Fish Screening Assay Discussion Paper
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Introduction

The Organisation for Economic Co-operation and Development (OECD) Validation Management Group for Ecotoxicity Testing (VMGeco) agreed to recommend to the OECD Task Force on Endocrine Disrupter Testing and Assessment (EDTA) and the Working Group of the National Co-ordinators of the Test Guidelines Programme (WNT) that an OECD Test Guideline be developed for a fish screening assay based on the Phase 1B validation trial protocol using vitellogenin (VTG) and secondary sex characteristics (SSC) as core endpoints. The objective of this assay, as recommended, is to detect estrogenic and androgenic agonists and aromatase-inhibiting substances. This agreement was conditioned upon the test guideline being able to accommodate additional endpoints (*e.g.*, fecundity and histopathology) once adequate data are made available to demonstrate their validity. The condition was needed because a minority view, which the U.S. held, was that the assay with only VTG and SSC would be inadequate to detect all known modes of action (*e.g.*, androgen receptor antagonism) and further limit the test guideline for other purposes, such as detecting reproductive toxicants through non-endocrine mechanisms.

The purpose of this paper is to briefly summarize the available data and present a rationale to support the U.S. contention that a broader suite of endpoints are ready to be included in the Fish Screening Assay Test Guideline. The U.S. asserts that the reliability of the additional endpoints is uniformly demonstrated for use in a screening assay, albeit some additional interlaboratory data are acknowledged will be needed with the standardized protocol to fully satisfy all validation requirements. The data presented focus on the fathead minnow and include results from over sixty (60) individual studies encompassing twenty (20) chemicals, seventeen (17) laboratories, and sixteen (16) protocol variations (see Addendum 1). At least eighteen (18) of these studies conducted in five (5) different laboratories were 21-day assays that included a suite of endpoints generally consistent with the proposed standardized protocol provided in Addendum 2. These studies effectively demonstrate the utility and reliability of this assay for its purpose in screening substances that potentially interfere with estrogen and androgen mediated processes. The summary of data presented in this paper is not intended to be exhaustive and detailed, only to emphasize that there is a fair and compelling body of information which is available that support the U.S. assertion for the fish screen with the fathead minnow. Data on medaka and zebrafish are not included because this would make the presentation more complex, but it should be understood that similar

supportive comparisons could be made for these species as well. It is expected that a detailed and exhaustive integrated summary validation report can be developed from the existing data sufficient to advance the protocol as presented in Addendum 2 for affirmation by a suitable peer review. That said, it is also unfortunately clear that an additional interlaboratory comparison is warranted with the standardized protocol to solidly demonstrate the reproducibility of the assay and this need will also be discussed.

Background

The OECD, in 1998, established the EDTA at the request of member countries as a Special Activity of the Test Guidelines Programme. The U. S. was one of the member countries who strongly encouraged this Special Activity due to its own legislative mandates and the recognized need to harmonize the development and validation of new and enhanced methods for evaluating endocrine disrupting chemicals (EDCs). The agreed focus of the EDTA, which is consistent with the U.S.'s and other member countries' interests, was on methods which could potentially cover the disrupting effects of chemicals on the reproductive system (e.g., estrogen agonists/antagonists and androgen agonists/antagonists) and on development (e.g., thyroid axis)(OECD 2004b).

In response to its own statutory obligations, the U. S. Environmental Protection Agency (EPA) implemented an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed to detect and characterize potential endocrine effects of environmental contaminants, industrial chemicals, and pesticides. The U.S. program is a two-tiered approach which employs a combination (e.g., battery) of *in vitro* and *in vivo* screens in Tier 1 for assessing the potential to affect endocrine-mediated processes and *in vivo* testing using two-generation studies in Tier 2 for characterizing dose-response and potential adverse effects. A fish assay in the Tier 1 screening battery was identified as an essential component. It is important because there is good evidence finfishes are being affected by EDCs in the field (Ankley and Giesy 1998; Crisp et al 1998). Additionally, estrogenic and androgenic controls in reproduction and development in fishes differ enough from those in higher vertebrates that screening limited to a single taxon (e.g., rodents) may not adequately identify potential EDCs across all vertebrate classes.

A Detailed Review Paper (DRP) has been completed which provides a thorough overview and scientific basis for a fish screening assay to be used in the detection of endocrine active substances (OECD 2004b). The purpose of the assay as expressed in the DRP is "to provide an indication of the potential of a chemical to behave as an endocrine-active substance on the organism studied

and thus to guide on whether additional testing will be needed to better characterize the potential endocrine disrupter". Further, the DRP states that it "is not designed to identify specific mechanisms of hormonal disruption (such as hormone receptor agonistic or antagonistic effects, or disturbance of hormone synthesis or metabolism)".

It is important that the purpose of the Fish Screening Assay is clearly articulated and understood for adequate protocol validation. In the EDSP, screening is defined as "the application of assays to determine whether a chemical substance or mixture may interact with the endocrine system" (US EPA 1998). The fish short-term reproduction assay which is the fish screening assay method advocated by the U.S., is one of a coordinated battery of assays that support this purpose. The specific role established for the fish assay as part of the EDSP is to assess the potential for a chemical substance to affect estrogen- and androgen-mediated processes in this vertebrate class. "The test is designed as a short-term reproduction assay suitable for identifying chemicals that affect reproduction or, potentially, development through disruption of any number of pathways, including those controlled by estrogens and/or androgens." (US EPA 2002) By using intact mature and actively spawning fishes, the assay can assess the entire hypothalamus-pituitary-gonadal (HPG) axis which directly affects these sex steroids. Essential endpoints included in the assay cover biochemical (VTG and steroids), tissue (gonad histopathology), and organism (SSC and fecundity) level responses influenced by these endocrine pathways. The assay has an added benefit of capturing the potential for a chemical substance to affect reproductive output through broader endocrine and non-endocrine mediated processes as well. This additional role for the assay has been accepted as integral to the assay purpose for EPA regulatory needs. This is consistent with what is also expressed in the DRP (OECD 2004b):

> "Endpoints should be reflective of specific disturbances at the (sub-) organism level and will be a combination of apical, histological and biochemical measurements. This is to establish a link between a response at the sub-organism level and a change observed at the organism level. Such a combination of measurements will also aid in the interpretation of test results and inform on the additional testing needs."

The screening assays in the EDSP were recommended (US EPA 1998) to capture all known modes by which the estrogen, androgen, and thyroid hormone systems can be affected. As stated, a screening assay is not intended to define a substance's mode of action or the specific biological mechanism/path with which it interacts toxicologically. Such an objective is more suited to a dedicated research program than to a simple screen. It should be noted that a single chemical may have multiple modes of action which thereby require

screening assays to be capable of detecting multiple modes of action. It is also recognized that not all mode of actions (MOAs) have been yet recorded or examined for the endocrine system. A screening assay is intended only to provide suggestive information that an endocrine-mediated process is involved, and certainly not confirm it. It is therefore not necessary to resolve whether an effect to an endpoint of regulatory concern such as reproductive output is due to endocrine or non-endocrine mechanisms. The outcome of a screen which identifies an effect on reproductive output, for example, under existing U.S. pesticide regulations (40 CFR Part 158) will be to derive a dose-response relationship in a definitive trial regardless of the mechanism involved. However, the combination of endpoints within the assay and across the battery of assays employed does provide some information to corroborate whether an endocrine or non-endocrine process is more likely. It is this weight-of-evidence approach to the data interpretation that is critical for determining the need for further tier testing progression.

The standardized fish screening assay protocol, based on the short-term reproduction method as appropriate to the fathead minnow (*Pimephales promelas*), which includes a comprehensive suite of endpoints and which is being advocated by the U.S. for the final steps of validation is presented as Addendum 2.

Chemicals employed in validation

Chemicals which have been employed in evaluation of the 21-day fish reproduction assay for fathead minnow (*Pimephales promelas*) are grouped by their predominant (anticipated) HPG MOA:

Androgen receptor agonists -
        Methyltestosterone
        17β-trenbolone
        17α-trenbolone

Androgen receptor antagonists -
        Flutamide
        Vinclozolin
        p,p′-DDE

Estrogen receptor agonists -
        17β-estradiol
        4-*tert*-pentylphenol
        Bisphenol A
        Methoxychlor

Steroid metabolism modulators -
      Fadrozole
      Fenarimol
      Ketoconazole
      Prochloraz
      Cadmium chloride

Uncertain modes of action -
      Atrazine
      Prometon
      Perfluorooctane sulfonate (PFOS)

Negative compounds -
      Potassium permanganate
      Perchlorate

Among these chemicals, those with possible multiple MOAs (based both on *in vivo* and *in vitro* analyses) include:
      Methoxychlor (estrogen receptor agonist/androgen receptor antagonist)
      Methyltestosterone (androgen receptor agonist/estrogen receptor agonist through aromatization)
      Fenarimol (aromatase inhibitor/estrogen receptor antagonist/androgen receptor antagonist)
      Prochloraz (multiple steroidogenesis enzyme inhibitor/androgen receptor antagonist)
      Ketoconazole (multiple steroidogenesis enzyme inhibitor)

The chemicals chosen to represent androgen receptor agonist modes of action in the fish screen include methyltestosterone, 17β-trenbolone, and 17α-trenbolone. Methyltestosterone is a synthetic derivative of testosterone which is aromatizable (to methylestradiol; Hornung et al 2004), and therefore may have estrogenic effects in addition to androgenic effects. 17-β and 17-α trenbolone are active metabolites of trenbolone acetate, a chemical used in food animal production that improves weight gain, and can be found in the environment. 17β-trenbolone has been shown to be highly androgenic in fathead minnow, resulting in masculinization and reduction of vitellogenesis in females (Ankley et al 2003). There is no evidence to suggest that trenbolone is aromatizable. Effects of androgen receptor agonists in fathead minnow include masculinization of SSC, reduced fecundity, impaired vitellogenesis, and pathological effects on the gonads. Potential histopathological changes associated with androgen agonists include suppression of oocyte maturation coupled with increased oocyte atresia and reduction in post-ovulatory follicles (Ankley et al 2001). Exposure to strong

androgen receptor agonists may also manifest as lowered yolk deposition in oocytes (Ankley et al 2003). In males, secondary sex characteristics may be magnified, and spermatogenesis may be inappropriately stimulated (Ankley et al 2001).

Androgen receptor antagonists, or anti-androgens, can exert feminizing effects on SSC. They also may affect spawning, gonadal development (especially testicular development), and gonadal histopathology including changes in spermatogenesis or changes in the ratio of spermatogenic cells. Potential effects of anti-androgens in the fathead minnow include effects on spermatogenesis and yolk deposition in oocytes (Makynen et al 2000), and increased oocyte atresia (Jensen et al 2004). Representative chemicals for androgen receptor antagonism used in the evaluation of the short-term fish reproduction assay include flutamide, vinclozolin, and p,p'-DDE. Flutamide and its 2-hydroxyflutamide metabolite are antagonists of the mammalian androgen receptor, and they bindsto the receptor of the fathead minnow (Ankley et al 2004; Makynen et al 2000). Vinclozolin, a fungicide thought to promote Leydig cell tumors, and which exerts anti-androgenic influences on sexual differentiation in mammals, is thought to interfere at the level of the androgen receptor (Gray et al 1994). p,p'-DDE is a metabolite of the pesticide dichlorodiphenyltrichloro-ethane (DDT). Past studies in rodents suggest that p,p'-DDE may interfere with binding of endogenous androgens at the androgen receptor (Gray et al 1999; Kelce et al 1995, 1997).

The short-term fish reproduction assay also has the ability to detect estrogenic substances. The most active endogenous estrogen, 17β–estradiol, is necessary for the normal growth and development of the female sex organs, and in some species contributes to the development and maintenance of female secondary sex characteristics. Estrogens also influence the release of gonadotropins from the pituitary gland. Reported changes in fathead minnow due to estrogen exposure include SSC changes, sex ratio changes, VTG induction, steroid hormone changes, changes in fertility and fecundity, and histologic alterations in gonadal tissues (Lange et al 2001; Lattier et al 2002; Miles-Richardson et al 1999b; Parrott and Blunt 2005; Pawlowski et al 2004b; Sohoni et al 2001). Compounds that were used in the evaluation that represent estrogen receptor agonists include 17β-estradiol as a strong estrogenic substance, and 4-*tert*-pentylphenol, methoxychlor, and bisphenol A as weakly estrogenic compounds. Methoxychlor is a relatively non-persistent organochlorine insecticide registered for insect control in post-harvest applications in a variety of crops, foliar treatment, soil, and seed treatment. It has been shown to have estrogenic influences in several fish species, including induction of vitellogenesis (in males) and negative impacts on fecundity (Ankley et al 2001; Hemmer et al 2001; Kobayashi et al 2003; Versonnen et al 2004). Bisphenol A is a high-

production chemical used to make polycarbonate plastic and epoxy resins. A previous study in which fathead minnows were exposed to various concentrations of bisphenol A for up to 164 days reported VTG induction in males at an approximate exposure level of 160 µg/L. Higher exposure levels (640 µg/L) also significantly raised VTG levels in females and diminished egg hatching success (Sohoni et al 2001). Additional effects reported were a decrease in fecundity at exposure levels of 1280 µg/L. Four-*tert*-pentylphenol is a member of the alkylphenol family. It has been shown to demasculinize male fish and induce vitellogenesis (Gimeno et al 1998; Panter et al 2002). Estrogenic substances may also induce histopathologic changes of the gonads, including testicular oocyte formation, necrosis of testicular germ cells and spermatozoa in conjunction with hyperplasia and hypertrophy of Sertoli cells (Miles-Richardson et al 1999b), increased oocyte atresia (Ankley et al 2001) or alteration in oocyte maturation, and increased interstitial fluid (Lange et al 2001; Miles-Richardson et al 1999a). Pathologic changes may also occur in the kidney of males due to the ineffective removal of excessive vitellogenin from the blood.

Steroidogenesis is the production of the steroid hormones required for reproduction. In the male, steroidogenesis occurs in the Leydig cells of the testis and, in the female, this pathway is found in the follicle of the ovary. Steroidogenesis also takes place in the adrenal tissue. At the cellular level, a series of biochemical reactions are initiated upon stimulation of gonadotropin receptors located in the membranes of these cells. Activation of the luteinizing hormone/follicle stimulating hormone (LH/FSH) receptors initiates a series of enzymatic reactions that culminate in the biosynthesis of end-product hormones, *i.e.*, primarily testosterone (11-ketotestosterone) in males, and primarily estradiol in females. Due to its ability to detect changes in estrogens and androgens, the short-term fish reproduction assay can also detect interference in the steroidogenesis pathways. Potential findings in fathead minnow that are associated with steroidogenesis inhibition include suppression of vitellogenesis through decreasing serum estradiol levels, decreased numbers of post-ovulatory follicles, changes in the normal maturation processes of oocytes and spermatozoa, and effects on the ability of female fish to spawn (Ankley et al 2002).

Chemicals used in the evaluation that interfere with normal steroidogenesis include fadrozole, fenarimol, ketoconazole, prochloraz, and cadmium chloride. Fadrozole is a therapeutic chemical developed to treat estrogen-dependent diseases, including breast cancer. It is a competitive inhibitor of P-450 aromatase (CYP19) interfering with the conversion of C19 androgens to C18 estrogens. Fenarimol, ketoconazole and prochloraz are all conazole fungicides designed to inhibit fungal growth by blocking a cytochrome P-450 (CYP) enzyme that makes ergosterol. Although this particular CYP is not

found in vertebrates, these compounds can affect CYP enzymes involved in steroidogenesis such as P-450scc, lyase, and aromatase. This causes reductions in steroid synthesis. Prochloraz, for instance, may affect aromatization of testosterone to estrogen in addition to its effects on other CYPs (Ankley et al 2005a). Cadmium has been suggested as inhibiting testosterone production in Leydig cells of male gonads, in addition to inhibition of progesterone, testosterone, and estradiol production in ovaries (Leblond and Hontela 1999; Piasek and Laskey 1994). Potential effects of steroidogenesis inhibitors in fathead minnow include impaired vitellogenesis, pronounced oocyte atresia and suppression of oocyte maturation, and ultimately a reduction in fecundity (Ankley et al 2002; Ankley et al 2005a).

