FISH SHORT-TERM REPRODUCTION ASSAY
TEST PROTOCOL – Fathead Minnow

1.0 INTRODUCTION

The U.S. Environmental Protection Agency (US-EPA 2002) has described a short-term reproduction assay with the fathead minnow *(Pimephales promelas)* that considers reproductive fitness as an integrated measure of toxicant effects, and also enables measurement of a suite of histological and biochemical endpoints that reflect effects associated with [anti-] estrogens and androgens. The assay (Ankley et al. 2001) is initiated with mature male and female fish. During a 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics are observed, and fecundity is monitored. Assessments of fertility and F1 development can be made, if desired. At the end of the test, measurements are made of a number of endpoints reflective of the status of the reproductive endocrine system, including the gonadal-somatic index (GSI), gonadal histology, and plasma concentrations of vitellogenin and steroids.

1.1 Principle of the Assay

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

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

The assays will be initiated with newly mature spawning adults. Spawning performance will be established during a pre-exposure period of at least 14 days.

The pre-exposure observations will occur in the same system/tanks as will be utilized for the chemical test (e.g., fish will not be transferred between tanks

between the pre-exposure and exposure periods, which could induce stress). An overview of the tests and relevant test conditions are provided in Table 1.

Table 1. Experimental Design for the Assay Method

Parameter	**Assay Protocol**
Test species:	Reproductively active fathead minnows *Pimephales promelas*(4.5-6 months old)
Pre-exposure evaluation	Duration: minimum of 14 days; Data Collected: fecundity (daily)
Dilution water	Clean, surface, well, or reconstituted water Total alkalinity = > 20 mg/L (as $CaCO_3$) Total organic carbon = ≤ 5 mg/L Unionized Ammonia = ≤ 35 mg/L
Test material	NS
Test chamber size	18 L (40 x 20 x 20 cm)
Test volume:	10 L
# Exchanges/day	6 tank volume exchanges
Flow rate:	2.7 L / hr
# Concentration / chemical	3
# Replicates:	4
Weight of each fish	NS. If possible, ± 20% arithmetic mean weight of same sex
# Fish/vessel	4 females and 2 males
Total # fish/concentration	4 adult females and 2 adults males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)
Feeding regime	Frozen brine shrimp, twice a day
# Controls	1 Dilution water control and a solvent control added if a solvent used
# Fish/control	4 adult females and 2 adults males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)
Photo period:	16 h light : 8 h dark
Temperature:	25°C ± 1°C
Light intensity	540 - 1080 lux
Aeration:	None unless D.O. <4.9 mg/L
pH	6.5 - 9.0
Biological endpoints:	Adult survival, reproductive behavior, secondary sexual characteristics, GSI, gonadal histology, VTG, fecundity, fertility, and plasma sex steroids
Test validity criteria:	D.O. ≥ 4.9 mg/L (≥ 60% saturation); mean temp. 25°C ± 1°C; 90% survival in the controls and successful egg production in controls. Spawning occurs at least every 4 days in each control replicate, or approximately 15 eggs/female/day/ replicate. Fertility > 95%. Measured exposure concentration CV < 20% for all replicates.

NS = Not specified in procedure.

2.0 DESCRIPTION OF THE METHOD

2.1 Selection of Test Organisms

The exposure phase will be started with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals. Based on the technical judgment of experienced laboratory personnel, fish will be reproductively mature (namely, with clear secondary sexual characteristics visible) and capable of actively spawning. For the whole batch of fish used in the test, the range in individual weights by sex at the start of the test will be kept, if possible, within ± 20% of the arithmetic mean weight of the same sex. A subsample of fish will be weighed before the test in order to estimate the mean weight. For general guidance only (and not to be considered in isolation from observing the actual reproductive status of a given batch of fish), fathead minnows should be 4.5 to 6 months of age, and should have been cultured at 25 ± 2°C.

Test fish will be selected from a laboratory population, preferably from a single stock, which has been acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test. (Note, this acclimation period is not an *in situ* pre-exposure period). Test fish must be reared in an in-house culture, as shipping of adult fish is stressful and will interfere with reliable spawning. Fish will be fed brine shrimp twice per day throughout the holding period and during the exposure phase.

Mortalities in the culture fish will be recorded and the following criteria applied:

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

Fish will not receive treatment for disease in the two-week acclimation period preceding the test, during pre-exposure, or during the exposure period.

2.2 Water

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

2.3 Assay System

Eighteen-liter glass exposure vessels are used for the test system. As recommended by Ankley et al. (2001), dimensions of the test chambers must be such that the animals can interact in a fashion conducive to successful spawning. The test chamber contains 10 L of test solution, which is renewed once every 4 hours (6 volume exchanges per day). This particular animal loading/water renewal rate is within recommended guidelines and in studies conducted according to this method, has maintained acceptable water quality while not utilizing an excessive amount of test material.

A randomized complete block design (4 blocks with one replicate of each treatment) will be used for the reproductive assay. This design is intended to randomize out the effects associated with the local environment (i.e., light and water) and possible trends associated with the diluter during testing. All fish will be impartially assigned to tanks before pre-exposure, then tanks will be randomly assigned to treatments within a block after spawning is established in the pre-exposure period. The blocks are filled in a random order, with the four tanks with the highest per-female fecundity (established during pre-exposure) being assigned first, followed by the second-highest spawners, etc. Thus, when one evaluates the difference between treatment means, the variability associated with experimental environment, experimental containers, and organisms being treated is removed and only the effect of the treatment remains.

2.4 Preparation and Testing of Chemical Exposure Water

Test chemicals will possess some varied physicochemical properties that will likely require different approaches for preparation of chemical exposure water. When possible, direct addition to water will be utilized if the test chemical has sufficiently high water solubility. For test chemicals with reduced water solubility, the use of a saturator column will be required to prepare the concentrated stock solution (US-EPA 2002). For poorly soluble substances, a solvent may be employed, but only as a last resort (see OECD 2000, Guidance

Document 23: *Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures*). All aqueous stock solutions will be encased in black tarpaulin during agitation and subsequent storage to prevent photo-oxidation.

