Validation of the Fish Short-Term Reproduction Assay: Integrated Summary Report

U.S. Environmental Protection Agency
Endocrine Disruptor Screening Program
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OBJECTIVE: The objective of this integrated summary report is to provide a detailed account of the validation process for the Fish Short-term Reproduction Assay so that peer reviewers may address the attached charge questions.

1 INTRODUCTION

Section 408(p) of the Federal Food Drug and Cosmetic Act (FFDCA) requires the U.S. Environmental Protection Agency (EPA) to:

> *develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect as the Administrator may designate [21 U.S.C. 346a(p)].*

Subsequent to passage of the Food Quality Protection Act in 1996, which amended FFDCA and FIFRA, the EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), a committee of scientists and stakeholders that was charged by the EPA to provide recommendations on how to implement its Endocrine Disruptor Screening Program (EDSP). The EDSP is described in detail at the following website: http://www.epa.gov/scipoly/oscpendo/. Upon recommendations from the EDSTAC (EDSTAC 1998), the EPA expanded the EDSP using the Administrator's discretionary authority to include the androgen and thyroid hormonal systems as well as the endocrine systems of wildlife. Following broader international concerns and the creation of similar programs in other countries, the Organisation for Economic Co-operation and Development (OECD) established the Endocrine Disrupters Testing and Assessment (EDTA) Task Force in 1998 within its Test Guidelines Programme. EDTA is charged with developing an internationally harmonized testing strategy for the screening and testing of endocrine disrupting chemicals, taking into account the consequences of such a testing strategy on the development and validation of Test Guidelines, and on existing regulatory systems for new and existing substances.

The Fish Short-term Reproduction Assay (or Fish Assay) is one of the (anti-)estrogen- and (anti-)androgen-relevant screening assays proposed as part of the EDSP and is the subject of this report. This report complements the attached supporting materials and is meant to provide the peer reviewers with the necessary information, and/or direction to necessary information, to address the peer review charge questions. It introduces the purpose of the Fish Short-term Reproduction Assay and how it fits into the EDSP, the scientific rationale for the assay, and an historical account of the development and optimization of the assay protocol. It also synthesizes the information gained during the validation process and addresses the advantages and limitations of the Fish Assay based on its strengths and weaknesses, practicality, reproducibility, reliability, and protocol transferability.

1.1 Endocrine Disruptor Screening Program

To comply with its mandate, the EPA chartered a Federal advisory committee (EDSTAC) to provide advice and guidance on the development of a screening and testing program. In 1998,

it recommended to the EPA a conceptual two-tiered approach that involved screening and testing chemical compounds for effects on the estrogen, androgen, and thyroid (EAT) hormone axes. The ultimate goal of these assays is to provide input into hazard identification to assess risk of adverse consequences to humans and wildlife (EDSTAC 1998).

The EPA submitted a proposal of the EDSP for public review and comment (FRN 1998) as well as peer review by a joint subcommittee of the EPA Science Advisory Board and FIFRA Scientific Advisory Panel (SAB, SAP 1999). A complete description of the program proposal can be found in the Federal Register Notice (FRN 1998). Briefly, the EDSP proposed by EDSTAC allows for: 1) initial sorting and prioritization of chemical compounds, 2) identification of chemicals for further testing using a Tier 1 screening battery that includes *in vitro* and *in vivo* mammalian, amphibian and fish assays, and 3) characterization of adverse consequences resulting from possible endocrine disruption and establishment of dose-response relationships for hazard identification using Tier 2 testing.

In comparison to the more refined, detailed, and definitive tests in Tier 2, the EDSTAC indicated that the *in vitro* and *in vivo* screening assays in the Tier 1 battery should:

- be relatively fast and efficient;
- be standardized and validated;
- be more sensitive than specific to minimize false negatives without an unreasonable rate of false positives;
- be comprised of multiple endpoints that reflect as many modes of endocrine action as possible;
- have a sufficient range of taxonomic groups among test organisms represented; and
- yield data that can be interpreted as either negative or positive for determining the necessity and manner in which to conduct Tier 2 tests.

Together, the suite of Tier 1 assays will become a battery in which some endocrine axis redundancy is incorporated (e.g. two different assays may cover some similar aspects of the estrogenic response). This redundancy will allow for a weight-of-evidence approach, as recommended by EDSTAC, to determine whether a chemical shall undergo further, more definitive, testing.

The following assays, recommended by EDSTAC, were to undergo validation prior to their inclusion in the Tier 1 screening battery (**Table 1-1**, **Table 1-2**). The recommended assays are meant to detect chemicals that may affect the estrogen, androgen and thyroid hormone axes through any known modes of action.

Table 1-1. Assays recommended for consideration for the Tier 1 screening battery.

Assay	Reason for Inclusion
Estrogen Receptor Binding or transcriptional activation assay	An *in vitro* test to detect chemicals that may affect the endocrine system by binding to the estrogen receptor.
Androgen Receptor Binding or Transcriptional Activation Assay	An *in vitro* test to detect chemicals that may affect the endocrine system by binding to the androgen receptor.
In vitro Steroidogenesis Assay	An *in vitro* test to detect chemicals that interfere with the synthesis of the sex steroid hormones.
Uterotrophic Assay	An *in vivo* assay to detect estrogenic chemicals.
Hershberger Assay	An *in vivo* assay to detect androgenic and anti-androgenic chemicals.
Pubertal Female Assay	An *in vivo* assay to detect chemicals that act on estrogen or through the HPG axis that controls the estrogen and androgen hormone systems. It is also enhanced to detect chemicals that interfere with the thyroid system.
Amphibian Metamorphosis Aassay	An *in vivo* assay to detect chemicals that interfere with the thyroid hormone system.
Fish Short-term Reproduction Assay	An *in vivo* assay to detect chemicals that interfere with the HPG axis.

In addition, EDSTAC recognized there were other combinations of assays that might substitute for some components of the recommended battery and also recommended that EPA validate the following assays as alternatives.

Table 1-2. Alternative assays for Tier 1.

Assay	Reasons for Inclusion
Placental Aromatase Assay	An assay to detect interference with aromatase.
Pubertal Male	An *in vivo* assay to detect chemicals that act on androgen or through the HPG axis that controls the estrogen and androgen hormone systems. It is also enhanced to detect chemicals that interfere with the thyroid system.
Adult Male	An *in vivo* assay designed to detect chemicals that act on androgen or through the HPG axis that controls the estrogen and androgen hormone systems. It is also enhanced to detect chemicals that interfere with the thyroid system.

1.2 Validation

Validation has been defined as "the process by which the reliability and relevance of a test method are evaluated for a particular use" (NIEHS 1997; OECD 1996).

Reliability is defined as the reproducibility of results from an assay within and between laboratories.

Relevance describes whether a test is meaningful and useful for a particular purpose (OECD 1996). For Tier I EDSP assays, relevance can be defined as the ability of an assay to detect chemicals with the potential to interact with the endocrine system.

Validation is generally recognized as necessary for the regulatory acceptance of new and revised test methods, and is now an integral component of the international development and acceptance of these methods (OECD 2005a). The criteria used to guide the validation process for the Fish Short-term Reproduction Assay were based on the principles of validation developed by the U.S. Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) (NIEHS 1997) and the OECD (OECD 2005a). These criteria as stated by ICCVAM (NIEHS 1997) are as follows:

1. The scientific and regulatory rationale for the test method, including a clear statement of its proposed use, should be available.

2. The relationship of the endpoints determined by the test method to the *in vivo* biologic effect and toxicity of interest must be addressed.

3. A formal detailed protocol must be provided and must be available in the public domain. It should be sufficiently detailed to enable the user to adhere to it and should include data analysis and decision criteria.

4. Within-test, intra-laboratory and inter-laboratory variability and how these parameters vary with time should have been evaluated.

5. The test method's performance must have been demonstrated using a series of reference chemicals preferably coded to exclude bias.

6. Sufficient data should be provided to permit a comparison of the performance of a proposed substitute test to that of the test it is designed to replace.

7. The limitations of the test method must be described (e.g., metabolic capability).

8. The data should be obtained in accordance with Good Laboratory Practices (GLPs).

9. All data supporting the assessment of the validity of the test methods including the full data set collected during the validation studies must be publicly available and, preferably, published in an independent, peer-reviewed publication.

The EPA has adopted these various validation criteria for the EDSP as described in attachment A (EDSP 2005). Although attempts have been made to thoroughly comply with all validation criteria, various *in vitro* and *in vivo* screening assays under consideration for the Tier 1 battery are not replacement assays (Validation Criterion No. 6). Many of them are novel assays; consequently, large data bases do not exist as a reference to establish their predictive capacity (e.g., determination of false positive and false negative rates).

In general, the EPA is following a five-part or -stage validation process outlined by ICCVAM (NIEHS 1997). The EPA believes that it is essential to recognize that this process was

specifically developed for *in vitro* assays intended to replace *in vivo* assays. A rudimentary problem confronting the EPA is how to adapt and work with this process for rodent and ecological *in vivo* assays in Tiers 1 and 2 that have no suitable *in vitro* substitute. Nonetheless, the stages of the process outlined by the ICCVAM are as follows:

The first stage of the process was *test development*, an applied research function which culminated in an initial protocol. As part of this phase, EPA drafted a Detailed Review Paper (DRP) to explain the purpose of the assay, the context in which it will be used, and the scientific bases upon which the assay's protocol, endpoints, and relevance rest (attachment B). The DRP reviewed the scientific literature for candidate protocols and evaluated them with respect to a number of considerations, such as whether candidate protocols meet the assay's intended purpose, costs, and other practical considerations. The DRP also identified the developmental status and questions related to each protocol; the information needed to answer the questions; and, when possible, recommended an initial protocol for the initiation of the second stage of validation, *standardization and optimization*. During standardization and optimization, studies were performed geared towards refining, optimizing, and standardizing the protocol, and initially assessing protocol transferability and performance. The OECD Phase 1B reports summarized the optimization studies for the Fish Short-term Reproduction Assay and can be found in attachments C and D. In *inter-laboratory validation,* studies were conducted in several independent laboratories with the refined protocol. The results of these studies were used to determine inter-laboratory variability and to set or cross-check performance criteria. The report on the inter-laboratory trials for the Fish Short-term Reproduction Assay is provided in attachment E. Inter-laboratory validation is followed by *peer review*, an independent scientific review by qualified experts, and by *regulatory acceptance*, adoption for regulatory use by an agency. EPA has developed extensive guidance on the conduct of peer reviews because the Agency believes that peer review is an important step in ensuring the quality of science that underlies its regulatory decisions (US EPA 2006).

It should be remembered that even though assays are being validated and peer reviewed individually (i.e., their strengths and limitations are being evaluated as stand-alone assays), the Tier 1 assays will, in fact, be used in a complementary battery of screens.

The purpose of this Integrated Summary Report is to provide a historical summary of the development and validation of a standardized protocol for the Fish Short-term Reproduction Assay (attachment F) proposed as an *in vivo* assay for the Tier-1 screening battery. The reasoning and judgments leading to the various studies, and conclusions concerning the strengths and weaknesses of the assay in its current form, are presented.

2 HISTORICAL OVERVIEW OF THE FISH SHORT-TERM REPRODUCTION ASSAY

2.1 Relevance of Fish Short-term Reproduction Assay

Purpose of the Fish Short-term Reproduction Assay

The fish short-term reproduction assay with fathead minnows is designed to detect changes in spawning, morphology and specific biochemical endpoints that reflect disturbances in the HPG axis. It is important to recognize that the assay is not intended to quantify or confirm endocrine disruption, or to provide a quantitative assessment of risk, but only to provide suggestive evidence that certain endocrine regulated processes may be sufficiently perturbed to warrant more definitive testing. Although some endpoints may be highly diagnostic (*e.g.*, vitellogenin induction in males and tubercle formation in females), not all endpoints in the assay are intended to unequivocally identify specific cellular mechanisms of action, but collectively the suite of endpoints observed do allow inferences to be made with regard to possible endocrine disturbances and thus provide guidance for further testing.

Rationale for the assay

The process of gametogenesis and maturation of the gonads is regulated by hormones produced by the hypothalamus, pituitary and gonads, collectively termed the hypothalamus-pituitary-gonadal (HPG) axis. Environmental signals (i.e. photoperiod, temperature) trigger production of gonadotropin releasing hormone (GnRH) by the hypothalamus, which signals the pituitary gland to produce gonadotropins, including follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones enter the bloodstream and travel to the gonads where they bind to receptors and stimulate the gonads to synthesize the sex steroid hormones (estrogens, androgens, and progestins). Sex steroids act within the gonads to stimulate gametogenesis and feed back upon the pituitary gland and hypothalamus to regulate gonadotropin secretion. Thus, a complex environmental and endocrine signaling network controls gametogenesis, regulates gamete maturation, and induces changes in external morphology (secondary sex characteristics) and behavior that result in spawning. Exposure to contaminants that disturb any portion of this signaling network may lead to impaired gametogenesis and/or altered spawning. Because spawning represents the culmination of proper HPG axis functioning, changes in circulating levels of sex steroids or other biochemical or morphological endpoints controlled by the HPG axis may precede or occur simultaneously with impaired spawning, hatching, and larval survival.

2.2 Scientific Bases for the Assay

Overview of Fathead Minnow Life History & Reproductive Endocrinology

General Biology

The fathead minnow (*Pimephales promelas*) is a common freshwater cyprinid native to central North America, but has now been introduced throughout most of the continent (Pflieger 1975). Fathead minnows can live in a wide range of habitats, including shallow turbid waters which are less favorable to many fishes. Fathead minnows are an important bait-fish species and as a result have a long history as a cultured fish and are readily available from commercial sources. The fathead minnow has been used extensively in aquatic toxicity testing in the United States, and a number of testing guidelines exist, including detailed information on their laboratory culture (Denny 1987).

Fathead minnows are small (35 to 75 mm in total length), but are approximately 5-10 times larger than Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), two other fish models commonly used for toxicity testing. This size difference is significant as it allows for more reliable collection of individual blood samples of sufficient volume for biochemical analyses (Ankley et al., 2001; Seki et al., 2006). Fathead minnows tolerate wide variations in water quality, but for optimal growth and reproduction, water temperatures should be within a range of 24 - 25ºC, and dissolved oxygen should remain above 60% saturation. Adult fathead minnows are sexually dimorphic and can easily be sexed in the aquarium. The development of aggressive behavior and secondary sex characteristics in males and the presence of an ovipositor in females are signs of sexual maturity. Adult males are territorial, but will tolerate other males and females in an aquarium.

Fathead minnows are gonochoristic (gonads develop either as testes or ovaries and remain the same throughout the life span). Ovarian differentiation occurs between days 10 - 25 post-hatch at 25^{o}C (van Aerle et al., 2004). Testes differentiation is more difficult to distinguish, but appears to have begun by day 25 post-hatch and by day 60, the appearance of lobules and sperm ducts is clearly evident (van Aerle et al., 2004). For both sexes, complete sexual maturation occurs by day 120 - 150 post hatch at 25^{o}C (Jensen et al., 2001, van Aerle et al., 2004).

Spawning Behavior & Fecundity

Once sexual maturity is attained, fathead minnows spawn repeatedly over an extended period (Gale and Buynak 1982; Jensen et al., 2001). In the laboratory, fathead minnows can be kept in a reproductively-active condition by maintaining them at 25°C on a 16 h light:8 h dark photoperiod. Under these conditions, fathead minnows can be induced to spawn by placing a spawning substrate in the aquarium. Early studies by Till (1977) and Benoit and Carlson (1977) explored the effect of various substrate materials on spawning behavior and concluded that glass and stainless steel should be avoided due to reduced egg adhesion. More recent studies have achieved better adhesion using PVC tubing or terra cotta tiles as the spawning substrate (Jensen et al., 2001; Battelle 2002; Thorpe et al., 2006). A screened tray can be placed beneath the spawning substrate to collect non-adherent eggs (Battelle 2003a, 2005; Thorpe et al., 2006).

The daily timing of spawning and spawning frequency is well characterized in fathead minnows. Spawning is usually initiated just before light and can last through the morning (Harries et al., 2000; Jensen et al., 2001). Therefore, it is advisable not to disturb the fish during

the morning hours except to feed and collect embryos. On average, spawning will occur every 3 - 4 days although intervals up to 15 days have been observed (Jensen et al., 2001). The number of eggs per spawn can vary considerably from <50 up to ~ 400 (Jensen et al., 2001; Thorpe et al., 2006). Female body size directly influences fecundity and larger females typically produce more eggs per spawn (Gale and Buynak 1982; see control data in Battelle 2003a). Fecundity may also increase slightly with increasing time in the experiment, but this effect is not apparent when fish are acclimated to experimental conditions and partners before fecundity data are collected (Thorpe et al., 2006). There appears to be little age-related difference in fecundity among females aged five to sixteen months (Thorpe et al., 2006). Thus, for highest consistency in fecundity measurements, it is important to select fish, especially females, of similar size and known age.

After spawning, the eggs can be collected easily by removing the spawning substrate. A screened tray, if used, can also be removed to collect any eggs not adhering to the substrate. The embryos can be incubated on the spawning substrate or they can be removed from the substrate and incubated in a suitable container (Denny 1987; Ankley et al., 2001). Fertilization can be assessed immediately with light magnification or 24 hours after spawning by counting the number of transparent (fertile) and opaque or white (non-fertile) embryos. Fertilization rates of ≥ 95% are typical (Ankley et al., 2001). The time to hatch for embryos is 4.5 to 5 days at 25ºC (US EPA, 1996). The hatching rate typically is in the range of 95 to 98% (Ankley et al., 2001). The larvae are best reared in separate aquaria and should be offered live brine shrimp nauplii (Artemia) immediately after hatch (Denny 1987). Juvenile fish can be fed a mix of frozen brine shrimp and live nauplii, whereas adults can be fed a mixture of frozen brine shrimp and commercial flake food. All life stages should be fed ad libitum two to three times per day.

External and Gonad Morphology

Sexual dimorphism in fathead minnows is quite pronounced, with the mature male exhibiting dark vertical banding, distinct breeding tubercles on the snout and a thick fatpad located on the cranium, which may extend beyond towards the anterior dorsal surface. Mature females normally lack these features and typically exhibit a swollen abdomen and an enlarged ovipositor. Certain male secondary sex characteristics can be readily quantified, specifically the number of breeding tubercles and the thickness of the fatpad. A recent analysis of tubercle number relative to body weight (tubercle score index) found this parameter requires the least amount of change from control values to detect a significant effect (Watanabe et al., in review).

The relative mass of the ovaries can vary as much as 45% during a spawning cycle, with minimum values occurring the day of spawning and peak values occurring two days post spawn (Jensen et al., 2001). In contrast, the relative mass of the testes is relatively constant in reproductively-active males (Jensen et al., 2001). Consistent with asynchronous spawning behavior, histological examination of fathead minnow gonads shows gametes in all stages of maturation. Quantitative staging of gamete development within the gonads suggests that the relative numbers of gametes at specific developmental stages are dependent upon whether the fish is actively spawning and the time since the last spawning act. Immediately after spawning, ovaries are comprised primarily of oocytes in the primary growth stage (~55% of total oocytes), which progressively decreases in successive days as the number of late vitellogenic oocytes increases (Jensen et al., 2001). In healthy females, few atretic oocytes are observed in the ovaries (Jensen et al., 2001). In the mature testes of reproductively-active males, the majority of

the sperm are in more advanced stages of maturation consisting of spermatocytes, spermatids and spermatozoa (Battelle 2003a). In sexually-mature but non-spawning males, there is a shift towards increased numbers of spermatozoa (see Battelle 2003a, non-spawning control male data).

Reproductive Endocrinology

The process of gametogenesis and gonadal maturation is regulated by hormones produced by the HPG axis. GnRH released by the hypothalamus signals the pituitary gland to produce FSH and LH. FSH and LH are heterodimer glycoproteins possessing a common, species-specific α-subunit and a hormone-specific β-subunit (Swanson et al., 2003). Recent isolation and sequencing of the genes for the fathead minnow FSHβ and LHβ subunits indicate close sequence similarity (> 86%) with other cyprinids such as the carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) (Villeneuve et al., 2006a). In females, the primary cellular targets of the gonadotropins are the granulosa and theca cells surrounding the oocyte within the ovarian follicle (Nagahama 1987, 1994). These cells are stimulated by the gonadotropins to synthesize the sex steroids 17β-estradiol (E2), testosterone (T), and maturation inducing steroids. In many fish, the maturation-inducing steroid is 17α,20β-dihydroxy-4-pregnen-3-one (17,20 DHP) (Sakai et al., 1987; Rinchard et al., 1997). Of the two gonadotropins, the role of LH in stimulating granulosa cells to produce E2 and 17,20β-DHP is more clearly established (Iwamatsu et al., 1994; Senthikumaran et al., 2004). Additional studies in asynchronous spawning fishes indicate that pituitary expression of LH β mRNA is higher than FSH β mRNA during oocyte development (Gen et al., 2000, 2001), although expression of both genes is increased during the later stages of oocyte maturation (Degani et al., 1997; Jackson et al., 1999). In fathead minnows, synthesis of FSH β mRNA appears to be greater than LH β mRNA throughout sexual maturation (Villeneuve et al., 2006a). The reproductive function of FSH has been best characterized in synchronous spawning fish, where it is the primary gonadotropin responsible for directing growth of the gonads during the majority of the reproductive cycle (Swanson 1991). In other fishes with reproductive behavior more similar to fathead minnows, FSH synthesis is associated with the onset of gonadal recrudescence and subsequent early stages of oocyte maturation (Fan et al., 2003; Shimizu et al., 2003; Kumakura 2004). This would be consistent with the recent observation that FSHβ mRNA expression is higher in three month old male minnows compared to four and five month old males (Villenueve et al., 2006a). Similarly, FSHβ expression was higher in males with less mature testes and declined as maturity stage increased (Villenueve et al., 2006a).

The sex steroids may exert both positive and negative feedback control of gonadotropin secretion. A number of studies involving gonadectomy have generally indicated that circulating sex steroids exert negative feedback on gonadotropin release (Kobayashi and Stacey 1990; Larsen and Swanson 1997; Kumakura et al., 2003). Other studies have demonstrated that steroid treatment can increase the expression of gonadotropin beta subunit mRNA and pituitary content of the hormones (Querat et al., 1991; Antonopoulou et al., 1999; Mateos et al., 2002), implying that steroid feedback upon the pituitary is primarily mediated at the gene transcription level.

Sex Steroids

The production of sex steroids is a critical component of sexual maturation and spawning. The common precursor of the gonadal steroids is cholesterol which is converted to a series of intermediate steroids to form androstenedione, the direct precursor of T. T is converted to E2 by

the cytochrome P450 enzyme aromatase (CYP19). This is a major pathway in fish ovaries and a minor one in testes. T can also be converted to 11-ketotestosterone (11-KT) via the intermediate steroid 11β-hydroxytestosterone. This two-step process is mediated by 11β-hydroxylase (T → 11βOHT) and 11β-hydroxysteroid dehydrogenase (11βOHT → 11-KT). 11-KT production is the predominant pathway in fish testis and is the primary regulator of spermatogenesis and male secondary sex characteristics in most fish (Kime, 1998).

In fathead minnows the primary sex steroids are T and E2 in females and T and 11-KT in males. Low levels of 11-KT (e.g., pg/ml range) are sometimes reported in female minnows (Jensen et al., 2001) and in other female fishes (Lokman et al., 2002), although the physiological significance in minnows and other cyprinids is unclear. Testosterone is produced in significant quantities in both sexes and can reach levels equivalent to E2 in females but in males T concentrations are normally less than half those of 11-KT (Jensen et al., 2001). As in other iteroparous fishes, circulating levels of E2 and 11-KT vary as much as two-fold depending on the time period since last spawning and the overall maturational state of the gonads. In female minnows, plasma levels of E2 are at peak values one day after spawning and then gradually decrease until the next spawning (Jensen et al., 2001). Peak E2 values may be twice that of minimum levels. In male minnows, 11-KT levels are near minimum values on the day of spawning and then gradually increase (Jensen et al., 2001).

A third class of sex steroid produced in fish gonads is the progestins. Progestins are typically produced during the final period of gamete maturation in both sexes and are often referred to as the maturation-inducing steroids. Several different progestins including 17,20-DHP, and 17α, 20β, 21-trihydroxyprogesterone (20βS) have been identified in both synchronous and asynchronous spawning teleosts. 17,20-DHP is the most potent sex steroid involved in the initiation of oocyte maturation in zebrafish and medaka (Selman et al., 1994; Kobayashi et al., 1996). To date, the maturation-inducing steroid that is most important in fathead minnows has not been identified.