Several chemicals with uncharacterized modes of action were also used to evaluate the fish screen. These chemicals include atrazine, prometon, and perfluorooctanesulfonate (PFOS). Atrazine is a widely used chlorotriazine herbicide. Because of its worldwide agricultural usage, the toxicity of atrazine has received considerable study. The suspected endocrine mode of action is considered to be at the neuroendocrine level (hypothalamus-pituitary axis), although this is based primarily on mammalian studies (Cooper et al 1999). Prometon is a structurally-related methoxytriazine herbicide. PFOS is a stable metabolite of fluorinated surfactants and has been found in myriad wildlife species (Giesy and Kannan 2001). There is some evidence that structurally similar chemicals to PFOS can alter plasma concentrations of both androgens and estrogens in fathead minnow (Oakes et al 2004). Due to the relative scarcity of information regarding the effects of atrazine, prometon, and PFOS in fathead minnow, these chemicals were chosen to evaluate the ability of the fish screening assay to detect effects of uncharacterized potential endocrine modulators.

To test the specificity of the fish screen, two chemicals were used that are not known to be disruptive of reproductive endocrine processes. These two chemicals include potassium permanganate and perchlorate. Potassium permanganate is an oxidizing agent that reacts with organic matter and is used to treat several common fish diseases. Perchlorate is a by-product of rocket fuel production widely found in groundwater; it is known to competitively inhibit iodine uptake by the thyroid gland (Wolff 1998). Decreased iodine uptake alters thyroid gland function and lowers production of thyroid hormones. The goal of testing potassium permanganate and perchlorate was to determine whether general, biological stress responses affect the estrogen and androgen related endpoints in the fish screeninh assay.

Endpoints

Vitellogenin

Vitellogenin is a female-specific yolk-precursor protein that is primarily regulated through the interaction of β-estradiol and the estrogen receptor (Kime 1998; Sumpter and Jobling 1995). It is expressed in sexually mature females of oviparous species and is required for development and maturation processes of oocytes. In males, the VTG genes are normally inactive; however, they retain the ability to produce VTG in response to estrogenic compounds, and in fathead minnow, induction by exposure to estrogen receptor agonists has repeatedly been demonstrated (Brodeur et al 2005; Hansen et al 1998; Harries et al 2000; Korte et al 2000; Kramer et al 1998; Lattier et al 2002; Panter et al 1998; Parks et al 1999; Schmid et al 2002). In addition, it has been shown that exposure of female fathead minnow to androgens can suppress vitellogenesis, potentially by depressing the endogenous androgens which are converted to estradiol (Ankley et al 2003; Miracle et al 2006).

There are several advantages to the use of VTG as an endpoint in the fish screen. One advantage is that it is primarily controlled through estrogen interaction with the estrogen receptor, and hence is directly related to a mechanism of concern (Korte et al 2000; Mori et al 1998). As mentioned above, it may also respond secondarily to androgenic compounds through suppression of natural androgens and subsequent reduction in endogenous estrogens. This secondary 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. Another advantage to the vitellogenin endpoint is the increasing commercial availability of ELISA kits that are specific to fathead minnow vitellogenin (Jensen et al 2006).

Because the endocrine system is integrative, by definition, and the mechanisms of vitellogenesis are not fully characterized, there are some issues to consider in the interpretation of vitellogenin results. First, vitellogenesis relies on the presence of adequate numbers of functional estrogen receptors in hepatocytes, as well as a clinically normal liver capacity. Hence, there is potential for changes in vitellogenesis to occur secondarily if compounds affect the number of estrogen receptors, or the ability of the liver to produce proteins. There is some evidence that cortisol may affect vitellogenesis through reduction of expression of the estrogen receptor in trout (Lethimonier et al 2000), however in fathead minnow, others have demonstrated that cortisol may potentiate, rather than suppress, vitellogenesis (Brodeur et al 2005). Also, in theory,

compromised liver capacity could affect the ability of a fish to synthesize vitellogenin. While it is likely that if a compound is toxic enough to compromise liver capacity, other aspects of the assay will also be affected and the fish would likely show clinical signs of disease, the possibility of liver toxicity should not be excluded as a potential cause of decreased vitellogenesis. Prolactin and thyroid hormones have also been postulated to influence vitellogenin synthesis (Sumpter and Jobling 1995).

Histopathology

Toxicologic histopathology is used to identify and describe morphologic differences between unexposed (negative control) and treated animals. Histopathology is a necessary endpoint in the fish screen assay because it is a direct evaluation of the reproductive organs of interest, and histopathologic changes express 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. Other recognized benefits of histopathology are the 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.

Histopathology has served as a fundamental endpoint 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 (Ankley et al 2001, 2002, 2003, 2004; Ankley and Johnson 2004; Van der Ven et al 2003; Wester and Canton 1991; Wester and Van der Ven 2000) and is considered an integral part of the fish screen. In addition, much work has been accomplished that characterizes the normal histology of gonads in healthy fathead minnow and the typical changes that might be expected in gonads in response to endocrine disruptor exposure (Leino et al 2005).

Histopathology has been useful and informative during the validation effort for the fish screening assay. For example, during the OECD Phase 1A analysis of the fish screening assay validation effort, male and female fish were kept separated and gonadal response to exposure to EDCs was confounded by ovarian pathology. In recognition of the concern raised about the ovarian response of treated and untreated fish in the Phase 1A trial, the pathologists suggested that the forced non-spawning conditions were responsible and these stressed conditions created a high rate of atresia in ovaries of control animals. They recommended that the fish be allowed to spawn in OECD Phase 1B tests by housing the male and female fish in the same aquaria to remove this

confounding factor and improve the readability of gonad slides. Lothenbach et al. (2005) investigated the gonad histology of spawning versus non-spawning medaka and confirmed the hypothesis of the histopathology expert group. This example clearly demonstrates the utility of histopathology as it relates to determining the general health of the test population and identifying confounding factors that might affect the performance of the endpoints in the fish screening assay.

The gonad is the primary organ of reproduction and, as such, will reflect substantive disturbances to the HPG axis. The gonad also encompasses the many physiologic responses made by an endocrine-active substance that affects the reproductive system. It is more general than a biochemical measurement, and it changes in response to multiple stressors, both chemical and non-chemical. The fine structure of gonadal tissue provides tangible and verifiable evidence for the health or dysfunction of the organ.

For histopathological analysis to be most useful in reproductive screening tests, it is important to ensure maximum control of the subjective nature of the interpretations, and to limit the interpretation to repeatable results that can be verified by different investigators. In support of improving diagnostic consistency and reducing bias, an intensive effort has been made to standardize reading practices, diagnostic terminology, and severity grading approaches, which is documented in the draft guidance document on fish histopathology (OECD 2004a). For an in-depth description of the histopathology standardization efforts to support the validation effort for the fish screening assay, please refer to the OECD final report for pathologists post-Heidelberg, and the OECD guidance document for fish histopathology (OECD 2004a; OECD 2004c). Briefly, lists of primary and secondary diagnoses (Tables 1 and 2) were established which standardize the description of criteria used for diagnostic purposes, and that minimize the time and reporting effort required by pathologists reviewing studies. Each of the four primary diagnoses per sex is directly relevant to endocrine function within the gonads and pathologists would routinely assess these diagnoses. Secondary diagnoses will only be noted if present.

Table 1. Histopathological Primary Diagnoses

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

Table 2. Histopathological Secondary Diagnoses

Secondary Diagnoses	
Males	Females
Decreased proportion of spermatogonia	Interstitial fibrosis
Increased proteinacesou fluid withing testicular vessels or interstitium	Egg debris in the oviduct
Asynchronous gonad development	Granulomatous inflammation
Granulomatous inflammation	Decreased post-ovulatory follicles

A severity grading system was also established to allow pathologists to take into account discrete, spatial, and global pathologic changes in the gonads on a 5-point scale. Each severity grade is described in the guidance document, and pathologists can score lesions in comparison to controls, or in comparison to "normal" gonad histology based on the pathologists' experience.

It has been discussed repeatedly and determined that the appropriate reading practice to employ for the fish screen is one that is non-blinded initially, with knowledge of control and treatment groups, in accordance with guidance from the Society of Toxicologic Pathology (Crissman et al 2004). Informal blinded re-evaluation is then performed to confirm diagnoses.

There is some concern that histopathology is a time-consuming and expensive endpoint for a screening assay. Standardization of histopathology, as described above, greatly reduces the time and cost associated with this endpoint. In addition, there may be more costly consequences of not performing histopathology as part of the fish screen. Besides being a sensitive indicator of endocrine dysfunction, histopathology confirms that the test population is healthy and responding normally. Confounding factors that can alter the molecular, cellular, and physiologic responses that affect the fish, and that might influence the outcome of the assay, can be detected using histopathology. Also, histopathology 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.

Fecundity

Fecundity is defined as the capacity to produce offspring and very broadly encompasses not only egg production, but fertility and hatching. Specifically for this assay, fecundity is measured by the total number of eggs, number of spawns, and number of eggs per spawn. Fertility is considered separately and recorded as the number of fertile and infertile eggs or proportion of fertile eggs. This determines the numbers of potential offspring. Fecundity is a relevant endpoint for the fish screening assay because the definitive measure of reproductive success is the number of surviving progeny that carry genes from the parents, and who will in turn reproduce successfully (Kime 1998).

Fecundity as an endpoint is also important to the fish screening assay because the number of eggs spawned is mediated, in large part, by the endocrine system. Changes in hormonal equilibrium could decrease the numbers and quality of eggs spawned. Fecundity can also be affected by gonadal development. It can be increased by recruitment of additional oocytes into vitellogenesis or it can be reduced by atresia (Hunter and Macewitz 1985). Certain EDCs can affect lifetime fecundity, survival of offspring to adulthood, and the fertility of the offspring.

Some advantages of the use of fecundity as an endpoint in the fish screen are that it can be collected non-invasively with minimal effort and does not require additional animal use. Fertility data can be collected easily at the same time egg counts are made, with minimal effort or time necessary when using fathead minnow. Fecundity data, when combined with gonadal histopathology, provides a good indicator of reproductive health of the fish. The biological relevance of fecundity far outweighs other potential endpoints, as it demonstrates potential population-level effects (Miller and Ankley 2004). Active spawning (egg production) can be used effectively as an acceptance or performance criterion of parental control fish for the study to ensure that fish are in an acceptable reproductive state for the duration of the study. Therefore, if egg production is already being collected for a portion of the fish used in the study, it could easily be collected for the fish used in the exposures in addition to those in the controls. Even though fecundity can be influenced by many chemical and non-chemical factors, its inclusion as an endpoint ensures that more endocrine effects will be caught, hence decreasing the incidence of false negatives. An added benefit of fertility determinations in conjunction with the fecundity endpoint is that this is an indication of male reproductive function (sperm quality).

Without question, fecundity represents the principal integrative endpoint in the fish short-term reproduction assay. The endpoint is easily and

unambiguously collected from actively spawning fishes. Egg production data
are needed to establish the acceptable spawning condition of test fish prior to
exposure, and to establish acceptable control production during exposure.
Reduced egg production has been demonstrated to be a sensitive response to
exposure to known endocrine active substances including estrogen receptor
agonists, androgen receptor agonists, androgen receptor antagonists, and
steroidogenesis inhibitors. It is also ethically incumbent to collect all available
data practical from animals used in testing, especially where this information in
one test will reduce the likelihood that repeated or additional tests would be
necessary.

   Secondary Sex Characteristics

   Exposure of fish to chemicals with androgenic or estrogenic activity may
result in degrees of abnormal sexual differentiation or function (Kime 1998).
Certain characteristics particularly relevant to the fathead minnow in the
screening of EDCs include body color (*i.e,* light or dark), coloration patterns (*i.e.,*
presence or absence of vertical bands), body shape (*i.e.,* shape of head and
pectoral region, distension of abdomen), and specialized secondary sex
characteristics (*i.e.,* number and size of nuptial tubercles, size/weight of the
dorsal nape pad and ovipositor) (US EPA 2002).

   Secondary sex characteristics are used in the start of the screen to identify
sexually mature fish to be used in the assay. General observations of physical
appearance of adults and alterations of secondary sex characteristics (*i.e.,* nuptial
tubercles, dorsal nape pad, darkened coloration in males; distended abdomen
and swollen ovipositor in females) can be made daily over the duration of the
study and also at the conclusion of the screen.

   The adult fathead minnow is sexually dimorphic, with males and females
readily distinguishable from one another when in breeding conditions (US EPA
2002). Sexually mature males develop large nuptial tubercles on the snout and a
dorsal pad that extends as a narrow band from the nape to the dorsal fin.
Nuptial tubercles are "visible external horny outgrowths on the surface of the
head" and dorsal nape pads are "soft enlargements of flesh on top of the head
that extend onto the back of the fish to, or near, the anterior margin of the dorsal
fin" which can be removed and weighed as an indication of effects of androgen
agonists or antagonists, as well as estrogenic compounds (US EPA 2002; K.
Thorpe, University of Exeter, personal communication). Males' bodies become
black on the sides except for two wide light-colored vertical bars (US EPA 1987).
However, females' bodies do not usually change in color or morphology, but
they do develop a fleshy ovipositor that can be used to identify juvenile males
from females (Flickinger 1969). Ovipositors are "urogenital structures present in

females for egg production" (US EPA 2002). Females could also develop a dorsal pad which can be quantitatively measured in weight to assess androgen exposure.

Some EDCs can cause abnormal occurrence of certain secondary sex characteristics in the opposite sex. In the fathead minnow, external morphological features are under the control of sex steroids and therefore could be affected by estrogen or androgen receptor agonists or antagonists (Ankley et al 2001; Ankley et al 2003; Harries et al 2000; Lange et al 2001; Miles-Richardson et al 1999a; Smith 1974). Androgens can elicit certain biomarker sex characteristics in females whereas estrogens, anti-androgens, and steroidogenesis inhibitors suppress them in males. For example, androgen receptor agonists, such as methyltestosterone, can cause female fathead minnows to develop nuptial tubercles (Ankley et al 2001; Smith 1974) and/or dorsal nape pads (K. Thorpe, University of Exeter, personal communication). Also, some estrogen receptor agonists may decrease the number or size of nuptial tubercles in males (Harries et al 2000; Lange et al 2001; Miles-Richardson et al 1999a).

Some of the advantages of the use of secondary sex characteristics as endpoints in the fish screen with the fathead minnow are that these endpoints are biologically relevant and have been relatively reproducible. OECD and U.S. EPA data support consistent and reproducible results with potent androgens and estrogens. Male secondary sex characteristics provide the fish screening assay with indicative androgenic/antiandrogenic endpoints that may not be observed with other endpoints. Some of the weaknesses of using secondary sex characteristics as endpoints are that the sensitivity and specificity to certain MOAs are unknown. Although these characteristics are relatively easily measured, not all of them are as quantitative as some other endpoints. Also, some alterations to physiology that manifest in morphological changes may not appear in the short duration of the fish screening assay. In December 2005, the VMG-Eco group of the OECD advised that secondary sex characteristics in the fish screen were adequately developed in the fathead minnow and medaka for inclusion in the assay (OECD 2005).

Sex steroids

An endpoint which should be considered as an option in the protocol is the measurement of plasma sex steroid concentrations. A number of studies have been conducted documenting sex steroid levels in the fathead minnow under normal conditions and after exposure to endocrine-active compounds (Ankley et al 2001, 2003, 2005a; Giesy et al 2000; Jensen et al 2001; Jensen et al 2004; Makynen et al 2000) and this endpoint is included as a core endpoint in the EPA (2002) fish screening assay description.

Steroids measured in these studies include estradiol and testosterone and, occasionally, the fish-specific androgen 11-ketotestosterone. In terms of overall alterations in endocrine function (and reproductive success), steroid concentrations tend to reflect changes in other endpoints (*e.g.,* changes in VTG or fecundity), but offer additional "value added" in two regards, as: (a) additional information in a weight-of-evidence analysis for identifying a chemical as endocrine-active, and (b) providing insights as to mechanisms associated with responses. For example, through analysis of steroid profiles it is possible to ascertain specific aspects of steroidogenesis that are affected in fathead minnows exposed to different model chemicals (Ankley et al 2002; 2005a).

Reported results

Available data are only briefly summarized in the body this paper. The current intent is to provide a simple overview of the studies supporting the assertion that the broader suite of endpoints are demonstrated to be reliable. The data are not critically analyzed in detail as would be expected in an integrated validation report. The tables in this section address four core endpoints (histopathology, VTG, SSC, and fecundity). Just those tests using mature spawning fathead minnow and histopathology and/or fecundity endpoints are reported. Data were considered comparable whether a spawning group (*e.g.,* 2 males and 4 females) or a spawning pair (*e.g.,* one male and one female) was employed. The tables indicate only which endpoints were notably affected by the test compound. Histopathology findings are listed by the predominant diagnosis(ses) that were consistent among the laboratories reporting. The figures provide selected graphs and images from the studies to illustrate examples of endpoint responses. The listing of "additional diagnoses" (in conjunction with histopathology) notes that other pathologies were observed that may not have been specifically consistent across studies. Details of the specific pathologies reported can be found in Addendum 1. Addendum 1 also provides a more complete, though still limited, tabulation of the variety of tests completed with fathead minnow in the development of the fish screening assay.