2.6 Analytical Determinations

After preparation of the stock solution, determinations of the concentration will be made using appropriate methods. The concentrations of the test chemical in the exposure chambers will be measured prior to adding fish to verify that target concentrations are reached. Additionally, water samples will be collected weekly thereafter and analyzed for the test chemical, testing two of the four chambers per exposure level each time (High, Med, Low, and Control, in half of the four replicates at each sampling). More frequent sampling is required for chemicals with known lability, if the chemical concentrations are not maintained between sampling intervals, or disturbances to the delivery system occur

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

96 Hour Range-finding: A range-finding test may be necessary to establish test chemical concentrations when 96 hour LC50 data for fathead minnow are not available.

The highest target test concentration for the range-finder will be based upon toxicity data for other fish species. If such information is lacking, the highest concentration will be 100 ppm or the solubility limit of the chemical in water if less than 100 ppm.. The test concentration will then be decreased by a factor of 10 for each successively lower exposure (five exposure concentrations total). Range-finding tests will be performed with fish of similar age and size to those that will be utilized in the test using a 96-hour exposure to five test concentrations plus a control (six total), four females and two males per exposure tank (36 fish total). The number of mortalities that occur will be used to develop a dose response curve. Based upon the results, the highest concentration that does not result in increased mortality or signs of overt morbidity compared to controls, or 1/3 the derived 96-hr LC50 will serve as the highest exposure concentration in the 21-day test.

2.7 Biochemical Determinations

Vitellogenin (VTG): Enzyme-linked immunosorbent assay (ELISA) tests will be conducted using commercially available test kits or equivalents. The methods

used for the bioanalytical measurements of VTG will follow manufacturer's specifications. Any non-detect samples should be recorded as ½ the limit of quantification (LOQ).

Sex steroids: Plasma concentrations of 17β-estradiol and testosterone should be determined using radioimmunoassay (RIA) techniques optimized for the relatively small sample volumes obtained from the fathead minnow (Jensen *et al*. 2001). Any non-detect samples should be recorded as ½ the LOQ.

2.8 21-day Assay Initiation and Conduct

Selection and weighing of test fish

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

Conditions of exposure

The test duration is 21 days.

Feeding

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

Pre-exposure

The pre-exposure phase will last a minimum of 14 days. The assay will use fish that are approximately 4.5-6 months, previously maintained in communal culture tanks. Four females and two males will be randomly assigned to the replicate exposure chambers at each treatment concentration. Additional exposure chambers should be set up for pre-exposure to account for a lack of spawning in some chambers and/or mortality during the pre-exposure phase. Any specimens whose sex cannot be identified will be excluded from the assay. It has been reported that, at 4.5 to 6 months of age, males are larger and darker and exhibit nuptial tubercles, while females possess an ovipositor. The pre-

exposure phase of the assay will be conducted under conditions (temperature, photo period, feeding, etc.) identical to those used during the chemical exposure. The animals will be fed frozen brine shrimp twice daily. Fecundity data will be collected daily. For each assay, successful pre-exposure (suitability for testing) is established when regular spawning occurs in each replicate test chamber at least two times in the immediately preceding 7 days and egg production exceeds 15 eggs/female/day/replicate group.

Chemical Exposure

After successful spawning is verified during pre-exposure as per the requirements of the assay, the chemical exposure will be initiated and continued for 21 days. The assays will be conducted at three chemical concentrations, as well as a diluent water control and solvent control (if a solvent is necessary), with four experimental units (replicates) per treatment. Each replicate tank will contain four female and two male fish. The replicate tanks will be assigned to treatment levels according to randomized complete block design, with the four highest-spawning replicates assigned (one each) to one treatment level or control first, followed by the assignment of the next four highest-spawning replicates by distribution to the four test groups, until all groups are established. The test chemical will be delivered to the exposure chamber using a proportional diluter or other appropriate delivery systems. The exposure will be conducted for 21 days, during which time the appearance of the fish, behavior, and fecundity will be assessed daily. At termination of the exposure, blood samples will be removed from adults and analyzed for VTG and sex steroids. Secondary sex characteristics will be recorded, and the gonads will also be removed for GSI determination and later histological analysis.

Frequency of analytical determinations and measurements

Prior to initiation of the exposure period, proper function of the chemical delivery system will be ensured. Additionally, all analytical methods necessary will be established, including sufficient knowledge of the substances' stability in the test system. During the test, the concentrations of the test substance will be determined at regular intervals, as follows: the flow rates of diluent and toxicant stock solution will be checked at intervals, at least once per week, and will not vary by more than 20% throughout the test. Actual test chemical concentrations will be measured in all vessels at the start of the test and at weekly intervals thereafter, sampling two of the four replicates per group each time.

- Results will be based on measured concentrations and will be included in reporting.

- Samples may need to be centrifuged or filtered (e.g., using a 0.45 μm pore size). If needed, then centrifugation is the recommended procedure; however, if the test material does not adsorb to filters, filtration may also be acceptable.
- During the test, dissolved oxygen, temperature, and pH will be measured in all test vessels at least once per week. If dissolved oxygen reaches 5 mg/L, aeration should be started for all test vessels immediately. Total hardness and alkalinity will be measured in the controls and one vessel at the highest concentration at least once per week. Temperature should preferably be monitored continuously in at least one test vessel.

3.0 OBSERVATIONS AND MEASUREMENTS

3.1 Endpoints

A number of endpoints will be assessed over the course of, and/or at conclusion of the assay. For those endpoints measured at test termination, length and weight measurements will be taken immediately after anesthetization of an individual fish, collection of fresh tissues (e.g., blood) will also be performed, then gonads will be perfused with fixative.

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

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

Body weights: Recorded at test termination.

