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REPORT OF THE VALIDATION OF THE 21-DAY FISH SCREENING ASSAY FOR THE DETECTION OF ENDOCRINE SUBSTANCES (PHASE 1B)

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No. 61

**REPORT OF THE VALIDATION OF THE 21-DAY
FISH SCREENING ASSAY FOR THE DETECTION OF
ENDOCRINE ACTIVE SUBSTANCES**

(PHASE 1B)

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

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**OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France**

**Fax: (33-1) 44 30 61 80
E-mail: ehscont@oecd.org**

FOREWORD

This document is the report of the OECD Phase 1B study to validate the 21-day Fish Screening Assay for the detection of some endocrine active substances. The Phase 1B enabled to investigate the capacity of the assay for the detection of weak estrogen, aromatase inihitors and anti-androgen, which represent the key modes of action of interest, beyond those already considered in Phase 1A. The protocol was amended for Phase 1B to improve the biological relevance of the assay. Fourteen laboratories participated; they selected one of the three fish species and conducted the test with generally two test substances. A total of thirty one experiments were successfully performed and reported here. The test substances were distributed from a central chemical repository, situated at the United States Environmental Protection Agency.

The proposed studies, the model protocol, the test substances and the concentrations were agreed by the Fish Drafting Group and approved by the Validation Management Group for Ecotoxicity Testing (VMG-eco). Each of the fourteen laboratories submitted the raw data to a technical lead laboratory for the fish species used. The technical lead laboratory centralised data for the same species and transmitted the data files to the lead laboratory for analysis and to the OECD Secretariat for archiving. Dr. Masanori Seki from the lead laboratory (CERI, Japan) prepared the initial draft of the report. Comments and input to the draft report were also contributed from the participating laboratories and from members of the VMG.

The VMG approved the draft report in December 2005. The Task Force on Endocrine Disrupters Testing and Assessment (EDTA) then endorsed the Phase 1A report, together with the Phase 1B report which constitutes a separate document, in January 2006. At its 18th Meeting, the Working Group of the National Coordinators of the Test Guidelines Programme agreed to the submission of the report to the Joint Meeting with a view to its declassification. This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

Contact for further details

Environment, Health and Safety Division
Environment Directorate
Organisation for Economic Cooperation and Development
2, rue André-Pascal
75775 Paris Cédex 16, France

Tel : +33 1 45 24 1674 or 9843
Fax : +33 1 45 24 1675
Email : env.edcontact@oecd.org

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ACKNOWLEDGEMENTS

SUMMARY

i)   This report provides the results from an OECD inter-laboratory study conducted in 2004 to examine the relevance and reproducibility of a standardized OECD protocol for the 21-day fish assay. The work carried out under Phase 1B follows initial work conducted in Phase 1A where the protocol transferability had been evaluated. The Phase 1B study was primarily aimed at experimentally establishing the relevance of the assay for the detection of weakly active substances acting as estrogen, anti-androgen and aromatase inhibitors, when conducted with one of three generally used fish species. The second objective of Phase 1B was to check the reproducibility of the assay by comparing test results obtained in a variety of laboratories in geographical diverse locations, using the same fish species, the same test substances, the same measurement method for vitellogenin and the same procedures for gonadal histology. A combination of three fish species and three test substances were repeatedly used throughout fourteen laboratories to allow a sufficient number of repeats (3 in average per species and chemical) for inter-laboratory comparison of test results.

ii)   The protocol for the 21-day fish assay for the detection of endocrine active substances used in this phase differed from that used in Phase 1A and can be summarised by the following description: reproductively active male and female fish were housed in groups of 5 males and 5 females and exposed to test chemical for 21 days. Three core endpoints as indicators of endocrine disrupter activity were measured, namely: i) gross morphology (i.e., secondary sexual characteristics), ii) vitellogenin (VTG) levels, and iii) gonadal histology. Additionally the spawning status was checked daily in all groups, and quantified in some. Three fish species, i.e., fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) were used as test fish. Test chemicals were 4-*tert*-pentylphenol (100, 320 and 1000 µg/l) as a weak estrogen, flutamide (100, 500, 1000 µg/l) as an anti-androgen, and prochloraz (20, 100, 300 µg/l) as an aromatase inhibitor; 17β-estradiol (100 ng/l) and fadrozole (100 µg/l) were used as positive controls.