Three androgen agonists have been investigated with a fathead minnow short-term reproduction assay comparable to the standardized protocol presented in Addendum 2. A summary of the findings for the core endpoints from these investigations are presented in Tables 3 – 5. All four endpoints were affected by the androgen agonist β-trenbolone in both laboratories which performed the assays (Table 3). The responses were also similar in both 14-day and 21-day versions of the assay performed by Battelle (2003a) indicating that, at the concentrations tested, a response occurs fairly rapidly for a potent androgen.

The two laboratories investigating another androgen methyltestosterone also reported generally consistent findings on the four core endpoints (Table 4). Both laboratories reported an estrogen-like increase in VTG in both sexes at the same concentrations. Methyltestosterone, unlike β-trenbolone, is aromatizable, which would explain the observed induction of VTG in males and females for this compound. A third androgen agonist (α-trenbolone) also affected three endpoints (VTG, SSC, fecundity) which were included in the study observations (Table 5). All three androgens affected plasma sex steroid concentrations in the fish in a fashion consistent with feedback inhibition of steroid production (Ankley et al 2001, 2003; Jensen et al 2000b).

Table 3. β-Trenbolone

Endpoints	(Ankley et al 2003)	(Battelle 2003a) (21d)	(Battelle 2003a) (14d)
Histopathology			
Male			
additional diagnoses	+	+	+
Female			
increased oocyte atresia	+	+	+
additional diagnoses	+	+	+
Vitellogenin			
Male	+	-	-
Female	+	+	+
Secondary sex characteristics			
Male	-	-	-
Female	+	+	+
Fecundity	+	+	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 4. Methyltestosterone

Endpoints	(Ankley et al 2001)	(Pawlowski et al 2004a)
Histopathology		
Male		
additional diagnoses	+	NR
Female		
increased oocyte atresia	+	+
additional diagnoses	+	-
Vitellogenin		
Male	+	+
Female	+	+
Secondary sex characteristics		
Male	+	-
Female	+	+
Fecundity	+	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 5. α-Trenbolone

Endpoints	(Jensen et al 2006)
Histopathology	
Male	NR
Female	NR
Vitellogenin	
Male	-
Female	+
Secondary sex characteristics	
Male	-
Female	+
Fecundity	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

The OECD Phase 1A was conducted with a non-spawning version of the fish screening assay in four laboratories. 17β-trenbolone was one of two compounds evaluated in this initial interlaboratory trial. The 17β-trenbolone was observed to induce male SSC in fathead minnow (tubercles) and medaka females (anal fin papillary processes), but not zebrafish, and also decreased VTG in the females in all three species. However, gonadal histopathology results were seemingly compromised by high incidence of abnormalities in controls probably caused by "forced" cessation in spawning (Lothenbach et al 2005).

Overall, fecundity and SSC appear to be quite sensitive endpoints for androgen agonists (Figures 1-4). Fecundity responds quickly to an androgen agonist exposure, as is consistently seen across laboratories (Figures 1, 2, and 4). Androgen-characteristic masculinization of females is visibly evident in appearance of male nuptial tubercles (*e.g.,* Figure 3).

Figure 1.

Effects of 17β-Trenbolone on Fathead Minnow Fecundity

Ankley et al. (2003) Environ.Toxicol. Chem.
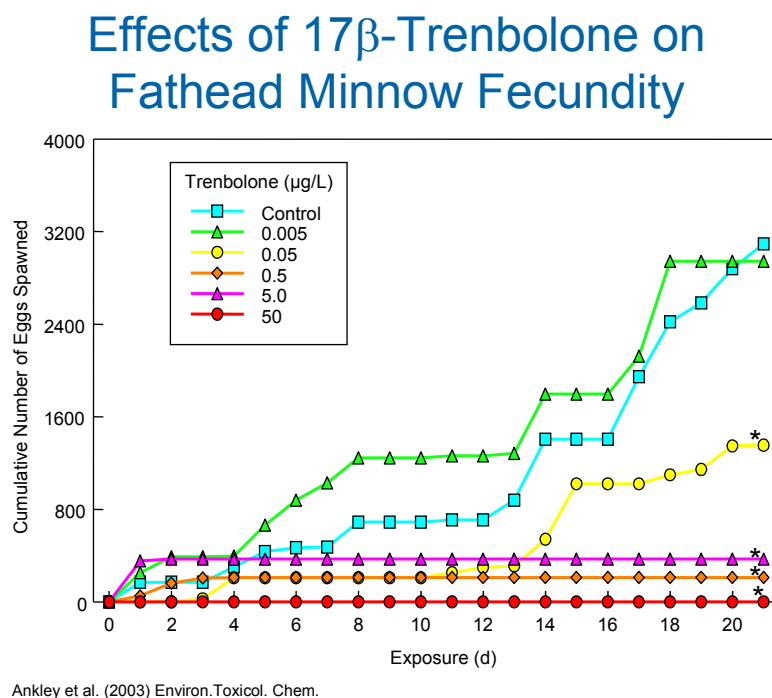
Figure 2.

17β-Trenbolone Effects in Fathead Minnow Assay

Battelle (2003a)
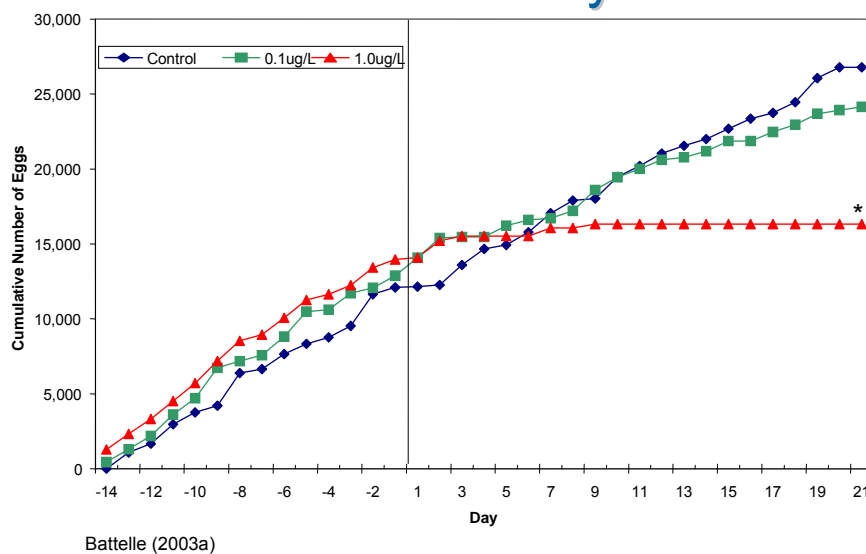
Figure 3.

Masculinization by 17β-Trenbolone

control male

control female

exposed female
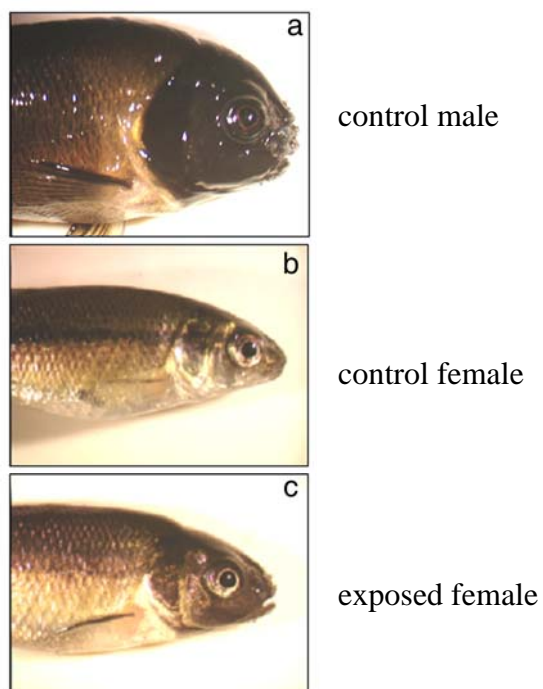
Ankley et al. (2003) Environ.Toxicol. Chem.

Figure 4.

Effects of Methyltestosterone on Fecundity
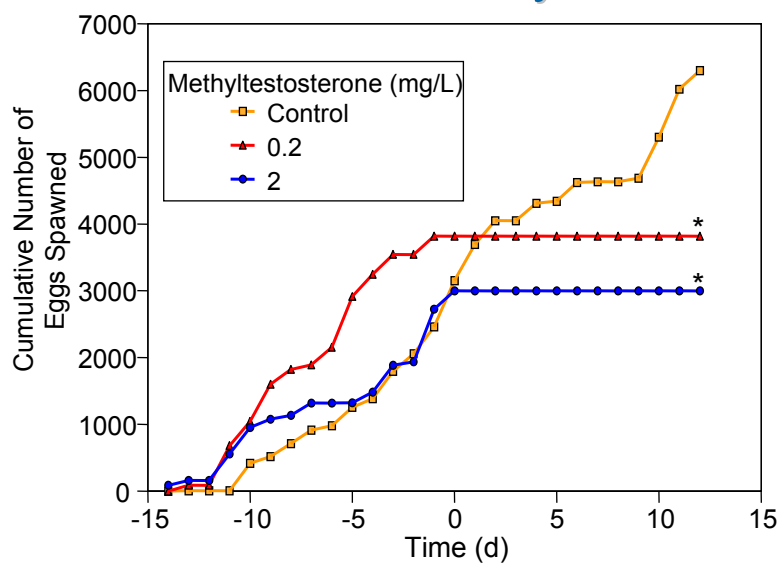
Ankley et al. (2001) Environ.Toxicol. Chem.

Fecundity also seems to be the most consistent endpoint responding to the androgen receptor antagonist flutamide (Table 6 and Figure 5). In the OECD Phase 1B trials (OECD 2006), the spawning configuration employed for fathead minnow (5 male:5 female per replicate) was sub-optimal and resulted in poor spawning in the control fish. In spite of this confounding factor, fecundity was still observed to be reduced in treated groups, which is consistent with the studies where spawning was acceptable (*e.g.*, Jensen et al 2004).

Table 6. Flutamide

Endpoints	(Jensen et al 2004)	(Battelle 2003a) (21d)	(Battelle 2003a) (14d)	(Battelle 2006) (a)	(Battelle 2006) (b)	(OECD 2006) Lab 9	(OECD 2006) Lab 14	(OECD 2006) Lab 13
Histopathology **Male**								
additional diagnoses	+	-	-	+	-	-	+	-
Female increased oocyte atresia	+	+	+	+	-	-	-	+
additional diagnoses	+	-	-	-	-	-	-	-
Vitellogenin **Male**	+	-	-	-	-	-	-	-
Female	+	-	-	-	-	-	-	-
Secondary sex characteristics **Male**	-	-	-	+	+	-	-	-
Female	-	-	-	-	-	-	-	-
Fecundity	+	+	+	+	?	+	+	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
? = statistical reanalysis underway
NR = not reported, endpoint not included in study

Flutamide was also observed to substantially impair fertility in one study (Figure 6). Tubercle score and morphology were not significantly affected by flutamide. However, fatpad weight which was measured in two studies (Battelle 2006) was decreased by flutamide. Although histopathology was inconsistent in detecting flutamide responses, this could have been influenced by protocol deviations and by the fact that the experimental fish were not always in optimal spawning condition in all the studies. At least one lab found subtle, but consistent, alterations in plasma VTG and steroid concentrations in fish exposed to flutamide (Jensen et al 2004; Jensen and Ankley 2006). Importantly, flutamide,

in a dual treatment version of the assay, was demonstrated to block the nuptial tubercle induction of 17β-trenbolone co-treated female fathead minnows, thus confirming the chemical as an androgen receptor antagonist (Ankley et al 2004).

Figure 5. Plot of Cumulative Fecundity by Treatment for the 21-Day Flutamide Assay

Cumulative Fecundity

 [Battelle 2006](Treatment groups diverge at day 0 with the medium and high treatment groups diverging from the control in a dose responsive pattern. The fecundity of the low treatment group is greater than control.)

Figure 6. Plot of Cumulative Fertility by Treatment for the 21-Day Flutamide Assay

Cumulative Fertility
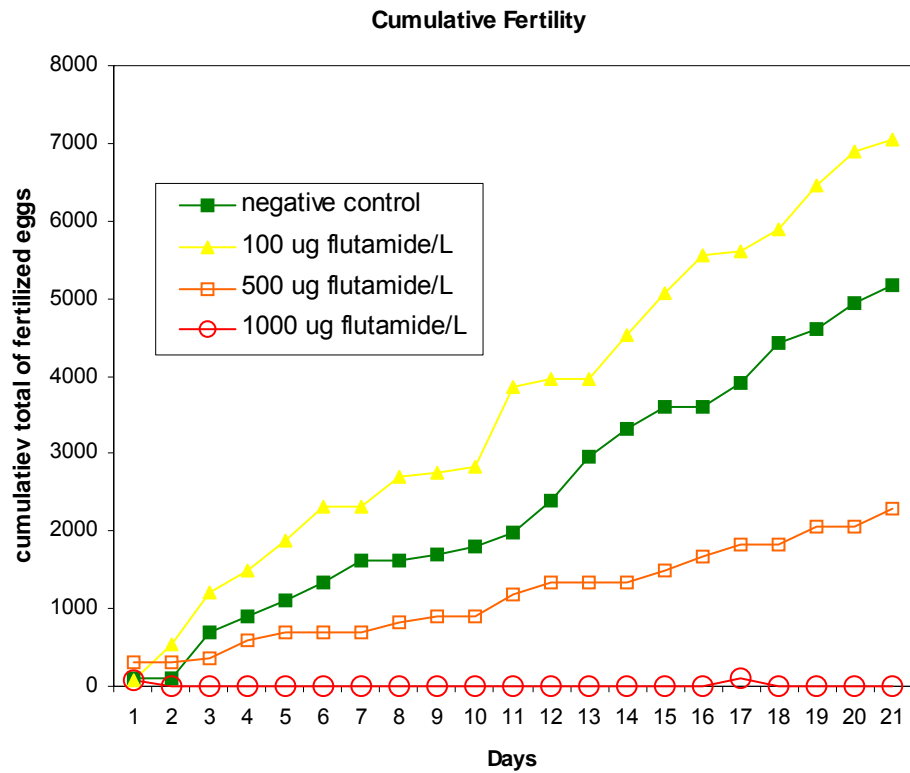
 [Battelle 2006](Treatment groups diverge at day 0 with the medium and high treatment groups diverging from the control in a dose responsive pattern. The fertility of the low treatment group is greater than control.)

Figure 7. Increased oocyte atresia observed in flutamide treated fish.
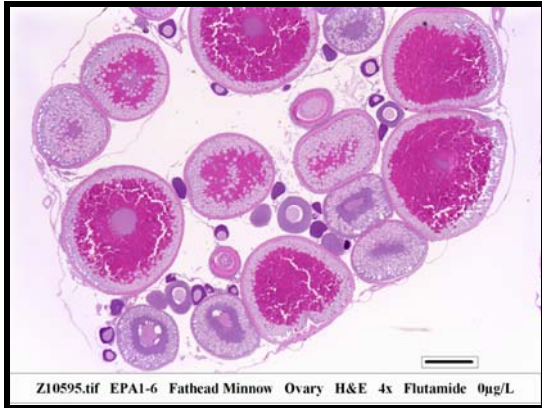
Ovary from a control group female. H&E, bar = 250 microns.
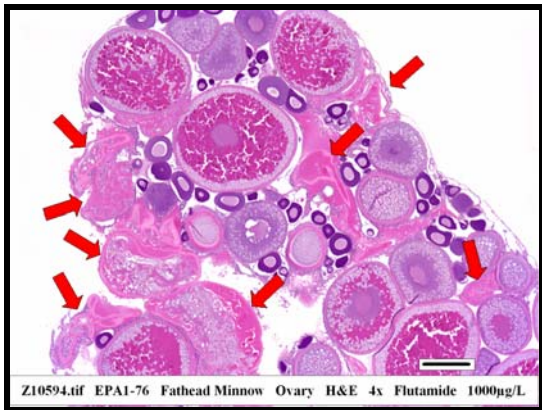
Ovary from a 1000 μg/L flutamide group female. Numerous atretic oocytes are evident (arrows). H&E, bar = 250 microns.

Figure 8.