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

Fecundity: Egg production will be determined daily. Because fathead minnows spawn within a few hours after the lights are turned on, they will not be disturbed (except for feeding) until late morning. This will allow time for

spawning and fertilization to be completed and for eggs to water-harden. The spawning substrates will be removed from the tanks to enumerate any eggs that are present. It is expected that one spawn typically will be composed of 50 to 250 eggs (EPA, 2002). If no eggs are present, the substrate is left in the tank; new substrates should be added to replace any that are removed. Fecundity will be expressed on the basis of surviving females per reproductive (test) day per replicate. It will also be evaluated as cumulative eggs laid over the test duration.

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

Appearance and observation of secondary sex characteristics: Secondary sexual characteristics are under endocrine control; therefore, observations of physical appearance of the fish should be made over the course of the test, and at conclusion of the study (Appendix C). Experience to date with fathead minnows suggests that some endocrine active chemicals may initially induce changes in the following external characteristics: body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in male fathead minnow, ovipositor size in females). Notably, chemicals with certain modes of action may cause abnormal occurrence of secondary sex characteristic in animals of the opposite sex; for example, androgen receptor agonists, such as methyltestosterone and dihydrotestosterone, can cause female fathead minnows to develop pronounced nuptial tubercles (Ankley et al. 2001; Smith 1974; and Panter et al., 2004). It also has been reported that estrogen receptor agonists and androgen receptor antagonists can decrease nuptial tubercle numbers and size of the dorsal nape pad in adult males (Miles-Richardson et al., 1999; Holbech et al., 2001).

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

In measuring the dorsal nape pad, dissection of females is necessary only if a fatpad is visible. In males (that have been confirmed to be males by observations of tubercles or coloration) where no fatpad is visible, confirmation (via dissection) should be done to confirm the absence of a fatpad. It may be possible to excise fatty adipose tissue without the outward visible presence of a fatpad. If none is present (as confirmed by dissection), then a score of 1 is appropriate.

Blood sampling: Blood will be collected from the caudal artery/vein (Appendix A) with a heparinized microhematocrit capillary tubule. Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 20 to 60 µL. Plasma is separated from the blood via centrifugation (approximately 3 minutes at 15,000 x g) and stored with protease inhibitors at -75°C to -85°C until analyzed for VTG and sex steroids. If limited plasma volume is available, VTG should be analyzed first, followed by β-estradiol for females and testosterone in males, then by β-estradiol in males and testosterone in females if possible. Any non-detect samples should be recorded as ½ the limit of quantification (LOQ).

Gonad size and histology: The first step of gonad histological analysis is necropsy and rapid gonad fixation in Davidson's fixative to prevent autolysis and cellular deterioration. Immediately after anesthetization of an individual fish, length and weight measurements will be taken, collection of fresh tissues (e.g., blood) will also be performed, and gonads will be perfused with fixative (Appendix B).

The following steps will be followed for gonad fixation:

1. Using a syringe, approximately 0.5 mL of Davidson's fixative will be gently applied to the gonads *in situ*. Approximately 90 seconds following the application of fixative, the liquid fixative within the abdomen will be removed with a gauze sponge, and the gonads will be excised in a manner similar to the abdominal viscera:
    a. Using microdissection scissors, the spermatic ducts or oviducts will be severed proximal to the genital pore.
    b. Microdissection forceps will then be applied to the spermatic ducts/oviducts. Using gentle traction, the gonads will be dissected out of the abdominal cavity in a caudal to cranial direction, severing the mesorchial/mesovarial attachments as needed using the microdissection scissors. The left and right gonads may be excised individually or they may be

excised simultaneously and subsequently divided at their caudal attachment.

2. The gonads (right and left) will be placed into a pre-labeled plastic tissue cassette which will then be placed into an individual container of Davidson's fixative accompanied by the abdominal viscera. The volume of fixative in the container will be at least 20 times the approximated volume of the tissues. The fixative container will be gently agitated for 5 seconds to dislodge air bubbles from the cassette.

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

Using the carcass, the secondary sex characteristics will be assessed (e.g., dorsal nape pad, nuptial tubercles – see Appendix C).

Routine histological procedures will be used to assess the condition of testes and ovaries from the fish using procedures provided in Histopathology Guidelines for the Fathead Minnow (*Pimephales promelas*) 21-day Reproduction Assay, and will be performed by a toxicologic pathologist with experience with small fishes. The Guidelines details the post mortem and histotechnical procedures that will be used.

Vitellogenin (VTG): The measurement of VTG in plasma samples will be performed using an enzyme-linked immunosorbent assay (ELISA). Polyclonal fathead minnow (*Pimephales promelas*) VTG antibody and purified VTG protein, also from the fathead minnow, should be utilized. Any non-detect samples should be recorded as ½ the limit of quantification (LOQ).

Sex steroids: Plasma concentrations of β-estradiol and testosterone should be determined using radioimmunoassay (RIA) techniques optimized for the relatively small sample volumes obtained from the fathead minnow. Detailed instructions for the measurement of fathead minnow plasma sex steroids using RIA are provided in US-EPA (2002). Given that limited plasma volume may be available, analysis priority should be given to β-estradiol for females and testosterone in males, then β-estradiol in males and testosterone in females if possible. Any non-detect samples should be recorded as ½ the limit of quantification (LOQ).

Table 2. Measurement Endpoints and Associated Criteria

Parameter	Units	Expected Results
Survival: Daily assessment of survival will be made to provide a basis for expression and interpretation of reproductive output.	Percentage	90% or greater survival in controls. Mortality is expected to be low based on previous studies at these exposure rates.
Behavior of Adults: Abnormal behavior (relative to controls), during the daily observations will be noted.	Not Applicable	Expected observations may include: hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and feeding abstinence. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males. Qualitative anecdotal observations.
Fecundity: Egg production will be determined daily, but only during the late morning.	Fecundity will be expressed on the basis of number of eggs laid by surviving females per reproductive (test) day	It is expected that one spawn typically will be composed of 50 to 250 eggs. If no eggs are present, the substrate will be left in the tank; new substrates should be added to replace any that are removed.
Fertilization Success: If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate is easily achieved.	Number embryos/number of eggs x 100%	Fertilized eggs will be apparent within a few hours of fertilization. Infertile eggs will be opaque or clear with a white dot where the yolk has precipitated. Control fertilization should be ≥95%.
Appearance of Adults: The external appearance of the adults will be assessed as part of the daily observations, and any unusual changes will be noted. These observations are especially important for assessing endocrine active agents that are (anti)-androgenic.	Not Applicable	External features of particular importance include body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females).