iii)   Fourteen laboratories in 7 countries participated in this Phase 1B of the validation work. Participation was on a voluntary basis and laboratories committed their own resources and time in this work. A total of 31 studies were planned to ensure a minimum of three repetitions per fish species per substance. On one occasion, an invalid study reduced the number of repetitions to 2 (fathead minnow, 4*tert*-pentylphenol).

iv)   Measured concentrations of the test substances and positive controls in the 31 studies showed that 19 studies remained within the range 80%-120% of nominal values; one study was above 120% nominal concentration; 11 studies were below 80% nominal values. One study (not included in the 31 studies) using the anti-androgen flutamide with fathead minnow was invalid because of high mortality in the control group. Test results from that study are not presented in this report.

v)   An extensive draft guidance document on gonadal histotechniques and histopathology was developed and available to the participating laboratories. Following Phase 1B, pathologists reviewed results and agreed to streamline histological evaluation for a future standard test by reducing exposure-related diagnoses to a minimum of four per male and four per female.

vi)   In Phase 1B, 4-*tert*-pentylphenol exposure induced dose-dependent VTG synthesis in males of three fish species, indicating that this protocol can detect effects of a weak estrogen. Prochloraz exposure decreased VTG levels dose-dependently in females of three fish species simultaneous with spawning cessation. These findings suggest that these responses follow a cascade triggered by aromatase inhibition. Consistency and reproducibility across studies and species was generally good for vitellogenin measurements. Concerning the anti-androgen, whereas the applicability of secondary sex characteristics

and vitellogenin measurement might be limited in detecting a treatment-related response, histological changes in the gonads, especially in zebrafish were needed for detecting this mechanism of action. However, issues of consistency and reproducibility of the reported histopathological findings across laboratories and fish species arose and made it difficult to affirm, both at this stage and on the basis of data collected in Phase 1B, on the readiness of the endpoint for regulatory acceptance and use in an assay aiming at the detection of endocrine active substances.

vii)   Phase 1B demonstrated that there is a good reproducibility across laboratories for both vitellogenin measurements and secondary sex characteristics (not applicable for zebrafish) when the protocol was followed, reproductively active fish were used and test concentrations were kept close to nominal values,. These two endpoints enable the detection of estrogenic substances, aromatase inhibitors and a potent androgenic substance. Phase 1B also showed that the sex ratio 5:5 is sub-optimal for the fathead minnow species; the crowding creates territoriality among males, thus disturbing normal spawning behaviour in the group.

viii)   Following the outcome of Phase 1B, it was recommended by the Validation Management Group to further evaluate the 21-day fish screening assay using putative negative substances, in order to gauge the potential for false positive results. The outcome of the negative testing, Phase 2, will be reported in a separate document.

1. INTRODUCTION

1. The need to develop and validate a fish assay capable of detecting endocrine active substances originates from the concerns that environmental levels of chemicals may be causing adverse effects in both humans and wildlife due to the interaction of these chemicals with the endocrine system. Several cases were reported where the exposures of exogenous chemicals have resulted in effects in wildlife, and in particular in fish (1)(2)(3). In 1997, OECD member countries advised that existing test methods were insufficient to identify such substances and characterise their effects. As part of the OECD Test Guidelines Programme a *Special Activity on the Testing and Assessment of Endocrine Disrupters* was initiated to revise existing, and develop new OECD Test Guidelines for the screening and testing of potential endocrine disrupters. A Task Force on Endocrine Disrupters Testing and Assessment (EDTA) was subsequently established to provide a focal point within OECD to consider and recommend priorities for the development of testing methods for endocrine disrupters.