Vitellogenesis

The biological role of the egg yolk protein VTG and regulation of its biosynthesis is well established in fish, including fathead minnows. Synthesis of VTG occurs in the liver under E2 control, mediated by the estrogen receptor. There are two distinct estrogen receptor (ER) subtypes in fathead minnows, ERα or esr1 and ERβ or esr2 (Filby and Tyler 2005). ERα appears to be the primary mediator of VTG synthesis. After E2 binding, the activated ERα serves as a transcription factor to regulate expression of the two primary VTG genes (vtg1, vtg2) as well as a third VTG gene (vtg3) (Miracle et al., 2006). After its synthesis in hepatocytes, VTG is secreted into the bloodstream and transported to the ovaries where it is actively absorbed by maturing oocytes. The highest plasma concentrations of VTG occur on the day of spawning (Jensen et al., 2001). In reproductively-active female minnows, plasma levels of VTG are quite high, approximately 10 - 20 mg/ml, and vary less than E2 during a spawning cycle. Some studies have reported much lower values for VTG in female minnows (Harries et al., 2000). This discrepancy is likely due to differences in immunoassay methods, specifically the use of carp anti-VTG antibodies as opposed to fathead minnow specific anti-VTG (Mylchreest et al., 2003).

In healthy male fathead minnows, VTG levels are normally extremely low (< 0.004 mg/ml) or undetectable (Jensen et al., 2001). However, both male and female fathead minnows

can be induced to synthesize VTG after estrogen or estrogen-mimic exposure. Thorpe et a. (2007) showed that high levels of VTG induction or suppression in female fathead minnow are associated with alterations in health status and reproductive fitness. VTG, therefore, has the potential to act as a health measure for exposure to chemicals that alter the HPG axis (Miller et al., 2007; Thorpe et al., 2007). This response forms the basis for using circulating VTG concentration as a specific biomarker of estrogen exposure (Kramer et al., 1998; Pawlowski et al., 2004a). Past studies on VTG induction and elimination from the blood of male fishes administered a single bolus dose of an estrogen, suggest a consistent pattern of synthesis rate, secretion and elimination. For example, when adult male fathead minnows were given an intra-peritoneal injection of E2 at 0.5 mg/kg or 5.0 mg/kg, the plasma concentration of VTG peaked after 48 to 144 hours post-injection and declined slowly, with plasma levels still within 50% of maximal values after 18 days (Korte et al., 2000). Another study determined the half-life of VTG in male fathead minnow plasma to be 21 days (Schmid et al., 2002). During a 35-day waterborne exposure to a synthetic estrogen, ethinylestradiol (50 ng/L), plasma VTG in males peaked after 14 days of exposure, remained elevated until 3 days after exposure ended, and declined thereafter (Schmid et al., 2002). Thus in male minnows, unnaturally expressed VTG can persist in blood for several weeks, minimizing the probability that transient induction of VTG will be missed with single time point sampling.

3 PROTOCOL DEVELOPMENT, OPTIMIZATION AND FEASIBILITY

3.1 Summary of the Fish Short-term Reproduction Assay Protocol

A description of the 21-day fish reproduction assay discussed herein was published by Ankley et al. (2001) (attachment G). This short-term test measures aspects of reproductive function of fathead minnows as an indicator of disruption of the HPG axis and, in addition to reproductive output, features measurements of morphology, histopathology and biochemical endpoints to help identify mode(s) of action of the test chemicals.

The test is started with recently matured adults (four – six months in age, though older fish can be used) that have a record of reproductive success measured by fecundity (number of eggs spawned) and embryo viability (hatch rate). This is established during a 14 to 21 day pre-exposure period in the same system/tanks as will be utilized for the chemical test. The test should be conducted with a minimum of two chemical concentrations, as well as appropriate control(s), with four experimental replicates per treatment. Each replicate tank contains four female and two male fish. Three spawning substrates, made from PVC tubing or clay pots longitudinally sectioned are placed into each tank. Under these conditions a female will deposit eggs on the inside portion of the spawning substrate. A tray covered by a mesh screen can be placed directly underneath to collect any eggs not adhering to the spawning substrate.

The exposure route for the test chemical is normally via the water using an appropriate flow-through delivery system such as that achieved with a proportional diluter. The exposure is conducted for 21 days, during which appearance of the fish, behavior, fecundity, and fertilization rate (percentage of eggs that are fertilized) are assessed daily. The percentage of embryos

hatched and 24-hr post-hatch viability of larvae are optional endpoints that are typically assessed once during the exposure and at the end of the exposure period. At termination of the exposure, secondary sex characteristics are assessed and blood samples collected and analyzed for VTG and sex steroids. The gonads are removed and weighed for gonadosomatic index (GSI) determination, then placed in appropriate fixative for later histological analyses.

The optimized Fish Assay protocol is designed to obtain the maximum amount of data from the minimum number of animals in the least amount of time necessary to detect a potential endocrine active substance. The incorporation of several endpoints to detect disruption of male and/or female reproductive systems ensures that the most information possible is obtained from each test. As with most *in vivo* animal tests, the fish employed in this assay must be humanely euthanized at the end of the test.

3.2 Study Design, Test Chemicals and Dose Selection

Equipment and Supplies (Husbandry, Endpoint Measurements, Analyses, etc.)

The equipment needed to perform the fish short-term reproduction assay can be separated into in-life and post-exposure phases of the study. During the in-life phase of the test, an appropriate aquatic animal culture facility capable of providing the minimal husbandry requirements (Table 3-1) is required. This includes providing an adequate supply of warm (25^{o}C) fresh water that is either naturally or artificially de-chlorinated (if a municipal water supply is used). Some form of emergency generator power is desirable in case of power outage. Performance of the test can generate large quantities of waste effluent so it is important that the facility has an approved method or procedure for proper disposal. Five gallon (19L) glass aquaria are the preferred test chambers and sufficient quantities of tanks are needed to house fish both before and during the test. For a one chemical test, this would be a minimum of 18 tanks, to allow a fish surplus during pre-exposure spawning assessment. These tanks are in addition to those needed for pre-test holding and acclimation or rearing on-site. Sufficient numbers of spawning substrates to provide three per tank are needed. These can be made from PVC pipe or clay pots (Battelle 2002). Actively-spawning fathead minnows need to be fed adult brine shrimp at least twice daily. Frozen brine shrimp are an acceptable feed. A toxicant delivery system is also needed to generate test solutions at the specific exposure concentration. This is normally performed using a proportional diluter such as that originally described by Mount and Brungs (1967) or other acceptable approaches (Garton 1980), such as pump-driven systems.

During the post-exposure phase of the test, biochemical and histological analyses are performed. Both during and immediately after the assay, assessment of the chemical exposure level is made. Each of these analyses has specialized equipment needs. Direct determination of chemical concentrations in the exposure media requires the availability of an appropriately equipped analytical chemistry laboratory. Standard equipment used for routine analysis would include high performance liquid chromatography (HPLC) and gas chromatography (GC) with a variety of detection methods that should include at a minimum, UV detection for the HPLC and flame ionization and electron capture detectors for the GC. A mass spectrometer (MS) either linked with the HPLC or GC is also desirable. For a few compounds, it may be possible to quantify exposure concentrations using analytical methods that do not involve chromatography, such as

immunoassay. However, immunoassay methods are unlikely to be available for the majority of test compounds.

Table 3-1. Summary of animal husbandry requirements for the optimized Fish Short-term Reproduction Assay.

Parameter	Assay Protocol
Test species:	Reproductively active fathead minnows (minimum 120 day old)
Pre-exposure evaluation	Duration: 14 days; Data Collected: fecundity
Dilution water	Clean, surface, well or reconstituted water
Test chamber size	19 L (40 x 20 x 20 cm)
Test volume:	10 L
# Exchanges/day	6 tank volume exchanges
Flow rate:	2.5 L / hr
# Replicates:	4
Weight of each fish	Not specified, but preferable to use males < 5-6 g
# Fish/vessel	4 females and 2 males
Total # fish/concentration	16 females and 8 males
Feeding regime	Frozen brine shrimp, twice a day
# Controls	1; Dilution water control, solvent control if solvent is used
# Fish/control	4 adult females and 2 adults males per replicate = 24 fish total per exposure concentration (96 fish per test chemical)
Photo period:	Constant day length: 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	6.8 – 8.3
Biological endpoints:	Adult survival, reproductive behavior, secondary sexual characteristics, GSI, gonadal histology, VTG, fecundity and fertility
Test validity criteria:	D.O. = 60% saturation; Mean temp. 25˚C ± 2˚C ; 90% survival in the controls and successful egg production in controls.

The core biochemical endpoints (VTG, sex steroids) can be measured using commercially available immunoassay kits if desired. These kits rely on absorbance, fluorescence, or radiological detection. Thus, essential equipment would include spectrofluorometer capable of working with 96-well plates (the standard format for most enzyme-linked immunoassay [ELISA] kits) or a liquid scintillation counter (for ^{14}C or ^{3}H counting). Other equipment needed for the biochemical measurements are standard items found in well-equipped biological laboratories

such as digital pipettors, centrifuges, a vortex mixer, fume hood, refrigerator, -20 and -80 ^{o}C freezers, pH meters and analytical balances.

Histological analyses of the gonads require specialized equipment for processing, embedding, and sectioning tissue samples. Other necessary equipment includes a compound light microscope for examination of tissue sections.

Animals (Species/Strain, Sex, Age, Weight, Number)

The assay is preferably started with newly-mature fish (typically 4 to 6 months old), as opposed to older animals that have been actively reproducing for an extended period of time. To ensure a sufficient supply of healthy fish fully acclimated to the test facility, maintaining a fathead minnow culture on-site is preferable to purchasing or rearing fish at an off-site location. To achieve genetic heterogeneity among the test fish, it is desirable to draw test fish from a pool of offspring produced by 10 – 20 breeding pairs in the culture facility. Field-collected fathead minnows should not be used to initiate cultures or for testing. A weight range for test fish is not specified, although maintaining loading densities < 1-2 g (total fish weight)/L of tank volume minimizes crowding. Thus, male fish below ~ 5-6 g in body weight are preferable. Because of the smaller size of females, it is not advisable to limit their size other than to achieve overall consistency in body size. The fish are fed frozen adult *Artemia ad libitum* twice daily. Table 3-1 provides a tabular summary of the experimental design for the optimized group spawning protocol.

Test protocols that include spawning need to establish the reproductive competence of the fish prior to chemical exposure. This is called the pre-exposure phase and should be started with fish that have achieved reproductive maturity, as evidenced by initial development of secondary sex characteristics, but have not been held in a culture/test situation conducive to routine spawning. Groups or breeding pairs should be housed identically to the set-up used for chemical exposures. The fish should be monitored daily for obvious alterations in secondary sex characteristics (breeding tubercles in males, ovipositor in females), reproductive behavior and spawning activity. The pre-exposure phase should last at least 14 days; if acceptable spawning has not occurred within 28 days, an assessment should be made as to why satisfactory biological performance had not been achieved. This might entail examination of water quality or condition of the fish. Some criteria for acceptable pre-exposure performance of tanks to be selected for exposures include 1) 100% survival of all adults, 2) presence of eggs in each replicate tank every 3 to 4 days, and 3) >90% fertility of the spawned embryos (Ankley et al., 2001).

Experimental Design

Three different types of experimental designs have been used during optimization studies. These can be divided into group spawning, pair breeding and non-spawning designs. More than sixteen variations in these protocols have been used due to different tank configurations, numbers of fish and tank replicates and test duration. An overview of the merits and drawbacks to each design is provided in this section and more specific details on performance are described in Section 2.3.

Group Spawning

Several test configurations have been studied using equal numbers of males and females or a 1:2 ratio of males to females. Both approaches have provided acceptable results although a 1:1 sex ratio can increase the potential for aggressive behavior among males, which may exhibit territorialism towards females (Martinovic et al., 2007). The optimum configuration appears to be two males and four females per tank for the Fish Short-term Reproduction assay. Because females can typically spawn every 3-4 days, this improves the likelihood that at least one female will spawn each test day if all females are spawning. Another advantage of having excess females in each tank replicate is to increase the sample size for biochemical measurements. Typically, less blood is collected from females compared to males, which can limit the number of E2, T and VTG assays performed. The use of two males and four females per tank replicate allows tank replication to be limited to four units per treatment while still providing sufficient statistical power in the analysis (Ankley et al., 2001). This is a convenient aspect of the protocol when performed with proportional diluters, which are typically designed to deliver water for up to 12 tanks. Thus, two treatment levels and a control group can be exposed from one diluter.

The primary drawback to group spawning protocols is the inability to associate fecundity with a specific female. This prevents normalizing fecundity data to female body weight and directly linking changes in biochemical endpoints with fecundity for a specific female. However, this drawback is not detrimental in the context of a short-term assay and careful selection of fish to insure uniform size minimizes these concerns.

Pair Breeding

For some aspects of the analysis of fecundity data and behavior, pair breeding protocols can prove superior to group spawning designs. Reproductive performance of specific fish before and after chemical exposure can be compared directly as opposed to comparing pre- and post-exposure performance of a group (Harries et al., 2000). Total and daily fecundity can be normalized to female body weight, which may improve statistical comparisons across tank replicates. Abnormal spawning behavior also may be easier to assess in a breeding pair as opposed to a group. Drawbacks to pair breeding are primarily greater cost and the inevitable compromises that must be made with numbers of fish and tank replication. Ideally, at least eight breeding pairs per treatment should be used to provide fish numbers similar to those used in group spawning protocols. Maintenance of eight tanks per treatment can be difficult and costly, so some have used multiple pairs in a tank separated by a divider (Harries et al., 2000; Ankley et al., 2005a; Villeneuve et al., 2006a), reducing the number of tank replicates. If an insufficient number of breeding pairs is used, high individual variation in fecundity or spawning interval may reduce the statistical power to differentiate treatment effects from controls. Another concern is unscheduled mortality, which reduces the number of replicates during the test. The latter is less of a problem with group spawning protocols, which can endure some mortality and still provide useful fecundity data.

Non-Spawning

The advantage of non-spawning protocols is the reduced labor needed to maintain an exposure as fecundity measurements can be highly labor intensive. Both sex-specific and mixed sex protocols (whereby each sex is separated by a mesh screen in the tank) have been used (Battele 2003a; OECD 2006). A key drawback, particularly to sex-specific exposures is the

effect on gonad histology and circulating sex steroids. Both quantitative and qualitative staging of gamete development in fish maintained in non-spawning configurations shows a preponderance of mature gametes. Also, high numbers of atretic follicles may be observed in control females. This can make it difficult to detect chemical-induced changes in gonad histology. Other evidence suggests greater variability in biochemical endpoints measured in non-spawning compared to group spawning fish (Battelle 2003a).

The overall conclusion based on results from over sixty individual studies encompassing twenty (20) chemicals and seventeen (17) laboratories is that the optimum protocol utilizes group spawning using two males and four females housed in one tank replicate containing three spawning substrates. Each treatment has four tank replicates. The preferred test duration is 21 days with 14 days being the minimum duration. At least eighteen (18) studies conducted in five (5) different laboratories used the 21-day exposure period in optimization studies. Throughout the remainder of this document, the term "optimized protocol" refers to the 21-day group spawning protocol with two males and four females per tank replicate, four tank replicates per treatment (see attachment F).

Dose Selection Procedures, Number of Dose Levels, and Range-finding Studies

For most optimization studies discussed herein, particularly those with a previously untested substance, three to five different test concentrations or treatment levels were used. These treatment levels typically covered a minimum 10-fold change in concentration and frequently were in excess of 100-fold changes in concentration. In some instances, only one or two test concentrations were used due to limited availability of the test substance or when evaluating comparability of findings. Essentially all optimization studies used sub-lethal concentrations. In some cases, water solubility limitations provided an upper limit for the exposure concentration. Nominal exposure levels above water solubility were never performed. For many chemicals tested during the optimization phase, sufficient data from previously published studies were available to guide selection of non-lethal concentrations.

For new test chemicals or negative control chemicals, range-finding studies were necessary. The general approach employed one to two tank replicates per concentration, with exposure lasting four to seven days (Ankley et al., 2001; Battelle 2006). From the range-finding tests, the highest concentration that did not result in increased mortality or signs of overt morbidity compared to controls served as the highest exposure concentration in subsequent reproductive tests. The low concentrations were determined by step-wise 10-fold reductions of the high concentration, as suggested by U.S. EPA (2001).

Because of the potential for large discrepancies between nominal and measured concentrations of a test chemical, all studies included some direct measure of the exposure level using an accepted analytical procedure. The preferred method was to sample directly from the exposure chamber on at least a weekly basis during the exposure. Exceptions to this requirement are noted in the text. Concentration– response evaluations of test results are based on the mean measured value unless noted otherwise.

Route of Test Substance Administration

Essentially all optimization studies described herein relied upon water as the route of exposure. The rationale for this approach is based on several factors. Water exposure is both ecologically relevant and the most common laboratory route for exposing fish to chemicals, thus facilitating transferability of the protocol and inter-laboratory studies. From a practical perspective, water exposures are generally easier to maintain at specified treatment levels, facilitating concentration-response evaluations. An additional advantage of water exposures is the passive nature of dosing; fish-handling for injections is avoided and all fish are exposed regardless of whether they consume feed.

Although aqueous exposures have been the preferred route for optimization studies, it should be noted that both controlled oral dosing and intra-coelomic (IC) injection (equivalent to intra-peritoneal injection in mammals) can be used for short-term reproduction assays with small fish such as the fathead minnow (Kahl et al., 2001; Muirhead et al., 2006). Dietary exposure is ecologically relevant and can be an advantageous route with compounds of poor water solubility or high instability in aqueous solutions (Schultz et al., 2007).

Dosing Schedule

The preferred dosing schedule for a short-term fish assay is continuous daily exposure. This approach improves confidence that desired test concentrations are achieved and that fish experience the target concentration of the test agent. All studies included in the optimization analysis utilized this approach. A few studies used a static renewal system as opposed to a continuous flow-through exposure system and are noted in the text. With regard to test duration, the preferred upper limit is 21 days. In some studies a shorter duration of 14 days was used. The 14 to 21 day exposure duration is based on the need to balance assay performance costs with the requirement to provide a sufficiently long exposure period to allow physiological responses to develop. Exposure must be continued long enough to evaluate toxicant effects on gametogenesis and fertilization over multiple spawning cycles or intervals. The extensive database on chemical bioaccumulation in fathead minnows suggests 21 days is a sufficient time period for pseudo-equilibrium to be achieved between the exposure water and fish tissues for most substances (Veith et al., 1979). Additional confidence that 14 to 21 days is sufficiently long to detect changes in core endpoints comes from results of several optimization studies described below. In some cases, reduction in spawning and alterations in secondary sex characteristics became apparent within 1 and 6 days of exposure respectively (Ankley et al., 2001, 2003), indicating the capacity of these endpoints to respond rapidly to endocrine perturbations.

3.3 Endpoints

Survival

Daily assessment of survival is necessary to provide a basis for interpreting reproductive output (number of eggs/female/day) and to quantify chemically-induced mortality.

Behavior

General observations should be made daily and unusual behavior (relative to controls) should be noted. Signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding are important to document. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, should also be noted.

Fecundity

Spawning and the quantity of fertilized embryos can be the most useful indicators of the general reproductive condition of mature fish as these endpoints reflect successful integration of a variety of physiological processes. Successful spawning requires production of mature gametes and performance of appropriate mating behavior to achieve fertilization and deposition of eggs on the spawning substrate. The general spawning strategy of fathead minnows is to produce many eggs that are initially protected by males, allowing large numbers of eggs to be produced in a relatively short time. Thus, disturbances in the HPG axis that directly or indirectly impair gamete maturation and/or interfere with reproductive behavior will reduce spawning frequency and fecundity.

During the chemical exposure, egg production should be determined daily. The spawning substrates can be removed from the tanks and inspected under appropriate magnification to count the eggs. If no eggs are present, the substrate is left in the tank; new substrates should be added immediately to replace any that are removed. Fecundity should be expressed on the basis of surviving females per day per replicate.

Fertilization Success

After the spawning substrate has been removed from the tank, the embryos should be carefully rolled off with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, determination of the fertility rate [100 x (number embryos/number of eggs)] is 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. An alternative to the microscopic approach to determining fertilization success is to enumerate eyed embryos at this time. If the latter approach is used for fertility determination, the embryos should be placed in incubation chambers and held in a system apart from the adults to avoid possible predation. Fertility is expressed as a percentage of fecundity and is typically $\geq$ 95%.

Hatchability, Larval Appearance, and Survival

If information concerning hatching success and/or development is desired, embryos can be removed from the substrate and maintained in incubation chambers. To maintain adequate water quality, the incubation system should either provide a continuous flow of water, or the test solution renewed daily. During this period of time, alterations in normal embryologic development can be assessed. At 25°C, untreated animals will hatch in 4.5 to 5 days. Incubation chambers should be evaluated daily and any dead animals counted and removed. Potential

endpoints include time to hatch, total number of embryos hatched, and number of normal larvae at hatch. The hatching rate of control animals typically is in the range of 95% to 98%, and should be expressed as a percentage of those eggs deemed fertile.

The appearance and behavior of hatched larvae can be evaluated, and results described either qualitatively or quantitatively (e.g., malformation rate). Gross morphological anomalies that may be observed include lordosis, scoliosis, kyphosis, retarded swim bladder development, and craniofacial abnormalities. Survival of the larvae may be assessed through yolk sac absorption (ca. 32-h post-hatch at 25°C); if estimates of survival beyond this are required, the animals must be fed (generally live *Artemia*).

Appearance and Secondary Sex Characteristics

Observations of the external appearance of the adults should be made throughout the test and at conclusion of the study. Noteworthy changes to document include changes in body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and especially secondary sex characteristics (size of dorsal fat pad, number/appearance of nuptial tubercles in males; ovipositor size in females).

The growth and formation of male secondary sex characteristics such as the fat pad and nuptial tubercles can be modulated by estrogen and androgen mimics and antagonists. Thus, this endpoint can provide information about a chemical's mode of endocrine activity. For example, as early as 1974 it was demonstrated that female fathead minnows will develop both rudimentary fat pads and nuptial tubercles when exposed to a strong androgen such as 17α-methyltestosterone (Smith 1974). Estrogenic chemicals can cause regression of these characteristics in males (Miles-Richardson et al., 1999a). Thus, formation of these structures in females is indicative of androgen exposure and reduction in males implies a disturbance in androgen signaling, possibly caused by estrogenic or anti-androgenic modes of action.

Gonad Histology

Histological methods have been recognized as the most accurate approach for staging reproductive development in fish (West 1990). However, a primary difficulty in applying histological analysis is that interpretation may vary from one investigator to another due to the qualitative to semi-quantitative approaches used to describe tissue alterations. In recent years, substantial effort has been placed on developing quantitative methods for histological analysis of the gonads in fathead minnows (Wolf et al., 2004; Leino et al., 2005). Characterizing the frequency distribution of gamete stages present in the gonads allows statistical analysis of any toxicant-induced changes (Wolf et al., 2004). In the testes, other measures such as the diameter of the seminiferous tubules and hyper/hypoplasia of Sertoli cells have also been used (Miles-Richardson et al., 1999a, b; Battelle 2003a). A recent review of the histological changes caused by exposing fathead minnows to various endocrine disruptors concluded that a general pattern of response is typically observed, especially in the ovaries, and is not specific to toxicant mode of action (Leino et al., 2005). Although histological analysis by itself cannot differentiate modes of action, it has proven to be especially valuable for separating chemically-induced effects from artifacts caused by poor animal husbandry (OECD 2006) and for establishing the physiological significance of changes in biochemical endpoints (see attachment H).

The general procedure for histological analysis is to preserve the gonads in a suitable fixative (e.g., 10% neutral buffered formalin or Davidson's fixative) and later embed in paraffin. Serial sections 4 to 5 µm thick are cut along the long axis of each gonad. A minimum of two serial sections are collected from three steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six tissue sections per sample. Sections can then be stained with hematoxylin and eosin, and submitted to qualified personnel for interpretation.

Histopathology is an important endpoint in the test because it is a direct evaluation of the reproductive organs of interest, and histopathologic changes embody the integration of several molecular, cellular and physiologic processes. In addition, it provides insight on the potential reproductive impacts of chemical disruption, and it is useful to confirm changes in other endpoints of the assay.

Histopathology has served as a "gold standard" in the mammalian toxicology testing arena. Even though it has not received as much attention in other vertebrate taxa, it has proven to be useful in understanding and assessing the effects of endocrine active substances in fish (Wester and Canton 1991; Wester and Van der Ven 2000; Ankley et al., 2001; Ankley et al., 2002; Ankley et al., 2003; Van der Ven et al., 2003; Ankley et al., 2004a; Ankley and Johnson 2004b) and is an integral part of the fish screen. Due to the qualitative nature of the science of histopathology, standardization of reading practices, diagnostic terminology, and severity grading approaches has been necessary to reduce time and effort required by pathologists, as well as to improve diagnostic consistency and reduce individual bias while reading studies. For an in depth description of the standardization efforts that have been put forth in the validation effort for the fish screen, please refer to attachment H. Briefly, a list of primary and secondary diagnoses has been established that minimizes the time and reporting effort required by pathologists reviewing studies, and standardizes the description of criteria used for diagnostic purposes included in the tables below.

Table 3-2a. Primary diagnostic criteria for histological analyses of male and female gonads.