Parameter	Units	Expected Results
Body Weights	Grams	Normal/increased/decreased relative weights to control animals.
Blood Samples: will be collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule and analyzed for VTG	Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 20 to 60 µL.	Plasma will be separated from the blood sample via centrifugation (approx. 3 minutes at 15,000 x g) and stored with protease inhibitors at -75ºC to -85ºC until analysis.
Vitellogenin (VTG) Concentration	ng/mL	The measurement of VTG in plasma samples will be performed using an enzyme-linked immunosorbent assay (ELISA). For the ELISA, polyclonal Fathead minnow (FHM) *(Pimephales promelas)* VTG antibody and purified VTG protein, also from the FHM, will be utilized.
Gonad Size: After sampling the blood, the fixed gonads removed and weighed (to the nearest 0.1 mg) to determine the GSI (GSI=100% x gonad wt/body wt).	Not Applicable	Typical GSI values for reproductively active fathead minnows range from 8 to 13% for females and from 1 to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes.
Gonad Morphology: Routine histological procedures will be used to assess the condition of testes and ovaries from the fish. Gonads will be placed in fixative (Davidson's fixative). A toxicologic pathologist with experience with small fish will perform histology procedures and will refer to *Histopathology Guidelines for the Fathead Minnow (*Pimephales promelas*) 21-Day Reproduction Assay*.	Not Applicable	Refer to *Histopathology Guidelines for the Fathead Minnow (*Pimephales promelas*) 21-Day Reproduction Assay*.

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

3.2 Statistical Methods

Descriptive statistics, including the mean, standard deviation, minimum, maximum, and quartiles, will be used to characterize each endpoint measured in the tests. Statistical significance for each endpoint and chemical will be evaluated based on the difference in the mean characteristics between the treated and control groups using analysis of variance, Tukey's multiple comparisons test, and the non-parametric Kruskal-Wallis test. Chemical dosing regimes will be considered classifications of fixed effects (i.e., control, low dose, mid dose, and high dose).

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

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

3.3 Performance Criteria

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

- There will be more than 90% survival of control animals over the duration of the chemical exposure

- Control fish in each replicate will spawn, at a minimum, every 4 days and there will be an average of at least 15 eggs/female/day/test chamber.

- There will be greater than 95% fertility of eggs from the control animals during the exposure.

3.4 Data Reporting

Test report: The test report will include the following information:

Test substance: physical nature and relevant physical-chemical properties, chemical identification data including purity and analytical method(s) for quantification of the test substance where appropriate, source, CAS number, lot number.

Test species: (at a minimum) scientific name, supplier, and any pretreatment.

Test conditions: test procedure used (test type, loading rate, stocking density, etc.); method of preparation of stock solutions and flow-rate; nominal test concentrations, means of the measured values and standard deviations in test vessels and method by which these were attained and evidence that the measurements refer to the concentrations of test substance in true solution; dilution water characteristics (including pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels, total organic carbon, suspended solids, and any other measurements made); water quality within test vessels: pH, hardness, temperature, and dissolved oxygen concentration; detailed information on feeding (e.g., type of food(s), source, amount given and frequency, and analyses for relevant contaminants if necessary, e.g., PCBs, PAHs and organochlorine pesticides), source and treatment of dilution water, average and ranges of water chemistry parameters, photo period, light intensity, chamber size, numbers of male and female fish per replicate, number and composition of spawning substrate, lot number of feed, number of daily water volume exchanges.

Results: evidence that controls met the validity criterion for survival, and data on mortalities occurring in any of the test concentrations; statistical analytical techniques used, statistics based on fish, treatment of data and justification of techniques used; tabulated data (using data template provided) on biological observations of gross morphology (including secondary sex characteristics), GSI, vitellogenin and sex steroids; detailed report on gonadal histology (using template provided); results of the statistical analysis preferably in tabular and graphical form; incidence of any unusual reactions by the fish and any visible

effects produced by the test substance; average, standard deviation, and range for each test endpoint.

4.0 CONTINGENCIES

The three problems most likely to be encountered are related to insufficient numbers of spawning tanks that are successfully "pre-validated," unplanned mortality in control or exposure tanks, and low or high measured concentrations relative to the nominal level of the test chemicals. These problems will be dealt with in the following manner:

- For each chemical, extra spawning tanks (up to 8 additional tanks) will be pre-validated to ensure an adequate supply of spawning fathead minnows.

- If there is excessive unscheduled mortality in any tank, the experiment should be terminated and the cause investigated.

- Prior to initiation of the chemical exposure, each diluter will be tested for at least 1 week for its ability to maintain the desired concentration. If, during the exposures the measured concentration becomes unacceptably low or high, adjustments will be made to the diluter to correct the problem.