Flutamide Effects on Steroids and Vitellogenin in Fathead Minnow
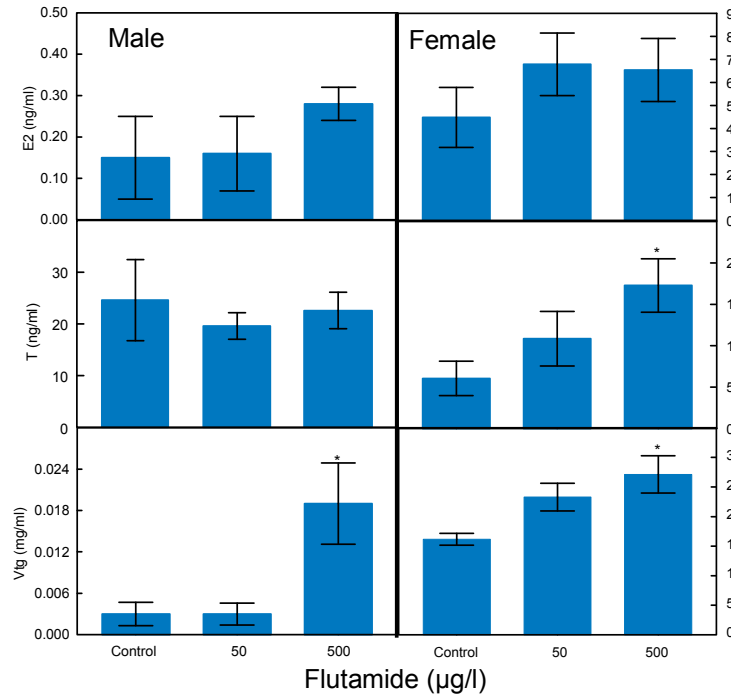
Jensen et al. (2004) Aquat. Toxicol.

Two other androgen receptor antagonists have been tested using the basic assay described in Addendum 2. For the weak mammalian androgen antagonist p,p′-DDE, histopathology was the only assay endpoint to exhibit a significant response (Table 7). In the case of vinclozolin, another established mammalian androgen receptor antagonist, histopathology and fecundity in the fathead minnow were both responsive (Table 8).

Table 7. p,p′-DDE

Endpoints	(Battelle 2004)
Histopathology	
Male	-
Female	
increased oocyte atresia	+
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	-
Female	-
Fecundity	-

+ = significant or pathologist observed change from control
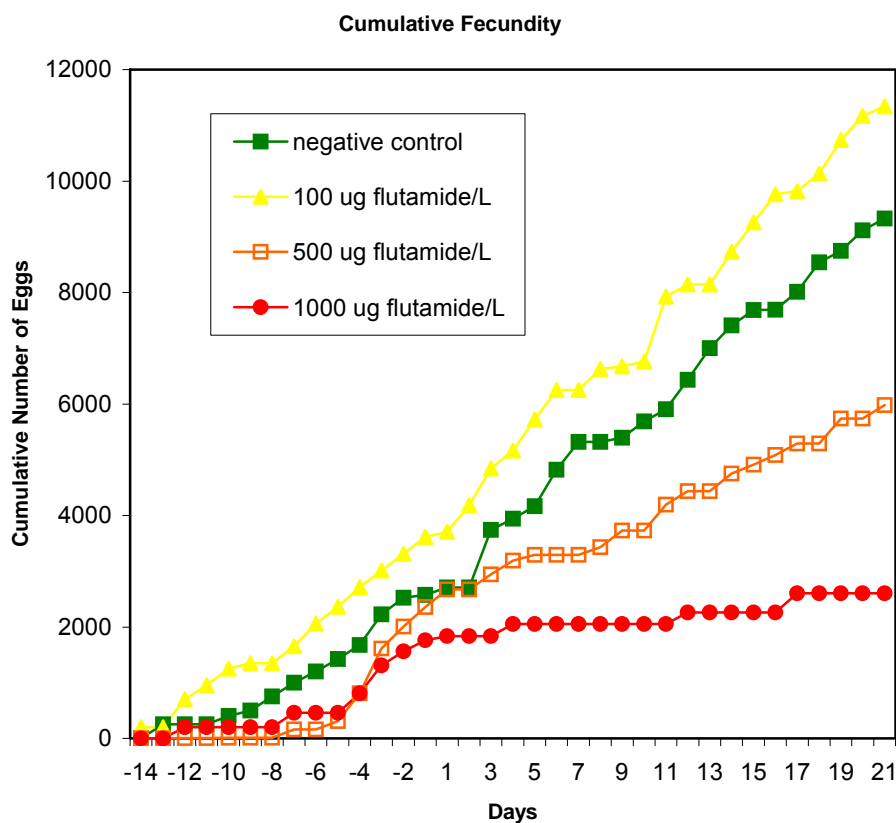- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 8. Vinclozolin

Endpoints	(Makynen et al 2000), (Ankley et al unpublished data)
Histopathology	
Male	-
Female	
altered gonadal stage (ovary)	+
Vitellogenin	
Male	NR
Female	NR
Secondary sex characteristics	
Male	NR
Female	NR
Fecundity	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Induction of vitellogenin in male fish is well established as a response to estrogen receptor agonists (Brodeur et al 2005; Hansen et al 1998; Harries et al 2000; Korte et al 2000; Kramer et al 1998; Lattier et al 2002; Panter et al 1998; Parks et al 1999; Schmid et al 2002). This was confirmed with the estrogen receptor agonists used in the various 21-day fathead minnow studies summarized in Tables 9 -12.

Effects of 17β-estradio, a strong estrogen, were consistently detected for nearly every endpoint (Table 9). The only exceptions were in the Phase 1B trials for 17β-estradiol, in which effects on SSC were not detected by one lab, and alterations in fecundity were not observed by two labs. Again, however, the Phase 1B experimental design spawning conditions were suboptimal, which could account for the seeming inconsistency.

For two more weakly estrogenic chemicals that have been tested, Bisphenol A and 4-*tert*-pentylphenol, the assay also consistently detected effects for each endpoint, an exception being for SSC in one study with Bisphenol A (Table 11). One example of findings include reduced or absent nuptial tubercles in males, similar to those reported for a study with 17β-estradiol by Miles-Richardson et al (1999a) (Figure 9). An example of increased proportion of

spermatogonia found in a study with 4-*tert*-pentylphenol is given in Figure 10 (OECD 2006).

Studies with the organochlorine pesticide methoxychlor that used fish in an optimized spawning condition (Table 12) also detected induction of VTG in male fathead minnows, changes in plasma sex steroid concentrations, histopathological alterations, and reductions in fecundity. Estrogenicity is one of several modes of action for methoxychlor, yet these estrogenic effects were the primary findings.

The fact that 17β-estradiol, a strong estrogen used as a positive control, was not always detected by changes in SSC or fecundity demonstrates the desirability of a suite of endpoints that mutually bolster findings. This need is further evident in instances in which problems arise with one or more evaluation. Lab 11 (Table 9), for example, experienced difficulty in measuring VTG concentrations, and because neither SSC nor fecundity detected any effects, only histopathological evaluations confirmed effects of this positive control.

Table 9. 17β-estradiol

Endpoints	(OECD 2006) Lab 9	(OECD 2006) Lab 10	(OECD 2006) Lab 11
Histopathology **Male**			
increased proportion of spermatogonia	+	+	+
additional diagnoses	+	+	+
Female	-	-	+
Vitellogenin **Male**	+	+	NR
Female	+	+	NR
Secondary sex characteristics **Male**	+	+	-
Female	-	-	-
Fecundity	-	+	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Figure 9.

E2 Effects on Male Tubercles

| control male | control female | treated male |
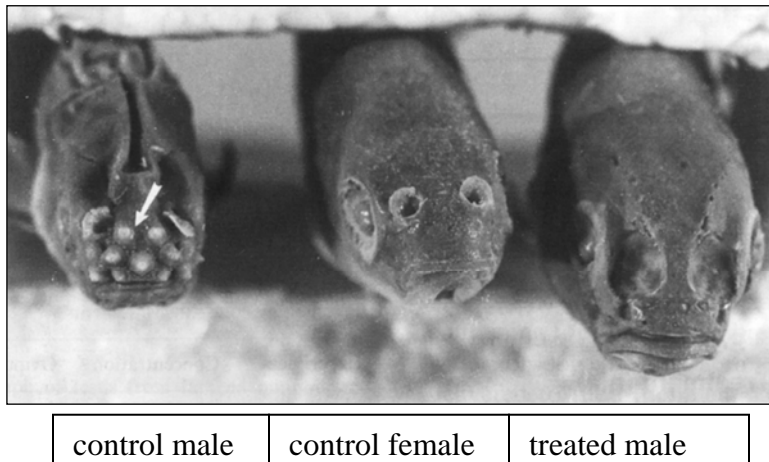
Miles-Richardson et al. (1999) Aquat. Toxicol.

Altered expression of secondary sex characteristics in male fathead minnows: Nuptial tubercles are present in control males, but absent in males treated with 17β-estradiol, similar to untreated females (Miles-Richardson et al 1999a).

Table 10. 4-*tert*-pentylphenol

Endpoints	(OECD 2006) Lab 9	(OECD 2006) Lab 10	(OECD 2006) Lab 11
Histopathology			
Male			
increased proportion of spermatogonia	+	+	+
additional diagnoses	+	+	+
Female			
altered gonadal stage	+	+	-
additional diagnoses	-	+	-
Vitellogenin			
Male	+	+	NR
Female	+	-	NR
Secondary sex characteristics			
Male	+	+	+
Female	-	-	-
Fecundity	+	+	+

+ = significant or pathologist observed change from control

- = no significant or pathologist observed change from control

NR = not reported, endpoint not included in study

Figure 10. Example of increased proportion of spermatogonia in 4-*tert*-pentylphenol treated male fathead minnows.

Z10688.tif   01A1-1   Fathead Minnow   Testis   H&E   40x   4tPP   0 ug/L
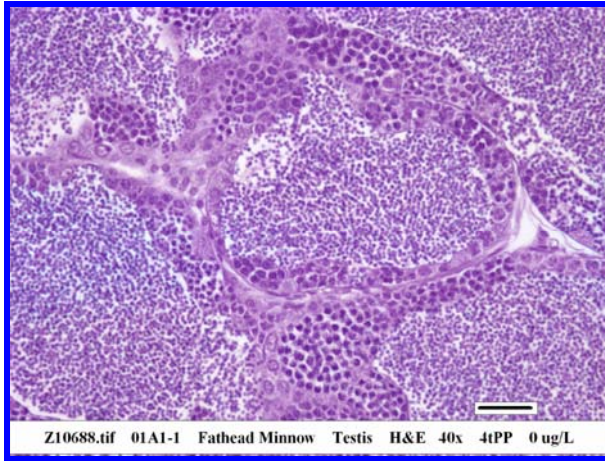
Testis from a control male FHM. H&E, bar = 25 microns.

Z10693.tif   03E1-1   Fathead Minnow   Testis   H&E   40x   4tPP   1000 ug/L

Testis from a male FHM exposed to 1000 µg/L 4tPP. Spermatogonia are the dominant cell type within the germinal epithelium. A large vein contains abundant proteinaceous fluid (plasma) (arrow). H&E, bar =

Table 11. Bisphenol A

| Endpoints	(Battelle 2004)
Histopathology	
Male	-
Female	
granulomatous inflammation	+
Vitellogenin	
Male	+
Female	+
Secondary sex characteristics	
Male	-
Female	-
Fecundity	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 12. Methoxychlor

Endpoints	(Ankley et al 2001)	(Battelle 2003a) (21d)	(Battelle 2003a) (14d)
Histopathology			
Male	-	-	-
Female			
increased oocyte atresia	+	-	-
additional diagnoses	-	+	-
Vitellogenin			
Male	+	?	?
Female	-	-	+
Secondary sex characteristics			
Male	-	-	-
Female	-	-	-
Fecundity	+	+	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
? = statistical analysis should be reviewed, apparent large difference between
control and high dose group

Steroid synthesis modulators can cause several responses in the 21-day
assay through disruption of normal androgen and estrogen production,
including alterations in plasma steroid levels, suppression of vitellogenesis,
decreased post-ovulatory follicles, impaired maturation of oocytes and
spermatozoa, pronounced oocyte atresia, and reduced fecundity. The fish
screening assay captures all these responses with three relevant core endpoints
histopathology, VTG, and fecundity (Tables 13 – 16, Figures 10 – 11). Secondary
sex characteristics appear to be unresponsive to this mechanism. The chemicals
used to investigate the fish short-term reproduction assay's ability to detect
steroid synthesis modulators include fadrozole, fenarimol, prochloraz,
ketoconazole and cadmium chloride. Abbreviated results for these chemicals
are presented in Tables 13 – 17.

Table 13. Fadrozole

Endpoints	(Ankley et al 2002)	(Battelle 2003a) (21d)	(Battelle 2003a)(14d)	(OECD 2006) Lab 10	(OECD 2006) Lab 11	(OECD 2006) Lab 12	(OECD 2006) Lab 13
Histopathology							
Male							
interstitial cell hyperplasia	NR	+	+	-	+	+	+
Female							
increased oocyte atresia	+	+	+	+	+	+	+
altered gonadal stage	+	+	+	+	+	+	+
Vitellogenin							
Male	-	-	-	-	NR	-	-
Female	+	+	+	+	NR	-	+
Secondary sex characteristics							
Male	-	-	-	-	-	-	-
Female	-	-	-	-	-	-	-
Fecundity	+	+	+	-	NR	-	-

+ = significant or pathologist observed change from control

- = no significant or pathologist observed change from control

NR = not reported, endpoint not included in study

Table 14. Fenarimol

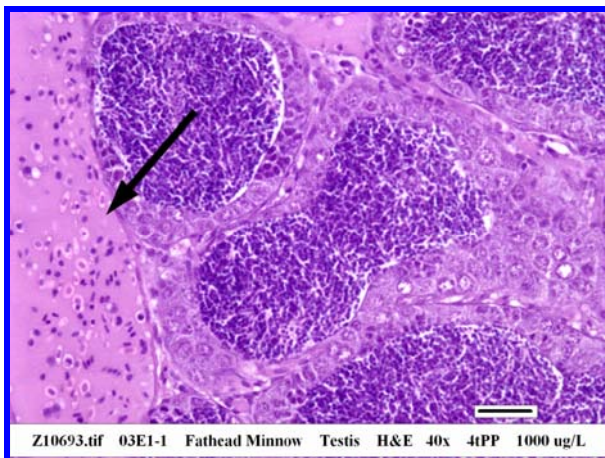| Endpoints	(Ankley et al 2005a)
Histopathology	
Male	
increased proportion of spermatagonia	+
Female	
increase oocyte atresia	+
Vitellogenin	
Male	-
Female	+
Secondary sex characteristics	
Male	-
Female	-
Fecundity	+

+ = significant or pathologist observed change from control

- = no significant or pathologist observed change from control

NR = not reported, endpoint not included in study

Table 15. Prochloraz

Endpoints	(Ankley et al 2005a)	(OECD 2006) Lab 10	(OECD 2006) Lab 11	(OECD 2006) Lab 12	(OECD 2006) Lab 13
Histopathology					
Male					
additional diagnoses	+	+	-	-	+
Female					
increased oocyte atresia	+	+	+	+	+
additional diagnoses	+	+	-	-	-
Vitellogenin					
Male	-	-	-	-	-
Female	+	+	NR	+	+
Secondary sex characteristics					
Male	-	-	-	-	-
Female	-	-	-	-	-
Fecundity	+	-	+	-	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 16. Ketoconazole

Endpoints	(Ankley and Villeneuve 2006)	(Battelle 2006)
Histopathology		
Male		
interstitial cell hyperplasia	+	+
Female	-	-
Vitellogenin		
Male	-	+
Female	NR	-
Secondary sex characteristics		
Male	NR	-
Female	NR	-
Fecundity	+	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 17. Cadmium Chloride

Endpoints	(Battelle 2004)
Histopathology	
Male	-
Female	-
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	-
Female	-
Fecundity	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

The VTG endpoint consistently was affected for fadrozole, prochloraz, and fenarimol in that female vitellogenesis was inhibited. For fadrozole (Table 13), female vitellogenesis was reported as significantly impaired in five out of six laboratories, and at a dose level as low at 2 µg/L (nominal) (Ankley et al 2002) (Figure 11).   The reduced VTG was consistent with decreased plasma concentrations of 17β-estradiol, which is consistent with inhibition of aromatase (Figure 11). Prochloraz (Table 15) suppressed female vitellogenesis in four out of four laboratories at dose levels between 100 and 300 µg/L (actual), and fenarimol (Table 14) also significantly suppressed female vitellogenesis (Ankley et al 2005a). Male vitellogenesis was not significantly affected for any of the three compounds. In contrast, with ketoconazole (Table 16), female vitellogenesis was unaffected, whereas male vitellogenesis induction was found in one out of two laboratories. This result was unanticipated because ketoconazole purportedly depresses testosterone synthesis, and therefore should seemingly suppress the production of both estrogens and androgens. The vitellogenesis endpoint was not affected by cadmium chloride (Table 17) exposure.