Males	Females
Increased proportion of spermatogonia	Increased oocyte atresia
Presence of testis-ova	Perifollicular cell hypertrophy/hyperplasia
Increased testicular degeneration	Decreased yolk formation
Interstitial cell hypertrophy/hyperplasia	Gonadal staging

Table 3–2b. Secondary diagnostic criteria for histological analyses of male and female gonads.

Males	Females
Decreased proportion of spermatogonia	Interstitial fibrosis
Increased proteinaceous fluid within the testicular vessels or interstitium	Egg debris in the oviduct
Asynchronous gonad development	Granulomatous inflammation
Altered proportions of spermatozoa or spermatocytes	Decreased post-ovulatory follicles
Altered gonadal staging	
Granulomatous inflammation	

In addition to determining the incidence of diagnostic criteria in the fish gonads, the severity of the lesions is also evaluated. In toxicologic pathology, it is recognized that compounds may exert subtle effects on tissues that are not adequately represented by simple binary (positive or negative) responses. Severity grading involves a semi-quantitative estimation of the degree to which a particular histomorphologic change is present in a tissue section (Shackelford et al., 2002). The purpose of severity grading is to provide an efficient, semi-objective mechanism for comparing changes (including potential compound-related effects) among animals, treatment groups, and studies. The severity grading scheme employed includes: not remarkable, grade 1 (minimal), grade 2 (mild), grade 3 (moderate) and grade 4 (severe), where the severity is evaluated using broad categories based on discrete changes, changes that are spatial, and changes that are global.

Biochemical Endpoints

Collection of Blood

An often overlooked factor in methodological comparisons of biochemical endpoints is blood sampling technique and subsequent representativeness of the plasma collected. This problem is especially important with small fish as the potential for hemolysis and/or inadvertent dilution with water from the surface of the fish or interstitial fluid is increased compared to larger animals (Congleton and Lavoie, 2001). Non-lethal or in-life sampling of blood is impractical with small fish such as fathead minnows because of the high stress imposed upon the animal. Thus, terminal blood sampling is performed at conclusion of the exposure by immersion in an anesthetic solution such as tricaine methanesulfonate (MS-222; used at 100 to 250 mg/L and buffered with 200 mg $NaHCO_3$/L). There are several approaches for terminal collection of blood such as severing the dorsal gill arches, decapitation and sampling from the heart, direct cardiac puncture or severing the caudal peduncle and sampling from the caudal vein (Ikeda and Ozaki 1981; Watson et al., 1989; Allen 1994). Of these methods, caudal vein sampling is normally

used with fathead minnows because of the relative technical ease and transferability between labs. Blood is collected from the caudal vein by severing the vein externally with a scalpel and collecting the blood with a heparinized microhematocrit capillary tube. Prior to severing the vein, the fish should be patted dry to minimize dilution of the sample. Plasma is then separated via centrifugation (3 min) and stored with or without protease inhibitors (i.e. aprotinin) at -80ºC. Depending on the size of the fathead minnow, blood volumes generally range from 5 µL to 25 µL for females and 20 µL to 60 µL for males.

Sex Steroids

Quantification of sex steroids in blood plasma is normally performed using an immunoassay such as radioimmunoassay (RIA) or enzyme-linked immunoassay (EIA). The reagents for sex-steroid RIAs have been commercially available either in complete kit form or as individual components for over 30 years, whereas EIA kits are a more recent development. Current gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC)-MS protocols that do not rely on ligand-antibody reactions have also been used for simultaneous quantification of naturally-occurring steroids in plasma (Dorgan et al., 2002). Depending on the specificity of the antisera, some form of sample preparation is occasionally applied to remove interfering substances. This can be helpful for immunoassay-based detection of androgens, where Sephadex LH-20 chromatography has been shown to improve analysis of T and 11-KT, which are prone to some cross reactivity with some antibodies (Lokman et al., 2002). However, direct analysis of E2 and T in female fathead minnow plasma and T and 11-KT in males without prior chromatographic separation has been reliably demonstrated (Ankley et al., 2001; Jensen et al., 2001). An informal inter-laboratory comparison of sex steroid values in control fathead minnows from EPA-Duluth and Battelle studies indicated that values were typically lower in the Battelle studies by a factor of 1 to 2 (Battelle 2003a). A more formal inter-laboratory study of sex steroid levels in carp plasma also observed high inter-laboratory variation in reported values (McMaster et al., 2001). Despite the inter-laboratory variation in measurements, both studies noted internal consistency in measurements of differences between control and exposed fish and with respect to hormone variation due to reproductive status.

Vitellogenin

Among the variety of direct and indirect methods to detect VTG in blood plasma, the most widely applied methods are immunoassays, ELISA and RIA. These methods exploit the highly specific interaction of antibodies and the VTG antigen. RIA and ELISA use homologous polyclonal or monoclonal anti-VTG antibodies to quantify VTG in fathead minnows, although heterologous antibody cross-reactivity with fathead minnow VTG allows for heterologous anti-VTG antibody use. Prior to the recent commercial availability of VTG ELISA kits specific for fathead minnow, carp or goldfish anti-VTG were often used as surrogates. The latter approach limits comparisons of absolute VTG levels across ELISA methods (for example, see Battelle 2003a), but has nonetheless proven useful in optimization studies for characterizing changes in VTG expression due to chemical exposure. The commercial availability of fathead minnow VTG ELISA kits is likely to improve reproducibility between assays (Jensen and Ankley 2006). A limitation of ELISA is the need for sample dilution to reduce interference in the assay, effectively raising the minimum detectable concentration in plasma compared to RIA which does not require plasma dilution. The small volume of plasma available from individual fathead minnows theoretically limits sensitivity, but in practice, the requirement for dilution means that

small plasma volumes (< 10 µl) provide sufficient dilute sample volume. Overall, ELISA is used more frequently because no radioactive isotopes are needed and it is relatively easy to set up and use.

Although immunoassays are now well developed and validated for a wide range of fishes including fathead minnows, alternative analytical methods such as LC-MS have been applied (Zhang et al., 2004; Wunschel et al., 2005). In general, MS approaches to fathead minnow VTG quantification have either measured the protein in its intact form (Wunschel et al., 2005) or quantified specific peptide fragments after chemical or enzymatic digestion of the intact protein (Zhang et al., 2004). Brodeur et al. (2006) compared values obtained by LC-MS or ELISA and found a general similarity in values comparable to that obtained using different ELISA kits, although LC-MS methods typically provide lower values. These authors also found that sampling and storage conditions affected concentrations of fathead minnow VTG. Specifically, the addition of protease inhibitors such as aprotinin and of the protein stabilizer, polyethylene glycol at the time of sampling improved the performance of ELISA kits.

An alternative to measuring the VTG protein is to quantify the messenger ribonucleic acid (mRNA) that codes for the VTG protein. In fathead minnows, the liver contains at least two VTG genes (vtg1, vtg3) that appear to be actively transcribed as part of VTG synthesis (Miracle et al., 2006). Expression of both vtg1 and vtg3 is upregulated after exposure to estrogen and xeno-estrogens, although vtg1 mRNA appears to be a more sensitive indicator of VTG protein induction (Miracle et al., 2006; Korte et al., 2000). Induction of VTG mRNA can be detected prior to increases in plasma VTG protein levels (Korte et al., 2000). Four methods for measuring absolute or relative amounts of VTG mRNA have been used: reverse transcription-polymerase chain reaction (RT-PCR), ribonuclease protection assay (RPA), hybridization protection assay and Northern analysis (Wheeler et al., 2005). Of these, RT-PCR is now the most widely used method. In RT-PCR (or quantitative PCR), the VTG mRNA in a liver sample is copied into complementary DNA (cDNA) using a fathead minnow specific VTG 3'-primer and reverse transcriptase (RT). The next step uses PCR to preferentially amplify the cDNA. Subsequent detection of the PCR products relies on the use of fluorescent probes, specific to the amplified DNA sequence, to detect the amount of product produced. The amount of fluorescence is monitored at the end of each cycle of PCR (i.e., real-time), and the amplification curve is recorded. The amount of DNA product measured is assumed to be directly proportional to the amount of starting mRNA. This measurement is compared with known amounts of VTG mRNA that have been included in the assay to determine the amount of starting mRNA target.

Despite the potential for improved sensitivity of VTG mRNA measurements, the more common practice in optimization studies is to quantify the VTG protein using an immunoassay. To date, immunoassays have been technically easier and have provided better inter-laboratory reproducibility relative to RT-PCR. An informal inter-laboratory comparison of plasma VTG and hepatic VTG protein and mRNA measurements using aliquots from a common plasma or tissue pool isolated from unexposed and E2 exposed male and female fathead minnows found greater inter-laboratory differences for mRNA measurements compared to VTG protein measurements (Battelle 2003b).

4 PROTOCOL OPTIMIZATION AND DEMONSTRATION

Formal optimization studies evaluated three different test configurations (as described above) of sexually-mature male and female minnows for use in a short-term assay. Since original publication of the group spawning protocol (Ankley et al., 2001), this and additional variations on the 21-d design have been used to test a variety of known and suspected endocrine active chemicals for purposes other than optimization of the protocol. Initial studies, some conducted before the approach described by Ankley et al. (2001), focused mostly on estrogenic compounds, which appeared to represent the greatest environmental concern based on field observations (e.g., Kramer et al., 1998; Miles-Richardson et al., 1999a, b). Subsequent efforts have investigated other modes of action especially androgenic, anti-androgenic and steroid biosynthesis inhibition. Collectively, these studies form the basis for final protocol recommendations, and provide the rationale for and support the merits of including the specific endpoints described above. Results of the formal optimization studies using the 21-d protocol plus pertinent findings from other studies using variations of the protocol are discussed in this section. Strong and weak estrogen receptor agonists and antagonists are discussed first followed by strong and weak androgen receptor agonists and antagonists. Next, steroid metabolism modulators, multi-modal chemicals, chemicals with unknown mechanisms of action, and chemicals with no direct endocrine mechanism of action are discussed.

4.1 Assay Performance with Specific MOA Chemicals

Estrogen Receptor Agonists

Estrogen and synthetic estrogens have been the most widely studied endocrine active chemicals in most biological systems, including fish. There have been several studies measuring the responses of fathead minnows to waterborne E2. Both spawning and non-spawning test configurations of minnows have been used. An early study (Kramer et al., 1998) used a group spawning design (three males / three females per tank) to study the effects of a 19-day exposure to E2 at concentrations between 13.7 – 1470 ng/L, with 1 - 3 tank replicates per exposure. At exposure concentrations at or above 870 ng/L, excessive mortality began to occur, particularly in males. Decreases in fecundity were observed at most exposure levels, with a calculated E2 exposure median effective concentration (EC_{50}) of 120 ng/L. Significant induction of VTG (indirectly determined by the alkaline labile phosphorus method) was also observed in both males and females. There was high variability in fecundity in the control groups, emphasizing the need for tank replication. In subsequent studies using a one male and two female test configuration, exposure to E2 caused histologic changes such as a loss of germ cells and the presence of degenerate spermatozoa (Miles-Richardson et al., 1999a). Additional testicular histopathology occurred, specifically the proliferation of Sertoli cells that in extreme cases led to complete occlusion of seminiferous tubules. Estradiol-induced ovarian histopathology was, in general, less pronounced when compared with testicular lesions, but E2 appeared to cause a significant increase in primary follicles with diminished numbers of secondary follicles. Changes in secondary sex characteristics were observed in male minnows, which had atrophied nuptial tubercles after nominal E2 exposures at or above 545 ng/L (Miles-Richardson et al., 1999a).

A more recent study (Bringolf et al., 2004) measured reproductive effects using the optimized group spawning protocol and a measured E2 exposure of 299 ng/L (n = 12). The E2 exposure was maintained as a static renewal system with 25% of exposure water replaced daily. A solvent-only tank was included to provide a control for methanol and acetone (50:50 v/v) added at 0.001% as a dosing vehicle for E2. In this study, E2 exposure stopped all spawning activity within seven days, but eggs collected during the brief spawning period had normal fertilization rates and larval survival. The E2 exposure caused an almost complete loss of nuptial tubercles in males and a significant decrease in GSI for both sexes. Quantitative gonadal staging indicated a decrease in mature oocytes and spermatozoa. There was significant induction of VTG in both males and females (Bringolf et al., 2004).

Other studies have used a non-spawning configuration to measure changes in morphology and VTG levels in adult male minnows exposed to E2. A 21-day exposure to nominal concentrations of E2 at 320 or 1000 ng/L (n = 7 per tank; one tank replicate/treatment level) significantly decreased the GSI compared with control fish (Panter et al., 1998). Lower E2 concentrations of 10, 32, and 100 ng/L did not alter the GSI in male fathead minnows (Panter et al., 1998). A subsequent study by these authors confirmed these observations and reported that the GSI in males is significantly decreased during a 21-day exposure only at nominal E2 concentrations greater than 60 ng/L (Panter et al., 2000). Collectively, these studies indicate that the effects of E2 in adult male fathead minnows change from reduction in testis mass to lethality over a relatively narrow concentration range: 60 to 1000 ng/L. Plasma VTG concentrations in adult male fathead minnows increased approximately 10-fold after exposure to nominal E2 concentrations as low as 30 ng/L for 21 days (Panter et al., 2000). Similar findings were reported in a study exposing adult male fathead minnows to nominal E2 concentrations as low as 27 ng/L, which caused an approximately 10- to 100-fold increase in VTG plasma levels after 7 to 21 days exposure (Parks et al., 1999). This latter study measured VTG using an ELISA based on fathead-minnow-specific anti-VTG with fathead minnow VTG as standard (Parks et al., 1999).

More recent inter-laboratory studies used male and female minnows either separately housed or separated by a mesh screen (*n* not specified) and exposed to measured E2 levels between 9-94 ng/L for 14 and 21 days (OECD 2006). All labs consistently detected significant elevations in male VTG at measured exposure levels ≥ 20 ng/L after 14 d of exposure. After 21 d of exposure, significant increases in male VTG were observed for all exposure levels of ≥ 9 ng / L. Increases in VTG in female minnows were primarily observed at the highest exposure level (94 ng/L). Both homologous and non-homologous fathead minnow VTG ELISA assays were used for measurement. There was no change in GSI or secondary sex characteristics in either sex at any exposure level. Changes in gonadal histology were noted in both sexes, although inter-laboratory comparability was hindered by differences in tissue preparation and interpretation. The most comprehensive analysis indicated E2 exposure increased the number of spermatogonia and decreased the number of spermatocytes at the highest exposure level (94 ng/l) (OECD 2006). In females, there was a trend toward increased numbers of atretic oocytes.

There have been several studies with fathead minnows exposed to the highly potent synthetic estrogen, ethynylestradiol (EE2). These studies did not use the optimized protocol but are noteworthy because of the inclusion of several reproductive endpoints and because they provide a broader context for interpreting findings obtained from the optimized, short-term exposure protocol. A full life-cycle study was performed with EE2 with exposure beginning at the embryonic stage and continuing through 28-day post-hatch of the F1 offspring (Lange et al.,

2001). Nominal concentrations of EE2 were between 0.2 ng/L and 64 ng/L. Reproductive performance of the F0 adults could only be assessed in the 0.2-ng/L and 1.0-ng/L treatment groups (n = 8 pairs, two tank replicates per exposure) because a progressive increase in the number of individuals with ova-testis occurred from 1.0 ng/L to 16 ng/L, with 94% of all fish phenotypically female at the 64-ng/L exposure (Lange et al., 2001). At higher exposure concentrations (≥ 4 ng/L), male secondary sex characteristics failed to develop, consistent with histological analysis showing the development of testis-ova. In the 0.2 – 1.0 ng/L exposed F0 adults, no differences in fecundity were observed. A significant reduction in the F0 female body weight was observed at the 1.0 ng/L treatment. The plasma and whole fish VTG concentrations measured in the 0.2 and 1.0 ng/L F0 adults were largely unchanged from controls. Assessment of VTG in fish exposed to higher concentrations of EE2 was complicated due to phenotypic reversal of male fish. Hatching success tended to be lower (79%, 65.5% hatching rate, 0.2 and 1.0 ng/L treatments respectively) relative to control (90.6% hatching rate), but the decrease was not statistically significant. However the F1 offspring had significantly reduced body weight and length when measured at 28 days post-hatch (Lange et al., 2001).

Reduced hatching success in the Lange et al. (2001) study appears to be a biologically-relevant finding despite the lack of statistical significance, as other studies with EE2 have also reported this phenomenon at exposure levels where no other reproductive effects occurred. For example, in a full life cycle study described by Parrott and Blunt (2005) fathead minnows exposed to nominal EE2 concentrations of 0.32 and 0.96 ng/L had statistically significant reductions in the numbers of fertilized eggs. This effect of EE2 exposure on fertilization appeared to be unrelated to clutch size as both large and small spawnings were equally affected (Parrott and Blunt 2005). Another study exposed fathead minnows to EE2 during a period of artificially induced gonadal recrudescence (Pawlowski et al., 2004b). After a 21-d exposure to a nominal EE2 concentration of 0.1 ng/L and measured EE2 concentrations of 0.7 – 100 ng/L, three pairs of minnows from each exposure group (one or two tank replicates / treatment) were placed in clean water and fecundity and fertilization success measured for 21 d. Minnows exposed to the 8.1 ng/l and higher treatment had reduced fecundity and significant reductions in fertilization success – down to ~30% of control values (Pawlowski et al., 2004b). Both induction of VTG and significant reductions in nuptial tubercles were also observed in males exposed to EE2 at or above 0.7 ng/L. A summary of the findings from the studies testing E2 is presented in Table 4-1.

Table 4-1. Responses to Estradiol-17β (E2) and synthetic estrogens (ethinylestradiol, EE2).

Numbers represent dose at which a significant effect occurred for the listed endpoint. * Indicates an EC50 value calculated when regression analysis was used by the authors. (-) indicates that the endpoint was measured, but was not statistically different from control. Blanks indicate that a particular endpoint was not assessed. Unless otherwise indicated, data are results of 21-day exposures. Details are provided in the text.

Test Configuration	Treatment	Dose Range (ng/L)	Fecundity	Gonad Histology	GSI	E2	T	11-KT	VTG	Male Tubercles	Male Fat Pad	Reference
Group spawn (3M, 3F)[1]	E2	13 – 1470	120*						251*			Kramer et al., 1998
Group spawn (1M, 2F)	E2	17 – 272,380		M ↑ Sertoli cell proliferation ≥ 136 F ↓ maturity ≥ 27						↓ ≥ 272	↓27, 238	Miles-Richardson et al., 1999a
Group spawn (2M, 4F)	E2	299	↓	↓maturity M & F	M↓ F ↓				M ↑ F ↑	↓		Bringolf et al., 2004
Group Spawn (3M, 3F) post-exposure[2]	EE2	0.1 – 100	↑ 0.1, 0.7 ↓100 ↓fertilization ≥ 8.1		M ↓ ≥ 10 F ↓ 100				M/F ↑0.7	↓ ≥ 0.7		Pawlowski et al., 2004
Full Life Cycle[3]	EE2	0.2 – 64	- 0.2, 1	M, ≥1 ovotestis					M ↓0.2	↓≥ 4	↓≥ 4	Lange et al., 2001
Full Life Cycle[4]	EE2	0.32 – 22.7	↑0.32, 0.96 ↓fertilization ≥ 0.32		F ↓ ≥ 3.5					↓0.96	↓0.96	Parrott & Blunt, 2005
Adult male non-breeding	E2	10 – 1000			↓ ≥ 320				↑≥ 100			Panter et al., 1998
Adult male non-breeding	E2	30 - 120			↓ 120				↑ ≥30			Panter et al., 2000
Adult male non-breeding	E2	27 – 2723							↑ ≥27			Parks et al., 1999
Adult M & F non-breeding	E2	9 – 94							M↑ ≥9 F ↑ ≥94			OECD 2006

* EC_{50}

[1]19 day exposure

[3]Beginning with embryos through 28 days post-hatch of F1

[4]Continuously exposed 2 -150 dph.

[2] 21 day exposure, endpoints measured during 21 days spawning post-exposure

Weak Estrogen Agonists

There have been several studies using pair or group spawning fathead minnow configurations to assess responses to weak estrogens. Methoxychlor is a relatively persistent organochlorine insecticide shown to be a weak estrogen mimic in fish (Denny et al., 2005) and has both estrogenic and anti-androgenic properties in mammals (Murono et al., 2006). Using the optimized group spawning design, a 21 day exposure to measured methoxychlor concentrations of 0.55 µg/L and 3.56 µg/L caused a decrease in fecundity from 20.5 to 8.3 eggs/female/day (control versus 3.56 µg/L treatment) (Ankley et al., 2001). Reduced fecundity was attributed to both a decrease in clutch size and a prolonged interval between spawns. The GSI in both sexes was unchanged, although the highest concentration caused an increase in the numbers of atretic follicles in the ovaries. Male minnows exposed to methoxychlor at 3.56 µg/L had significantly elevated plasma VTG concentrations (>4000 fold) and decreased plasma levels of testosterone and 11-KT. In females, VTG was not significantly changed, but E2 levels were decreased (Ankley et al., 2001). A subsequent study using a similar experimental design also observed reduced fecundity after a 21 day exposure to methoxychlor at a measured concentration of 3.22 µg/L (Battelle, 2003a).

The alkylphenol ethoxylates and their environmental degradation products, alkylphenols, are a large class of contaminants considered to be weakly estrogenic. These contaminants are particularly relevant as excessive environmental releases have been hypothesized to be responsible for endocrine disruption in some wild fish populations (Jobling et al., 1998). The original basis for the presumption of an estrogenic mode of action for these contaminants came from both *in vitro* and *in vivo* studies measuring induction of VTG in salmonid species (Jobling and Sumpter, 1993; White et al., 1994). Subsequent studies with fathead minnows have used both group spawning and pair breeding designs with exposure durations lasting up to 42 days. In a pair spawning study, Harries et al. (2000) found that breeding pairs of fathead minnows ($n = 2$ pairs per tank, 2 tanks per treatment) exposed for 21 days to 4-nonylphenol at measured concentrations of 0.62, 8.1 and 57.7 µg/L exhibited reduced fecundity (total eggs spawned) that was 60%, 45% and < 5% of control values, respectively. In males, the number of nuptial tubercles was decreased at the highest exposure concentration while the thickness of the dorsal fat pad was significantly decreased at all concentrations. GSI was a less sensitive endpoint, remaining unchanged after most exposures. Consistent with an estrogenic mode of action, significant induction of VTG occurred in both sexes. In male minnows, induction of VTG could be observed at concentrations as low as 8.1 µg/L, but in females only at the highest concentration. A carp (*C. carpio*) based VTG assay was used in these measurements. This same study also tested another suspected weak estrogen, butyl benzyl phthalate, at a mean measured concentration of 75.5 µg/L. The experimental design and measured endpoints were the same as for the 4-nonylphenol studies, however there were no observable effects on reproduction other than a decrease in spawning frequency and an increase in clutch size (Harries et al., 2000). It is noteworthy that *in vitro* studies suggest that 4-nonylphenol is 10 – 100 times more potent as an estrogen than butyl benzyl phthalate (White et al., 1994; Harris et al., 1997).

An earlier study of 4-nonylphenol using a pair breeding design (two pairs/tank; 3 tank replicates/treatment) focused on changes in gonadal histology and secondary sex characteristics after 42-day exposures to measured concentrations of 0.05 – 3.4 µg/L (Miles-Richardson et al., 1999b). At these lower concentrations, no changes in secondary sex characteristics were noted

although increased Sertoli cell numbers and necrotic spermatozoa were observed at 1.1 and 3.4 µg/L (Miles-Richardson et al., 1999b). A companion study used group spawning minnows (2 males, 2 females per tank; 3 tanks/treatment) exposed for 42 days to measured concentrations of 4-nonylphenol ranging from 0.05 to 3.4 µg/L (Giesy et al., 2000). Spawning was monitored daily for the initial 21 and final 12 days of the exposures. Fecundity decreased at concentrations as low as 0.16 µg/L, although only the 3.4 µg/L treatment was statistically different from controls (Giesy et al., 2000). There was no consistent induction of VTG in male minnows at any treatment level. However, significant decreases in VTG appeared to have occurred in females at higher concentrations (Giesy et al., 2000). A goldfish (*C. auratus*) based VTG assay was used in these measurements. Finally, an additional study using a similar group spawning design (three males and females; three tank replicates/treatment), assessed the impact of a mixture of 17 different nonylphenol ethoxylates in fathead minnows at measured concentrations between 0.21 µg/L and 7.9 µg/L (total of all oligomers) (Nichols et al., 2001). At these exposure levels, no significant effects on fecundity, VTG, or sex steroids (E2, T) were detected. This study also noted that high variation in spawning combined with a relatively small sample size (*n*= 4-8 / treatment level) reduced the statistical power to discriminate between treatments (Nichols et al., 2001).