5.0 LITERATURE

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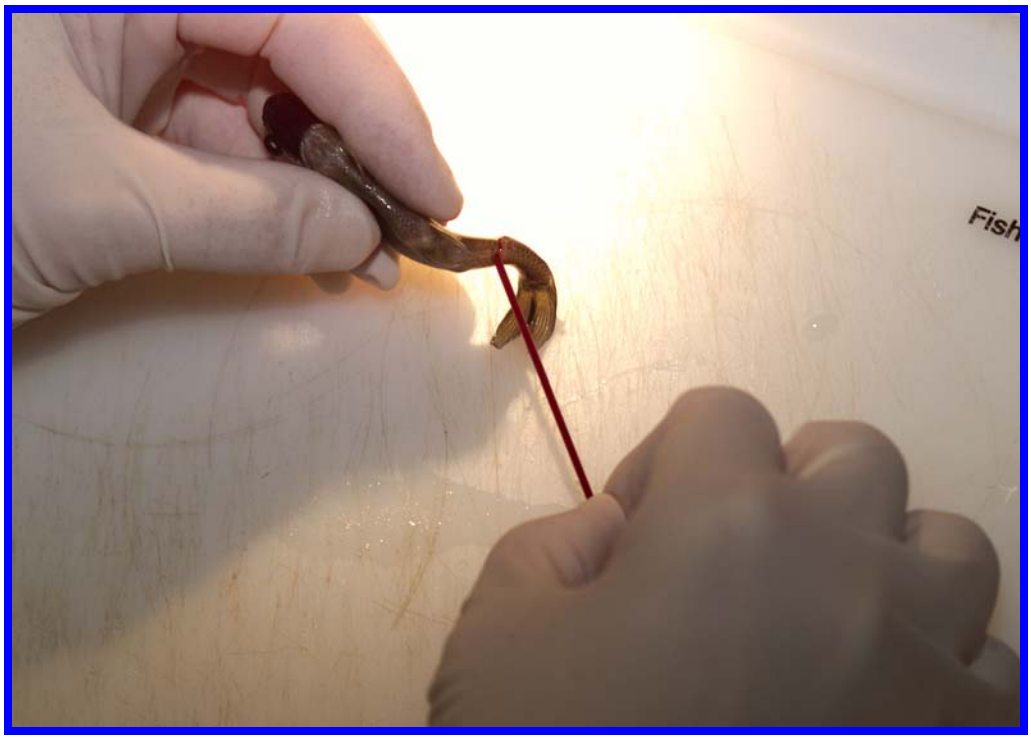
APPENDIX A

SAMPLE COLLECTION PROCEDURES FOR VITELLOGENIN ANALYSIS

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



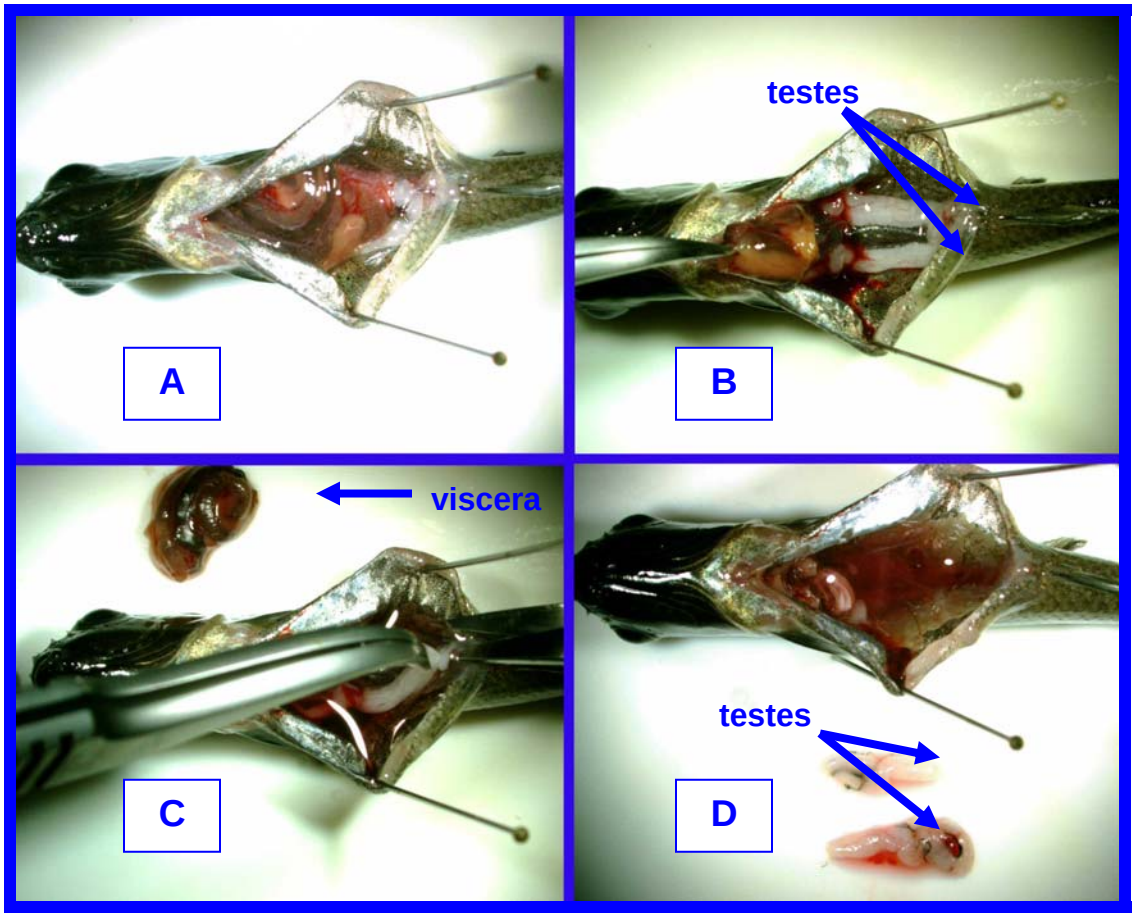
After anaesthetization, the caudal peduncle is partially severed with a scalpel blade and blood is collected from the caudal vein/artery with a heparinized microhematocrit capillary tube. After the blood has been collected, the plasma is quickly isolated by centrifugation for 3 min at 15,000 *g*. If desired, percent hematocrit can be determined following centrifugation. The plasma portion is then removed from the microhematocrit tube and stored in a centrifuge tube with 0.13 units of aprotinin (a protease inhibitor) at -75°C to -85°C until determination of vitellogenin and sex steroid concentrations can be made. Depending on the size of the fathead minnow (which is sex-dependent), collectable plasma volumes generally range from 20 to 60 microliters per fish (Jensen *et al.* 2001).

