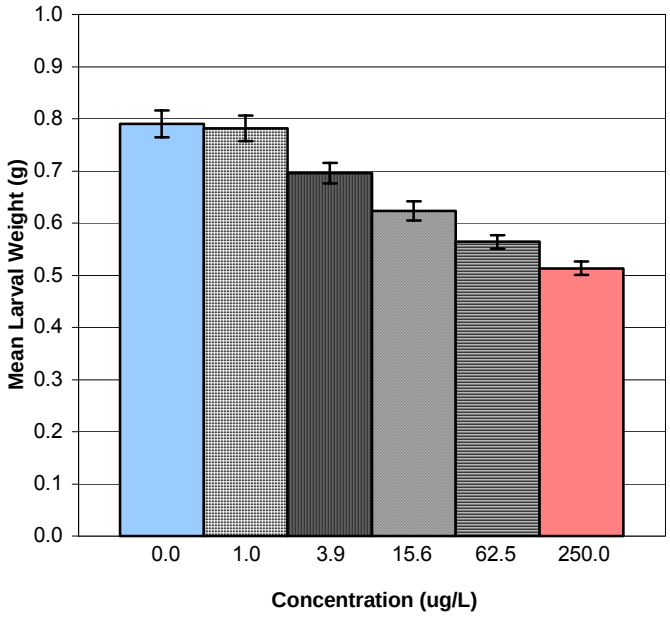

Figure 5a. Effect of 21-d Phenobarbital Exposure on *X. laevis* Developmental Stage - T_0 = Stage 51

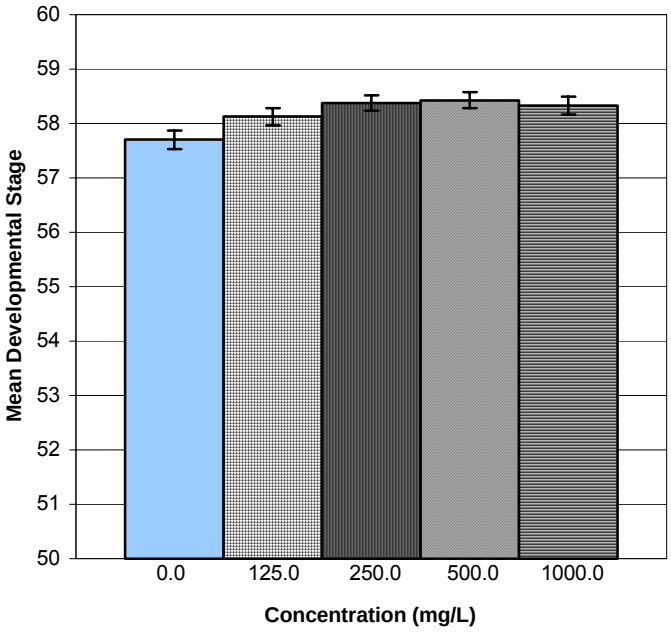

Figure 5b. Effect of 21-d Phenobarbital Exposure on *X. laevis* Larval Weight - T_0 = Stage 51

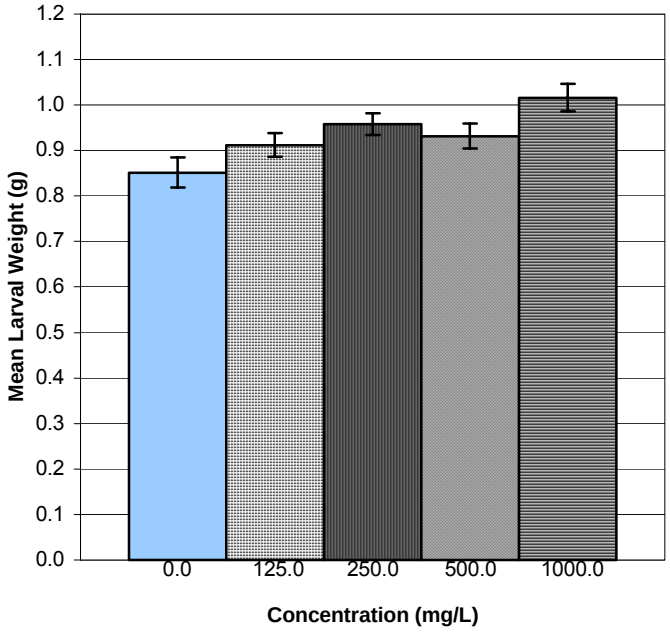

Figure 5c. Effect of 14-d Phenobarbital Exposure on *X. laevis* Developmental Stage - T_0 = Stage 54