An inter-laboratory evaluation of 4-*tert*-pentylphenol was performed by three different laboratories using a group spawning protocol consisting of five male and five female minnows (*n*=2 tank replicates / treatment) exposed for 21 days (OECD 2005b). Three different 4-*tert*-pentylphenol concentrations were tested with measured values of 80.1 – 85.7, 270 – 298 and 857 – 887 µg/L (minimum – maximum values for participating laboratories). Endpoints measured were qualitative daily assessment of spawning, quantitative assessment of nuptial tubercles, plasma VTG and gonad histology. All laboratories observed significant, dose dependent decreases in spawning frequency after the exposures with a complete lack of spawning observed in the high 4-*tert*-pentylphenol treatment group. There were also dose dependent decreases in the numbers of male nuptial tubercles, with significant decreases observed by all laboratories after the high treatment and by one laboratory after the intermediate treatment. All laboratories observed significant increases in VTG levels in males after the intermediate and high 4-*tert*-pentylphenol treatments. Elevated VTG was also observed in females by one laboratory after the high treatment. Several changes in gonad histology were noted in both sexes. In the testes, all laboratories observed increased numbers of spermatids and spermatocytes and an increase in testicular degeneration. In the ovaries, all laboratories observed increases in oocyte atresia (OECD 2005b).

Another weakly estrogenic alkylphenol with a potency comparable to 4-nonylphenol, is bisphenol A (Anderson et al., 1999). This chemical was tested using the optimized group spawning protocol (four tank replicates per treatment) at two different treatment levels, 56.6 and 344 µg/L (Battelle 2005). At the high concentration, fecundity was significantly reduced to approximately half that of the control and low treatment groups. The hatching percentage of fertilized eggs was 77 and 83% for the low and high treatment groups, respectively, as compared to 99% for the control group. With regard to the biochemical endpoints, there was significant induction of VTG in males at both treatment concentrations and elevated levels in females at the high concentration. In males, the mean VTG levels was approximately 2 mg/ml in the low exposure and 92.7 mg/ml in the high treatment group, a value higher than that observed for spawning female fathead minnows. A carp (*C. carpio*) based VTG ELISA was used in these

measurements. In both sexes E2 levels were reduced, and in females the reduction was concentration- dependent with levels in the high treatment less than half those of the control females. Testosterone concentration in females was unaffected by treatment, but in males the high exposure group had T levels that were roughly 15% of control values. Similarly, 11-KT levels in males from the high treatment were 5 - 10% of the values observed in control fish. Quantitative staging of the ovaries indicated an increase in early stages (stage 1A, defined as oocytes in nests with small cytoplasmic volume) (Battelle 2005) and a decrease in stage 3 oocytes (early vitellogenic oocytes). In males, no significant changes in testicular histology were observed despite the large decreases in circulating androgen levels caused by exposure. These findings are consistent with a previous study that exposed fathead minnows to bisphenol A for up to 164 days at nominal concentrations ranging from 1 - 1,280 μg/L (Sohoni et al., 2001). In that study, both pair breeding (n = 8 pairs; two tank replicates) and non-spawning experimental designs were employed. In pair breeding minnows, decreased fecundity (as total eggs laid between exposure days 43 – 164) was only observed at the highest exposure concentration. However, reduced hatching success was observed at exposure levels down to 640 μg/L. Vitellogenin induction in males was observed at exposure levels at or above 160 μg/L and in females at 640 μg/L. In non-spawning males, quantitative staging of the gonads indicated concentration-dependent decreases in the numbers of spermatozoa at exposure levels of 16 μg/L and higher (Sohoni et al., 2001). A summary of the findings from the studies testing weak estrogen agonists is presented in Table 4-2a (female responses) and Table 4-2b (male responses).

Table 4-2a. Weak estrogens: Female responses.

Numbers represent dose at which a significant effect occurred for the listed endpoint. (-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Blanks indicate that a particular endpoint was not assessed. Unless otherwise indicated, data are results of 21-day exposures. Details are provided in the text.

Test Configuration	Treatment	Dose Range (µg/L)	Fecundity	Gonad Histology	GSI	E2	T	11-KT	VTG	Tubercles	Fat Pad	Reference
Group spawn	Methoxychlor	0.55, 3.56	↓ 3.56	↑ atresia 3.56	↓ 0.55	↓ 3.56			-			Ankley et al., 2001
Group spawn	Methoxychlor	3.22	↓ 3.22									Battelle, 2003
Pair spawn	4-NP	0.65, 8.1, 57.7	↓ ≥ 57.7		↓ 8.1				↑ 57.7			Harries et al., 2000
Pair spawn[1]	4-NP	0.05 – 3.4		-								Miles-Richardson et al., 1999
Group spawn (2M, 2F)[2]	4-NP	0.05 – 3.4	↓ 3.4			↑ < 3.4			↓,↑			Giesy et al., 2000
Group spawn (3M, 3F)	mix-17 NPEO	0.21 – 7.9	-			-	-		-			Nichols et al., 2001
Group spawn (5M, 5F)[3]	4-PP	80.1 - 85.7 270 – 298 857 - 887	↓ 80.1 abolished 857-887*(3)	↑atresia (3)					↑ 857-887 (1)			OECD, 2005
Group spawn	BPA	56.6, 344	↓ 344 ↓ hatch	↓maturity		↓	-		↑ 344			Battelle, 2005
Pair spawn[4]	BPA	1 - 1280	↓ 1280 ↓ hatch ≥ 640	-	↓ ≥ 640				↑ 640			Sohoni et al., 2001
Pair spawn	Butyl benzyl phthalate	75.5	-		-				-			Harries et al., 2000

Abbreviations: 4-NP, 4-nonylphenol; mix-17 NPEO, a mixture of 17 nonylphenol ethoxylates; 4-PP, 4-*tert* pentylphenol; BPA, bisphenol-A

[1]Exposed for 42 days

[2]Exposed for 42 days, spawning monitored on days 8-21 and days 30-42.

[3]Numbers in parentheses indicate number of participating laboratories that observed the effect. Three laboratories participated.

[4]Exposed for 164 days

*Qualitative assessment of fecundity as presence/absence of spawning

Table 4-2b. Weak estrogens: Male responses.

Numbers represent dose at which a significant effect occurred for the listed endpoint. Blanks indicate that a particular endpoint was not assessed. Unless otherwise indicated, data are results of 21-day exposures. Details are provided in the text.

Test Configuration	Treatment	Dose Range (µg/L)	Fecundity	Gonad Histology	GSI	E2	T	11-KT	VTG	Tubercles	Fat Pad	Reference
Group spawn	Methoxychlor	0.55, 3.56	↓ 3.56	-	-		↓ 3.56	↓ 3.56	↑ 3.56			Ankley et al., 2001
Group spawn	Methoxychlor	3.22										Battelle, 2003
Pair spawn	4-NP	0.65, 8.1, 57.7	↓ ≥ 57.7		-				↑ ≥ 8.1	↓ ≥ 57.7	↓ ≥ 0.65	Harries et al., 2000
Pair spawn[1]	4-NP	0.05 – 3.4		↑ Sertoli cell proliferation ≥ 0.33						-	-	Miles-Richardson et al., 1999
Group spawn (2M, 2F)[2]	4-NP	0.05 – 3.4	↓ 3.4			↑ < 3.4			-			Giesy et al., 2000
Group spawn (3M, 3F)	mix-17 NPEO	0.21 – 7.9	-			-	-		-			Nichols et al., 2001
Group spawn (5M, 5F)[3]	4-PP	80.1 - 85.7 270 – 298 857 - 887	↓ 80.1 abolished 857 - 887*(3)	↓ maturity (3)					↑ ≥ 270 (3)	↓ ≥ 270 (1) ↓ 857 (2)		OECD, 2005
Group spawn	BPA	56.6, 344	↓ 344 ↓ hatch	-		↓	↓ 344	↓ 344	↑ ≥ 56.6			Battelle, 2005
Pair spawn[4]	BPA	1 - 1280	↓ 1280 ↓ hatch ≥ 640	↓ maturity ≥ 16	↓ ≥ 640				↑ ≥ 160			Sohoni et al., 2001
Pair spawn	Butyl benzyl phthalate	75.5	-		-				-	-	-	Harries et al., 2000

Abbreviations: 4-NP, 4-nonylphenol; mix-17, a mixture of 17 nonylphenol ethoxylates; BPA, bisphenol-A

[1]Exposed for 42 days

[2]Exposed for 42 days, spawning monitored on days 8-21 and days 30-42.

[3]Numbers in parentheses indicate number of participating laboratories that observed the effect. Three laboratories participated.

[4]Exposed for 164 days

[*]Qualitative assessment of fecundity as presence/absence of spawning

Estrogen Receptor Antagonists

The best characterized estrogen antagonist in vertebrates is the pharmaceutical tamoxifen (and its analogs), which bind directly to the estrogen receptor and inhibit its function as a transcription factor (MacGregor and Jordan 1998). The relative binding affinity of tamoxifen in fathead minnow liver cytosol (IC_{50} = 1.7 ± 0.7 x 10^{-7} M) is 4.2 times higher than that of estrogen (Denny et al., 2005). Tamoxifen has been studied using a pair breeding design (n= 8 pairs / treatment) that lasted 42 days with measured concentrations (mean of weekly analysis) of 0.11, 0.41, 1.65, 5.97 and 18.2 μg/L (Williams et al., 2007). For these exposures, 0.0002% triethylene glycol was added as a carrier solvent. During the 42-day exposure, 1 male and 10 females died and fecundity data was calculated based on total egg production per reproductive day (surviving female days / total fecundity). Fecundity was significantly higher (nearly 2-fold higher) and hatching success was significantly lower (80 vs 97%) in the triethylene glycol control group than in the dilution water control (Williams et al., 2007). Subsequent statistical comparisons were made between tamoxifen treatments and the solvent control. Tamoxifen treatment did not cause significant changes in body weight or standard length of the fish. After the 1.65 and 18.2 μg/L treatments, fecundity was significantly reduced (Williams et al., 2007), while hatchability was not significantly different at any tamoxifen concentration (Williams et al., 2007). There were no significant differences in plasma VTG levels between control and tamoxifen treated fish (Williams et al., 2007). Continued exposure of F1 offspring at the same measured tamoxifen levels for 28 days post hatch (DPH) significantly reduced standard length and body weight of the F1 fish at 5.6 and 18.2 μg/L tamoxifen compared to the pooled values from the dilution water and solvent controls. The whole fish VTG concentrations were also significantly reduced (relative to pooled controls) in F1 fish exposed to tamoxifen concentrations of 0.56 μg/L and higher (Williams et al., 2007).

A study by Panter et al. (2002) exposed juvenile fathead minnows (45 - 100 days post hatch) to the pharmaceutical E2 antagonist ZM189,154 for 4, 7, 14 and 21 days at measured concentrations of 5.0, 24.4, and 76.6 μg/L. A significant decrease in the whole body VTG concentration was observed at all exposure levels after 14 d and at the high exposure level after only 4 d. No concentration caused significant changes in body weight or total length of the fish however, the condition factor was reduced in fish exposed to the two higher treatment levels.

Androgen Receptor Agonists

Androgens regulate male differentiation, as well as other key aspects of reproduction in both male and female fish (Borg 1994). Receptor binding studies using recombinant fathead minnow androgen receptor inserted into a mammalian cell line indicated the following rank order of binding affinities (IC_{50}) for natural and synthetic androgens (highest to lowest): methyltestosterone > 17α-trenbolone >17β-trenbolone > 17-dihydrotestosterone > 11-KT > T = androstenedione (Wilson et al., 2004). Other chemicals tested in this system were the model anti-androgens *p,p,*DDE, which had an affinity 550 times less than 11-KT and vinclozolin and associated metabolites (20-300 times lower binding than 11-KT). Many of these chemicals have been studied using pair breeding, group spawning and non-spawning protocols and thus provide a comprehensive data set for assessing the response of fathead minnows to androgens (and anti-

androgens) of varying potency and assessing reliability of assay endpoints when measured in different laboratories.

An initial assessment of the effects of androgen exposure in fathead minnows using the optimized protocol was reported for methyltestosterone (Ankley et al., 2001). In this study, minnows were exposed for 12 days to measured concentrations of 120 µg/L and 1700 µg/L. Some mortality was observed at both concentrations, with only 20% of the fish surviving in the highest treatment. Nonetheless, both concentrations caused an immediate cessation in spawning. A number of morphological changes in the gonads also occurred, including a reduction in the GSI in both sexes, an increase in atretic follicles in the ovaries, and only scattered spermatogenic activity in the testes. Methyltestosterone exposure also reduced plasma concentrations of T in both sexes and 11-KT in males. Curiously, methyltestosterone strongly induced VTG synthesis in both sexes (Ankley et al., 2001). A subsequent study demonstrated that methyltestosterone is aromatized to methylestradiol which stimulates the E2 receptor and hence can induce VTG synthesis (Hornung et al., 2004). A particularly important observation was the formation of nuptial tubercles on female minnows. The formation of tubercles was noticeable after only six days exposure, demonstrating the potential for rapid change of this endpoint in female minnows. This finding is consistent with earlier work by Smith (1974), who also reported methyltestosterone-stimulated nuptial tubercle growth in female fathead minnows.

Several different laboratories have studied the effects of 17β-trenbolone in both group spawning and non-spawning protocols. Using the optimized group spawning protocol, exposure to measured concentrations from 0.0015 to 41 µg/L reduced fecundity at concentrations at or above 0.026 µg/L (Ankley et al., 2003). For most exposure levels, changes in fecundity were noticeable within 2 - 3 days of the exposure. A similar sensitivity was noted for induction of male secondary sex characteristics in females, with increasing numbers of nuptial tubercles appearing as the exposure increased from 0.026 to 41 µg/L. Associated with these effects were decreased circulating E2, T and VTG in females, at test concentrations as low as 0.27 µg/L (E2 and T) or 0.026 µg/L (VTG). In males, there was little change in biochemical endpoints except at the highest exposure of 41 µg/L, which decreased 11-KT and increased E2 and VTG. Histological examination of the ovaries indicated increased oocyte atresia in exposed fish (Ankley et al., 2003).

A subsequent study in another lab found similar effects, using the same protocol with measured 17β-trenbolone concentrations of 0.04 - 0.07 and 0.6 - 0.86 µg/L (concentration ranges for the low and high treatment levels, respectively) (Battelle 2003a). Fecundity was significantly reduced at the high treatment level and the decrease was noticeable by day 2. The high exposure level caused an increase in female body weight, GSI, and number of atretic ovarian follicles, while resulting in decreased female E2 and VTG concentrations (Battelle 2003a). Two other 17β-trenbolone experiments were performed by Battelle that used either a reduced exposure period (14 days) or a non-spawning protocol consisting of sex-specific groups exposed for 21 days. Exposure levels were comparable to those used for the optimized protocol, except that a third, mid-level exposure of 0.45 µg/L was included in the non-spawning protocol. Results from the 14-day exposure were entirely consistent with those obtained from the optimized 21-day protocol. However, results from the non-spawning protocol were somewhat different – only the biochemical endpoints changed in a similar manner as that observed for the group spawning protocols (Battelle 2003a). Specifically, no significant changes in GSI or gonad morphology were observed at any exposure level, in contrast to findings of increased GSI and atretic follicles

in females exposed in the spawning protocols (Ankley et al., 2003; Battelle 2003a). Unfortunately, secondary sex characteristics were not measured as part of these studies.

A more recent inter-laboratory study of 17β-trenbolone also identified problems associated with histological analysis in non-spawning minnows (OECD 2006). In these studies, non-spawning minnows were exposed to measured concentrations (approximate mean from four different labs) of 0.04, 0.40 and 4.50 µg/L. Decreased VTG concentrations in females were observed by all four labs at the high exposure, but only two of the four labs detected a decrease in VTG at the lower treatment levels (OECD 2006). There was little change in GSI at any exposure level in contrast to findings using group spawning protocols. However, increased numbers of nuptial tubercles were observed in females at the two higher exposure levels. Histological analysis did not demonstrate consistent findings among the participating labs in that an increase in number of atretic follicles was only reported at the high test concentrations by some labs and not at all in another lab. Interpretation of chemical-induced histological responses was hindered by higher than expected incidence of atresia in control females. The report authors concluded that future evaluations should not separate fish by sex, thus allowing for normal spawning behavior to occur (OECD 2006).

17α-trenbolone is a reversibly formed metabolite of 17β-trenbolone that has been studied using a 21-d pair breeding protocol (n = 8 pairs / treatment) at measured concentrations varying between 0.003 and 0.10 µg/L (Jensen et al., 2006). In preliminary studies using the optimized group spawning protocol, the authors reported exposure concentrations ≥ 0.177 µg/L completely blocked all spawning activity within the first day of treatment. At the lower test concentrations used in the subsequent pair breeding studies, 17α-trenbolone significantly decreased fecundity at exposure levels down to 0.01 µg/L and decreased circulating sex steroid and VTG levels in females at exposure levels down to 0.03 µg/L. Formation of nuptial tubercles in females became apparent at 0.03 µg/L and was significantly increased at the 0.1 µg/L treatment. No endpoints in males were affected by low concentrations of 17α-trenbolone. Analysis of tissue residues for 17α-trenbolone and 17β-trenbolone after the exposures indicated that both isomers were present in roughly equal concentrations determined in fish exposed to 0.094 µg/L of 17α-trenbolone (Jensen et al., 2006). A summary of the findings from the studies testing androgen agonists is presented in Table 4-3a (female responses) and Table 4-3b (male responses).

Table 4-3a. Androgen receptor agonists: Female responses.

Numbers represent the lowest dose at which a significant effect occurred for the listed endpoint. (-) indicates that the endpoint was measured, but was not statistically different from control. Blanks indicate that a particular endpoint was not assessed. Unless otherwise indicated, data are results of 21 day exposures. Details are provided in the text.

Test Configuration	Treatment	Dose Range (µg/L)	Fecundity	Gonad Histology	GSI	E2	T	11-KT	VTG	Tubercles	Fat Pad	Reference
Group Spawn[a]	Methyl-testosterone	120, 1700	abolished	↑ atresia ↓ maturity	↓ 120	↓ 120	↓ 120		↑ 120	↑ 120		Ankley et al., 2001
Group Spawn	17β-Trenbolone	0.0015 - 41	≥ 0.026 ↓	≥ ↑ atresia; ↓ yolk deposition	-	↓ ≥ 0.27	↓ ≥ 0.27		↓ ≥ 0.027	↑ ≥ 0.026	thicker	Ankley et al., 2003
Group Spawn[b]	17β-Trenbolone	0.04/0.06 0.6/0.86	0.6/0.86 ↓	↑ atresia	↑ 0.6/0.86	↓ 0.6/0.86			↓ 0.6/0.86			Battelle, 2003
Non-spawning	17β-Trenbolone	0.04/0.06, 0.45, 0.6/0.86		-	-	↓ 0.6/0.86			↓ 0.6/0.86			Battelle, 2003
Non-spawning	17β-Trenbolone	0.04 - 4.5		↑ atresia* ≥ 4.5	-				↓ ≥ 4.5	↑ ≥ 0.45		OECD, 2006
Pair Spawn	17α-Trenbolone	0.003 - 0.10	≥ 0.01** ↓			↓ ≥ 0.10	↓ ≥ 0.03		↓ ≥ 0.03	↑ ≥ 0.10		Jensen et al., 2006

[a]12 day exposure

[b]21 day and 14 day exposure with similar results

*, high atresia in controls due to exposure protocol

** EC_{50}

Table 4-3b. Androgen receptor agonists: Male responses.

Numbers represent the lowest dose at which a significant effect occurred for the listed endpoint. Doses enclosed in parentheses indicate a trend. (-) indicates that the endpoint was measured, but was not statistically different from control. Blanks indicate that a particular endpoint was not assessed. Unless otherwise indicated, data are results of 21-day exposures. Details are provided in the text.

Test Configuration	Treatment	Dose Range (µg/L)	Fecundity	Gonad Histology	GSI	E2	T	11-KT	VTG	Tubercles	Fat Pad	Reference
Group Spawn[a]	Methyl-testosterone	120, 1700	abolished	↓spermatogenesis; germ cell necrosis	↓ 120	-	↓ 120	↓ 120	↑ 120	↑ size		Ankley et al., 2001
Group Spawn	17β-Trenbolone	.0015 - 41	↓ ≥ 0.026	Hyperproduction of sperm	-	↑ 41	-	↓ 41	↑ (41)	-		Ankley et al., 2003
Group Spawn	17β-Trenbolone	0.04/0.06 0.6/0.86	0.6/0.86↓		↑ 0.6							Battelle, 2003
Non-spawning	17β-Trenbolone	0.04/0.06, 0.45, 0.6/0.86				↓ 0.04		↓ 0.04				Battelle, 2003
Pair Spawn	17α-Trenbolone	0.003 - 0.10	↓ ≥ 0.01*			-	-	-	-	-		Jensen et al., 2006

* EC50

[a]12 day exposure

Androgen Receptor Antagonists

Several different anti-androgenic chemicals have been tested using the optimized protocol. These chemicals represent diverse types of chemicals such as pharmaceutical / clinical agents, persistent environmental pollutants and pesticides. The best studied anti-androgen in fathead minnows is the pharmaceutical agent flutamide, which has been used primarily to treat prostate cancer (Sohani et al., 1984). Flutamide is generally considered to be a pro-drug and bioactivation to the 2-hydroxy metabolite is believed necessary for full biological activity (Simard et al., 1986). The latter conclusion appears to be applicable to fathead minnows as *in vitro* binding studies using recombinantly expressed fathead minnow androgen receptor indicated 2-hydroxy flutamide had approximately a 16-fold higher binding affinity than flutamide (Ankley et al., 2004).

Initial assessment of flutamide using the optimized protocol was described by Jensen et al. (2004). In this study, minnows were exposed for 21 days to measured concentrations of flutamide at 62.7 and 651 µg/L. The tissue concentration of flutamide in minnows was determined to be 12 - 24 times that of the water concentration. Confirmation of 2-hydroxy flutamide formation was made in a single male and female tissue extracts, demonstrating the potential of fathead minnows to convert flutamide to its more biologically potent metabolite. Exposure to both concentrations of flutamide decreased cumulative fecundity, although only the high treatment level was statistically significant (Jensen et al., 2004). Hatching success of embryos was significantly decreased in the high treatment, to approximately 60% of control values. Testosterone levels in females were increased at both concentrations (statistically significant at the high concentration) and there was an apparent increase in E2 levels (not statistically significant). Other sex steroid levels did not appear to be altered nor was GSI affected. There did appear to be a slight induction of VTG in males and an approximately 2-fold increase in females at 651 µg/L. There were also histological changes in the gonads, most notably in females where an increase in less mature follicles and atretic follicles was observed after the high flutamide treatment (Jensen et al., 2004). Other notable findings were the lack of changes in external morphology including male nuptial tubercle growth.

A subsequent assessment of flutamide was performed by Battelle (2003a) using the optimized protocol, a 14-day group spawning protocol, and the non-spawning protocol. The group spawning protocols used similar treatment levels to the Jensen et al. (2004) study (43-47 and 504-519 µg/L) and the non-spawning protocol included a third, intermediate treatment level, 260 µg/L. Cumulative fecundity was significantly decreased at the high exposure level in both the 14- and 21- day exposures (Battelle 2003a). Histological analysis indicated an increase in atretic follicles in group spawning fish after the high treatment. No significant histological changes were observed at the low treatment in the spawning fish or at any treatment level in non-spawning fish. With regard to sex steroid levels, increased testosterone in females and 11-KTin males were observed at the high exposure level in the 21-day but not the 14-day group spawning fish (Battelle 2003a). There was no evidence for induction of VTG (ELISA, carp VTG antibody) in either sex in any treatment in any protocol. No statistically significant differences in sex steroid levels were observed in fish exposed under the non-spawning protocol. Overall, the group spawning protocols provided results generally consistent with the previous flutamide study (Jensen et al., 2004), except that no VTG increase was observed at high exposure levels. An additional study of flutamide was performed by Panter et al. (2004) using a non-spawning

protocol where fish were housed in sex-specific groups (n = 18 / treatment level). Three flutamide exposures at measured concentrations of 95.3, 320.4 and 938.6 µg/L (mean values of weekly sampling) were conducted for 14 or 21 days. Endpoints measured were numbers of nuptial tubercles, GSI and plasma vitellogenin (ELISA, carp VTG antibody). The only significant findings were obtained using the high treatment level, which decreased the numbers of tubercles in males and caused an increase in plasma vitellogenin in females, but not males (Panter et al., 2004).

Recently, an inter-laboratory evaluation of flutamide was performed by three different laboratories using a group spawning protocol consisting of five male and five female minnows (*n*=2 tank replicates / treatment). Three different flutamide concentrations (low, mid and high) were tested with measured values of 68.7 – 83.5, 354-464 and 552-875 µg/L (minimum – maximum values for participating laboratories) lasting for 21 days (OECD 2005b). Endpoints measured were qualitative daily assessment of spawning, quantitative assessment of nuptial tubercles, plasma VTG and gonad histology. All laboratories observed significant decreases in spawning frequency after the high flutamide exposure. One laboratory noted a significant decrease in male nuptial tubercles after the high treatment. There was little treatment related effect on plasma VTG levels with the exception that a significant increase was observed by one laboratory in females after the mid exposure concentration. Several changes in gonad histology were noted in both sexes. In the testes, two of the three laboratories observed dose-dependent proliferation of Leydig cells and increased numbers of spermatogonia with an overall increase in testicular degeneration. In the ovaries, all laboratories observed increases in oocyte atresia (OECD 2005b).