Procedure 1B: Fathead Minnow, Blood Collection from Heart

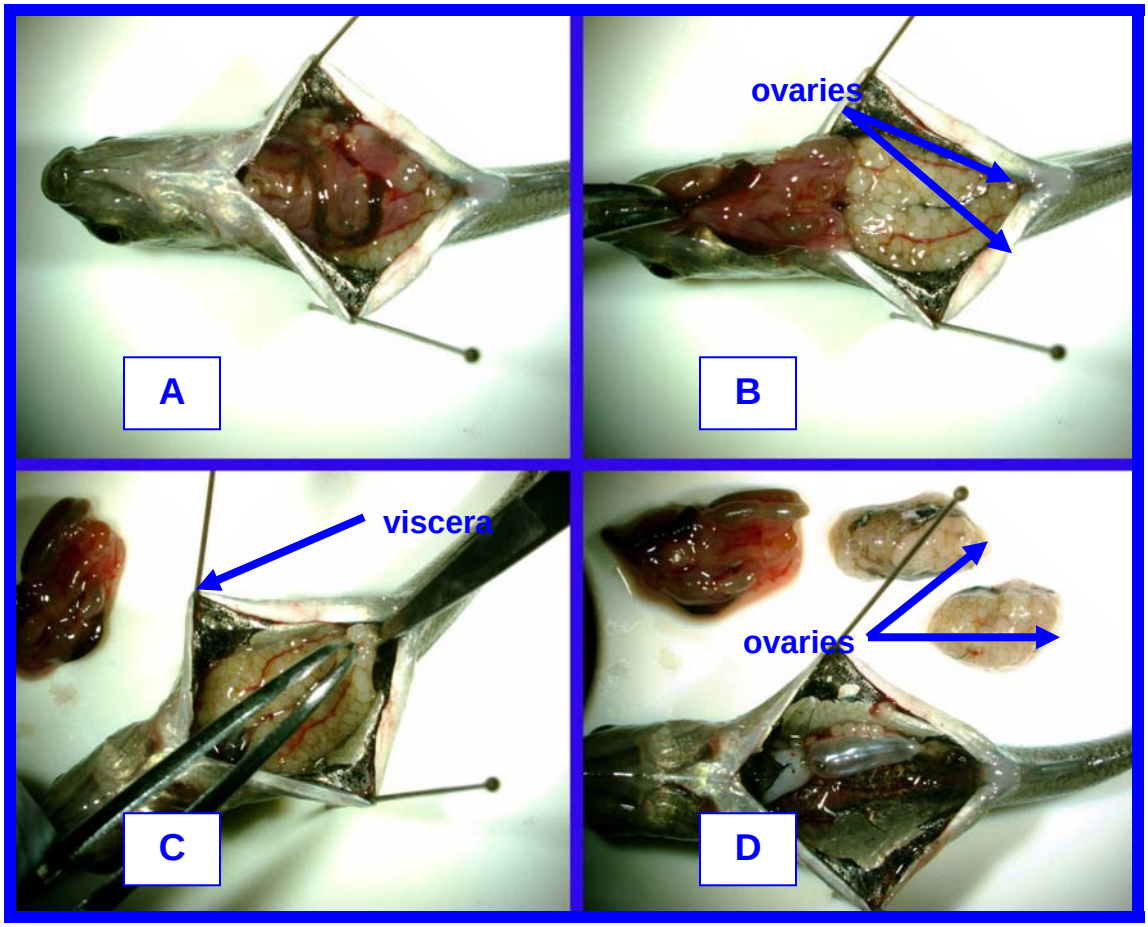
Alternatively, blood may also be collected by cardiac puncture using a heparinized syringe (1000 units of heparin per ml). The blood is transferred into Eppendorf tubes (held on ice) and then centrifuged (5 min, 7,000 g, room temperature). The plasma should be transferred into clean Eppendorf tubes (in aliquots if the volume of plasma makes this feasible) and promptly frozen at -75°C to -85°C until analyzed (Panter et al., 1998).

APPENDIX B

Removal of gonads from Fathead minnows



Fathead Minnow, Male: Excision of the testes during necropsy.
A. The abdominal wall is pinned laterally. B. The terminal intestine is severed and retracted prior to removal. C. The testes are grasped near the spermatic ducts. D. Removal of the testes is complete.



Fathead Minnow, Female: Excision of the ovaries during necropsy. A. The abdominal wall is pinned laterally. B. The terminal intestine is severed and retracted prior to removal. C. The ovaries are grasped near the oviducts. D. Removal of the ovaries is complete.

APPENDIX C

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

Michael Kahl and Gerald Ankley

Nuptial Tubercle Scoring

Overview

Potentially important characteristics of physical appearance in adult fathead minnows in endocrine disrupter testing include body color (i.e., light/dark), coloration patterns (i.e., presence or absence of vertical bands), body shape (i.e., shape of head and pectoral region, distension of abdomen), and specialized secondary sex characteristics (i.e., number and size of nuptial tubercles, size of dorsal pad and ovipositor).

Nuptial tubercles are located on the head (dorsal pad) of reproductively active male fathead minnows, and are usually arranged in a bilaterally symmetric pattern (Jensen et al. 2001). Control females and juvenile males and females exhibit no tubercle development (Jensen et al. 2001). There can be up to eight individual tubercles around the eyes and between the nares of the males. The greatest numbers and largest tubercles are located in two parallel lines immediately below the nares and above the mouth. In many fish there are groups of tubercles below the lower jaw; those closest to the mouth generally occur as a single pair, while the more ventral set can be comprised of up to four tubercles. The actual numbers of tubercles is seldom more than 30 (range, 18-28; Jensen et al. 2001). The predominant tubercles (in terms of numbers) are present as a single, relatively round structure, with the height approximately equivalent to the radius. Most reproductively-active males also have at least some tubercles which are enlarged and pronounced such that they are indistinguishable as individual structures.