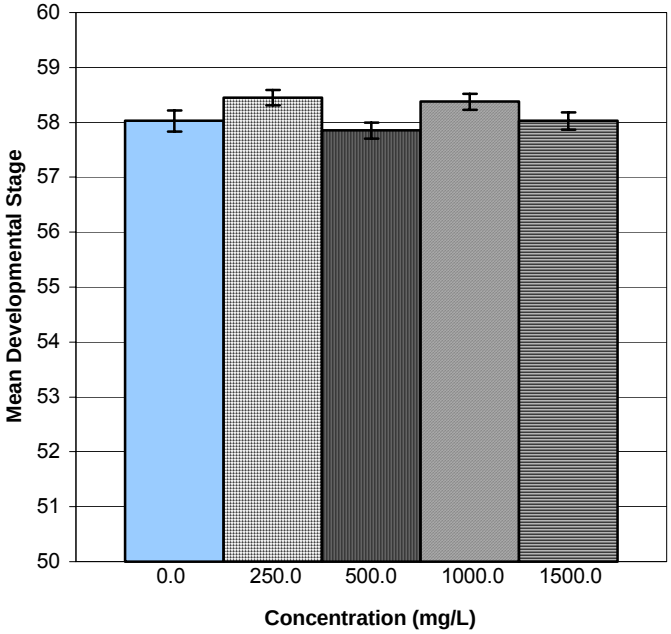

Figure 5d. Effect of 14-d Phenobarbital Exposure on *X. laevis* Larval Weight - T_0 = Stage 54

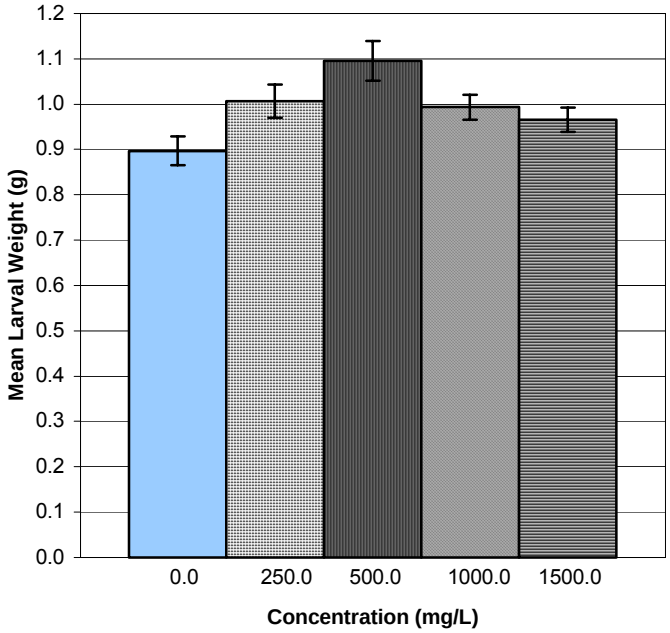

Figure 6a. Effect of 21-d Pregnenolone-16-a-Carbonitrile (PCN) Exposure on *X. laevis* Developmental Stage - T_0 = Stage 51