Figure 11.

Fadrozole Effects on E2, Vtg
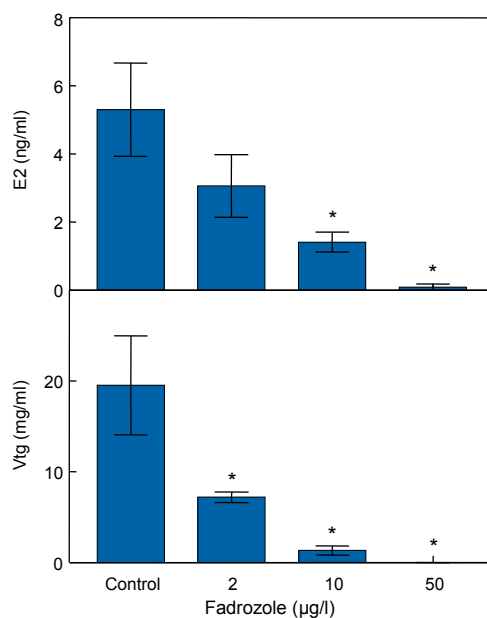
Ankley et al. (2002) Toxicol. Sci.

Histopathology consistently detected effects of all of the steroidogenesis inhibitors except cadmium chloride. For fadrozole, prochloraz, and fenarimol, the most consistent diagnostic criterion was increased oocyte atresia. Seven out of seven labs reported a significant increase in oocyte atresia for fadrozole (see Figure 12 as an example), and five of five laboratories reported increased oocyte atresia for prochloraz. Altered gonadal stage (ovaries) was also reported in seven out of seven laboratories that tested fadrozole. An additional significant histopathological lesion reported for fadrozole, was decreased deposition of VTG (Figure 12, 13), a finding consistent with female vitellogenesis suppression. The testes of the fathead minnow were significantly affected by both fenarimol and ketoconazole. With fenarimol, a significant increase in spermatogonia was detected, potentially as a result of suppressed levels of testosterone leading to alteration of the ability for the spermatogenic cells to mature. With ketoconazole, both laboratories reported interstitial cell hyperplasia (Figure 14), again probably due to suppressed levels of testosterone.

Figure 12. Example of increased oocyte atresia from exposure to fadrozole

Fadrozole Effects on Fathead Minnow Ovary
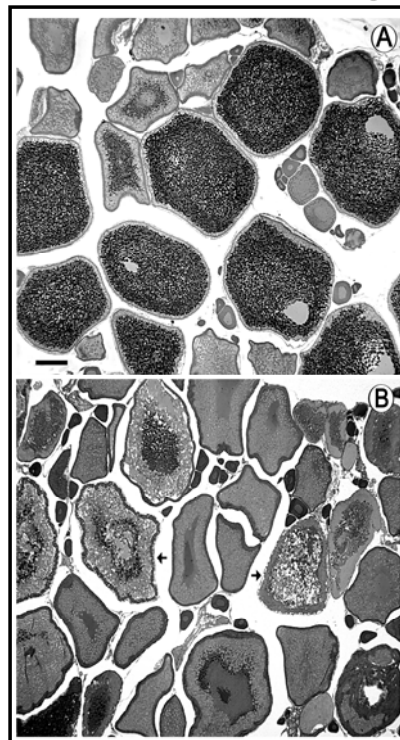
Ankley et al. (2002) Toxicol. Sci.

Figure 13. Example of decreased vitellogenin deposition in fadrozole treated fish.

Z10607.tif EPA2-6 Fathead Minnow Ovary H&E 4x Prochloraz 0µg/L
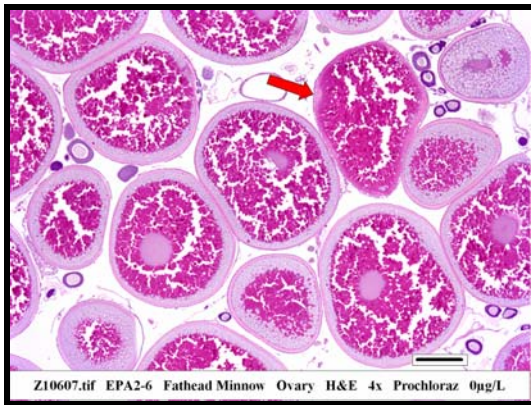
Ovary (Stage 3) from a control group female. A single atretic ovary is evident (arrow). H&E, bar = 250 microns.

Z10605.tif EPA2-97 Fathead Minnow Ovary H&E 4x Fadrozole 100µg/L
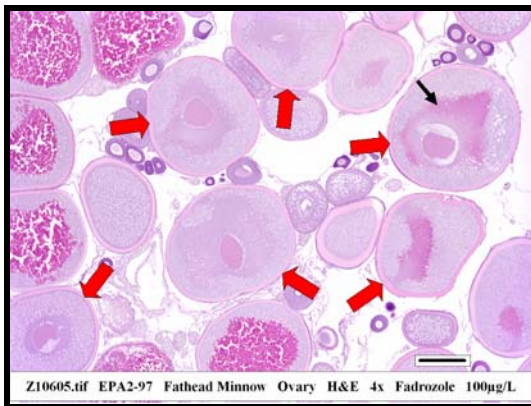
Ovary from a 100 µg/L fadrozole group female. Fadrozole was used as a positive control in the prochloraz study. Decreased vitellogenesis is characterized by the presence of oocytes in which yolk material is not present despite their relatively large size (large arrows). Note that oocytes are affected to varying degrees. Some affected oocytes have extremely fine vitellogenic granules (small arrow), and this is

(OECD 2006)

Figure 14. Example of Interstitial cell hyperplasia in ketoconazole treated fish.
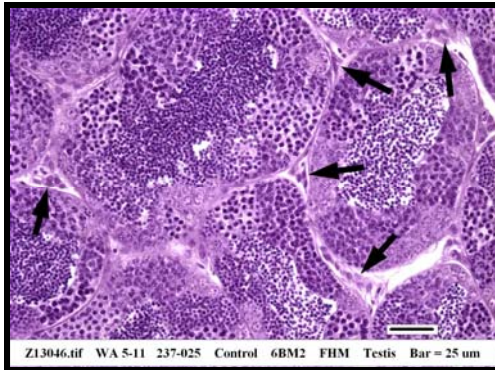
Testis from a control group male. Interstitial areas contain small aggregates of interstitial (Leydig) cells (arrows). Most interstitial cells have wispy, pale cytoplasm
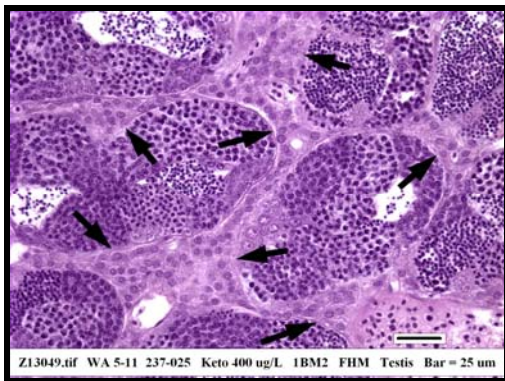
Testis from a 400 µg/L ketoconazole group male. Interstitial cell aggregates (arrows) in the testis of this fish are larger than in control fish, and the cells tend to fill and expand the interstitial spaces. This was diagnosed as Increased Cells, Interstitial Cells, Grade 2 (mild) severity. H&E

(Battelle Memorial Institute, *unpub.*)

Fecundity was significantly affected (decreased) by exposure to fadrozole (three out of six labs, Figures 16A and B), fenarimol, prochloraz (two out of five labs, Figure 17), and ketoconazole (one out of two labs). Again, much of the fecundity data (for fadrozole and prochloraz) are derived from the OECD Phase 1B study which, due to suboptimal spawning design, renders interpretation of fecundity data somewhat problematic. As expected, secondary sex characteristics were not significantly affected by exposure to any steroidogenesis inhibitors. Cadmium chloride did not affect either endpoint in the fish.

Figure 15.

Effects of Fadrozole on Fathead Minnow Fecundity

A
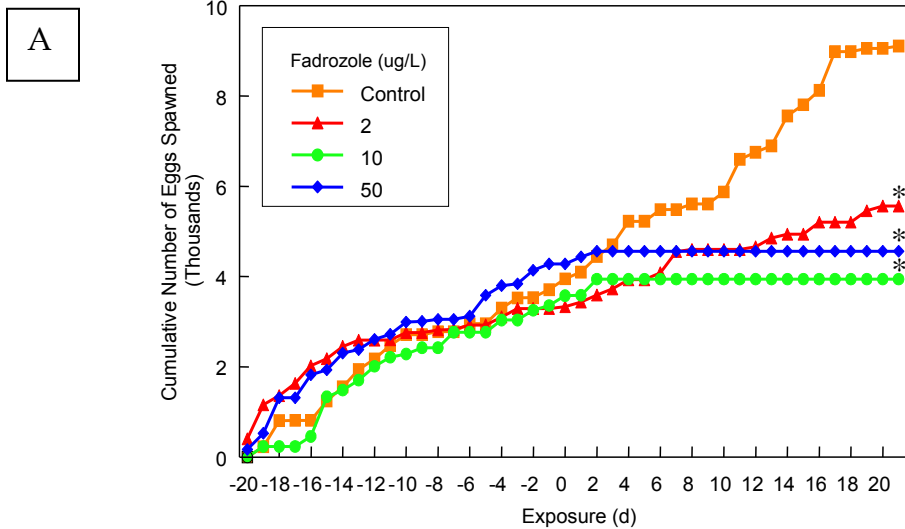
Ankley et al. (2002) Toxicol. Sci.

B.
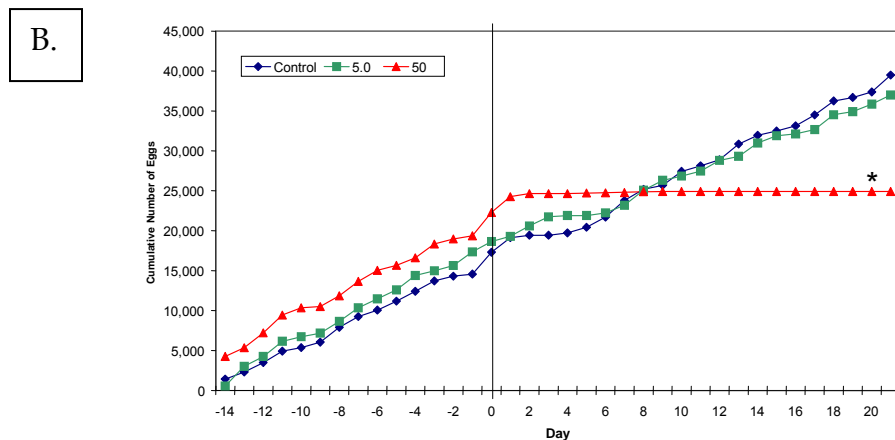
Battelle (2003)

Figure 16. Effects of prochloraz and fenarimol on fecundity

Effects of Two Fungicides on Fathead Minnow Fecundity
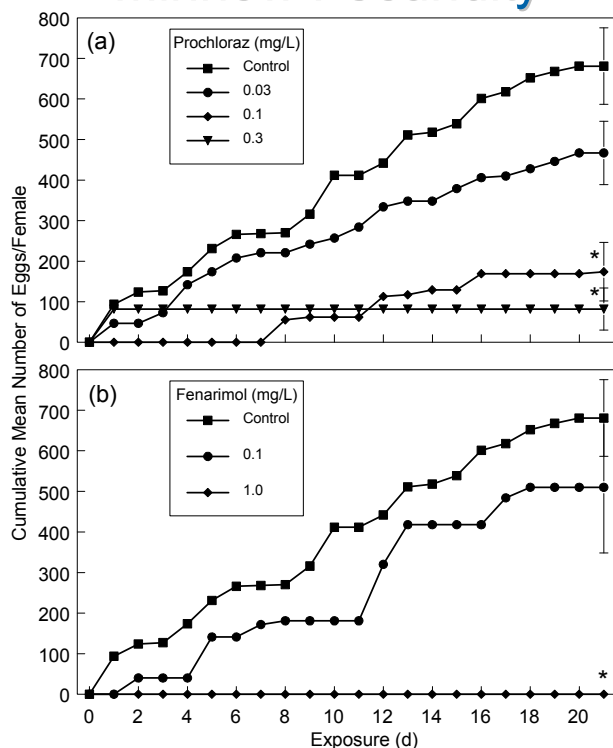
Ankley et al. (2005) Toxicol. Sci.

Overall, the endpoint that most consistently detected (four out of five) purported steroid synthesis modulators was histopathology. Vitellogenin (decreases in females) consistently detected three of the steroid synthesis modulators (fadrozole, fenarimol and prochloraz), and four of five chemicals affected fecundity, albeit in a less consistent inter-laboratory manner. Only cadmium chloride failed to produce responses in the fish short-term reproduction assay.

Two studies have used atrazine as a test chemical in the fish screening assay, with one laboratory finding a significant effect (Table 18). It might be anticipated that the fish's reproductive system would be affected by atrazine, as it is considered to be active at the neuroendocrine level (hypothalamus-pituitary axis) in mammals (Cooper et al 1999). Although male fathead minnows showed an increased proportion of spermatogonia following exposure to atrazine in one study, this was not accompanied by changes in fecundity, VTG, or SSC (Table 18).

Table 18. Atrazine

Endpoints	(Battelle 2004)	(Bringolf et al 2004)
Histopathology		
Male		
increased proportion of spermatogonia	+	-
additional diagnoses	+	-
Female	-	-
Vitellogenin		
Male	-	-
Female	-	-
Secondary sex characteristics		
Male	-	-
Female	-	-
Fecundity	-	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Villeneuve et al. (2006) found that the related triazine herbicide prometon caused significant effects on histopathology as seen in increased oocyte atresia as well as decreased post-ovulatory follicles (Table 19). The chemical also caused significant changes to SSC in male fathead minnows. Male fat pad weights relative to body weights were significantly reduced, which might be indicative of anti-androgenic activity. However, there was no clear mechanism of action, as no response was observed in plasma VTG or sex steroid concentrations, fecundity or fertility measurements, aromatase activity, or tubercle index.

Table 19. Prometon

Endpoints	(Villeneuve et al 2006)
Histopathology	
Male	-
Female	
increased oocyte atresia	+
additional diagnoses	+
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	+
Female	-
Fecundity	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Ankley et al. (2005b) speculated that PFOS might affect reproduction in fish through alterations to the HPG axis. Chemicals structurally similar to PFOS can alter plasma concentrations of sex steroids in fathead minnow (Oakes et al 2004). Endpoints that have traditionally been sensitive to alterations to the HPG axis in the fathead minnow include measurements of fecundity and plasma levels of sex steroids and vitellogenin (Ankley et al 2005b). As demonstrated in this study, exposure to PFOS reduced cumulative fecundity in the fathead minnow and caused increased oocyte atresia, altered gonadal stage, and decreased post-ovulatory follicles in females (Table 20). Histopathological changes in females were consistent with the failure to develop and release eggs. Fecundity and histopathological criteria were shown to be sensitive endpoints for this chemical, demonstrating reproductive effects. Although Ankley et al. (2005b) found some evidence of altered plasma steroid concentrations in exposed fish, it is uncertain whether or not these effects were modulated through the HPG axis.

Table 20. PFOS

Endpoints	(Ankley et al 2005b)
Histopathology	
Male	-
Female	
increased oocyte atresia	+
additional diagnoses	+
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	-
Female	-
Fecundity	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Potassium permanganate and perchlorate were selected as toxic negative compounds to evaluate how general stress responses would affect estrogen and androgen-mediated processes (Tables 21 and 22).   The results of the tests indicate that when exposures exceed lethal thresholds, these processes are indeed disturbed. With the exception of a positive histology response (oocyte atresia) for perchlorate, exposure concentrations below lethal limits did not significantly affect the four core endpoints.