2. Two Fish Expert Consultations were organised, in London in 1998 and in Tokyo in 2000, to review the state-of-the-knowledge and science in the area of fish screening and fish testing in relation to endocrine disrupters. The outcome of these Expert Consultations was a recommendation on three promising core endpoints that should, at a minimum, be part of the future fish screening assay. These endpoints were the vitellogenin level, the gross morphology (including secondary sex characteristics and the gonado-somatic index) and gonad histology. In 2001, during the First Meeting of the Validation Management Group for Ecotoxicity Testing (VMG-eco) proposals for candidate protocols were made. On that basis, a Fish Drafting Group was established to agree a common OECD protocol and to prepare a proposal to the VMG-eco for validation of the test method. One constraint for the protocol was the need to check its applicability to the three fish species commonly used in OECD countries for regulatory testing: fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). Following an agreement on use of a phased approach to validation, a protocol for Phase 1A was selected and aimed at verifying the feasibility of the assay in general from one laboratory to another. The Phase 1B protocol was altered based on the Phase 1A to evaluate the relevance and reproducibility of the assay and is the subject of this report.

3. The fish screening assay fits into the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupters, discussed and agreed at the sixth Meeting of the EDTA Task Force. This framework identifies approaches, assays and long-term tests of increasing biological complexity, meant to gather information on potential endocrine disrupters. Each of the tools added to the framework will require validation to ensure its relevance and reliability, the two main validation principles. The OECD Guidance Document 34 on Validation and Acceptance of New and Updated test methods for Hazard Assessment provides definitions, principles and concrete examples of validation, applied in different areas of hazard assessment.

4. The 21-day fish assay, evaluated in Phase 1A and Phase 1B validation trials, is intended for the detection of individual chemicals acting as estrogens, androgens, aromatase inhibitors or anti-androgens. Reproductively active adults males and females are exposed together, but then analyzed separately. The assay is based on the principle that certain sex characteristics and proteins are under the control of endogenous hormones, and that exposure to exogenous substances in the water can induce these characteristics in animals of the opposite sex to which they are normally observed, or reduce their normal occurrence in individuals of the sex in which they are usually observed. The aim of the validation was to develop a robust, relevant and reliable test method for the detection of chemicals mentioned above. It is also the purpose of the validation to understand and define the area of application of the assay and any limitation to its use.

Scientific rationale for the endpoints

Vitellogenin

5.   Vitellogenin is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active females of all oviparous species; the production of vitellogenin is controlled by interaction of estrogens, predominantly 17β estradiol with the estrogen receptor (4). Significantly, males maintain the capacity to produce vitellogenin in response to stimulation with estrogen receptor agonists; as such, induction of vitellogenin in males has been successfully exploited as a biomarker specific for estrogenic compounds in a variety of fish species, including the fathead minnow, the medaka and the zebrafish. A number of vitellogenin measurement methods have been developed and standardised for each of these species (5)(6)(7)(8); criteria for selecting methods in this validation work are described further in the report.

Secondary sex characteristics

6.   Nuptial tubercles in fathead minnow and papillary processes in medaka are among the sexual characteristics observed under normal conditions in males. Chemicals with certain endocrine-mediated action will cause the abnormal occurrence of these secondary sex characteristics in the opposite sex. For example, androgen receptor agonists will induce formation of nuptial tubercles in female fathead minnow (9), and formation of papillary processes in female medaka (10). No secondary sex characteristics are available for the zebrafish.

Gonad histopathology

7.   There is interest in developing and applying toxicological pathology for lower vertebrates, such as fish, particularly in the case of endocrine active substances. Examination of gonadal histopathology has been beneficial in understanding and assessing the effects of endocrine active substances in fish (11)(12) because histopathological changes are the result of the integration of a large number of interactive physiological processes. However, it is recognised that there is inherent subjectivity in histopathological evaluation, and some difficulty in having clearly defined profiles associated with each mode of action and consistency across species. Efforts are being made to reduce the bias associated with individual pathologists, to streamline histotechniques, to identify exposure-related diagnoses and to harmonise the reporting of observed changes. These aspects are critical for the regulatory acceptance and use of histopathology. The diagnostic value of the endpoint, the amount of effort necessary and the time required for a reliable evaluation will, together, determine the role of gonadal histopathology in a screening assay for endocrine active substances. This endpoint represents a major challenge in the validation effort.