A subsequent inter-laboratory study of flutamide was performed by two independent laboratories using the optimized group spawning protocol, which includes quantitative fecundity measurements (Battelle 2006). The nominal exposure concentrations were 100, 500 and 1000 µg/L. A significant decrease in fecundity was only observed by one laboratory at the high treatment level (Battelle 2006). The same laboratory also noted decreases in male body weight and male GSI after the intermediate and high exposures. Both laboratories observed a significant decrease in the percentage of fertilized eggs laid during the high treatment. Changes in male secondary sex characteristics were also observed by both laboratories. The male fat pad index was decreased after the high exposure and in one laboratory, significant decreases in the numbers of nuptial tubercles at all flutamide treatment levels was observed (Battelle 2006). With regard to gonad histology, one laboratory observed significant increases in the numbers of spermatogonia at the intermediate and high flutamide treatment levels (Battelle 2006). No significant changes in VTG in females or males were observed.

Because different types of VTG ELISA assays have been used (fathead minnow and carp-based), it is worth noting that a recent study of VTG levels in female minnows exposed to flutamide at 100, 500 and 1000 µg/L (nominal values) observed significant increases at the 500 µg/L exposure but not at the lower or higher exposure level (Jensen and Ankley 2006). This study measured VTG using two different ELISA assays incorporating fathead minnow specific anti-VTG antibodies. Also of interest is a previous study of flutamide exposure in fathead minnows that determined relative change in hepatic VTG mRNA after 1- and 14-day exposures to the chemical in the presence or absence of 17β-trenbolone, nominal levels = 400 µg/L, 0.5 µg/L, measured levels within ± 12% for flutamide and trenbolone respectively (Ankley et al., 2004). As expected, the trenbolone treatment significantly decreased VTG mRNA expression in

both the 1- and 14-day exposure periods with and without co-exposure to flutamide. After 14 d, there was a slight increase in VTG mRNA in the flutamide only treatment that was not statistically significant (Ankley et al., 2004). The trenbolone exposure also significantly induced nuptial tubercles in the females after 7 d of exposure, as previously demonstrated. Importantly, co-exposure with flutamide completely blocked trenbolone-induced formation of nuptial tubercles, a response that confirms the anti-androgenic nature of flutamide in fish (Ankley et al., 2004). These studies indicate that a reliable endpoint for detecting anti-androgens is reduction of male secondary sex characteristics – either in males with endogenous androgens or in females treated with androgens. Increased oocyte atresia also appears to be a consistent finding. Changes in biochemical endpoints, specifically increased T and perhaps 11-KT in males and T, E2 and VTG in females may also occur as a consequence of disturbances in androgen feedback regulation of steroidogenesis by the pituitary (Jensen et al., 2004).

p,p'-Dichlorodiphenyldichloroethylene (*p,p*-DDE) is a biologically persistent metabolite of the pesticide DDT. Past studies in rodents suggest DDE may act via an anti-androgenic mode of action, based on competitive inhibition of testosterone binding to androgen receptors (Gray et al., 1999). As previously mentioned in the androgen section, *p,p*-DDE has an affinity for the fathead minnow androgen receptor that is approximately 550 times less than 11-KT (Wilson et al., 2004). *p,p*-DDE was studied using the optimized 21-d protocol and measuring fecundity, biochemical parameters and gonad histology as study endpoints. Two exposure concentrations were used, 0.022 and 0.167 µg/L (mean value of weekly measurements). At these exposure levels, no effect on fecundity or any of the biochemical changes were observed (Battelle 2005). The only significant histological change observed was an increase in the numbers of atretic follicles in the high treatment group.

A well-characterized chemical known to antagonize androgen action is vinclozolin, a fungicide used for over 20 years. The anti-androgenic activity of vinclozolin was first described in developing rats exposed *in-utero* or lactationally via oral dosing of the pregnant mother (Gray et al., 1994; Kelce et al., 1994). The anti-androgenic effect of vinclozolin is attributed to the competitive inhibition of testosterone binding to the androgen receptor by two main metabolites: 2-[(3,5-dichlorophenyl)]-carbamoyloxy-2-methyl-3-butenoic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2; Kelce et al., 1994). Vinclozolin and its metabolites also bind to recombinantly-expressed fathead minnow androgen receptor, with the M2 metabolite exhibiting ~ 15 fold higher affinity than M1 (Wilson et al., 2004). Vinclozolin was evaluated at measured concentrations of 200 µg/L or 700 µg/L using a 21-d paired spawning protocol (Makynen et al., 2000). At the high exposure level, vinclozolin had little effect on males other than a significant increase in E2 concentrations. In females, no changes in sex steroid levels were observed. However, the high exposure caused a significant decrease in the GSI and qualitative histological analysis indicated an overall reduction in oocyte maturation (Makynen et al., 2000). Both the M1 and M2 metabolites were measured in whole fish homogenates after the 21-day exposure and were approximately 2% to 6% (M1) or 0% to 1.2% (M2) of the total vinclozolin body burden (Makynen et al., 2000). The latter finding established that fathead minnows were able to bioactivate vinclozolin.

A subsequent more extensive study of vinclozolin also used a pair breeding protocol (ten pairs / treatment; five tank replicates / treatment) in conjunction with measured exposure levels of 60, 255 and 450 µg/L (mean of twice weekly measurements) (Martinovic et al., 2008). Fecundity was decreased by all three treatment levels with complete cessation of spawning

occurring in pairs in the high treatment level. There was no effect of vinclozolin treatment on fertilization or hatching success. Interestingly, vinclozolin exposure significantly increased female body weight but not GSI, while in males GSI was significantly increased. Male secondary sex characteristics were significantly decreased – nuptial tubercles by the 255 and 450 μg/L treatments and fatpad thickness by 450 μg/L. The predominant histological change was a significant increase in the number of atretic ovarian follicles. With regard to the biochemical endpoints, a notable change was an increase in VTG levels in females at the 255 and 450 μg/L treatment levels. Additional endpoints characterized in this study were the ex-vivo production of sex steroids in isolated testes and ovaries cultured for 14.5 hr after addition of 1 μg/L hydroxycholesterol. In these supplemental endpoints, 11-KT formation was significantly decreased in testes after the 60 μg/L treatment and significantly increased in testes after the 255 and 450 μg/L vinclozolin treatments. In cultured ovaries, T formation was significantly increased after the 255 and 450 μg/L treatments. The high treatment level also up-regulated androgen receptor gene expression in the testes (Martinovic et al., 2008). In a separate experiment, the effects of co-exposure to a mixture of vinclozolin (177 or 591 μg/L) and 17β-trenbolone (457 μg/L) were investigated (Martinovic et al., 2008). Both vinclozolin treatments abolished the trenbolone induced formation of nuptial tubercles in females. The co-exposures did not significantly impact changes in the fatpad thickness in males, which is increased by trenbolone, although the 591 μg/L vinclozolin partially blocked this effect in a non-significant manner. Vinclozolin exposure did not prevent the trenbolone induced decrease in plasma VTG in females (Martinovic et al., 2008). A summary of the findings from the studies testing anti-androgens is presented in Table 4-4a (female responses) and Table 4-4b (male responses).

Table 4-4a. Androgen receptor antagonists: Female responses.

Numbers represent the lowest dose at which a significant effect occurred for the listed endpoint. Blanks indicate that a particular endpoint was not assessed. Unless otherwise indicated, data are results of 21-day exposures. Group spawn denotes the optimized 21-day protocol with 2 males, 4 females per tank, 4 tank replicates per treatment. Details are provided in the text.

Test Configuration	Treatment	Dose Range (µg/L)	Fecundity	Gonad Histology	GSI	E2	T	11-KT	VTG	Tubercles	Fat Pad	Reference
Group Spawn	Flutamide	62.7, 651	↓ 651 ↓ hatch 651	↑ atresia, ↓ maturity 651	-	-	↑ 651		↑ 651			Jensen et al., 2004
Group Spawn[1]	Flutamide	43/47, 504/519	↓ 504	↑ atresia 504		-	↑ 504*		-			Battelle, 2003
Group Spawn[2]	Flutamide	100, 500, 1000	↓ 1000 (1) ↓ fertilization 1000 (2)	-	-				-			Battelle, 2006
Group Spawn[3] (5M, 5F)	Flutamide	68.7 - 83.5 354 - 464 552 - 875	↓ 552 (3)[#]	↑ atresia (3)					↑ 354 (1)			OECD, 2005
Non-spawning	Flutamide	43/47, 260, 504/519		-		-	-		-			Battelle, 2003
Non-spawning	Flutamide	95.3, 320.4, 938.6			-				↑ 95.3			Panter et al., 2004
Group Spawn	P,p'DDE	0.022, 0.167	-	↑ atresia 0.167		-	-		-			Battelle, 2005
Pair Spawn	Vinclozolin	200, 700		↓ maturity 700	↓ 700	-	-					Makynen et al., 2000
Pair Spawn	Vinclozolin	60, 255, 450	↓ ≥ 60	↑ atresia ≥ 60	-		-		↑ ≥ 255			Martinovic et al., 2008

[1]Exposure for 21 days or for 14 days, * effects that occurred only in fish exposed for 21 days, but not for 14 days

[2]Numbers in parentheses indicate number of laboratories finding a particular results, 2 laboratories participated

[3]Numbers in parentheses indicate number of laboratories finding a particular results, 3 laboratories participated

[#]Fecundity measured as presence/absence of daily spawning

Table 4-4b. Androgen receptor antagonists: Male responses.

Numbers represent the lowest dose at which a significant effect occurred for the listed endpoint. Doses enclosed in parentheses indicate a trend. Blanks indicate that a particular endpoint was not assessed. Unless otherwise indicated, data are results of 21-day exposures. Details are provided in the text.

Test Configuration	Treatment	Dose Range (µg/L)	Fecundity	Gonad Histology	GSI	E2	T	11-KT	VTG	Tubercles	Fat Pad	Reference
Group Spawn	Flutamide	62.7, 651	↓ 651 ↓ hatch 651	Spermatocyte degeneration/necrosis ↑ 62.7	-	-	-	-	↑ 651	-		Jensen et al., 2004
Group Spawn[1]	Flutamide	43/47, 504/519	↓ 504	-				↑ 504*	-			Battelle, 2003
Group Spawn[2]	Flutamide	100, 500, 1000	↓ 1000 (1) ↓ fertilization 1000 (2)	↑ spermatogonia ≥ 500 (2)	↓ ≥ 500 (1)				-	↓ ≥ 100 (1)	↓ 1000 (2)	Battelle, 2006
Group Spawn[3] (5M, 5F)	Flutamide	68.7 - 83.5 354 - 464 552 - 875	↓ 552 (3)[#]	↑ Leydig cells (2) ↑ spermatogonia (2)					-	↓ 552 (1)		OECD, 2005
Non-spawning	Flutamide	43/47, 260, 504/519		-		-	-		-			Battelle, 2003
Non-spawning	Flutamide	95.3, 320.4, 938.6			-				-	↓ 938		Panter et al., 2004
Group Spawn	P,p'DDE	0.022, 0.167	-			-	-		-			Battelle, 2005
Pair Spawn	Vinclozolin	200, 700		-	-	↑ 700	-	-				Makynen et al., 2000
Pair Spawn	Vinclozolin	60, 255, 450	↓ ≥ 60	-	↑ ≥ 255		-		-	↓ 450	-	Martinovic et al., 2008

[1]Exposure for 21 days or for 14 days, * effects that occurred only in fish exposed for 21 days, but not for 14 days

[2]Numbers in parentheses indicate number of laboratories finding a particular results, 2 laboratories participated

[3]Numbers in parentheses indicate number of laboratories finding a particular results, 3 laboratories participated

[#]Fecundity measured as presence/absence of daily spawning

Steroid Metabolism Modulators

A recent review of sex steroid biosynthesis (steroidogenesis) concluded that many structurally diverse chemicals are capable of causing endocrine disruption by inhibiting the activity of key enzymes in androgen and estrogen synthesis (Sanderson 2006). A well-characterized steroidogenesis inhibitor in fish is fadrozole, which is a competitive inhibitor of CYP19 (aromatase), a key enzyme in the biosynthetic pathway for estrogen. Initial studies of fadrozole using the optimized group spawning protocol were reported by Ankley et al. (2002) using measured concentrations of 1.4, 7.3 and 57 µg/L. This study measured brain aromatase activity after the 57 µg/L treatment and observed a significant reduction in activity in both sexes. As expected, fadrozole treatment decreased both E2 and VTG levels in female minnows in a concentration-dependent manner (Ankley et al., 2002). In male fish, both 11-KT and T were increased after all treatments, significantly so at the two highest exposure levels. A significant increase in GSI was also observed in males but not females, at the two highest treatments (Ankley et al., 2002). There was no effect on external morphology, but increasing fadrozole concentration altered ovarian histology, producing an overall immature appearance characterized by the complete lack of stage 4 and 5 oocytes (late vitellogenic oocytes) and progressive increases in the number of atretic follicles (Ankley et al., 2002). In males, fadrozole treatments caused pronounced enlargement of the semeniferous tubule lumen, which was filled with sperm (Ankley et al., 2002). With respect to reproduction, fecundity decreased from mean control values of 20.5 eggs / female / day to 8.9, 1.5 and 1.2 eggs / female / day after treatment with 1.4, 7.3 and 57 µg/L fadrozole respectively (Ankley et al., 2002). At the two higher concentrations, the impact on fecundity was apparent within two days of initiating exposure, indicating how rapidly fadrozole was absorbed and impacted E2 synthesis and spawning.

A subsequent study of fadrozole was performed by another laboratory using the optimized 21-day group spawning protocol, the 14-d group spawning protocol, and a 14-day non-spawning protocol (Battelle 2003a). Measured low and high fadrozole concentrations were 5.1-5.5 and 55.7 – 60.0 µg/L (minimum and maximum levels for low and high concentrations from weekly sampling) with an intermediate level of 31.7 µg/L (overall mean of weekly sampling) included in the non-spawning protocol. Consistent with the Ankley et al. (2002) study, E2 and VTG levels in females were significantly decreased in all high exposure groups, and in the low exposure group from the 21-day spawning protocol. Plasma T concentrations were increased in both males and females at all exposures in the group spawning protocols and in males only at the high treatment in non- spawning fish (Battelle 2003a). Cumulative fecundity was significantly decreased after the high treatment level in all group spawning fish. Quantitative histological examination of the ovaries indicated a statistically significant reduction in stage 4 and stage 5 oocytes in all treatments of group spawning fish, and at the two higher treatments with the non-spawning fish. In males, changes in GSI and testes histology were only observed in the 21-day group spawning fish, where increases in tubule diameter were associated with an overall increase in GSI (Battelle 2003a).

An inter-laboratory evaluation of fadrozole was performed by four different laboratories using a group spawning protocol consisting of five male and five female minnows (n=2 tank replicates / treatment) exposed for 21 days (OECD 2005b). A single test concentration was used with the mean measured value ranging from 93 – 105 µg/L among the laboratories. Qualitative assessment of spawning indicated no change in spawning frequency although generally low

spawning in the control groups limited interpretation of this endpoint. There was no change in the numbers of nuptial tubercles in male minnows. All laboratories observed significant decreases in VTG levels in females after the fadrozole treatment. Several changes in gonad histology were noted in both sexes. In the testes, three of the four laboratories observed proliferation of Leydig cells and two laboratories observed increased numbers of spermatozoa. In the ovaries, three of the laboratories observed increases in oocyte atresia (OECD 2005b).

An additional study of fadrozole was performed by Panter et al. (2004) using a non-spawning protocol where sex-specific groups of fish (n = 18 / treatment level) were exposed to measured concentrations of 24.8, 51.7 and 95.5 µg/L (mean values of weekly sampling) for 14 and 21 days. Consistent with the previous studies using spawning protocols, the two highest treatments increased the male GSI after 21 days (Panter et al., 2004). In females, both the GSI and plasma VTG were significantly decreased at all three exposure levels after 21 days. A summary of the findings from the studies testing fadrozole is presented in Table 4-5a (female responses) and Table 4-5b (male responses).

Table 4-5a. Fadrozole: Female responses.

Numbers represent the lowest dose at which a significant effect occurred for the listed endpoint. (-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Blanks indicate that a particular endpoint was not assessed. Unless otherwise indicated, data are results of 21-day exposures. Group spawn denotes the optimized 21-day protocol with 2 males, 4 females per tank, 4 tank replicates per treatment. Details are provided in the text.

Test Configuration	Dose Range (µg/L)	Fecundity	Gonad Histology	GSI	E2	T	11-KT	VTG	Tubercles	Fat Pad	Reference
Group Spawn	1.4, 7.3, 57	↓	↓ maturity ↑ atresia	-	↓ ≥ 7.3	-		↓ ≥ 1.4	-		Ankley et al., 2002
Group Spawn	5.1-5.5, 55.7 - 60	↓ 55.7	↓ maturity		↓ ≥ 55.7	↑ ≥ 5.1		↓ ≥ 5.1			Battelle, 2003
Group Spawn[1] (5M, 5F)	93 – 105	-*(4)	↑ atresia (3)					↓ (4)			OECD, 2005
Non-spawning	5, 32, 55		↓ maturity ≥ 32		↓ 55	-		↓ 55			Battelle, 2003
Non-spawning	24.8, 51.7, 95.5			↓ ≥ 24.8				↓ ≥ 24.8			Panter et al., 2004

[1]Numbers in parentheses indicate number of laboratories finding a particular results, 4 laboratories participated

*Fecundity measured as presence/absence of daily spawning

Table 4-5b. Fadrozole: Male responses.

Numbers represent the lowest dose at which a significant effect occurred for the listed endpoint. (-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Blanks indicate that a particular endpoint was not assessed. Unless otherwise indicated, data are results of 21 day exposures. Details are provided in the text.

Test Configuration	Dose Range (µg/L)	Fecundity	Gonad Histology	GSI	E2	T	11-KT	VTG	Tubercles	Fat Pad	Reference
Group Spawn	1.4, 7.3, 57	↓	↑ sperm production	↑ ≥ 7.3		↑ ≥ 7.3	↑ ≥ 7.3	-	-	-	Ankley et al., 2002
Group Spawn	5.1-5.5, 55.7 - 60	↓ 55.7	↑ sperm production	↑		↑ ≥ 5.1		-			Battelle, 2003
Group Spawn[1] (5M, 5F)	93 - 105	-*(4)	↑ sperm production (2) ↑Leydig cells (3)					- (4)			OECD, 2005
Non-spawning	5, 32, 55		-	-		↑ 55		-			Battelle, 2003
Non-spawning	24.8, 51.7, 95.5			↑ ≥ 51.7				-			Panter et al., 2004

[1]Numbers in parentheses indicate number of laboratories finding a particular results, 4 laboratories participated

[*]Fecundity measured as presence/absence of daily spawning

Multi-Modal Chemical Agents

For many of the studies described above, model compounds exhibited the expected predominant mode of endocrine action. Using these types of chemicals is clearly important to improve understanding of how reproductive endpoints will be affected by specific endocrine perturbations. However, many chemicals are also recognized as possessing multiple modes of toxic action that may or may not involve interactions with sex steroid receptors or disturbances in biosynthesis of sex steroids. In addition, a chemical may cause differential responses at different loci within the HPG axis. The multi-modal actions of a chemical may cause additive or synergistic responses of assay endpoints, or may counteract each other to the extent that little change is observed. Thus, it is also important to assess the performance of a short-term assay using chemicals known to possess multiple modes of toxic action. For the fish short-term reproduction assay, four different types of multi-modal chemicals have been evaluated: ketoconazole, prochloraz, cadmium chloride and fenarimol.

Ketoconazole is an anti-fungal drug known to inhibit multiple cytochrome P450 (CYP) enzymes, including CYP1A1 and CYP3A in both fish and mammals (James et al., 2005; Hegelund et al., 2004). Ketoconazole also inhibits 14α-demethylase, the enzyme responsible for cholesterol synthesis in mammals, and inhibits several P450 enzymes important for steroidogenesis in mammals, particularly CYP 11A (cholesterol sidechain cleavage), CYP17 (17α-hydroxylase and 17,20 lyase activity), and CYP11B1 (11β-hydroxylase) (Feldman 1986; Sonino, 1987). Clinical administration of ketoconazole as a therapy for hypercortisolism reduces plasma testosterone, enhances the estrogen:testosterone ratio in men and can induce gynecomastia (breast formation) (Sonino, 1987). More recently, ketoconazole has been demonstrated to inhibit the expression and activity of the orphan nuclear receptors, constitutive androstane receptor (CAR) and pregnane X receptor (PXR), which regulate the production of several enzymes important in the cellular uptake and elimination of sex steroids (Duret et al., 2006; Huang et al., 2007).

Ketoconazole was evaluated using the optimized group spawning protocol and four different exposure concentrations of 7.0, 25, 88 and 357 µg/L (mean value of six measurements) (Ankley et al., 2007). Spawning frequency and fecundity were significantly decreased at the 25 and 357 µg/L exposure levels but not at the intermediate exposure level of 88 µg/L due to high variability in the fecundity data within treatment replicates. Fertilization rate and hatching success were not affected by the exposures nor were there any changes in external morphology. The GSI for both sexes was increased at the highest exposure level and also for males at the 88 µg/L treatment. Changes in gonad histology were most noticeable in males, where a concentration dependent increase in the numbers of fish exhibiting proliferation of interstitial cells (steroidogenic cells) was observed. There were no significant changes or consistent trends in the biochemical endpoints in either sex (Ankley et al., 2007).

Additional endpoints characterized after 21-day exposures were gonadal gene expression for specific steroidogenic enzymes (CYP11A, CYP17, and CYP19A), *in vitro* formation of T in ovarian and testicular tissue removed from exposed fish, and hepatic gene expression of CYP1A1 and CYP3A (Ankley et al., 2007). Ketoconazole exposure decreased T formation in cultured gonads from both sexes, as might be expected based on clinical data cited above. In ovaries, significant decreases in T formation were observed at the 25, 88 and 357 µg/L exposure

levels and in testes at the 357 µg/L exposure. In contrast, ketoconazole treatment tended to increase gene expression of enzymes involved in steroidogenesis (CYP11A) and androgen formation (CYP17). These increases were statistically significant at the highest ketoconazole concentration. There were no significant changes in expression of aromatase (CYP19A) in the ovaries. Higher exposure concentrations decreased hepatic gene expression of CYP1A1 in male minnows (Ankley et al., 2007). Collectively, the increased gonadal expression of CYP11A and CYP17, proliferation of interstitial cells in the testis, and normal circulating T suggest a compensatory response to the tested doses of ketoconozole. *In vitro* data clearly indicate inhibition of steroidogenesis, while *in vivo* data show compensatory changes in gene expression and steroidogenic cell activity to maintain endocrine homeostasis.

An additional study of ketoconazole using the optimized group spawning protocol and three nominal ketoconazole test concentrations of 25, 100 and 400 µg/L obtained similar results (Battelle 2006). This study also observed significant increases in the numbers of interstitial (Leydig) cells in the testes after all ketoconazole treatments and a significant increase in male VTG levels after the high treatment (Battelle 2006).

Prochloraz is an azole-based fungicide that also inhibits the activity of several CYPs involved in steroidogenesis, such as CYP17 (Blystone et al., 2007). In addition to being a potent aromatase inhibitor, prochloraz has also been demonstrated to possess anti-androgenic and anti-estrogenic properties (Andersen et al., 2002). Prochloraz was studied using a 21-day pair breeding protocol (six pairs / treatment; three tank replicates) and measured concentrations of 32, 116 and 311 µg/L (mean value of six measurements) (Ankley et al., 2005a). Spawning was significantly decreased at the 116 and 311 µg/L treatment groups, where only limited, infrequent spawning occurred during the exposure. There was no change compared to controls in fertilization or hatching success. Prochloraz treatment increased both the numbers of atretic follicles in the ovaries and spermatogonia in the testes. Among the biochemical endpoints, the high prochloraz exposure caused significant decreases in T and 11-KT in males, while in females, concentration dependent decreases in VTG and E2 were observed. Brain aromatase activity was unaffected in females but was significantly decreased in males at the 311 µg/L treatment. *In vitro* experiments using post-mitochondrial supernatant prepared from unexposed brain and ovarian tissue demonstrated that prochloraz inhibited aromatase activity with IC_{50} values of 11.2 and 7.2 µM respectively (Ankley et al., 2005a). These inhibitory constants are similar to those calculated for fadrozole in fathead minnow brain and ovarian tissue, which were 8.8 and 6.9 µM respectively (Villeneuve et al., 2006b). Prochloraz was also demonstrated to bind recombinantly expressed fathead minnow androgen receptor with an IC_{50} of 10 µM (Ankley et al., 2005a). Collectively, these results indicate that prochloraz acts *in vivo* as an inhibitor of steroidogenesis, rather than as an anti-androgen. The endpoint responses in females are consistent with a suppression of aromatase activity, as decreased E2, VTG and increased atresia are similar to results from fadrozole studies. In males there was evidence for inhibition both of aromatase activity and CYP17 lyase, as plasma T and 11-KT concentrations were reduced by prochloraz.