Table 21. Perchlorate

Endpoints	(Battelle 2004)
Histopathology	
Male	-
Female	
increased oocyte atresia	+
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	-
Female	-
Fecundity	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 22. Potassium permanganate

Endpoints	(Battelle 2006) Lab 15	(Battelle 2006) Lab 17
Histopathology		
Male		
increased proportion of spermatogonia	+(m)	+
additional diagnoses	+(m)	-
Female		
altered gonadal stage	+(m)	-
Vitellogenin		
Male	-	-
Female	+(m)	-
Secondary sex characteristics		
Male	+(m)	-
Female	-	-
Fecundity	+(m)	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
m = observed only at concentrations exhibiting mortality
NR = not reported, endpoint not included in study

Test method reliability

The fish short-term reproduction assay with the combination of endpoints has been demonstrated by efforts in the U.S. to be capable of detecting known endocrine-active compounds. In the OECD Phase 1A, a non-spawning protocol was used which did not include a fecundity endpoint, but did include the histopathology endpoint. In fact, the histopathology identified disturbances in the gonads of control fishes that were determined to be related to the non-spawning status, and therefore led to changes in Phase 1B to allow spawning (Lothenbach et al 2005). This supports the idea that histopathology is useful to determine the general health of the fish, and acts as an internal control for the experiment. However, the VTG endpoint in the Phase 1A did respond to the potent estrogen agonist (17β-estradiol) and the potent androgen agonist (17β-trenbolone) and SSC in female medaka and fathead minnow were similarly responsive to 17β-trenbolone. In the OECD Phase 1B study, the VTG endpoint responded consistently to both the weak estrogen and steroidogenesis inhibitor. The VTG endpoint performed poorly for the anti-androgen (flutamide), and consistent responses in SSC were not observed for any of the test compounds.

The spawning and histopathology endpoints in Phase 1B provided mixed results, which were likely due to the inconsistencies in spawning observations (qualitative or quantitative), spawning configuration for fathead minnows, maturation/spawning condition of the fish, and inconsistent reporting for histopathology. Nonetheless, spawning and histopathology were generally the most sensitive endpoints (or at least comparable to VTG). For flutamide (an anti-androgen), the only two endpoints that identified activity for this compound in Phase 1B were spawning in the fathead minnow and histopathology in zebrafish.

The variability for egg production measures in the fathead minnow assay, as performed in the U.S. trials, is often less than what is observed for plasma VTG measures (Table 23). In considering the variability associated with histopathology, it must be understood that the diagnostic criteria or specific lesions do not represent separate discreet endpoints and should not be evaluated in this context. It is the summary pathology reading and overall response of the organ that represents the endpoint. In this regard, the pathology experts at the Phase 1B histopathology consultation "…concluded that overall exposure-related findings were generally consistent among different laboratories" in the Phase 1B trials (OECD 2004c).

Table 23.

Mean values and variability of fecundity & VTG endpoints in control females

Control	Fecundity			Vitellogenin		
Treatment	Eggs/♀/day			(mg/mL)		
Group	Mean	SD[a]	CV[b]	Mean	SD	CV
Atrazine	53.6	13.1	24%	6.6	2.4	36%
Bisphenol A	57.8	10.6	18%	11.1	3.4	31%
Cadmium	63.9	18.3	29%	10.7	2.5	23%
p,p-DDE	65.5	9.1	14%	11.5	2.6	22%
Perchlorate	61.3	8.3	14%	8.3	2.0	24%
Study Mean	60.4	11.8	20%	9.6	3.1	32%

Battelle (2004)

Data interpretation

The U.S. endocrine screening and testing framework intends to use a weight-of-evidence (WOE) approach for data interpretation, which includes WOE of the endpoints within an assay (with some endpoints more important than others) and WOE of findings from a battery of assays (*in vitro* and *in vivo*). It is the battery assessment which dictates the conclusions relevant to endocrine disruption and triggers for further testing. However, an apical endpoint such as fecundity has relevance beyond EDCs. Impaired fecundity is an adverse effect with regulatory importance whether it is due to endocrine-mediated activity or another MOA. Evaluation of a possible fecundity effect would have to consider the relative concentration which elicits the effect. If fecundity alone is affected and it is only affected at concentrations which also cause mortality, weight loss, or other obvious sign of toxicity, then it would likely be discounted. If it occurs at sublethal (toxic) levels, on the other hand, then a dose-response would likely be sought with additional higher tier testing.

A suite of endpoints (VTG, SSC, histopathology, and fecundity) are needed (in the U.S. opinion) to allow a conclusion of "no effect" in the fish short-term reproduction assay. A "no effect" conclusion would pass a compound through the screen and relieve it from further consideration. The endpoints together buttress each other for WOE review. The utility of the assay extends beyond potential endocrine-active substances to also include possible fish reproductive toxicants and meets regulatory needs currently unfulfilled in both cases.

Assay validation status

It should be clear from the information presented that there are ample data available which can support the reliability of a fish screening assay that includes a comprehensive suite of endpoints to detect compounds known to interfere with endocrine-mediated processes or otherwise affect reproduction. Likewise, the available information demonstrates the inability of the assay to detect all known ED substances when limited to just VTG and SSC endpoints. Even though no single endpoint has proven to be the most sensitive and most consistently responsive for all known estrogen- and androgen-mediated process-disrupting agents, the suite of endpoints has been shown to effectively capture and inform the need for further testing of these agents. It is also recognized that the assay is able to serve broader hazard identification purposes. Although these data are only briefly and generally summarized in this paper, it should be apparent that there is a solid basis to develop an integrated validation summary report to advance to a peer review on validation status for the intended purpose of the assay as it is defined in this paper. It remains a prerogative of a peer

review panel to agree or disagree with the validity of the assay and the suite of endpoints as recommended.

In considering what conclusions may be drawn from the available data, the following points are provided:

(1) Androgens cause clear, unambiguous changes in secondary sex characteristics in females in the assay and trigger further testing. The fact that the other endpoints are altered, vitellogenin, fecundity, histology (and steroids) is added information, in terms of the screen's ability to flag a compound, *only* to better understand toxicity pathways (VTG, steroids, histology) or reproductive impacts (fecundity).

(2) Estrogens cause a clear, unambiguous increase in VTG in males. The fact that the other endpoints can be altered, secondary sex characteristics, fecundity, histology (and steroids) is added information *only* to better understand toxicity pathways (steroids, histology) or reproductive impacts (fecundity).

(3) Several chemicals known as inhibitors of different steps in steroidogenesis (fadrozole, prochloraz, fenarimol) decrease VTG in females, although the *in vivo* steroid data are somewhat ambiguous that this is by inhibition of steroidogenesis. Again, the fact that other endpoints can be altered is important only to better understand toxicity pathways (*e.g.*, we can identify which enzymes might be inhibited by looking at steroids) or specific reproductive impacts.

The exception here-and it may be an important one- is ketoconazole, which we know from mammalian studies *should be* an ED compound. The fish assay is positive for fecundity effects indicating that it is a reproductive toxicant. It also is positive for gonad histology effects that are specific enough (interstitial cell hyperplasia) to suggest an endocrine pathway etiology.

(4) Based on all the data in the analysis, a strong case cannot be made that androgen receptor antagonists are effectively identified by the assay as "endocrine active". The exception may be the US-EPA MED laboratory study which includes serum steroid analysis. In considering flutamide, there seems to be a consistent effect on fecundity, which means that it would be flagged as a reproductive toxicant. However, neither secondary sex characteristics nor vitellogenin would identify it as a potential ED compound, and the primary histological effects (oocyte atresia) are too general to do so. Oocyte atresia can be caused by many things in addition

to ED, including flawed experimental designs (see OECD Phase 1A). The vinclozolin fecundity and histology data that are presented, although more limited than flutamide, offer exactly the same picture-that of a reproductive toxicant, not an ED substance. And, the p,p-DDE data show only the general histopathology finding (oocyte atresia) and no fecundity response.

Overall, if you consider all the data, one may conclude that the assay-with all four endpoints-will detect anti-androgenic chemicals, as at least reproductive toxicants.

(5) Next, it is necessary to consider what is summarized for the three chemicals—atrazine, PFOS and prometon—with undefined "endocrine" modes of action. It should be understood that the primary mode of action for the three compounds may not in fact be endocrine-mediated, and their endocrine modalities only secondary to other action. The results for these compounds were somewhat mixed in demonstrating the assay's effectiveness. Two of the chemicals-atrazine and prometon-were not identified as reproductive toxicants (to the extent that fecundity was reduced). Both triazines caused oocyte atresia, a relatively non-specific histological response. PFOS both reduced fecundity and caused oocyte atresia, so it can be construed to be a reproductive toxicant. The only specific endocrine-mediated response from these three studies was a decrease in male fatpad weight in prometon-exposed males. However, there is limited experience with this measurement that has only recently be included in the secondary sex characteristics endpoint.

The analyses from the three chemicals with unknown modes of action show that all three cause oocyte atresia-the same response as seen for anti-androgens and a negative control (perchlorate)-and one chemical (PFOS) that also decreased fecundity.

(6) Finally, we need to consider the two chemicals identified as "toxic negatives". Potassium permanganate in one lab shows that all the endpoints respond when lethal thresholds are exceeded. In another lab, histology (in males), but not fecundity, is affected. Perchlorate also caused effects on (female) histology but not fecundity. However, when concentrations are below overt toxicity (lethal) levels, the endpoints do not spuriously respond.

The fish short-term reproduction assay, as proposed, can be considered to address two roles – to detect potential endocrine disrupting agents or to detect reproductive toxins. There is no debate that changes in VTG and SSC are

indicative of endocrine-disrupting effects. There should also be no debate that fecundity (and fertility, etc.) and histopathology are appropriate for identifying potential reproductive toxicants. The debate that exists is on the split purpose of the assay. The U.S. accepts both purposes and contends that these are not altogether mutually exclusive. Substances that interfere with the normal function of androgens and estrogens also affect reproduction. Additionally, reproductive toxicants which act through non-hormonally mediated action are also of potential regulatory concern. The only difference between a fish screening protocol specific to ED and one targeting reproductive toxins, are the observational endpoints included. The in-life conduct and numbers of fish used are the same. It is irresponsible to not collect and evaluate the fecundity data provided by spawning fish and collect the reproductive organ for assessing possible pathology at assay termination in addition to vitellogenin and secondary sex characteristics endpoints in a single assay especially where it would save the use of animals in a separate, virtually identical assay.

In addition to detecting an ED and/or reproductive effect and triggering higher tier testing, the fish screening assay should be able to inform the appropriate concentration range to be used in a definitive test. This would avoid the need for an additional range-finding study and further reduce the number of animals needed overall in hazard assessment. A screening assay limited to VTG and SSC would be inadequate in this matter, where information included for fecundity and histopathology would be invaluable.

The standardized protocol presented in Addendum 2 has not received a formal interlaboratory evaluation. Although the general design and components are by and large identical to many of the studies summarized in this discussion paper which support the assay reliability, there exist some elements which have been modified to improve the test guidance and practicality. For this reason, it is appropriate to evaluate the standardized protocol in a limited interlaboratory exercise. The U.S. EPA proposes to perform this interlaboratory trial with a minimum of 3 laboratories and 2 test chemicals using the fathead minnow. It is recommended that one of the chemicals will be well demonstrated to be consistently responsive across the four core endpoints in the assay, and the second chemical will be one with limited experience in the fathead minnow assay but expected to be responsive for one or more core endpoints. This exercise is intended to confirm the reproducibility of the assay for its designated purpose as indicated by the prevailing data. Information on the identity of the test chemicals will be limited (e.g., coded for certain endpoint analysis where practical) to avoid any potential bias.

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Addendum 1

Summary results of fathead minnow screening assays

Laboratory & protocol information for studies included

Laboratories

Lab 1	EPA MED-Duluth
Lab 2	Battelle-SEQUIM
Lab 3	Iowa State	static-renewal aquatic system as opposed to flow-through
Lab 4	Michigan State
Lab 5	Astra-Zeneca
Lab 6	Germany
Lab 7	Brunel University
Lab 8	Univ. of Guelph
Lab 9	Lab 7 of OECD Phase 1B
Lab 10	Lab 8 of OECD Phase 1B
Lab 11	Lab 9 of OECD Phase 1B
Lab 12	Lab 11 of OECD Phase 1B
Lab 13	Lab 4 of OECD Phase 1B
Lab 14	Lab 10 of OECD Phase 1B
Lab 15	ABC labs
Lab 16	Wildlife International
Lab 17	Springborn Smithers

Protocols

	Duration	Spawning/Non-spawning	Female:Male	
Protocol 1	21 days	spawning	4:2	
Protocol 2	21 days	spawning	1:1	
Protocol 3	14 days	spawning	4:2	
Protocol 4	14 days	spawning	1:1	
Protocol 5	9-12 days	spawning	4:2	
Protocol 6	14 days	non-spawning	sexes kept separate	
Protocol 7	10 days	sexually mature (6-8 months old)	4:2	
Protocol 8	21 days	spawning	3:3	
Protocol 9	21 days	non-spawning	unknown - 7 randomly chosen per tank	only a 24 hour acclimation period was used
Protocol 10	4, 7, 14, 21, 32 days	non-spawning	unknown - 8 randomly chosen per tank	14 day acclimatization period used
Protocol 11	21 days	non-spawning	sexes kept separate	
Protocol 12	21 days	fish kept in gonadal quiescence until start of test	1:1	
Protocol 13	42 days	spawning	1:1	
Protocol 14	28 days	spawning	4:2	
Protocol 15	21 days	spawning	5:5	
Protocol 16	7 days	spawning?	4:2	

Methyltestosterone

	Lab 1 - protocol 5 (Ankley et al 2001)	Lab 1 - protocol 16 (Hornung et al 2004)	Lab 6 - protocol 12 (Pawlowski et al 2004a)
Core Diagnostic Criteria			
Males Testis-ova	-	NR	NR
Increased proportion of spermatogonia	-	NR	NR
Interstitial cell hypertrophy/hyperplasia	-	NR	NR
Increased testicular degeneration	+ (0.2 mg/L$^{#}$)	NR	NR
Females Increased oocyte atresia	+ (0.2 mg/L$^{#}$)	NR	+ (0.09 µg/L)
Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR
Decreased yolk formation	NR	NR	NR
Altered gonadal stage	+ (0.2 mg/L$^{#}$)	NR	-
Secondary Diagnostic Criteria			
Males Decreased proportion of spermatogonia	+ (0.2 mg/L$^{#}$)*	NR	NR
Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR
Asynchronous gonad development	NR	NR	NR
Altered gonadal staging	+ (0.2 mg/L$^{#}$)	NR	NR
Granulomatous inflammation	NR	NR	NR
Females Interstitial fibrosis	NR	NR	NR
Egg debris in the oviduct	NR	NR	NR
Granulomatous inflammation	NR	NR	NR
Decreased post-ovulatory follicles	+ (0.2 mg/L$^{#}$)	NR	NR
Vitellogenin			
Males	+ (0.2 mg/L$^{#}$)	+ (20 µg/L$^{#}$)	+ (1 µg/L$^{#}$)
Females	+ (0.2 mg/L$^{#}$)	+ (20 µg/L$^{#}$)	+ (1 µg/L$^{#}$)
Secondary sex characteristics			
Males	+ (0.2 mg/L$^{#}$)	-	-
Females	+ (0.2 mg/L$^{#}$)	+ (20 µg/L$^{#}$)	+ (1 µg/L$^{#}$)
Fecundity	+ (↓ 0.2 mg/L$^{#}$)	NR	+(↓ 48.2 µg/L)

NR = Not reported

Lab 1 - protocol 2 reported a significant decrease in spermatogenesis in treated males

*Report of hyperproduction of sperm - is this associated with decreased proportion of spermatogonia?