Some types of endocrine-disrupting chemicals (EDCs) can cause the abnormal occurrence of certain secondary sex characteristics in the opposite sex; for example, androgen receptor agonists, such as 17α-methyltestosterone or 17β-trenbolone, can cause female fathead minnows to develop nuptial tubercles (Smith 1974; Ankley *et al.* 2001; 2003), while estrogen receptor agonists may decrease the number or size of nuptial tubercles in males (Miles-Richardson et al. 1999; Harries et al. 2000).

This protocol describes characterization of nuptial tubercles in fathead minnows based on procedures used at the U.S. Environmental Protection Agency lab in Duluth, MN. Specific products and/or equipment listed can be substituted with comparable materials.

Protocol

Anesthetic
MS-222 is used as an anesthetic for fish sampling/assessment. Sodium bicarbonate is used as a buffering agent for the sedative.

Reagents:
MS-222 - Fenquel™ (Tricaine Methanesulfonate, Argent Chemical Laboratories, Redmond, WA 98052, USA).
Sodium bicarbonate - $NaHCO_3$, (J.T. Baker Inc., Phillipsburg, NJ 08865, USA).

Procedure:
1. Collect 1 L of control test water at nominal test temperature (e.g., 25°C) in a beaker
- Allocate 100 mg of MS-222 and 200 mg of sodium bicarbonate to weigh pan
- Add weighed chemicals to control water and stir (ca. 1 minute)
2. Transfer dissolved chemical solution to stainless steel bowl for easy fish handling
- Solution will accommodate 20 to 30 organisms (added individually); fresh solution will need to be prepared for additional animals

Sampling Methods

Procedure:
1. Using a 12.5cmX10cm (125mm) fine mesh nylon net, carefully net organism from culture or test chamber.
- If handling toxicant-exposed fish, start with control fish and work up with increasing EDC concentrations.
2. Place organisms in MS-222 solution.
- Activity level may be momentarily high with rapid swimming or darting. Activity will decrease but gill ventilation rate may become elevated or rapid.
3. Within about 1 minute fish will start to show loss of equilibrium, spiral or erratic swimming, loss of movement, listlessness.
- Gentle probing with the net will cause little physical response. Organisms are still actively ventilating.
4. Remove fish from anesthetic with a net. Wipe excess moisture from net and fish into an absorbent towel. Gently place fish on petri dish.

- Fish should not be actively moving; muscle tissue should still be rigid without loss of character. Continued emersion into MS-222 may be required. If potency of MS-222 is not adequate, additional chemical (≤ 10 mg) may be added to strengthen effectiveness.

5. Viewing is best accomplished using an illuminated magnifying glass or 3X illuminated dissection scope. View fish dorsally and anterior forward (head toward viewer).

a. Place fish in small petri dish (e.g., 100 mm in diameter), anterior forward, ventral side down. Focus viewfinder to allow identification of tubercles. Gently and slowly roll fish from side to side to identify tubercle areas. Count and score tubercles.

b. Repeat the observation on the ventral head surface by placing the fish dorsal anterior forward in the petri dish.

c. Observations should be completed within 2 min for each fish.

d. Return fish to control water to revive, if desired.

6. If fish are handled in a gentle manner within a reasonable amount of time during tubercle assessment recovery will occur within a few minutes without lasting adverse effects. To avoid mortality during and after this procedure be alert to the following details.

- Keep fish moist during procedure.
- Limit the amount of time used to score tubercles.
- When placing fish into clean water gently move the fish back and forth, aiding water movement across the gill membranes.

7. If tubercles are assessed at test conclusion, animal may be subjected to additional sampling at this time (e.g., removal of blood for vitellogenin measurements; dissection of gonads).

Tubercle Counting and Rating

Six specific areas have been identified for assessment of tubercle presence and development in adult fathead minnows. A template was developed to map the location and quantity of tubercles present (see below). The number of tubercles is recorded and their size can be quantitatively ranked as: 1-present, 2-enlarged and 3-pronounced for each organism (Figure 1C).

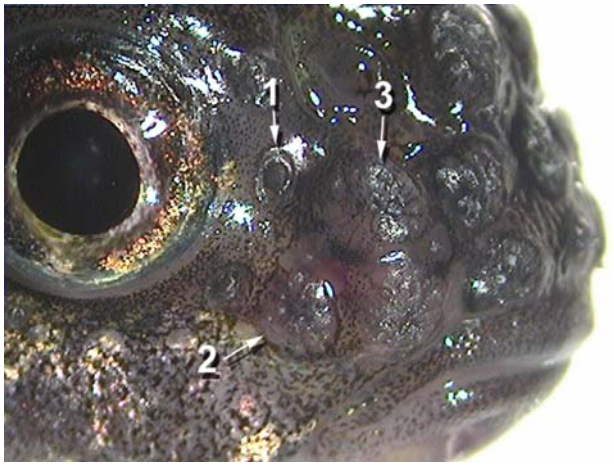


Figure 1C. The actual number of tubercles in some fish may be greater than the template boxes (see template below) for a particular rating area. If this happens, additional rating numbers may be marked within, to the right or to the left of the box. The template therefore does not have to display symmetry. An additional technique for mapping tubercles which are paired or joined vertically along the horizontal plane of the mouth could be done by double-marking two tubercle rating points in a single box.

Rating 1-present; identified as any tubercle having a single point whose height is nearly equivalent to its radius.
Rating 2- enlarged; identified by tissue resembling an asterisk in appearance; usually has a large radial base with grooves or furrows emerging from the center. Tubercle height is often more jagged but can be somewhat rounded at times.
Rating 3- pronounced; usually quite large and rounded with less definition in structure. At times these tubercles will run together forming a single mass along an individual or combination of areas (B, C and D, described below). Coloration and design are similar to rating 2 but at times are fairly indiscriminate. Using this rating system generally will result in overall tubercle scores of <50 in a normal control male possessing a tubercle count of 18 to 20 (Jensen et al. 2001).