An inter-laboratory evaluation of prochloraz was performed by four different laboratories using a group spawning protocol consisting of five male and female minnows (n=2 tank replicates / treatment) exposed for 21 days (OECD 2005b). Three different prochloraz concentrations were tested with measured values of 15.3 – 24.1, 68.9 – 121 and 275 – 382 µg/L (minimum – maximum values for participating laboratories). Endpoints measured were

qualitative daily assessment of spawning, quantitative assessment of nuptial tubercles, plasma VTG and gonad histology. Only one laboratory observed significant decreases in spawning frequency associated with prochloraz exposure with complete cessation of spawning observed after the high exposure level. For the other laboratories, poor spawning in control tanks limited assessment of the effects of prochloraz exposure. There were no significant changes in male nuptial tubercles observed after the prochloraz treatments. Two laboratories noted the occasional appearance of a single nuptial tubercle in females exposed to prochloraz. All laboratories observed significant decreases in VTG levels in females after the high prochloraz treatment with one laboratory also observed a decrease at the intermediate level. Several changes in gonad histology were noted in both sexes. In the testes, three of the four laboratories observed dose-dependent proliferation of leydig cells and increased numbers of spermatgonia. In the ovaries, all laboratories observed increases in oocyte atresia (OECD 2005b).

Cadmium (Cd) is a well studied contaminant that has been shown to cause a variety of sub-lethal effects in fish. An early study by Thomas (1989) reported 1 mg/L Cd increased circulating levels of E2 after 40 days in Atlantic croaker (*Micropogonias undulates*). A later study reported that similar Cd exposures increased the spontaneous release of gonadotropins from isolated croaker pituitaries (Thomas 1993). More recent studies have suggested that Cd may be weakly estrogenic (Choe et al., 2003; Johnson et al., 2003) and anti-androgenic (Martin et al., 2002). Thus, Cd appears capable of disturbing the HPG axis at multiple locations with multiple modes of action. Cadmium has been studied in fathead minnows using the optimized group spawning protocol and with a pair breeding protocol. In the latter study, 18, 15, 12 and 4 breeding pairs (corresponding to 9, 8, 6, 2 tank replicates) were exposed to measured Cd concentrations of 8.6, 24.3 and 39.7 µg/L respectively (Sellin and Kolok 2006). Decreased fecundity was observed at all exposure levels although only the highest treatment level was significantly different from controls (Sellin and Kolok 2006). Fertilization and hatching success were not altered. Overall, there were no differences in external morphology although a significant decrease in nuptial tubercles was noted in at an intermediate exposure level of 24.3 µg/L (Sellin and Kolok 2006). Other than an increase in 11-KT in males after the low treatment, there were no changes in biochemical endpoints (VTG was not reported in this study). Another study used Cd with the optimized group spawning protocol and measured concentrations of 1.2 and 11.0 µg/L (mean of weekly measurements). No changes in protocol endpoints including fecundity, qualitative and quantitative gonad histology or biochemical parameters (including VTG) were observed (Battelle 2005). Overall Cd – at the comparatively low concentrations used in these studies – appears to have little effect on HPG function in the fathead minnow.

A fungicide that is now established to act as an aromatase inhibitor in mammals is fenarimol (Andersen et al., 2002). Other actions of fenarimol include preganane X receptor binding (Lemaire et al., 2006) and anti-androgenic activity (Vinggaard et al., 2005). Fenarimol has been studied at measured concentrations of 96 and 569 µg/L (mean value of six measurements) using a 21-d pair breeding protocol (four pairs / treatment; two tank replicates) (Ankley et al., 2005a). Spawning was decreased in both treatment groups, with the high fenarimol treatment causing an immediate cessation of spawning. The fenarimol treatment appeared to increase the number of atretic follicles in most females and increase the number of spermatogonia in males. None of the biochemical endpoints were affected in male fish, however both exposure levels significantly increased E2 levels in females. Interestingly, VTG levels were significantly decreased in females after the high exposure despite the increase in circulating E2. An additional endpoint measured was brain aromatase activity, which was unaffected in both

sexes after the fenarimol treatments. In contrast, *in vitro* experiments using the post-mitochondrial supernatant prepared from unexposed brain and ovarian tissue demonstrated that fenarimol can weakly inhibit ovarian aromatase activity, though not to the extent that permitted calculation of an IC_{50} (Ankley et al., 2005a). Hence, the action of fenarimol as an aromatase inhibitor in fish is less clear than in mammals. Another interesting finding from this study was that fenarimol bound to recombinantly expressed fathead minnow androgen receptor with an IC_{50} of 25 μM. Overall, data from this study suggest that fenarimol may have multiple endocrine modes of action in the fish short-term reproduction assay.

Uncertain Modes of Action

Chemicals known to disturb normal estrogen and androgen signaling or steroid biosynthesis have been the major focus of most optimization studies using the fish short-term reproduction assay. However, other, less well characterized modes of endocrine interference exist. For example, chemicals can disturb progestin signaling or interrupt biosynthesis of hypothalamic and pituitary hormones in fish (Kime, 1998), but these mechanisms have been investigated in only a few fish species. Alternatively, other endocrine pathways important in coordinating maturation and reproduction such as growth hormone, insulin-like growth factor-I and corticosteroids may be targets of endocrine disruption (Filby et al., 2007). Although the fish short-term reproduction assay has been optimized primarily to detect estrogen and androgen agonists / antagonists, capacity to detect endocrine-mediated reproductive effects of chemicals with uncertain modes of action is desirable. In this section, the results of testing with four different chemicals that appear to impact reproduction by complex and uncertain modes of action are discussed.

Atrazine is a widely used chlorotriazine herbicide. In female rats, atrazine has been shown to induce mammary tumors among a variety of other effects characteristic of exogenous estrogen exposure (reviewed in Sanderson 2006). Atrazine does not appear to be a direct estrogen receptor agonist, but may induce aromatase activity and enhance endogenous estrogen synthesis (Sanderson et al., 2001). However, conflicting results have been obtained in amphibian studies and it is unclear whether atrazine modulates steroidogenesis (Hayes et al., 2002; Hecker et al., 2005). Another hypothesis for atrazine's mode of action is through disturbance of hypothalamic control of gonadotropin release from the pituitary (Cooper et al., 2000). Thus, exposure to atrazine (and potentially other triazine herbicides) could cause reproductive effects reflective of changes in circulating sex steroids and gonadal maturation in the fish test. Atrazine has been studied twice using the optimized group spawning protocol. Bringolf et al. (2004) measured atrazine effects at two treatment levels of 4.32 and 43.63 μg/L (mean value of weekly determinations). The atrazine exposure was maintained as a static renewal system with 25% of exposure water replaced daily. A solvent-only tank was included to provide a control for methanol and acetone (50:50 v/v) added at 0.001% as a dosing vehicle for atrazine. Atrazine exposure did not affect external morphology, fecundity, fertility or hatching success. The GSI in males was decreased compared to controls, but not to a statistically significant level. Quantitative staging of the gonads indicated atrazine had no significant differences from controls. Plasma VTG was significantly increased in males but not in females after the atrazine treatments and the solvent control (Bringolf et al., 2004). Thus, it is unclear whether this finding is attributable to the atrazine exposures.

An additional atrazine study using the optimized group spawning protocol was performed at two measured exposure concentrations of 24.5 and 223 μg/L (Battelle 2005). The overall results from this study indicated atrazine exposure had no statistically significant effects on important assay endpoints. The only noteworthy findings were subtle changes in testicular histology, which included a significant decrease in the semeniferous tubule diameter in the high exposure group and an increased number of sperm at developmental stage 2A. Other noteworthy but not statistically significant trends were slight decreases in fecundity and E2 levels in females and T and 11-KT in males (Battelle 2005).

Prometon is another chlorotriazine herbicide that has been recently studied using a pair breeding protocol (eight pairs / treatment; four tank replicates). Exposure concentrations were 19.6, 46.1, 199 and 999 μg/L (Villeneuve et al., 2006c). Some treatment related mortality of females occurred in the highest exposure group. Despite this, no treatment related changes in fecundity or fertility were observed. With regard to external morphology, most prometon treatments decreased the size of the fat pad in males as measured by its relative mass (fat pad index). However, no change in nuptial tubercles was observed nor were there any treatment effects on gonad histology. Among the biochemical endpoints, the notable change was increased levels of T at all treatment levels in females, although only the 19.6 μg/L exposure was statistically significant. Other endpoints assessed in this study were ovarian aromatase activity and FSH and LH β subunit gene expression in the pituitary. Although no statistically significant changes occurred in these endpoints, aromatase activity was decreased approximately three-fold in the 19.6 μg/L treatment and less so in the higher treatment levels. Pituitary expression of FSHβ was increased at the two exposure levels sampled (46.1 and 999 μg/L) but LHβ appeared to be unchanged (Villeneuve et al., 2006c). Collectively, the findings from the atrazine and prometon studies do not support a classical estrogenic / steroidogenesis modulator / anti-androgenic mode of action. However, some test results suggest these herbicides have more subtle effects upon the HPG axis but specific mode of action(s) cannot be discerned at present.

Perfluorooctane sulfonate (PFOS) is a perfluorinated alkyl compound where fluorine has been substituted for hydrogen. This type of chemical was used in many consumer and industrial products, is exceptionally stable in the environment, and accumulates in fish and other wildlife (Houde et al., 2006a,b). Perfluorooctane sulfonate is considered to be a weak peroxisome proliferator in rodents due to activation of the PPAR-α receptor (Takacs and Abbott 2007). Exposure to PFOS in rodent models has been associated with reproductive and developmental toxicity (Lau et al., 2004; 2006). Perfluorooctane sulfonate has been studied in controlled laboratory studies using a pair breeding protocol (eight pairs / treatment; four tank replicates) and in outdoor mesocosms using a group spawning design that consisted of one male and two females separated from other groups (n = 8 groups / treatment; three mesocosm replicates / treatment). In laboratory studies, four measured PFOS concentrations of 27.6, 101, 281 and 818 μg/L were tested (mean of weekly determinations; Ankley et al., 2005b). The 818 μg/L treatment was not well tolerated and was shortened to a 14-d exposure. Spawning was reduced by the three higher exposure levels, although fertilization rate was consistently high in all treatment groups. No effect on male gonadal histology was observed but in females, an increased number of atretic and late vitellogenic follicles was observed in the 281 μg/L treatments. No changes in biochemical endpoints were observed in females but in males, the 281 μg/L treatment increased both T and 11-KT. Brain aromatase activity measured in post-

mitochondrial supernatant was significantly decreased in males at 281 μg/L (Ankley et al., 2005b).

In the outdoor mesocosm exposures, a nominal concentration of 300 μg/L and three higher, measured concentrations of 2,800, 11,800 and 32,900 μg/L (time weight averages) were studied (Oakes et al., 2005). The mean water temperature for these exposures was 22.7 ^{o}C (range 20.1 - 29.2) and pH was 9.2 (8.9-10.9), which are somewhat outside the recommended range for the optimized group spawning protocol. Poor tolerance and excessive mortality occurred in the 11,800 and 32,900 μg/L exposures, giving an LC_{50} of 7200 μg/L for PFOS. In the lower treatments, the average daily fecundity per female was decreased compared to controls, though not in a statistically significant manner. Among biochemical endpoints, significant increases in T and 11-KT in males were observed after the 300 μg/L but not the 2,800 μg/L treatment groups. A significant increase in E2 in females was also observed. Another endpoint measured was hepatic fatty acid Acyl-CoA activity (a marker for peroxisome activity), which was not significantly altered after the 300 μg/L and 2,800 μg/L treatments (Oakes et al., 2005). Thus, both the laboratory and mesocosm study provided comparable results in that the two endpoints primarily affected by PFOS were fecundity and circulating androgen levels in male minnows.

Recently, several lipophilic chemicals used as UV filters in many consumer products have been identified in fish at parts-per-million levels (Balmer et al., 2005). One such chemical, 3-benzlidene camphor (3BC), has been shown to induce VTG in juvenile fathead minnows (Kunz et al., 2006). A study by this group assessed 3BC effects on reproduction using a modified version of the optimized group spawning protocol along with five nominal treatment levels of 1,10, 100, 250 and 500 μg/L (n=3 tank replicates; Kunz et al., 2006). The 3BC exposure was maintained as a static renewal system with 100% of the exposure water replaced every 48 hrs. Measured water concentrations were at or slightly above nominal levels initially but decreased to a level that was 18 – 27% of nominal after 48 hrs. This may have been due in part to bioaccumulation by the fish, which had whole-body levels of 3BC that were 100 – 500 times that of the averaged, measured water concentration during the 21 d study. 3BC exposure significantly decreased fecundity at the 250 and 500 μg/L nominal exposure levels. Fecundity appeared to be lower at lower treatment levels, but not to a significant extent. With regard to external morphology, there was a significant decrease in nuptial tubercles in males exposed to the two highest exposure levels. There was no effect on GSI in either sex but quantitative staging of the gonads indicated 3BC exposure at the 10 - 500 μg/L nominal levels significantly decreased the numbers of spermatocytes observed in the testes. In the ovaries, there was a significant increase in atretic follicles and a decrease in the numbers of early and late vitellogenic follicles. Perhaps the most striking result from this study is the concentration-dependent induction of VTG in males, but not females. In males exposed to the two highest treatment levels of 3BC, VTG levels exceeded those observed for females (Kunz et al., 2006). Thus, the weight of evidence from this study strongly suggests an estrogenic mode of action for 3BC although the lack of more specific responses in females raises some question as to whether a more novel mode of action may be occurring.

Complex Mixtures

Several industrial effluents, including bleached kraft mill effluent (BKME) and metal mining effluent (MME) have been studied using a pair spawning protocol (Rickwood et al.,

2006a;b). Exposure concentrations of BKME were 0, 1, or 100% in a flow-through system, nine pairs / treatment, three tank replicates (Rickwood et al., 2006a). Fecundity and secondary sex characteristics were compared within each treatment pre- and post-exposure and compared across treatments during exposure. Pre- and post-exposure fecundity was similar for all treatments, but post-exposure fecundity was significantly higher in the 1% BKME treatment. Both male and female external morphology were affected. At 100% BKME, females developed male-like coloration, but no nuptial tubercles and at both BKME dilutions, males developed ovipositors. Gonad histology showed a reduction in the proportion of oogonia in ovaries at the 100% BKME dilution, but no treatment effect on testicular morphology. Plasma T concentration was the only biochemical endpoint measured. Female T was significantly higher at 100% BKME, while male T was significantly elevated only at 1% BKME (Rickwood et al., 2006a). Collectively, the responses suggest the particular KME contains compounds with both estrogenic and androgenic modes of action. The increase in fecundity at low effluent concentrations is similar to the fecundity effects of low doses of ethinylestradiol (Pawlowski et al., 2004b), while the mild increase in male secondary sex characteristics in females and female secondary sex characteristics in males are consistent with the actions of an aromatizable androgen (Rickwood et al., 2006a).

Exposure to MME from a copper mine, 45% MME in a flow-through system, five pairs / treatment, five tank replicates decreased fecundity, but did not alter male or female secondary sex characteristics (Rickwood et al., 2006b). Gonad histology showed significant increases in apoptosis and fibrosis in testes. Like BKME, MME increased the proportion of oogonia in ovaries. GSI was unchanged in both sexes. None of the biochemical endpoints were changed in males, but exposure to MME significantly increased female T and VTG (600% increase) without changing circulating E2 (Rickwood et al., 2006b). The changes in male and female gonads suggest that reduced fecundity is due to impaired gamete production (Rickwood et al., 2006b). Changes in female biochemical endpoints indicate upregulation of VTG synthesis. Although the mechanism by which this occurs in unclear, the profile of changes is similar to the female biochemical responses to flutamide, an anti-androgen (Table 5a). Testicular changes are different from those observed in response to anti-androgens, indicating that multiple mechanisms of endocrine interaction are likely.

Negative Compounds

The fish short-term reproduction assay can identify chemicals that impact reproduction through non-lethal, endocrine pathways versus chemicals that impact reproduction through non-endocrine mechanisms associated with biological stress. To test the specificity of the fish assay, toxicants considered to have no direct interaction with sex steroid signaling were evaluated. Potassium permanganate and ammonium perchlorate were tested using the optimized group spawning protocol to assess the response of measurement endpoints during prolonged systemic toxicity.

Potassium permanganate is an oxidizing agent used to treat a variety of external fish parasites and was tested by two independent laboratories using the optimized group spawning protocol (Battelle 2006). The nominal exposure concentrations were 225, 400 and 900 μg/L, which represent 16 - 64% of a reported no-observable-effect-concentration value for acute potassium permanganate toxicity calculated from juvenile fathead minnow exposures (Hobbs et

al., 2006). However, one participating laboratory estimated the LC_{50} of the reproductively active fish used in their studies to be 662 µg/L (Battelle 2006). Thus, the high and perhaps the intermediate exposure levels were sufficient to be acutely toxic to the fish. This observation is consistent with the results from both laboratories that indicated survival was significantly decreased in the high exposure groups and also at the 400 µg/L exposure level at one laboratory (Battelle 2006). Not surprisingly, spawning was significantly decreased at both the mid and high exposure levels. The only other statistically significant finding was a decrease in female GSI at all three exposure levels. The latter was only observed in one of the three laboratories (Battelle 2006). Overall, there were no notable changes in measurement endpoints that did not occur at exposure levels which caused significant decreases in survival (Battelle 2006)

Perchlorate is a by-product of rocket fuel production, widely found in groundwater and is known to competitively inhibit iodine uptake by the thyroid gland (Wolff 1998). Decreased iodine uptake can alter thyroid gland function, and lower secretion of thyroid hormones. In fish, normal thyroid function is essential for maintaining growth and normal reproductive performance (Cyr and Eales 1996). Ammonium perchlorate was tested using the optimized group spawning protocol. The two measured exposure levels were 5,638 and 43,500 µg/L (mean of weekly measurements). There was no effect of perchlorate on fecundity, fertilization rate or larval survivorship (Battelle 2005) which is not surprising as thyroid-active chemicals may not affect the HPG axis in the fish short-term reproduction assay due to the short duration of the study. There was no significant effect on GSI of either sex, however histological examination identified a significant increase in atretic ovarian follicles in both perchlorate treatment groups. A significant decrease in primary growth oocytes was also observed. In males, no changes in testes histology occurred. Among the biochemical endpoints, the only change was a non-significant increase in T and 11-KT in males (Battelle 2005).

4.2 Comparison of Group Spawning and Non-spawning Protocols

A simultaneous comparison of the optimized group spawning protocol, a 14-day version of the group spawning protocol with minimal pre-exposure evaluation, and a 14-day non-spawning protocol using single sex groups of fathead minnows has been conducted (Battelle 2003a). As described above, four chemical agents were tested in each protocol: methoxychlor, flutamide, 17β-trenbolone and fadrozole. Only aspects of protocol performance are discussed in this section.

Both spawning protocols, 14- and 21-days, provided entirely consistent fecundity results and similar biochemical endpoint results, although biochemical endpoints showed more pronounced changes with flutamide and fadrozole using the optimized (i.e., longer) protocol. Quantitative fecundity estimates during the pre-exposure evaluation period did not improve the power of the statistical analysis of fecundity after chemical exposure.

Comparison of the group spawning and non-spawning protocols suggested that all protocols would provide similar conclusions about strong endocrine disruptors tested at high test concentrations. However, comparison of the results from the weaker endocrine disruptors and from all chemicals at low exposure concentrations indicated that the non-spawning protocol is less sensitive, so has a greater tendency to provide false negative results. For example, all endpoints were negative for both sexes in the non-spawning assay at high and low flutamide exposures.

4.3 Transferability

Comparison of results among laboratories that have conducted studies with the optimized group spawning 21-day assay shows that the protocol is successfully transferable. Across laboratories, compounds with a particular mechanism of action generate a similar profile of core endpoint responses. Estrogen and androgen agonists (Tables 4-2a,b and 4-3a,b) give consistent results with the core endpoints of fecundity, secondary sex characteristics, sex steroids, VTG and histopathology in both sexes. The anti-androgen flutamide has been tested in group spawning protocols in multiple laboratories and consistently reduces fecundity (6 of 7 laboratories), increases oocyte atresia (5 of 7 laboratories), and increases the proportion of spermatogonia, i.e. inhibits spermatogenesis (4 of 7 laboratories) at high concentrations (Tables 4-4a,b). Male secondary sex characteristics are relatively resistant to flutamide; high concentrations only reduced tubercle score in two of six spawning studies. VTG was the only biochemical endpoint measured in all laboratories. High flutamide concentrations increased female VTG in two studies and male VTG in only one study (Tables 4-4a,b). The core endpoints of fecundity and histopathology were most effective in detecting anti-androgenic effects of flutamide.

The steroidogenesis modulator fadrozole has also been tested in multiple laboratories. As predicted for a compound that blocks E2 production, female VTG concentrations are consistently reduced (6 of 6 laboratories) and in some cases ovarian maturity is retarded (2 of 6 laboratories) while oocyte atresia is increased (4 of 6 laboratories)(Table 4-5a). Two laboratories measured sex steroid concentrations, and in both fadrozole treatment suppressed female E2 and elevated male T and 11-KT as expected when E2 production is blocked. Fadrozole consistently alters testis histology, increasing sperm production (4 of 6 laboratories) and in some cases Leydig cell proliferation (2 of 6 laboratories, Table 4-5b). Fecundity was significantly decreased in two studies, probably because in the other four studies, only presence or absence of spawning was scored and spawning in controls was low (OECD 2005b). The core endpoints of sex steroids, VTG, gonad histology, and fecundity gave results consistent with the known mechanism of fadrozole action.

4.4 Lessons Learned From Optimization Studies

In addition to evaluating the fish assay with different model compounds, formal optimization studies also evaluated several aspects of the protocol related to test configuration, value of specific endpoints and measurement techniques. The insights gained from these efforts have helped to provide answers to important questions such as: Is the added expense of collecting quantitative fecundity and histopathological data warranted? What is an acceptable number and ratio of male and female minnows for each treatment replicate? Does inclusion of plasma sex steroid measurements provide any added benefit to the assay? Is there any benefit in measurement of secondary sex characteristics such as nuptial tubercles and fatpad mass? Is there any real advantage to measuring liver VTG mRNA as opposed to measuring plasma VTG? A summary of the evidence that answers these questions is presented below.

Fecundity

There is strong evidence from the formal optimization studies and related research activities reviewed above supporting fecundity as the most important endpoint in the fish

reproduction assay. There is little doubt that fecundity represents the principal integrative endpoint in the fish short-term reproduction assay. Fecundity measurements do not require high technical expertise, allowing excellent transferability and inter-laboratory comparisons. Reduced fecundity has been the most consistently observed finding after exposure to diverse endocrine active substances, including all of the primary modes of action the assay is designed to detect: estrogen receptor agonists, androgen receptor agonists, androgen receptor antagonists, and steroidogenesis inhibitors (see Tables 4-1 to 4-5). Although fecundity can be influenced by non-chemical factors, its inclusion will decrease the likelihood of false negative conclusions. An argument can also be made that it is ethically incumbent to collect all available data from animals used in testing, especially where this information in one test will reduce the likelihood that repeated or additional tests would be necessary.

Alternatives to the optimized group spawning protocol have been tried and found to be inferior. For example, a concern noted previously with non-spawning protocols is the tendency to cause artifactual changes in gonad histology, such as increased oocyte atresia, and increase variability in the biochemical endpoints, which ultimately reduces statistical power of the assay. Qualitative as opposed to quantitative characterization of spawning performed in one inter-laboratory study (OECD 2005b) provided inconsistent results and identified poor spawning behavior in control fish as a potential complication, which might otherwise be avoided if quantitative fecundity data was collected during the pre-validation phase of the study. Semi-quantitative measurement of fecundity has also been studied, whereby fecundity was initially estimated visually using a matrix (e.g., [0 (none), 10, 25, 50, 100, 150, 200, 250, 300, and >300]) and then all eggs counted as recommended in the optimized protocol (Battelle 2006). The results suggested the semi-quantitative counts were within 20% of the actual values when clutch size was < 250 eggs, but larger errors were noted when clutch size increased (Battelle 2006). Using equal numbers of male and female minnows in a test replicate as opposed to the two male and four females advocated in the optimized protocol has also been observed to be less than ideal due to excessive male aggressive behavior (OECD 2005b, Kramer et al., 1998).

Histopathological Analyses

Histopathological examination of the gonads has been consistently recognized as being diagnostically informative during optimization studies (attachment H). The microscopic structure of the gonads provides direct evidence for the overall health or dysfunction of the tissue. As previously noted, histopathological analysis was instrumental in identifying problems associated with non-spawning test configurations, which might otherwise have gone unnoticed. In addition, this endpoint has proven to be important because it provides a bridge between the more general whole fish endpoints (fecundity, morphology) with the more specific biochemical endpoints. This is particularly valuable when decreases in fecundity have occurred as it can provide additional confidence the decrease is associated with endocrine-mediated changes. In some studies such as those with ketoconazole, histopathological changes in the testes were the predominant endpoint affected by the treatment (Battelle 2006; Ankley et al., 2007). Thus, histopathological analysis can also help to reduce the number of false negative conclusions.