= nominal versus actual concentration

β–trenbolone

	Lab 1 - protocol 1 (Ankley et al 2003)	Lab 2 - protocol 1 (Battelle 2003a)	Lab 2 - protocol 3 (Battelle 2003a)	Lab 2 - protocol 6 (Battelle 2003a)
Core Diagnostic Criteria				
Testis-ova	-	-	-	-
Increased proportion of spermatogonia	-	-	-	-
Interstitial cell hypertrophy/hyperplasia	-	-	-	-
Increased testicular degeneration	-	NR	NR	NR
Increased oocyte atresia	+ (5 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	-
Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR	NR
Decreased yolk formation	+ (5 µg/L[#])	NR	NR	NR
Altered gonadal stage	+ (5 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	+ (0.071 µg/L)
Secondary Diagnostic Criteria				
Decreased proportion of spermatogonia	+ (5 µg/L[#])*	+ (0.60 µg/L)	-	-
Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR	NR
Asynchronous gonad development	NR	NR	NR	NR
Altered gonadal staging	+ (5 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	-
Granulomatous inflammation	NR	-	-	-
Interstitial fibrosis	NR	NR	NR	NR
Egg debris in the oviduct	NR	NR	NR	NR
Granulomatous inflammation	NR	-	-	-
Decreased post-ovulatory follicles	+ (5 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	NR
Vitellogenin				
Males	+ (50 µg/L[#])	-	-	+ (↓ 0.071 µg/L)
Females	+ (↓ 0.05 µg/L[#])	+ (↓ 0.60 µg/L)	+ (↓ 0.78 µg/L)	+ (↓ 0.071 µg/L)
Secondary sex characteristics				
Males	-	-	-	-
Females	+ (0.05 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	+ (0.86 µg/L)
Fecundity	+ (↓ 0.05 µg/L[#])	+ (↓ 0.60 µg/L)	+ (↓ 0.78 µg/L)	NA

Orange header row (Lab 2 – protocol 6) indicates 14-day non-spawning assay
NR = Not reported
Lab 1 - protocol 1 reported a significant increase in testis tubule lumen size with treated males
*Report of hyperproduction of sperm - is this associated with decreased proportion of spermatogonia?
= nominal versus actual concentration
Pink highlighting indicates that there is discrepancy between the published paper and the table

α-trenbolone

	Core Diagnostic Criteria	(Jensen et al 2006)
Males	Testis-ova	NR
	Increased proportion of spermatogonia	NR
	Interstitial cell hypertrophy/hyperplasia	NR
	Increased testicular degeneration	NR
Females	Increased oocyte atresia	NR
	Perifollicular cell hypertrophy/hyperplasia	NR
	Decreased yolk formation	NR
	Altered gonadal stage	NR
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	NR
	Asynchronous gonad development	NR
	Altered gonadal staging	NR
	Granulomatous inflammation	NR
Females	Interstitial fibrosis	NR
	Egg debris in the oviduct	NR
	Granulomatous inflammation	NR
	Decreased post-ovulatory follicles	NR
	Vitellogenin	
	Males	-
	Females	+ (↓ 0.032 µg/L)
	Secondary sex characteristics	
	Males	-
	Females	+ (0.032 µg/L)
	Fecundity	+ (0.0097 µg/L)

NR = not reported

p,p'-DDE

	Core Diagnostic Criteria	Lab 2 - Protocol 1 (Battelle 2004)
Males	Testis-ova	-
	Increased proportion of spermatogonia	-
	Interstitial cell hypertrophy/hyperplasia	-
	Increased testicular degeneration	NR
Females	Increased oocyte atresia	+ (0.022 µg/L)
	Perifollicular cell hypertrophy/hyperplasia	NR
	Decreased yolk formation	NR
	Altered gonadal stage	-
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
	Increased proteinaceous fluid within testicular vessels or interstitium	NR
	Asynchronous gonad development	NR
	Altered gonadal staging	-
	Granulomatous inflammation	-
Females	Interstitial fibrosis	NR
	Egg debris in the oviduct	NR
	Granulomatous inflammation	-
	Decreased post-ovulatory follicles	-
	Vitellogenin	
	Males	-
	Females	-
	Secondary sex characteristics	
	Males	-
	Females	-
	Fecundity	-

NR = not reported

Flutamide, part 1 (protocols 1 & 15) – continued on next page

		Lab 1 - protocol 1 (Jensen et al 2004)	Lab 2 - protocol 1 (Battelle 2003a)	Phase 1B studies Lab 9 - protocol 15 (OECD 2006)	Lab 14 - protocol 15 (OECD 2006)	Lab 13 - protocol 15 (OECD 2006)	Lab 16 - protocol 1 (Battelle 2006)	Lab 17 - protocol 1 (Battelle 2006)
Males	Testis-ova	NR	-	-	-	-	-	-
	Increased proportion of spermatogonia	-	-	-	+ (445 µg/L)	-	+ (388 µg/L)	-
	Interstitial cell hypertrophy/hyperplasia	NR	-	-	-	-	-	-
	Increased testicular degeneration	+ (50 µg/L#)*	NR	-	-	-	-	-
Females	Increased oocyte atresia	+ (500 µg/L#)	+ (510 µg/L)	-	-	+ (68.7 µg/L)	+ (822 µg/L)	-
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	-	-	-	-	-
	Decreased yolk formation	NR	NR	-	-	-	-	-
	Altered gonadal stage	+ (500 µg/L#)	-	-	-	-	-	-
	Secondary Diagnostic Criteria							
Males	Decreased proportion of spermatogonia	-	-	-	-	-	-	-
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	-	-	-	-	-
	Asynchronous gonad development	NR	NR	-	-	-	-	-
	Altered gonadal staging	-	-	-	-	-	+ (388 µg/L)	-
	Granulomatous inflammation	NR	-	-	-	-	-	-
Females	Interstitial fibrosis	NR	NR	-	-	-	-	-
	Egg debris in the oviduct	NR	NR	-	-	-	-	-
	Granulomatous inflammation	NR	-	-	-	-	-	-
	Decreased post-ovulatory follicles	NR	-	-	-	-	-	-
	Vitellogenin							
	Males	+ (500 µg/L#)	-	-	-	-	-	-
	Females	+ (500 µg/L#)	-	-	-	-	-	-
	Secondary sex characteristics							
	Males	-	-	+ (↓ 754 µg/L)	-	-	+ (↓ 388 µg/L)	+ (↓ 690 µg/L)
	Females	-	-	-	-	-	-	-
	Fecundity	+ (↓ 500 µg/L#)	+ (↓ 510 µg/L)	+ (↓ 754 µg/L)	+ (↓ 875 µg/L)	+ (↓ 940 µg/L)	+ (↓ 822 µg/L)	-

Flutamide, part 2 (protocols 3, 6, & 11) – continued from previous page

		Lab 2 - protocol 3 (Battelle 2003a)	Lab 2 - protocol 6 (Battelle 2003a)	Lab 5 - protocol 11 (Panter et al 2004)
Males	Testis-ova	-	-	NR
	Increased proportion of spermatogonia	-	-	NR
	Interstitial cell hypertrophy/hyperplasia	-	-	NR
	Increased testicular degeneration	NR	NR	NR
Females	Increased oocyte atresia	+ (519 µg/L)	-	NR
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR
	Decreased yolk formation	NR	NR	NR
	Altered gonadal stage	-	-	NR

Secondary Diagnostic Criteria

Males	Decreased proportion of spermatogonia	-	-	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR
	Asynchronous gonad development	NR	NR	NR
	Altered gonadal staging	-	-	NR
	Granulomatous inflammation	-	-	NR
Females	Interstitial fibrosis	NR	NR	NR
	Egg debris in the oviduct	NR	NR	NR
	Granulomatous inflammation	-	-	NR
	Decreased post-ovulatory follicles	+ (519 µg/L)	-	NR

Vitellogenin

Males	-	-	-
Females	-	+ (260 µg/L)	+**

Secondary sex characteristics

Males	-	-	+ (938.6 µg/L)
Females	-	-	-

Fecundity	+ (↓ 519 µg/L)	NA	NA

Orange headed indicates 14-day non-spawning assay
NR = Not reported
*Lab 1 - protocol 1 reported the increased presence of cells with pycnotic nuclei located in the tubule epithelium in flutamide-treated males (considered aborted spermatogonia or sperm precursor cells)
**Dilution water control showed a significant increase in female vitellogenin levels in addition to 95.3 µg/L test level
= nominal versus actual concentration
Purple highlighting indicates control groups did not spawn well

		Methoxychlor				
		Lab 1 - protocol 1 (Ankley et al 2001)	Lab 2 - protocol 1 (Battelle 2003a)	Lab 2 - protocol 3 (Battelle 2003a)	Lab 2 - protocol 6 (Battelle 2003a)	Lab 5 - protocol 10 (Panter et al 2002)
	Core Diagnostic Criteria					
Males	Testis-ova	-	-	-	-	NR
	Increased proportion of spermatogonia	-	-	-	-	NR
	Interstitial cell hypertrophy/hyperplasia	-	-	-	-	NR
	Increased testicular degeneration	-	NR	NR	NR	NR
Females	Increased oocyte atresia	+ (0.5 µg/L[#])	-	-	-	NR
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR	NR	NR
	Decreased yolk formation	NR	NR	NR	NR	NR
	Altered gonadal stage	NR	-	-	-	NR
	Secondary Diagnostic Criteria					
Males	Decreased proportion of spermatogonia	NR	-	-	-	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR	NR	NR
	Asynchronous gonad development	NR	NR	NR	NR	NR
	Altered gonadal staging	NR	-	-	-	NR
	Granulomatous inflammation	NR	-	-	-	NR
Females	Interstitial fibrosis	NR	NR	NR	NR	NR
	Egg debris in the oviduct	NR	NR	NR	NR	NR
	Granulomatous inflammation	NR	-	-	-	NR
	Decreased post-ovulatory follicles	NR	-	*	-	NR
	Vitellogenin					
	Males	+ (5 µg/L[#])	-**	-**	-	+ (0.16 µg/L)***
	Females	-	-	+ (↓ 0.79 µg/L)	-	NR
	Secondary sex characteristics					
	Males	-	-	-	-	NR
	Females	-	-	-	-	NR
	Fecundity	+ (↓ 5 µg/L[#])	+ (↓ 4.05 µg/L)	+ (↓ 3.98 µg/L)	NA	NA

Orange header (Lab 2, protocol 6) indicates 14-day non-spawning assay
NR = Not reported
NA = Not applicable
*Lab 2 - protocol 1 reported a significant difference in females with increased post-ovulatory follicles in high dose groups
** The statistical analysis of this result should be reviewed as it looks as if there is a large difference in vitellogenin concentrations between controls and high dose groups
*** Significance was achieved in after only four days of exposure to the test chemical, however, at 21 days, the dilution water control males also had significant changes in vitellogenin levels
# - nominal versus actual concentration

Vinclozolin

	Core Diagnostic Criteria	Lab 1 - protocol 2 (Makynen et al 2000)
Males	Testis-ova	NR
	Increased proportion of spermatogonia	-
	Interstitial cell hypertrophy/hyperplasia	NR
	Increased testicular degeneration	NR
Females	Increased oocyte atresia	-
	Perifollicular cell hypertrophy/hyperplasia	NR
	Decreased yolk formation	NR
	Altered gonadal stage	+ (1200 µg/L$^{\#}$)
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
	Increased proteinaceous fluid within testicular vessels or interstitium	NR
	Asynchronous gonad development	NR
	Altered gonadal staging	-
Females	Granulomatous inflammation	NR
	Interstitial fibrosis	NR
	Egg debris in the oviduct	NR
	Granulomatous inflammation	NR
	Decreased post-ovulatory follicles	NR
	Vitellogenin	
	Males	NR
	Females	NR
	Secondary sex characteristics	
	Males	NR
	Females	NR
	Fecundity	NR

NR = not reported
 # = nominal concentration

17β-estradiol

	Lab 9 - protocol 15 (OECD 2006)	Lab 10 - protocol 15 (OECD 2006)	Lab 11 - protocol 15 (OECD 2006)	Lab 4 - protocol 7 (Miles-Richardson et al 1999a)	Lab 4 - protocol 8 (Kramer et al 1998)	Lab 5 - protocol 9 (Panter et al 1998)
Core Diagnostic Criteria						
Testis-ova	-	-	-	NR	NR	NR
Increased proportion of spermatogonia	+ (90.6 ng/L)	+ (100 ng/L#)	+ (100 ng/L#)	NR	NR	NR
Interstitial cell hypertrophy/hyperplasia	-	-	-	+ (2 nM#)*	NR	NR
Increased testicular degeneration	-	+ (100 ng/L#)	-	+ (0.5 nM#)	NR	NR
Increased oocyte atresia	-	-	-	+ (2 nM#)**	NR	NR
Perifollicular cell hypertrophy/hyperplasia	-	-	-	NR	NR	NR
Decreased yolk formation	-	-	-	NR	NR	NR
Altered gonadal stage	-	-	+ (100 ng/L#)	+ (0.1 nM#)	NR	NR
Secondary Diagnostic Criteria						
Decreased proportion of spermatogonia	-	-	-	NR	NR	NR
Increased proteinaceous fluid within testicular vessels or interstitium	-	+ (100 ng/L#)	+ (100 ng/L#)	NR	NR	NR
Asynchronous gonad development	-	-	-	NR	NR	NR
Altered gonadal staging	-	-	+ (100 ng/L#)	NR	NR	NR
Granulomatous inflammation	-	-	-	NR	NR	NR
Interstitial fibrosis	-	-	-	NR	NR	NR
Egg debris in the oviduct	-	-	-	NR	NR	NR
Granulomatous inflammation	+ (90.6 ng/L)	-	-	NR	NR	NR
Decreased post-ovulatory follicles	-	-	-	NR	NR	NR
Vitellogenin						
Males	+ (90.6 ng/L)	+ (100 ng/L#)	NR	NR	+	+ (100 ng/L#)
Females	+ (90.6 ng/L)	+ (100 ng/L#)	NR	NR	+	NR
Secondary sex characteristics						
Males	+ (90.6 ng/L)	+ (100 ng/L#)	-	+ (100 nM#)***	NR	NR
Females	-	-	-	-	NR	NR
Fecundity	-	±& (100 ng/L#)	-	NR	+	NR

NR = Not reported
*Lab 4 - protocol 1 reported Sertoli cell hypertrophy/hyperplasia
**Lab 4 - protocol 1 reported increased oocyte atresia in comparison to controls in two of six treatment groups
***Lab 4 - protocol 1 reported significant atrophy of the nuptial tubercles in treated males
& = Difficult to determine if spawning status was affected
# = Nominal versus actual concentration
Labs 9 - 11 ran estradiol as a positive control for a prochloraz study, so there is only one concentration represented

4-*tert*-pentylphenol

Phase 1B studies

		Lab 9 - protocol 15 (OECD 2006)	Lab 10 - protocol 15 (OECD 2006)	Lab 11 - protocol 15 (OECD 2006)	Lab 5 - protocol 10 (Panter et al 2002)
	Core Diagnostic Criteria				
Males	Testis-ova	-	-	-	NR
	Increased proportion of spermatogonia	+ (862 µg/L)	+ (887 µg/L)	+ (820 µg/L)	NR
	Interstitial cell hypertrophy/hyperplasia	-	-	-	NR
	Increased testicular degeneration	+ (862 µg/L)	-	-	NR
Females	Increased oocyte atresia	-	-	-	NR
	Perifollicular cell hypertrophy/hyperplasia	-	-	-	NR
	Decreased yolk formation	-	-	-	NR
	Altered gonadal stage	+ (862 µg/L)	+ (887 µg/L)	-	NR
	Secondary Diagnostic Criteria				
Males	Decreased proportion of spermatogonia	-	-	-	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	+ (80.1 µg/L)	-	+ (820 µg/L)	NR
	Asynchronous gonad development	-	-	-	NR
	Altered gonadal staging	+ (862 µg/L)	-	-	NR
	Granulomatous inflammation	-	-	-	NR
Females	Interstitial fibrosis	-	-	-	NR
	Egg debris in the oviduct	-	-	-	NR
	Granulomatous inflammation	-	+ (887 µg/L)	-	NR
	Decreased post-ovulatory follicles	-	-	-	NR
	Vitellogenin				
	Males	+ (270 µg/L)	+ (298 µg/L)	NR	+ (10 µg/L)
	Females	+ (862 µg/L)	-	NR	NR
	Secondary sex characteristics				
	Males	+ (↓ 862 µg/L)	+ (↓ 887 µg/L)	+ (↓ 820 µg/L)	NR
	Females	-	-	-	NR
	Fecundity	+ (↓ 862 µg/L)	+ (↓ 887 µg/L)	+ (↓ 820 µg/L)	NR

NR = not reported

Purple highlighting indicates that control groups did not spawn well

Bisphenol A

	Core Diagnostic Criteria	Lab 2 - Protocol 1 (Battelle 2004)	Lab 7 - protocol 13 (Sohoni et al 2001)
Males	Testis-ova	-	-
Males	Increased proportion of spermatogonia	-	+*
Males	Interstitial cell hypertrophy/hyperplasia	NR	-
Males	Increased testicular degeneration	NR	-
Females	Increased oocyte atresia	-	-
Females	Perifollicular cell hypertrophy/hyperplasia	NR	-
Females	Decreased yolk formation	NR	-
Females	Altered gonadal stage	-	-
	Secondary Diagnostic Criteria		
Males	Decreased proportion of spermatogonia	-	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR	-
Males	Asynchronous gonad development	NR	-
Males	Altered gonadal staging	-	-
Males	Granulomatous inflammation	-	-
Females	Interstitial fibrosis	NR	-
Females	Egg debris in the oviduct	NR	-
Females	Granulomatous inflammation	+ (56.6 µg/L)	-
Females	Decreased post-ovulatory follicles	-	-
	Vitellogenin		
	Males	+ (56.6 µg/L)	+ (640 µg/L[#])
	Females	+ (344.1 µg/L)	-
	Secondary sex characteristics		
	Males	-	NR
	Females	-	NR
	Fecundity	+ (344.1 µg/L)	NR

NR = not reported

Lab 2 - protocol 2 reported significant differences in stage 1A (increase) and stage 3 (decrease) oocytes

*It is difficult to tell when this change was significant, it may be after 164 days rather than only 42. If this is the case, then it should be considered NR for the purposes of this table.