Mapping regions:

A - Tubercles located around eye. Mapped dorsal to ventral around anterior rim of eye. Commonly multiple in mature control males, not present in control females, generally paired (one near each eye) or single in females exposed to androgens.

B - Tubercles located between nares, (sensory canal pores). Normally in pairs for control males at more elevated levels (2- enlarged or 3- pronounced) of

development. Not present in control females with some occurrence and development in females exposed to androgens.

C - Tubercles located immediately anterior to nares, parallel to mouth. Generally enlarged or pronounced in mature control males. Present or enlarged in less developed males or androgen-treated females.

D - Tubercles located parallel along mouth line. Generally rated developed in control males. Absent in control females but present in androgen-exposed females.

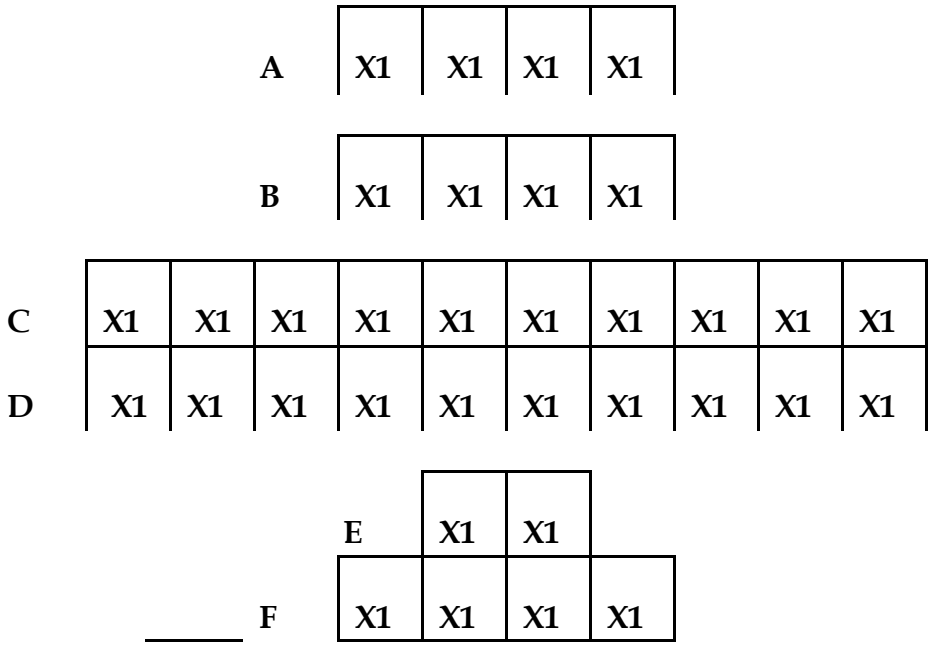
E - Tubercles located on lower jaw, close to mouth, usually small and commonly in pairs. Varying in control or treated males, and treated females.

F - Tubercles located ventral to E. Commonly small and paired. Present in control males and androgen-exposed females.

ID_____
Date_____
Total Score_____

Tubercle Template

Numerical Rating
1-present
2-enlarged
3-pronounced



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Fatpad Scoring

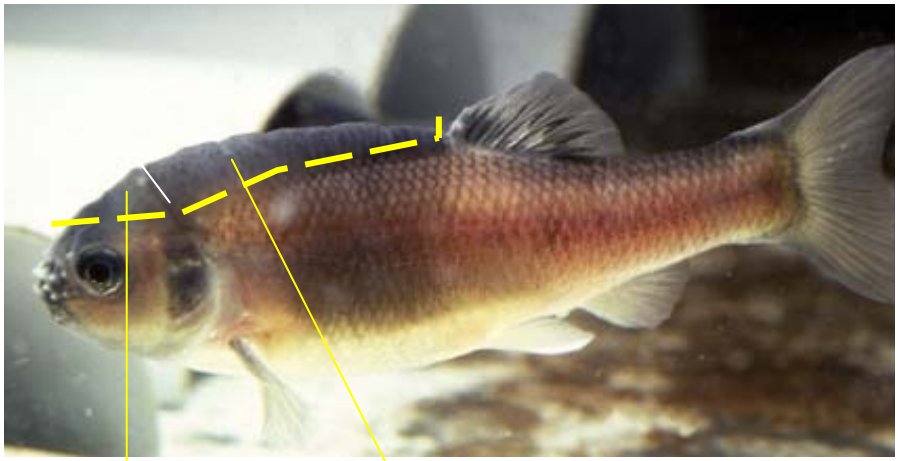
For evaluating the fatpads, use a very similar system as to that used in tubercle assessment:

1	No fatpad visible
2	Small fatpad evident
3	Fatpad is clearly visible and is just above body surface
4	Fatpad is prominent, and is clearly above the body surface, but not 'overhanging'
5	Fatpad is very prominent and is starting to 'overhang' the body surface

These evaluations are rather sufficient to identify chemical effects during exposure and should be accompanied by fatpad removal at the end of the experiment for a more accurate assessment of the fatpad (see fatpad index described below).

Fatpad Index (based on personal communication from Karen Thorpe)

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



Fatpad Index (FPI) – score around the edge of the fatpad using a scalpel and then starting from the dorsal fin and working towards the head, gently peel the fatpad away from the dorsal musculature. Be careful not to remove the muscle with the fatpad, as this will affect the overall weight. The fatpad consists of two regions, the head pad and the dorsal pad. Once you reach the head pad, stop, and then starting from the head and working back to the dorsal pad carefully slice of the head pad to the point where it is attached to the dorsal pad. Carefully sever any points at which the fatpad is still attached to the body of the fish. Weigh the fatpad.
Fatpad index (FPI) = (fatpad weight (mg)/total wet body weight (mg))*100%