With regard to the variability associated with histopathology, it should be recognized that diagnostic criteria such as increased oocyte atresia in the ovaries and proliferation of interstitial cells in the testes do not represent separate discreet endpoints. Rather, it is the summary of the pathology and overall response of the gonad that represents the endpoint. It is worth noting that

when comparable exposures have been performed, the pathology reports have proven to be consistent among different laboratories (Battelle 2003a; OECD 2005b).

Secondary Sex Characteristics

Inclusion of male secondary sex characteristics has been demonstrated to provide the fish short-term reproduction assay with an endpoint that is indicative of an androgenic / anti-androgenic mode of action, which may not be observed with other endpoints. Inter-laboratory comparisons of secondary sex characteristics as endpoints have been relatively reproducible. For example, in the OECD (2005) study with 4-tertpentylphenol, all participating laboratories observed decreases in the numbers of male nuptial tubercles. An early OECD-sponsored study demonstrated that trenbolone-induced formation of nuptial tubercles in females can be detected (OECD 2006). In another study, exposure to high concentrations of flutamide (1000 µg/L) was observed by all participating laboratories to decrease the thickness of the male fatpad (Battelle 2006). Although changes in secondary sex characteristics do not appear to be responsive to certain modes of action (for example, steroid metabolism modulators), its sensitivity towards (anti-) androgens strongly warrants its inclusion.

Vitellogenin

Measurement of the VTG endpoint is well established as the most valuable biochemical endpoint in the fish assay. Research in the fathead minnow and other fishes has indicated that changes in hepatic gene expression of VTG can be detected before increases in circulating levels of the VTG protein (Korte et al., 2000). These observations combined with the limited volumes of plasma that can be collected from a single fish raised the question of whether hepatic VTG mRNA measurements would make a better endpoint than plasma VTG. However, the recent availability of fathead minnow VTG ELISA kits and their demonstrated reproducibility and inter-laboratory comparability have greatly helped to standardize plasma VTG measurements even with small (e.g. ≤ 5 µl) volumes commonly used in the assay (Jensen and Ankley 2006; Battelle 2006). This is important particularly in regards to previous findings, which have found poor inter-laboratory comparability of VTG mRNA measurements (Battelle 2003b). Also, the advantage of hepatic VTG mRNA responding more rapidly is of diminished value in the context of a short-term assay, where measurements will be made after 21 days of exposure.

Sex Steroid Measurements

Changes in circulating sex steroid levels appear to typically coincide with alterations in other endpoints such as fecundity and VTG. However, measurement of sex steroid levels has proven to be helpful in two important ways: they 1) provide additional supportive information that an endocrine mediated as opposed to non-endocrine mediated mode of action is occurring, which is especially valuable when decreased fecundity is also observed; 2) provide important insights into the specific mode of action. For example, sex steroid measurements help to identify aromatase inhibitors among other potential steroid metabolism perturbations (Ankley et al., 2002, 2005a). The latter information can be particularly helpful for guiding subsequent studies in a multi-tiered testing program.

4.5 Exposure Analyses

Knowledge of the administered concentration is essential for any short-term assay to interpret accurately the biological significance of changes (or the lack thereof) in measurement endpoints. Some direct analytical measurement of the exposure media must be made prior to, during, and at completion of the assay. Large discrepancies between nominal and measured concentrations of test chemicals have been reported in some cases. This problem has been particularly noticeable with exposures using steroid analogues. For example, Nimrod and Benson (1998) used direct delivery of E2 (dissolved in acetone) to exposure tanks via a peristaltic pump and observed that measured concentrations of E2 in the tanks ranged from 11% to 17% of the predicted nominal concentration. These observations reinforce the need to measure chemical exposure levels directly. Chemical purity is also important to provide confidence that fish responses are attributable to the chemical of interest and not an impurity. Minimum acceptable standards of purity will vary across chemical class and commercial availability, but in general, the highest purity available such as ACS grade or equivalent is preferable. In the optimization studies reviewed for this report, high standards of chemical purity were consistently met and typically exceeded 98%.

Another important concern is the stability of the test agent during the exposure. During the optimization phase of fish short-term reproduction assay development, water exposure was the route of toxicant delivery. There are several processes that can remove a chemical from the water including adsorption to the test chamber, volatilization and degradation (photolysis, hydrolysis, oxidation and microbial). Both adsorption and volatilization can be anticipated to a certain extent based on quantitative structure activity relationships (QSAR) (Lokar and Ducker 2004; Basak and Mills 2005). Poorly water soluble substances and/or weakly charged substances are known to adsorb to glass surfaces and/or organic material (food) added to test tanks. For aqueous tests some of these concerns can be overcome by preconditioning the exposure tanks and using a continuous flow-through chemical delivery system as opposed to static renewal. Chemicals with high volatility may require special procedures, such as using a static renewal system and adding the test chemical directly to a test chamber that is sealed or partially closed (OECD 2000). Degradation of the test agent under conditions of the short-term assay can be difficult to predict without prior testing. Concerns about photolysis and hydrolysis may be addressed by minimizing light exposure to the chemical delivery system and preparing stock solutions immediately prior to use (e.g. maintain a short hold time for aqueous solutions). Microbial degradation can be minimized by using clean glassware that has been sterilized prior to use in the test. Chemicals undergoing rapid oxidation in water can be problematic due to the need to maintain adequate dissolved oxygen levels for fish health. For these chemicals, a decision to accept degradation as part of the test or to seek an alternative route of exposure such as oral or ip injection may be necessary. Without any prior knowledge of chemical stability for a new test agent, a stability test should be performed prior to performance of the fish assay. The tiered approach described in OECD Number 23 (2000) is a sensible procedure for establishing stability with minimal effort. The stability test begins by using an open, completely static test, followed by a 24 hr static renewal test and then an open flow-through system. If none of these tests provides acceptable stability (>80% after four days), then closed or partially-sealed systems are tested to determine whether loss due to volatilization is occurring.

An occasional challenge encountered during optimization studies was the preparation of aqueous solutions of high concentration relative to aqueous solubility limits. Organic co-

solvents have been used as carriers to assist in delivering a test substance in water. However, there are several drawbacks to this practice, which include the need for an additional control group (solvent control) and the observation that organic solvents enhance bacterial growth, which increases maintenance time during the exposure. Perhaps more importantly, some studies have found that solvent exposures can impact measurement endpoints, thus potentially reducing the sensitivity of the assay (Hutchinson et al., 2006; Mortensen and Arukwe 2006). To avoid the confounding effects of carrier solvents, most optimization studies used direct addition with mixing or a saturator column to prepare solvent-free, concentrated stock solutions at the upper limits of water solubility (Kahl et al., 1999).

4.6 Statistical Analyses

The fish short-term reproduction assay incorporates a diverse collection of whole animal, organ system and biochemical endpoints to assess the impact of chemical exposure on reproduction. The direct reproductive endpoints are fecundity, fertilization success, and embryo hatching. These endpoints can be quantified before, during and immediately after the exposure period, which allows for statistical testing of paired pre- and post-exposure rates for each treatment replicate, assuming there is no effect associated with time. For the remaining endpoints, which can only be collected at termination of the exposure, the post-exposure response is compared with the non-exposed control. Initial data reduction usually consists of calculating descriptive statistics such as the mean, standard deviation, minimum, maximum, and quartile values. Statistical significance is then evaluated based on the difference in the mean characteristics between the treated and control groups. It can be very helpful to determine the presence of outliers in the data and decide whether it is justifiable to exclude these data before proceeding with more detailed statistical analysis. As a guideline, outliers can be identified by values that exceed the mean plus three times the inter-quartile range (i.e., the difference between the 75th and 25th percentiles). Alternatively, it has been recommended to perform the Grubbs screening test with a heterogeneous variance generalization to detect outliers (Battelle 2006). However, the most common practice for most optimization studies has been to include all measured values if no reasonable explanation exists for the discrepancy.

The statistical approaches used to assess significant differences between treatment groups can be divided into parametric and non-parametric tests. The basis for selection depends on whether there is a deviation from the assumptions of normality and homogeneity of variance. It has been recommended that for endpoints other than survival, the Shapiro-Wilk normality test and Levene's heterogeneity of variance tests should be conducted first (Battelle 2006). Other tests such as the Kolmogorov-Smirnov test have also been used to test for normality (Kunz et al., 2006; Villeneuve et al., 2006c). If the data meet these assumptions then parametric procedures should be used, otherwise nonparametric procedures are used. Occasionally, some studies have used data transformation approaches such as square root or $\log_{10}$ to normalize values and allow the use of parametric analysis methods (Bringolf et al., 2004; Ankley et al., 2005a). A summary of the tests and approaches for each are provided below.

It is worth noting that statistical significance and biological significance are not always the same. This can be particularly pertinent to reproductive studies where statistically non-significant changes may be important biologically. Thus, statistical analyses should not be relied upon exclusively when a simple plot of the data can provide as much or more insight as a

statistical test. A good example of this approach is with fecundity data where visual inspection can often reveal a downward trend in reproduction.

Parametric tests

The most common approach is to begin by performing analysis of variance (ANOVA) using a *t*-test or the Dunnett's and/or F tests to determine if any concentration of the test chemical produces a significantly different mean response from the control response. Alternatively, the linear trend and the Williams ordered step down tests have been deemed more sensitive in some analyses (Battelle 2006). Battelle (2006) recommended that step down tests should be used unless there is statistical or toxicological indication that the dose response trend is not monotonic.

Non-parametric tests

Typically, the Kruskal-Wallis ANOVA on rank transformed data is the first test to be made. Alternatively, the Wilcoxon Mann Whitney (with Bonferroni's adjustment) general test can be used. However, statistical analysis of data collected by some of the optimization studies suggested that the test was "very insensitive" and was not recommended for use in analyzing reproductive data (Battelle 2006). Pair-wise multiple comparison tests such as Dunn's tests for unequal sample size have also have been employed to analyze fecundity data (Thorpe et al., 2007). The linear trend and the Jonckheere-Terpstra step down tests were deemed the most sensitive in analyzing fish assay data and recommended unless there is some indication that the dose response trend is not monotonic. Some general observations made as part of analysis of data from some of the optimization work include:

1) Parametric and nonparametric tests had about the same sensitivity when parametric tests were appropriate.
2) Linear trend tests appear to be a bit more sensitive than the Williams test or Jonckheere-Terpstra test, but the difference is slight.

It is worthwhile to note that many dose-response analyses assume a monotonic response. However, there are many examples of non-monotonic responses such as that described in Welshons et al. (2003). Thus, complex response profiles or the lack of a consistent dose-response relationship with respect to changes in assay endpoints and exposure concentration may be reflective of non-monotonic responses.

5 INTERLABORATORY VALIDATION

An interlaboratory evaluation of the test protocol is necessary to fully demonstrate the reproducibility of the assay. Therefore, an interlaboratory study was performed by three independent laboratories using the same five chemicals (attachment E). The three participating laboratories generally followed the protocol as presented in attachment F, but the specifics for each laboratory are detailed in the interlaboratory report (attachment E).

The five chemicals chosen for the study were selected to represent a range of endocrine modalities. 4-tert-Octylphenol is an alkylphenol, which has previously been presented as a weak estrogen agonist (section 4.1). Prochloraz and ketoconazole are azole-based fungicides that inhibit the synthesis of ergosterol a vital component of the fungal cell membrane. Prochloraz and ketoconazole also exhibit multiple endocrine disruptor modes of action (section 4.1). Vinclozolin is a dicarboximide fungicide that affects DNA synthesis and lipid metabolism. Metabolites of vinclozolin have been well demonstrated to antagonize *in vitro* and *in vivo* the androgen receptor (section 4.1). Sodium dodecyl sulfate (SDS) is an anionic surfactant which has not been previously tested in a fish short-term reproduction assay. The mechanism of action of this compound is to disturb cell membrane structure. It was selected with the expectation that it would be a non-endocrine active toxicant, and therefore serve as a toxic negative for the assay.

5.1 4-t-Octylphenol

Exposure levels

Nominal concentrations for the 4-t-octylphenol exposures were 1.0, 50 and 150 µg/L. The mean measured concentrations ranged from 57 to 84% of the nominal levels. Measured concentrations from the three laboratories were generally highest on day 0 and generally greater than 70% of nominal concentrations. Concentrations generally decreased by day 7 and remained relatively constant with recoveries generally ranging between 50 and 70% of nominal concentrations at Laboratories A and B and generally greater than 70% of nominal concentrations at Laboratory C.

Sensitive endpoints

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

The results from all three laboratories are summarized in Tables 5-1a and 5-1b. Most of the effects seen in the female fish were confined to the high test concentration. A decreased number of spawns was the only endpoint affected in all three laboratories. The number of eggs, the number of fertile eggs and the percent of fertile eggs per female were also significantly reduced in the female fish exposed at Laboratories A and B. The overall fecundity was noticeably greater in the Laboratory C exposure relative to the Laboratories A and B exposures, yet a greater the number of fecundity parameters were affected in the exposures at Laboratories

A and B. Due to mortality and some miss–sexed female fish that ended up being male fish, the sex ratios in the Laboratory A and C exposures were skewed from the 2:4 ratio of the protocol. As also seen in the prochloraz exposure, the screening assay experimental design is robust enough to compensate for some skewing of sex ratios and still detect effects in fecundity.

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

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

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

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

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

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

Conclusions

4-t-Octylphenol is an estrogen receptor agonist. Consistent with other estrogen mimics, 4-t-octylphenol induced vitellogenin production in male fish. Octylphenol also significantly reduced testosterone levels in the male fish. Consistent with the feminization, increased testicular degeneration was observed and secondary sexual characteristics in males were reduced. Reductions in tubercle counts and scores were found at all three laboratories. Fatpad weight,

score, and index also were reduced in the male fish in one laboratory. Vitellogenin induction and testosterone decreases were observed at the mid and high treatment concentrations in all laboratories, and impacts on secondary sex characteristics were also evident at the highest treatment level in all three laboratories and at the middle concentration in two laboratories. Testicular lesions were detected from all three laboratories at least at the highest treatment concentration. Testicular oocytes have been observed in male fish exposed to estrogen agonists, however, no testicular oocytes were observed in any of the male fish exposed to 4-t-octylphenol. This may be a function of exposure time or potency of 4-t-octylphenol.

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

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

Table 5-1a. 4-t-Octylphenol: Female responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Nominal test concentrations 1 μg/L (L), 50 μg/L (M), and 150 μg/L (H).

Laboratory	Mean Measured Concentrations (μg/L)	Fecundity	Gonad Histology	GSI	E2	VTG
A	0.57, 37, 120	↓(H)	↑ atresia (L, M, H)	↓ (H)	-	↑(M, H)
B	0.58, 31, 98	↓ (H)	↑ atresia (H)	-	-	-
C	0.84, 42, 120	↓(H)	-	-	↓ (H)	-

Table 5-1b. 4-t-Octylphenol: Male responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Nominal test concentrations 1 µg/L (L), 50 µg/L (M), and 150 µg/L (H).

Laboratory	Mean Measured Concentrations (µg/L)	Tubercles	Fatpad	Gonad Histology	GSI	Testosterone	VTG
A	0.57, 37, 120	↓(M, H)	↓(L,M,H)	↑ testicular degeneration (M, H) ↑increased spermatogonia, spermatocytes (L, M, H) ↑granulamatous inflammation (M, H)	-	↓ (L,M,H)	↑(M, H)
B	0.58, 31, 98	↓ (H)	-	↑increased spermatogonia, spermatocytes (L, M, H)	-	↓ (H)	↑(M, H)
C	0.84, 42, 120	↓(M, H)	-	↑ spermatogonia (H)	-	↓ (M, H)	↑(L, M, H)

5.2 Prochloraz

Exposure levels

Nominal concentrations for the prochloraz exposures were 20, 100 and 300 µg/L. The mean measured concentrations ranged from 75 to 112% of the nominal levels. Measured concentrations at Laboratories A and C remained relatively constant throughout the exposure period. Measured concentrations a Laboratory A exceeded nominal concentrations on day 0, then dropped considerably (4 to 5 times lower than day 0 at their lowest levels) on days 7, 9 and 13, before recovering to day 0 levels on day 21.

Sensitive endpoints

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

The results across all three laboratories are summarized in Tables 5-2a and 5-2b. Reductions in fecundity at the highest prochloraz test concentration were observed by all three laboratories. Laboratories B and C measured significant decreases in female vitellogenin concentrations at the two highest treatment levels, with Laboratory C also measuring significant reductions at the lowest treatment level. Decreases in female vitellogenin were not observed in Laboratory A even though fecundity was significantly reduced. The sex ratios throughout the exposure levels in Laboratory A were skewed toward male fish in a proportion greater than 2:4. This was due to mis-sexed females at initiation of the pre-exposure period that ended up being identified as male fish at the end of the exposure. Control fecundity was also lowest in the Laboratory A exposure. However, neither the skew ratio nor the relatively low fecundity

impacted the ability of the exposure to detect differences in fecundity at the high exposure level just as in Laboratories B and C.

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

Tubercle score was the only male endpoint affected at the highest exposure level at all three laboratories. It was also affected in the mid treatment level at Laboratories A and C. Tubercle count and fatpad score were affected at the highest exposure level in Laboratories A and B and Laboratories B and C, respectively. Gonad weight and GSI were significantly increased at the highest exposure level in Laboratories B and C and at the mid exposure level at Laboratory C. The fatpad index was a sensitive endpoint in one laboratory (Laboratory B) with significant reductions seen at all three treatment levels, however Laboratory A saw no signifant change and Laboratory C recorded a significant effect only at the highest test concentration.

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

Increased oocyte atresia was observed in the female fish at the mid and high exposures from Laboratory A and all three treatment levels from Laboratory B. In addition the female fish from Laboratory B had decreased post-ovulatory follicles at the mid and high prochloraz treatment levels. No treatment-related lesions were observed in the females exposed at Laboratory C. This may have been due the presence of microsporidian spores observed in some of these fish.

Conclusions

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

at the mid treatment level. Fatpad index was the most sensitive endpoint with significant decreases found at all three exposure levels, but in only one laboratory.

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

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

The prochloraz study in Laboratory A included juvenile females and several fish of ambiguous gonadal sex at necropsy. The fish in this study did not exhibit as many significantly affected endpoints as those in the prochloraz studies by the other two laboratories, perhaps due to this difference.

Table 5-2a. Prochloraz: Female responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Nominal test concentrations 20 µg/L (L), 100 µg/L (M), and 300 µg/L (H).

Laboratory	Mean Measured Concentrations (µg/L)	Fecundity	Gonad Histology	GSI	E2	VTG
A	16, 77, 220	↓(H)	↑ atresia (M, H)	-	-	-
B	15, 83, 230	↓ (H)	↑ atresia (L, M, H)	↑(H)	↓ (L, M, H)	↓ (M, H)
C *	23, 90, 270	↓(H)	-	-	↓ (H)	↓ (L, M, H)

* Occurrence of microsporidian spores.

Table 5-2b. Prochloraz: Male responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Nominal test concentrations 20 µg/L (L), 100 µg/L (M), and 300 µg/L (H).

Laboratory	Mean Measured Concentrations (µg/L)	Tubercles	Fatpad	Gonad Histology	GSI	Testosterone	VTG
A	16, 77, 220	↓(M, H)	-	↑ testicular degeneration (H)	-	-	-
B	15, 83, 230	↓ (H)	↓(L, M, H)	↑ testicular degeneration (M, H) ↑increased interstitial cells(M, H) ↑increased spermatogonia (M,H)	↑(H)	-	-
C	23, 90, 270	↓(M, H)	↓(H)	↑ testicular degeneration (L, M, H) ↑increased interstitial cells(L, M)	↑(M, H)	-	-

5.3 Ketoconazole

Exposure levels

Nominal concentrations for the ketoconazole exposures were 25, 100, and 400 µg/L. The mean measured concentrations ranged from 60 to 81% of the nominal levels. Measured concentrations at all three laboratories were generally near nominal concentrations on day 0. Measured concentrations dropped during the mid part of the exposure in all three laboratories and typically increased slightly by the end of the exposure.

Sensitive endpoints

Male endpoints affected by ketoconazole exposures included gonad weight, GSI, and increased interstitial cells. Female endpoints affected included vitellogenin concentration, gonad weight, GSI and spawns per day.

The results across all three laboratories are summarized in Tables 5-3a and 5-3b. Female GSI increased in the high ketoconazole exposure at Laboratories B and C and in the mid exposure at Laboratory B. Laboratory B also detected an increase in female gonad weight at the high ketoconazole exposure and a decrease in the number of spawns. Laboratory A measured decreases in vitellogenin concentration in the high and low exposure levels. The vitellogenin concentration in the mid exposure level also was less than the control concentration, but not statistically so.

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

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

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

Conclusions

Ketoconazole has been found to inhibit gonadal testosterone synthesis in males. Increased male GSI, increased testes weight and increased proportion of interstitial cells in the testis appear to be consistent with a compensatory response to reduced testosterone synthesis (Ankley et al. 2007). In addition, there were no significant decreases observed in the testosterone levels from the male plasma at any treatment level. Increased male GSI and increased proportion of interstitial cells in the male testis were the most robust endpoints, being observed at all three laboratories, and the increased interstitial cells was also the most sensitive observation.

Ketoconazole exposure increased oocyte atresia, increased female GSI, increased gonad weight and reduced the number of spawns in fathead minnows. However these effects were only observed by one or two laboratories. Microsporidian spores were observed in ovaries of fish from Laboratories A and B. Oocyte atresia related to ketoconazole exposure was distinguished from any impact due to the presence of the Microsporidia. Although control fecundity was less than 15 egg/female/day in all three laboratories, there was no correlation between the presence of Microsporidia and fecundity. Somewhat low fecundity in the control exposures also did not impact the evaluation of the fecundity endpoint, since most of the treatments had fecundity levels greater than the control levels.

Table 5-3a. Ketoconazole: Female responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Nominal test concentrations 25 µg/L (L), 100 µg/L (M), and 400 µg/L (H).

Laboratory	Mean Measured Concentrations (µg/L)	Fecundity	Gonad Histology	GSI	E2	VTG
A*	20, 74, 290	-	↑ atresia (L)	-	-	↓ (L, H)
B*	18, 81, 320	↓ (H)	↑ atresia (H)	↑(H)	-	-
C	16, 75, 240	-	-	↑(M, H)	-	-

* Occurrence of microsporidian spores.

Table 5-3b. Ketoconazole: Male responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Nominal test concentrations 25 µg/L (L), 100 µg/L (M), and 400 µg/L (H).

Laboratory	Mean Measured Concentrations (µg/L)	Tubercles	Fatpad	Gonad Histology	GSI	Testosterone	VTG
A	16, 77, 220	-	-	↑ testicular degeneration (L,M,H) ↑increased interstitial cells(L,M,H)	↑(H)	-	-
B	15, 83, 230	↓ (H)	-	↑increased interstitial cells(L,M,H)	↑(M,H)	-	-
C	23, 90, 270	-	-	↑increased interstitial cells(L,M,H)	↑(M,H)	-	-

5.4 Vinclozolin

Exposure levels

Nominal concentrations for the vinclozolin exposures were 100, 300 and 900 µg/L. The mean measured concentrations ranged from 75 to 150% of the nominal levels. Measured concentrations at Laboratory A ranged between 73 and 83% of nominal concentrations and increased through day 14 where they remained near nominal concentrations for the remainder of the exposure. Some intermediate measurements at the low and mid concentrations between days 11 and 21 suggest that the measured concentrations could vary day to day but the variability between replicates was negligible. Measured concentrations at Laboratory B ranged between 80 and 89% of the nominal concentrations and increased through day 14 to 160 to 190% of the nominal concentrations before decreasing slightly by day 21. Measured concentrations at Laboratory C started near nominal concentrations (86 to 93% of nominal concentrations) on day 0, increasing slightly on day 7 and dropping slightly below the day 0 measurements throughout the rest of the exposure.

Sensitive endpoints

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

The results across all three laboratories are summarized in Tables 5-4a and 5-4b. Fecundity was significantly reduced in the high vinclozolin concentration in all three laboratories and continued into the mid treatment level in Laboratory B. The effects seen in the mid treatment level at Laboratory B may be partially explained by the fact that the mean measured exposure concentrations were noticeably higher than those measured in Laboratories A and C. Decreased levels of beta-estradiol were also observed from the female fish exposed to the mid and high treatment levels in Laboratory B. Significant increases in gonad weight were observed

in the female fish exposed at Laboratories B and C, with an increase in GSI also observed at Laboratory C. Increased length and body weight were observed in the female fish exposed to the mid and high treatment levels at Laboratory B. Increased vitellogenin levels were measured from the mid and high treatment levels in the female fish exposed at Laboratory C.