= nominal concentration

Fadrozole, part 1 (protocols 1 & 15) – continued on next page

Core Diagnostic Criteria		Lab 1 - protocol 1 (Ankley et al 2002)	Lab 2 - protocol 1 (Battelle 2003a)	Lab 10 - protocol 15 (OECD 2006)	Lab 11 - protocol 15 (OECD 2006)	Lab 12 - protocol 15 (OECD 2006)	Lab 13 - protocol 15 (OECD 2006)
Males	Testis-ova	NR	-	-	-	-	-
	Increased proportion of spermatogonia	NR	-	-	-	-	+ (104 µg/L)
	Interstitial cell hypertrophy/hyperplasia	NR	+ (55.7 µg/L)	-	+ (93 µg/L)	+ (97.1 µg/L)	+ (104 µg/L)
	Increased testicular degeneration	NR	NR	-	-	-	-
Females	Increased oocyte atresia	+ (5 µg/L[#])	+ (5.2 µg/L)	+ (100 µg/L[#])	+ (93 µg/L)	+ (97.1 µg/L)	+ (104 µg/L)
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	-	-	-	-
	Decreased yolk formation	NR	NR	-	+ (93 µg/L)	-	+ (104 µg/L)
	Altered gonadal stage	+ (5 µg/L[#])	+ (55.7 µg/L)	+ (100 µg/L[#])	+ (93 µg/L)	+ (97.1 µg/L)	+ (104 µg/L)
Secondary Diagnostic Criteria							
Males	Decreased proportion of spermatogonia	NR	-	-	-	-	-
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	-	-	-	-
	Asynchronous gonad development	NR	NR	-	-	-	-
	Altered gonadal staging	+	-	+ (100 µg/L[#])	-	-	-
	Granulomatous inflammation	NR	-	-	-	-	-
	Interstitial fibrosis	NR	NR	-	-	-	-
Females	Egg debris in the oviduct	NR	NR	+ (100 µg/L[#])	-	-	-
	Granulomatous inflammation	NR	-	-	-	-	-
	Decreased post-ovulatory follicles	+ (5 µg/L[#])	+ (55.7 µg/L)	-	-	-	-
Vitellogenin							
Males		-	-	-	NR	-	-
Females		+ (↓ 2 µg/L[#])	+ (↓ 5.2 µg/L)	+ (↓ 100 µg/L[#])	NR	-	+ (↓ 104 µg/L)
Secondary sex characteristics							
Males		-	-	-	-	-	-
Females		-	-	-	-	-	-
Fecundity		+ (↓ 10 µg/L[#])	+ (↓ 55.7 µg/L)	-	NR	-	-

Orange header (Lab 2, protocol 6) indicates 14-day non-spawning assay
NR = Not reported
NA = Not applicable
Lab 1 - protocol 1 reported a significant increase in mean tubule diameter in treated males
Lab 2 - protocol 2 reported a significant increase in mean tubule diameter in treated males
= nominal versus actual concentration
Labs 10 - 13 ran fadrozole as a positive control for a prochloraz study, so there is only one concentration represented

Fadrozole, part 2 (protocols 3, 6, & 11) – continued on next page

Core Diagnostic Criteria		Lab 2 - protocol 3 (Battelle 2003a)	Lab 2 - protocol 6 (Battelle 2003a)	Lab 5 - protocol 11 (Panter et al 2004)
Males	Testis-ova	-	-	NR
	Increased proportion of spermatogonia	-	-	NR
	Interstitial cell hypertrophy/hyperplasia	+ (59.9 µg/L)	+ (57.0 µg/L)	NR
	Increased testicular degeneration	NR	NR	NR
Females	Increased oocyte atresia	+ (59.9 µg/L)	+ (57.0 µg/L)	NR
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR
	Decreased yolk formation	NR	NR	NR
	Altered gonadal stage	+ (59.9 µg/L)	-	NR

Secondary Diagnostic Criteria				
Males	Decreased proportion of spermatogonia	-	-	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR
	Asynchronous gonad development	NR	NR	NR
	Altered gonadal staging	-	-	NR
	Granulomatous inflammation	-	-	NR
Females	Interstitial fibrosis	NR	NR	NR
	Egg debris in the oviduct	NR	NR	NR
	Granulomatous inflammation	-	-	NR
	Decreased post-ovulatory follicles	+ (5.5 µg/L)	-	NR

Vitellogenin			
Males	-	-	+ (24.8 µg/L)
Females	+ (↓ 5.5 µg/L)	+ (↓ 31.7 µg/L)	+(24.8 µg/L)

Secondary sex characteristics			
Males	-	-	+ (24.8 µg/L)*
Females	-	-	-

Fecundity			
	+ (↓ 59.9 µg/L)	NA	NR

Orange header (Lab 2, protocol 6) indicates 14-day non-spawning assay
NR = Not reported
NA = Not applicable
Lab 1 - protocol 1 reported a significant increase in mean tubule diameter in treated males
Lab 2 - protocol 2 reported a significant increase in mean tubule diameter in treated males
*Dose response not achieved as there was no significant difference at fadrozole levels above 24.8 µg/L.
= nominal versus actual concentration
Labs 10 - 13 ran fadrozole as a positive control for a prochloraz study, so there is only one concentration represented

Fenarimol

		Lab 1 - protocol 2 (Ankley et al 2005a)
	Core Diagnostic Criteria	
Males	Testis-ova	NR
	Increased proportion of spermatogonia	+
	Interstitial cell hypertrophy/hyperplasia	NR
	Increased testicular degeneration	NR
Females	Increased oocyte atresia	+
	Perifollicular cell hypertrophy/hyperplasia	NR
	Decreased yolk formation	NR
	Altered gonadal stage	-
	Secondary Diagnostic Criteria	
	Decreased proportion of spermatogonia	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR
	Asynchronous gonad development	NR
	Altered gonadal staging	NR
	Granulomatous inflammation	NR
Females	Interstitial fibrosis	NR
	Egg debris in the oviduct	NR
	Granulomatous inflammation	NR
	Decreased post-ovulatory follicles	NR
	Vitellogenin	
	Males	-
	Females	+
	Secondary sex characteristics	
	Males	NR
	Females	NR
	Fecundity	+

NR = not reported

Ketoconazole

Core Diagnostic Criteria		Lab 1 - protocol 1 (Ankley and Villeneuve 2006)	Lab 17 - protocol 1 (Battelle 2006)
Males	Testis-ova	-	-
	Increased proportion of spermatogonia	-	-
	Interstitial cell hypertrophy/hyperplasia	+	+ (18 µg/L)
	Increased testicular degeneration	-	-
Females	Increased oocyte atresia	-	-
	Perifollicular cell hypertrophy/hyperplasia	-	-
	Decreased yolk formation	-	-
	Altered gonadal stage	-	-
Secondary Diagnostic Criteria			
Males	Decreased proportion of spermatogonia	-	-
	Increased proteinaceous fluid within testicular vessels or interstitium	-	-
	Asynchronous gonad development	-	-
	Altered gonadal staging	-	-
	Granulomatous inflammation	-	-
	Interstitial fibrosis	-	-
Females	Egg debris in the oviduct	-	-
	Granulomatous inflammation	-	-
	Decreased post-ovulatory follicles	-	-
Vitellogenin			
	Males	-	+ (320 µg/L)
	Females	NR	-
Secondary sex characteristics			
	Males	NR	-
	Females	NR	-
Fecundity		+	-

NR = not reported

		Prochloraz				Lab 1 - protocol 2 (Ankley et al 2005a)
	Core Diagnostic Criteria	Lab 10 - protocol 15 (OECD 2006)	Lab 11 - protocol 15 (OECD 2006)	Lab 12 - protocol 15 (OECD 2006)	Lab 13 - protocol 15 (OECD 2006)	
Males	Testis-ova	-	-	-	-	NR
	Increased proportion of spermatogonia	-	-	-	+ (382 µg/L)	+
	Interstitial cell hypertrophy/hyperplasia	-	-	-	-	NR
	Increased testicular degeneration	-	-	-	-	NR
Females	Increased oocyte atresia	+ (299 µg/L)	+ (341 µg/L)	+ (275 µg/L)	+ (382 µg/L)	+
	Perifollicular cell hypertrophy/hyperplasia	-	-	-	-	NR
	Decreased yolk formation	-	-	-	-	NR
	Altered gonadal stage	-	+ (341 µg/L)	-	-	-
	Secondary Diagnostic Criteria					
Males	Decreased proportion of spermatogonia	-	-	-	-	-
	Increased proteinaceous fluid within testicular vessels or interstitium	+ (299 µg/L)	-	-	-	NR
	Asynchronous gonad development	-	-	-	-	NR
	Altered gonadal staging	-	-	-	-	+
	Granulomatous inflammation	-	-	-	-	NR
Females	Interstitial fibrosis	-	-	-	-	NR
	Egg debris in the oviduct	+ (97.5 µg/L)	-	-	-	NR
	Granulomatous inflammation	-	-	-	-	NR
	Decreased post-ovulatory follicles	-	-	-	-	NR
	Vitellogenin					
	Males	-	-	-	-	-
	Females	+ (↓ 299 µg/L)	NR	+ (↓ 275 µg/L)	+ (↓ 121 µg/L)	+
	Secondary sex characteristics					
	Males	-	-	-	-	NR
	Females	-	-	-	-	NR
	Fecundity	-	+ (↓ 341 µg/L)	-	-	+

NR = not reported

Purple highlighting indicates that there was poor spawning in the controls.

Cadmium chloride

		Lab 2 - Protocol 1 (Battelle 2004)
	Core Diagnostic Criteria	
Males	Testis-ova	-
Males	Increased proportion of spermatogonia	-
Males	Interstitial cell hypertrophy/hyperplasia	-
Males	Increased testicular degeneration	NR
Females	Increased oocyte atresia	-
Females	Perifollicular cell hypertrophy/hyperplasia	NR
Females	Decreased yolk formation	NR
Females	Altered gonadal stage	-
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR
Males	Asynchronous gonad development	NR
Males	Altered gonadal staging	-
Males	Granulomatous inflammation	NR
Females	Interstitial fibrosis	NR
Females	Egg debris in the oviduct	NR
Females	Granulomatous inflammation	NR
Females	Decreased post-ovulatory follicles	-
	Vitellogenin	
	Males	-
	Females	-
	Secondary sex characteristics	
	Males	-
	Females	-
	Fecundity	-

NR = not reported

Atrazine

	Core Diagnostic Criteria	Lab 2 - protocol 1 (Battelle 2004)	Lab 3 - protocol 1 (Bringolf et al 2004)
Males	Testis-ova	-	-
	Increased proportion of spermatogonia	+ (2A) (24.5 µg/L)	NR
	Interstitial cell hypertrophy/hyperplasia	-	NR
	Increased testicular degeneration	NR	NR
Females	Increased oocyte atresia	-	NR
	Perifollicular cell hypertrophy/hyperplasia	NR	NR
	Decreased yolk formation	NR	NR
	Altered gonadal stage	-	-
	Secondary Diagnostic Criteria		
Males	Decreased proportion of spermatogonia	+ (2B) (24.5 µg/L)	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR
	Asynchronous gonad development	NR	NR
	Altered gonadal staging	-	NR
	Granulomatous inflammation	NR	NR
Females	Interstitial fibrosis	NR	NR
	Egg debris in the oviduct	NR	NR
	Granulomatous inflammation	NR	NR
	Decreased post-ovulatory follicles	-	NR
	Vitellogenin		
	Males	-	-
	Females	-	-
	Secondary sex characteristics		
	Males	-	-
	Females	-	-
	Fecundity	-	-

NR = not reported

Lab 2 - protocol 2 reported a significant decrease in testis tubule diameter in treated fish

PFOS

	Core Diagnostic Criteria	Lab 1 - protocol 4 (Ankley et al 2005b)	Lab 8 - protocol 14 (Oakes et al 2005)
Males	Testis-ova	NR	NR
	Increased proportion of spermatogonia	-	NR
	Interstitial cell hypertrophy/hyperplasia	NR	NR
	Increased testicular degeneration	NR	NR
Females	Increased oocyte atresia	+	NR
	Perifollicular cell hypertrophy/hyperplasia	NR	NR
	Decreased yolk formation	NR	NR
	Altered gonadal stage	+	NR
	Secondary Diagnostic Criteria		
Males	Decreased proportion of spermatogonia	-	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR
	Asynchronous gonad development	-	NR
	Altered gonadal staging	-	NR
	Granulomatous inflammation	NR	NR
Females	Interstitial fibrosis	NR	NR
	Egg debris in the oviduct	NR	NR
	Granulomatous inflammation	NR	NR
	Decreased post-ovulatory follicles	+	NR
	Vitellogenin		
	Males	-	NR
	Females	-	NR
	Secondary sex characteristics		
	Males	-	NR
	Females	-	NR
	Fecundity	+	-*

NR = not reported

*Lab 8 - protocol 1 reported trends of effects of fecundity, though they were not considered statistically significant

Prometon

	Core Diagnostic Criteria	Lab 1 - protocol 2 (Ankley and Villeneuve 2006)
Males	Testis-ova	-
	Increased proportion of spermatogonia	-
	Interstitial cell hypertrophy/hyperplasia	-
	Increased testicular degeneration	-
Females	Increased oocyte atresia	+
	Perifollicular cell hypertrophy/hyperplasia	-
	Decreased yolk formation	-
	Altered gonadal stage	-
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
	Increased proteinaceous fluid within testicular vessels or interstitium	-
	Asynchronous gonad development	-
	Altered gonadal staging	-
	Granulomatous inflammation	-
Females	Interstitial fibrosis	-
	Egg debris in the oviduct	-
	Granulomatous inflammation	-
	Decreased post-ovulatory follicles	+
	Vitellogenin	
	Males	-
	Females	-
	Secondary sex characteristics	
	Males	+
	Females	-
	Fecundity	-

NR = not reported

Potassium permanganate

	Core Diagnostic Criteria	Lab 15 - protocol 1 (Battelle 2006)	Lab 17 - protocol 1 (Battelle 2006)
Males	Testis-ova	-	-
	Increased proportion of spermatogonia	+ (389 µg/L)	+ (680 µg/L)
	Interstitial cell hypertrophy/hyperplasia	-	-
	Increased testicular degeneration	+ (389 µg/L)	-
Females	Increased oocyte atresia	+ (184 µg/L)	-
	Perifollicular cell hypertrophy/hyperplasia	-	-
	Decreased yolk formation	-	-
	Altered gonadal stage	+ (389 µg/L)	-
	Secondary Diagnostic Criteria		
Males	Decreased proportion of spermatogonia	-	-
	Increased proteinaceous fluid within testicular vessels or interstitium	-	-
	Asynchronous gonad development	-	-
	Altered gonadal staging	+ (389 µg/L)	-
	Granulomatous inflammation	-	-
Females	Interstitial fibrosis	-	-
	Egg debris in the oviduct	-	-
	Granulomatous inflammation	-	-
	Decreased post-ovulatory follicles	-	-
	Vitellogenin		
	Males	-	-
	Females	+ (↓ 389 µg/L)	-
	Secondary sex characteristics		
	Males	+ (850 µg/L)	-
	Females	-	-
	Fecundity	+ (↓ 389 µg/L)	-

NR = not reported

Perchlorate

	Core Diagnostic Criteria	Lab 2 - Protocol 1 (Battelle 2006)
Males	Testis-ova	-
	Increased proportion of spermatogonia	-
	Interstitial cell hypertrophy/hyperplasia	-
	Increased testicular degeneration	NR
Females	Increased oocyte atresia	+ (5638 µg/L)
	Perifollicular cell hypertrophy/hyperplasia	NR
	Decreased yolk formation	NR
	Altered gonadal stage	-*
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
	Increased proteinaceous fluid within testicular vessels or interstitium	NR
	Asynchronous gonad development	NR
	Altered gonadal staging	-
	Granulomatous inflammation	-
Females	Interstitial fibrosis	NR
	Egg debris in the oviduct	NR
	Granulomatous inflammation	NR
	Decreased post-ovulatory follicles	-
	Vitellogenin	
	Males	-
	Females	-
	Secondary sex characteristics	
	Males	-
	Females	-
	Fecundity	-

NR = not reported
*Lab 2 - protocol 2 reported significant decreases in the proportion of stage 1A oocytes

Addendum 2

FISH SCREENING ASSAY
TEST PROTOCOL – Fathead Minnow