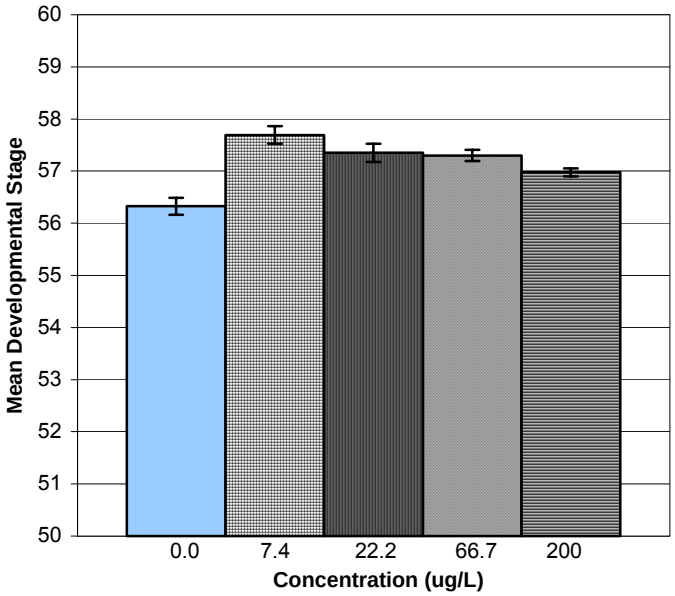

Figure 6b. Effect of 21-d Pregnenolone-16-a-Carbonitrile (PCN) Exposure on *X. laevis* Larval Weight - T_0 = Stage 51

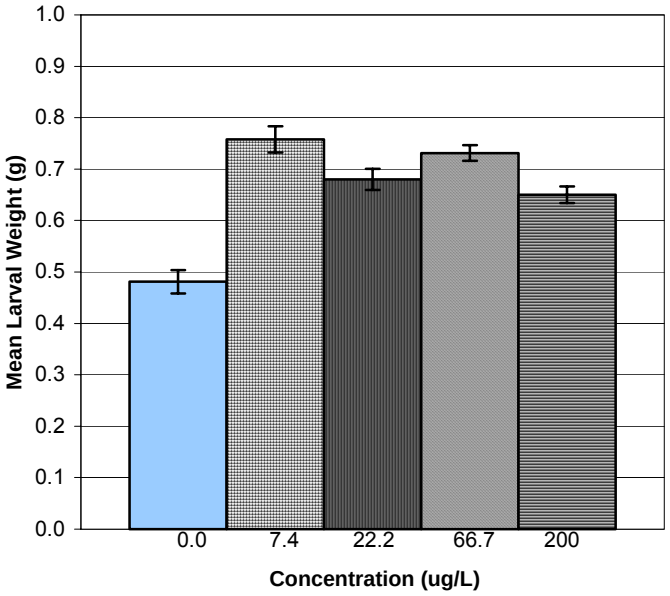

Figure 6c. Effect of 14-d Pregnenolone-16-a-Carbonitrile (PCN) Exposure on *X. laevis* Developmental Stage - T_0 = Stage 54

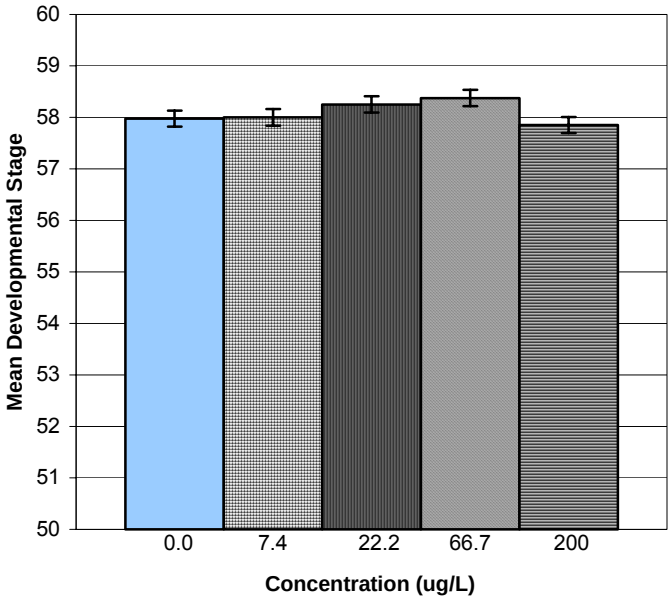

Figure 6d. Effect of 14-d Pregnenolone-16-a-Carbonitrile (PCN) Exposure on *X. laevis* Larval Weight - T_0 = Stage 54