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

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

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

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

Conclusions

Studies in rats and fish indicate that metabolites of vinclozolin are androgen receptor antagonists. In the present analysis, male secondary sex characteristics, testicular degeneration, and male gonad weight and GSI were the most robust and sensitive endpoints. At higher exposures, testosterone levels in male fish may have also been a robust endpoint. Tubercle score was significantly reduced in males exposed to the highest vinclozolin treatment at all three laboratories. Tubercle count was reduced in two of the three laboratories in the high exposure and empirically reduced in Laboratory C as well. Male gonad weight and GSI were significantly increased and increased incidence of testicular degeneration was observed in the male fish exposed to the high treatment level at two of the three laboratories. The presence of granulomatous inflammation in the testicular tissues of the fish from Laboratory C did not impact the effects of vinclozolin exposure on other endpoints (e.g., GSI, gonad weight and tubercle score). Only Laboratory C measured significant increases in male vitellogenin concentrations at the mid and high vinclozolin treatment levels.

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

Table 5-4a. Vinclozolin: Female responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Nominal test concentrations 100 µg/L (L), 300 µg/L (M), and 900 µg/L (H).

Laboratory	Mean Measured Concentrations (µg/L)	Fecundity	Gonad Histology	GSI	E2	VTG
A*	75, 280, 830	↓(H)	grannulomatus inflammation (L, H)	-	-	-
B	150, 370, 1200	↓ (M, H)	↑ atresia (H)	-	↓ (M, H)	-
C *	84, 270, 760	↓(H)	-	↑(H)	-	↑ (M, H)

* Occurrence of microsporidian spores.

Table 5-4b. Vinclozolin: Male responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls. Nominal test concentrations 100 µg/L (L), 300 µg/L (M), and 900 µg/L (H).

Laboratory	Mean Measured Concentrations (µg/L)	Tubercles	Fatpad	Gonad Histology	GSI	Testosterone	VTG
A	75, 280, 830	↓(H)	-	↑ testicular degeneration (L, M, H) ↑increased interstitial cells (L, H) ↑increased spermatogonia, spermatocytes (L, M, H)	-	-	-
B	150, 370, 1200	↓(L, M, H)	↓(L, M, H)	↑ testicular degeneration (L, H) ↑increased interstitial cells (L, M, H) ↑increased spermatogonia, spermatocytes (L,M,H)	↑(L, M, H)	↓(H)	-
C	84, 270, 760	↓(H)	-	inconclusive	↑(H)	-	↑ (M,H)

5.5 SDS

Exposure level

The nominal concentration for the SDS exposures ranged from 2 mg/L in two laboratories to 14 mg/L at one laboratory. SDS toxicity could in part be a function of water hardness; thus, each laboratory conducted acute testing with SDS to assist in choosing a nominal exposure concentration. Laboratory B conducted the first exposure using 14 mg/L as a nominal exposure concentration. Even though Laboratory B could not maintain nominal concentrations, they still saw significant reductions in reproduction. Laboratories A and C selected lower nominal concentrations recognizing that measured exposure levels would be lower than the selected nominal concentration.

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

2.0 mg/L. The mean measured concentration was 0.62 mg/L or 31% of the nominal level. At Laboratory C the SDS concentrations again started relatively high at approximately 0.76 mg/L, dropped to 0.39 mg/L on day 7, increased to 0.85 mg/L on day 14 and then dropping on day 21 to 0.46 mg/L.

Sensitive endpoints

The results from the SDS exposure varied according to the exposure levels maintained by each laboratory. The results across all three laboratories are summarized in Tables 5-5a and 5-5b. Laboratory A had the lowest mean measured concentrations with testicular lesions and oocyte atresia being the principal effects observed in the exposure. Laboratory B had the highest exposure levels and saw an increase in basal-level endpoint (length, body weight, organ weight) in both the males and females, increases in testicular degeneration and oocyte atresia and a decrease in fecundity endpoints. Laboratory C had a mean exposure about twice as high as Laboratory A and about seven times lower than Laboratory B. They reported effects on the male fatpad and increased vitellogenin concentration in both the male and female fish. Granulomatous inflammation in the testicular and ovarian tissues from Laboratory C confounded the evaluation of SDS exposure to the fish exposed by Laboratory C.

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

Conclusions

SDS proved to be a difficult substance and may have been a poor choice for a reference compound due to its instability in water even under flow-through exposure conditions. The results from the SDS exposure were additionally complicated by two significant factors: a single dose (thus no evaluation of a dose response was possible) and the single exposure levels were not consistent between the three laboratories. The effects seen in the SDS exposure were correlated with the mean exposure concentration tested, therefore drawing conclusions regarding the reproducibility of the assay and the suitability of SDS as a toxic negative is not possible. Laboratory B had the highest exposure concentration and saw the most effects centering around gonad degeneration (testicular degeneration and oocyte atresia), gonad weight and GSI as well as with reductions in fecundity. Laboratory A had the lowest exposure and only saw effects centering on gonadal degeneration; both testicular degeneration and increased oocyte atresia. Laboratory C had an exposure between Laboratories A and C. They found fatpad effects, and increased vitellogenin concentrations in both male and female fish. Although comparsions between laboratories can not be made in the SDS exposure, effects such as testicular degeneration, oocyte atresia, an increase in GSI (biological compensation for endocrine disruption), decreases in fatpad endpoints and vitellogenin induction in males (although a relatively small induction) suggest that SDS may affect the endocrine systems in fathead minnows.

Table 5-5a. SDS: Female responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls.

Laboratory	Mean Measured Concentrations (mg/L)	Fecundity	Gonad Histology	GSI	E2	VTG
A*	0.27	-	↑ atresia	-	-	-
B	<0.44 – 13	↓	↑ atresia	↑	-	-
C	0.62	-	-	-	-	↑

* Occurrence of microsporidian spores.

Table 5-5b. SDS: Male responses.

(-) Indicates an endpoint was measured, but was not statistically significantly different from controls.

Laboratory	Mean Measured Concentrations (mg/L)	Tubercles	Fatpad	Gonad Histology	GSI	Testosterone	VTG
A	0.27	-	-	↑ testicular degeneration ↑increased interstitial cells ↑increased spermatogonia, spermatocytes	-	-	-
B	<0.44 – 13	-	-	↑ testicular degeneration ↑increased interstitial cells ↑increased spermatogonia, spermatocytes	↑	-	-
C	0.62	-	↓	-	-	-	↑

6 DISCUSSION AND SUMMARY

6.1 Reproducibility of the assay

The reproducibility of the fish short-term reproduction assay, for screening purposes, has been broadly demonstrated using a number of representative endocrine-active chemicals across geographically diverse laboratories. In the interlaboratory study with the optimized protocol, five chemicals were tested which generally demonstrate the reproducibility of the assay. In addition, a number of studies conducted prior to the interlaboratory study have also shown reproducibility of the optimized assay.

For estrogen receptor agonists, the induction of VTG in male fish has proven especially effective. As expected, the most sensitive and reproducible endpoint in the interlaboratory octylphenol exposures was male plasma vitellogenin. This chemical also resulted in decreased tubercle counts and scores in males in all three labs, which is consistent with the feminization of males. There was also a decrease in the number of spawns in females exposed to octylphenol, reduced plasma testosterone titers in males, and testicular lesions observed in all three labs. This

suite of responses in the fish is similar to those seem with other types of weak and strong estrogen receptor agonists like methoxychlor, Bisphenol A and 17B-estradiol (Panter et al., 1998, 2000; Miles-Richardson et al., 1999a; Parks et al. 1999; Ankley et al., 2001; Sohoni et al., 2001; Battelle, 2003a; Bringholf et al., 2004; Battelle, 2005).

Exposures in the interlaboratory study with vinclozolin, which has anti-androgenic metabolites, caused a decrease in male tubercle score and a decrease in female fecundity in all three labs just as prochloraz did. These findings were generally consistent with what has previously been reported with vinclozolin in similar fathead minnow studies (Makynen et al., 2000; Martinovic et al., 2008). Similarly, the anti-androgen flutamide resulted in reduced cumulative fecundity and increased testosterone levels in female fathead minnows in other testing (Battelle 2003a; Jensen et al., 2004).

Although not included with this interlaboratory exercise, the assay has been demonstrated to be effective at detecting androgen agonists. Exposures with methyltestosterone, an androgen, resulted in tubercle growth in females in multiple laboratories (Smith 1974; Ankley et al., 2001) and 17 α-trenbolone and 17β-trenbolone, both synthetic androgens, in addition to promoting female tubercles, decreased fecundity by day 2 of exposure and caused oocyte atresia in females in two studies (Ankley et al., 2003; Battelle 2003a; Jensen et al., 2006).

The assay has been effective at detecting the mixed effects associated with chemicals that have multiple modes of action that may involve interaction with steroid receptors or disruption of sex steroid biosynthesis that may affect different points within the HPG axis. One of these chemicals, prochloraz, has been consistently detected with responses in several endpoints. Reduced tubercle score in males, increased testicular degeneration, and reduced fecundity were generally observed in all laboratories in the interlaboratory study, and similarly consistent responses were seen in related studies (Ankley et al., 2007a; OECD 2005b). Ketoconazole exposures resulted in an increase in male GSI in all three laboratories at the high concentration, but an increase in the proportion of interstitial cells in male testis proved to be the most sensitive with the response seen at all concentrations in all laboratories. The aromatase inhibitor fadrozole caused decreased fecundity, a reduction in mature oocytes, an increase in testosterone levels and GSI in males, and a decrease in E2 and VTG levels in females (Ankley et al, 2002; Battelle 2003a). The decrease in VTG in females was also seen in another study where fadrozole was employed as a positive control (OECD 2005b).

6.2 Data Interpretation

The fish short-term reproduction assay as presented is intended to serve in a screening capacity to provide an indication of potential endocrine activity, not to confirm any specific mechanism, mode of action, or adverse effect. Therefore, any significant effect in one or more of the core endpoints of this assay (fecundity, histopathology, GSI, sex steroid measurements, vitellogenin, and secondary sex characteristics) should be considered a positive response in the Fish Short-term Reproduction assay, and supports further testing of the compound in the Tier 2 assays of the EDSP. Because the statistical analysis methods recommended tend to favor detection of monotonic responses, it is important to consider any significant finding an indication of a positive response. Also, the suite of endpoints included is deemed necessary to provide a fully comprehensive assessment of the disrupting potential to the HPG-axis in a representative fish.

Responses may have many forms other than monotonic, so even if a general test of significance had $p>0.05$ but was low and the response showed a trend, it should be considered biologically relevant. Conversely, a significant global test indicates the need for further examination even if no multiple comparison tests are significant, to evaluate whether between-treatment differences suggest a meaningful response. These evaluation approaches are particularly appropriate for favoring false negatives rather than false positives in the Tier 1 screening battery.

It is important to note however that if a given exposure level results in substantial mortality or other overt signs of toxicity, responses in other endpoints may be due to general toxicity, not necessarily mediated primarily via interaction with the endocrine system. The lower treatment level(s) should be examined for effects outside of the range of general toxicity. If all test concentrations exhibit mortality, then the assay would need repeating before inference on possible endocrine activity can be made.

It is recognized that some endpoints may be responsive to non-endocrine stresses in addition to endocrine-mediated pathways, particularly fecundity. Although reductions in fecundity indicate adverse organismal and, potentially, population level effects (i.e., reproductive toxicity), these cannot be definitively distinguished from direct endocrine-mediated effects by this assay when changes in other core endpoints are not present. Nevertheless, reductions in fecundity are considered a positive effect in this assay because they may be endocrine-mediated and should be considered in concert with results of the other assays in the Tier 1 battery. Similarly, responses in secondary measurements (e.g., length, weight) also should be considered in light of other results. Results that would be considered equivocal for this single assay should be considered indications of potential endocrine activity and evaluated in light of the weight of the evidence from the other assays in the Tier I battery of assays for the EDSP.

6.3 Strengths and Limitations of the Fish Short-term Reproduction Assay

The optimized fish short-term reproduction assay protocol is designed to obtain the maximum amount of data from the minimum number of animals in the least amount of time necessary to detect a potential endocrine active substance. The incorporation of several endpoints to detect disruption of male and/or female reproductive systems ensures that the most information possible is obtained from each test. The fish short-term reproduction assay incorporates a diverse collection of whole animal, organ system and biochemical level endpoints to assess the impact of chemical exposure on reproduction.

As with any toxicological assay, there are strengths and limitations which must be recognized and considered when evaluating data and drawing conclusions. Significant advantages of the Fish Assay are:

General Advantages of Fish Short-term Reproduction Assay

- Incorporates a standard, easily acquired laboratory model species, *Pimephales promelas* and utilizes common aquatic toxicology methods;
- Straightforward, cost effective, and produces a relatively large volume of data for a reasonably rapid assay;

- Uses an intact HPG axis and hence is relevant to other taxa when conserved elements of the HPG axis are considered;
- Detects HPG-axis active compounds, including (anti-)estrogen and (anti-)androgen substances using reproductively active male and female fish;
- Multiple labs have experience using this assay and have demonstrated its reproducibility where compounds with a particular mechanism of action generate a similar profile of core endpoint responses.

Specific Endpoint Strengths

Vitellogenin as an endpoint: 1.) primarily controlled through estrogen interaction with the estrogen receptor, and hence is directly related to a mechanism of concern (Korte et al 2000); 2.) clear, unambiguous induction in male fish is well established as a response to estrogen receptor agonists (Brodeur et al 2006; Harries et al 2000; Korte et al 2000; Kramer et al 1998; Panter et al 1998; Parks et al 1999; Schmid et al 2002); 3.) VTG may also respond secondarily to androgenic compounds through suppression of natural androgens and subsequent reduction in endogenous estrogens; this mechanism may also manifest when fish are exposed to a steroidogenesis (e.g., aromatase) inhibitor due to the impaired ability to adequately produce endogenous estrogens; 4.) well-established endpoint and increasing commercial availability of ELISA kits that are specific to fathead minnow vitellogenin (Jensen et al 2006).

Fecundity as an endpoint: 1.) can be collected non-invasively with minimal effort and does not require additional animal use; 2.) fertility data can be collected easily at the same time egg counts are made with minimal effort or time necessary, and they do not require technical expertise, allowing excellent transferability and inter-laboratory comparisons; 3.) fecundity as an apical endpoint, when combined with gonadal histopathology, provides a good indicator of reproductive health of the fish as impaired fecundity is an adverse effect with regulatory importance whether it is due to endocrine-mediated activity or another mechanism of action (MOA); 4.) fertility provides an indication of male reproductive function (sperm quality); 5.) reduced fecundity has been the most consistently observed finding after exposure to diverse endocrine active substances, including all of the primary modes of action the assay is designed to detect: (anti-)estrogens, (anti-)androgens, and modulators of steroidogenesis.

Gonad Histopathology as an endpoint: 1.) sensitive indicator of endocrine dysfunction; 2.) provides a direct evaluation of the reproductive organs of interest; 3.) histopathologic changes express the integration of several molecular, cellular, and physiologic processes, 4.) provides insight on the potential reproductive impacts of chemical disruption; 5.) may decrease ambiguity when fish are exposed to chemicals with unknown modes of action, reconcile unexpected results from other endpoints and hence, may reduce the likelihood that assays must be run multiple times in such instances; 6.) ability to assess the general health of test populations, and the ability to identify causes of morbidity and mortality not associated with test compounds or reproductive endocrine activity, thus histopathological analysis can also help to reduce the number of false negative conclusions.

Secondary Sex Characteristics as an endpoint: 1.) biologically relevant, unique, robust and reproducible; 2.) male secondary sex characteristics provide indicative androgenic/ antiandrogenic effects that may not be observed with other endpoints; 3.) inter-laboratory comparisons of secondary sex characteristics as endpoints have been relatively reproducible; 4.)

sensitive endpoint for androgen agonists which cause clear, unambiguous changes in secondary sex characteristics in females in the assay and trigger further testing.

Sex Steroids as an endpoint: 1.) provide additional supportive information that an endocrine mediated as opposed to non-endocrine mediated mode of action is occurring, which is especially valuable when decreased fecundity is also observed; 2.) provide important insights into the specific mode of action.

Strengths of Fathead Minnow as a Species

Fathead minnows can be compared to other laboratory fish species (i.e. zebrafish and medaka) but for the purposes of this assay, fathead minnow have several strengths regarding endocrine active chemicals. Culture and handling is well defined and well documented for fathead minnow therefore, as a species they are relatively easy to culture, maintain, and use in exposures in the laboratory. Also, fathead minnow tolerate a wide range of water-quality and water-temperature conditions, require small culture space, and produce the number of embryos needed for testing. They have a high fertilization rate and are large enough from which to collect individual blood plasma samples. Fathead minnows have great application in toxicity evaluations and recent studies in reproductive endocrinology provide a larger database for comparison of endpoints in control versus treated fish. Guidance for standardized histological assessments of *Pimephales promelas* gonad tissues are available (Attachment H).

Limitations of the Fish Short-term Reproduction Assay

While there are many strengths of the Fish assay, there are also several limitations of the assay, both technical and general which should be considered. IN general, the assay uses adult fish which require substantial and sustained culture capabilities to generate sufficient numbers of fish at appropriate age and spawning condition.

Specific Endpoint Limitations

Vitellogenin as endpoint: 1.) requires large blood volume relative to amount routinely able to collect; 2.) dependent in contract laboratories on available commercial kits which have no consistent validation or quality assurance standards which necessitates additional calibration in the individual laboratory.

Fecundity as endpoint: 1.) can be influenced by many chemical and non-chemical factors, 2.) requires pre-exposure monitoring to ensure suitable compliance.

Histopathology as endpoint: 1.) requires additional time and services of a qualified pathologist.

Secondary Sex Characteristics as endpoint: 1.) sensitivity and specificity are limited to certain modes of action; 2.) not all are as quantitative as some other endpoints; 3.) some alterations to physiology that manifest in morphological changes may not appear in the short duration of the fish assay.

Sex Steroids as an endpoint: 1.) requires radio-immuno assay for quantification which may be challenging for some commercial laboratories.

Relevant Aquatic Toxicity Testing Related Issues

Inherent technical difficulties testing substances that are poorly soluble in water in aquatic systems, and methods for delivering such substances to the test system are well known and this issue and general guidance for dealing with difficult substances has been provided (OECD 2000).

Importantly, it should be recognized that the limitations listed above represent gaps in our knowledge that have been identified during the course of developing and refining the Fish Assay, and that similar gaps exist in the development and validation of any assay. These gaps in knowledge represent an understanding that additional advantages or limitations of the assay likely exist, but are not yet defined.

6.4 Complementary technologies to the Fish Short-term Reproduction Assay

A need exists to better understand the underlying cellular and sub-cellular processes within the HPG axis that precede reproductive failure and accurately predict which chemicals are likely to be endocrine disruptors. Although accurate prediction can seem daunting, computational toxicology in its various forms offers a practical approach to meet this challenge. Currently, genomic tools such as DNA arrays appear to offer good potential to aid both high throughput screening of chemicals and identification of endocrine disruptor mode(s) of action. Toxico-genomic, proteomic and metabolomic approaches are increasingly being used and recognized for their potential to provide useful data and guide the regulatory process (Ankley et al., 2006). At the same time, mathematical models of receptor-gene signaling and gene regulatory networks are being developed (Bolouri and Davidson 2002, Neves and Iyengar 2002, Schoeberl et al., 2002). In the future, these and other types of mathematical models can be linked with diverse types of biological data to allow more complex descriptions of a cell, tissue or whole organism.

The type of analysis which combines knowledge from many sources and seeks to integrate data from multiple levels of biological organization is termed systems biology. A particular advantage of systems biology is the ability to develop a model which links genomic and proteomic changes with whole fish responses, facilitating phenotypic anchoring. The integrative approach provided by systems biology will likely improve understanding of how endocrine disruptors affect fish reproduction (Villeneuve et al. 2007).

The experimental tools to assess changes in transcriptome, proteome and metabolome are now rapidly being applied to aquatic organisms including fathead minnows (Ekman et al., 2007; Dowling and Sheehan 2006; Denslow and Larkin 2006; Samuelsson et al., 2006). These technologies, although not fully standardized, can improve mechanistic understanding and better define relevance of the endpoint and overall test by providing additional data regarding a specific mode(s) of action (Denslow and Larkin 2006). However the current fish assay has been developed to address the goals and the objectives of the EDSP as Tier 1 assays are not intended to identify MOAs but to instead identify substances with the potential to interact with the estrogen, androgen and thyroid axes in the endocrine system.

Despite the potential for genomic tools to aid regulatory testing, many technical challenges exist and more research is clearly needed before their widespread application can occur in ecotoxicology studies and regulatory evaluations (Ankley et al., 2006). Perhaps the most intriguing aspect of a systems biology approach is the potential to expand the diagnostic capability of short-term assays to detect other classes of disturbances beyond estrogen and androgen signaling. Another important goal of ongoing research is to link gene – protein –

metabolite changes in fathead minnows with tissue and whole fish responses to endocrine disruptors (Miracle and Ankley 2005). There is much progress being made towards understanding genomic changes in fathead minnows exposed to endocrine disruptors and how these responses can be incorporated into a systems biology model of the HPG axis. Thus, the fish short-term reproduction assay described in this document has the potential for continual improvements in sensitivity, specificity and scope of toxicant class when additional knowledge about the fathead minnow HPG axis is obtained.

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APPENDIX A. GLOSSARY OF TERMS

The following are acronyms and definitions of terms used in the ISR:

Acute toxicity- Effects observed in tests of ≤96 h in duration
ANOVA analysis of variance
Chronic toxicity- Effects observed in tests ≥ 28 d in duration
DPH days post hatch
DDT dichlorodiphenyl trichloroethane
17,20-DHP 17α, 20ß-dihydroxy-4-pregnen-3-one
DRP detailed review paper
E2- 17β-estradiol; major estrogenic sex steroid regulating reproductive function
EAC endocrine-active chemicals
EC50 median effective concentration
ECD electron capture detector
EDC endocrine disrupting chemical
EDSP Endocrine Disruptor Screening Program
EDSTAC Endocrine Disruptor Screening and Testing Advisory Committee
EE2 17 α -ethynylestradiol
EIA enzyme immunoassay
ELISA enzyme-linked immunosorbent assay; analytical method used for determining plasma vitellogenin concentration
EPA United States Environmental Protection Agency
ER estrogen receptor
Fat pad- soft enlargement of flesh on top of the head of sexually-mature male fathead minnows that extends onto the back of the fish to, or near, the anterior margin of the dorsal fin.
Fecundity- measure of total egg production
Fertility- measure(s) of fertilization success as indicated for example by actively-dividing embryonic cells or occurrence of eyed embryos
FIFRA Federal Insecticide, Fungicide, and Rodenticide Act
FQPA Food Quality Protection Act
FSH, GTH I follicle stimulating hormone
GC gas chromatography
GnRH Gonadotropin releasing horomone
GSI gonadosomatic index; gonad weight relative to total body weight ((gonad wt(g)/ body wt(g)) x 100)
GTH gonadotropic hormones
HPG Hypothalamus-pituitary-gonadal axis
HPLC high performance liquid chromatography
11βOHT 11β-hydroxysteroid dehydrogenase
ICCVAM Interagency Coordinating Committee on the Validation of Alternative Methods
ip injection- interperitoneal injection; method of chemical delivery
11-KT 11-ketotestosterone; major male sex steroid in fish responsible for development of secondary sex characteristics as well as gonad development
LC liquid chromatography
LC50- concentration lethal to 50% of a group of organisms under specified conditions

LH, GTH II luteinizing hormone
M1 2-[(3,5-dichlorophenyl)]-carbamoyloxy-2-methyl-3-butenoic acid
M2 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide
MOA- Mechanism/ Mode of action; the mechanism or mode via which a chemical exerts a toxic response in an organism
MS mass spectrometry/ spectrometer
NIEHS National Institute of Environmental Health Sciences
NOEC (NOAEC)- No Observed Effect Concentration; highest concentration of a chemical that does not cause a significant adverse effect upon any life functions
NOEL no observed effects level
NRC National Research Council
Nuptial tubercles- visible external horny outgrowths on the surface of the head of the sexually-mature male fathead minnow in breeding condition
OECD Organization for Economic Cooperation and Development
Ovipositor- Urogenital structure present in sexually-mature females for egg deposition.
RIA radioimmunoassay; analytical method used for determining plasma steroid concentrations
RPD- Relative Percent Difference; calculation utilized to assess measurement precision
SAB Scientific Advisory Board
SAP Scientific Advisory Panel
Saturator- An apparatus capable of generating a saturated stock solution of a chemical that is relatively insoluble in water
T testosterone; androgenic sex steroid normally present in both sexes and necessary for development and maintenance of reproductive function.
TCDD 2,3,7,8-tetrachlorodibenzo-p-dioxin
20βS 17 α, 20 ß, 21-trihydroxyprogesterone
TSH thyroid stimulating hormone
Viability- Measure(s) of embryonic development subsequent to fertilization, including hatching success and normal larval maturation.
VTG vitellogenin; precursor to egg yolk protein that occurs normally in the blood of sexually-mature female fish; it can be induced by estrogen receptor agonists in male fathead minnows.