2. OUTCOME OF PHASE 1A

8.   Following discussions in 2002 on the protocol for the Fish Screening Assay for the detection of endocrine active substances, and out of concern for resource optimization and animal welfare, Phase 1 was split into Phase 1A and Phase 1B.

9.   Phase 1A was indeed considered to be a feasibility study; adult fish of both sexes were exposed to two known chemicals (an estrogen and an androgen) during 21 days. Males and females were kept separated by a mesh barrier in one tank to prevent spawning. Three core endpoints, recommended by two Expert Consultations and approved by the Validation Management Group for Ecotoxicity Testing (VMG-eco) were measured in the assay: vitellogenin (VTG) level, gross morphology (including secondary sex characteristics and gonado-somatic index (GSI)) and gonad histology. Measurements were analyzed separately for males and females. This first step was limited and conducted in four laboratories, each using at least two of the three possible fish species (fathead minnow, medaka and zebrafish) to ensure that repeats could be compared across laboratories. Each laboratory used the two relatively strong test substances: 17-β estradiol (estrogen) and 17-β trenbolone (androgen) with each fish species. Males and females fish were analyzed separately. A total of 20 tests were performed in 2002.

10.   The Fish Drafting Group of the Validation Management Group for Ecotoxicity Testing met in October 2003 to discuss the outcome of Phase 1A and to plan Phase 1B.

11.   It was concluded from Phase 1A studies that the fish screening assay was reliable for the detection of the strong estrogenic compound 17-β estradiol and the strong androgenic compound 17-β trenbolone in all three species, showing the potential relevance of this assay. Analysis of male VTG was valid for strong estrogen and measurement of female VTG was sensitive to androgenic exposure across all three species. In addition, secondary sex characteristics in two species, medaka and fathead minnow were responsive to the androgen. The GSI poorly responded to both 17-β estradiol and 17-β trenbolone, and variability among responses was high. Concerning the evaluation of gonadal histology, the outcome of Phase 1A showed that, despite exposure-related responses, female fish showed pathological symptoms (e.g. oocyte atresia) attributable to their non-spawning situation. Therefore, to improve the fitness of fish and the biological relevance of the assay, fish experts recommended holding males and females in the same test chamber, thus allowing fish to spawn. A detailed guidance document was prepared after Phase 1A for the harmonized evaluation of the histopathology. A revised draft Report of Phase 1A was made available for comments and approval of the VMG-eco in December 2003.

12.   An action plan including detailed standard operating procedures, test substances and concentrations, and timelines for the work was submitted to the VMG-eco for approval at the end of 2003. After agreement of the VMG-eco on the plan for Phase 1B, laboratories performed experimental work from March until September 2004. Fourteen laboratories from 7 countries and industry (Denmark, Germany, Japan, the Netherlands, Switzerland, the United Kingdom and the United States) took part in Phase 1B.

3. OBJECTIVES OF PHASE 1B

13.   The objective of Phase 1B was primarily to experimentally establish the relevance of the assay for the detection of weakly active substances acting as estrogen, anti-androgen and aromatase inhibitor, when conducted in a choice of three generally used fish species. The second objective of Phase 1B was to check the reproducibility of the assay by comparing test results obtained by a variety of laboratories in diverse geographic locations. Here is a summary of key goal of Phase 1B:

- Obtain additional information on the relevance of assay endpoints and in particular their ability to respond weakly active substances with diverse modes of action in the context of this assay.

- Collect a set of data for three weakly active substances;

- Obtain additional information on possible differences in species sensitivity to a weak estrogen, a mammalian anti-androgen weakly active in fish, and a weak aromatase inhibitor;

- Check that the protocol contains enough details to enable laboratories to conduct the assay in a reproducible manner;

- Check the reproducibility of test results in laboratories located in diverse geographical areas, and with diverse levels of experience in conducting this type of assay.

14.   The OECD Guidance Document 34 on the Validation and International Acceptance of New and Updated Test Methods for Hazard Assessment (64) provides the following important definitions:

> *Relevance:* Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method.

> *Reliability*: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability.

> *Repeatability*: The agreement among test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions. (see Reliability)

> *Reproducibility*: The agreement among results obtained from testing the same substance using the same test protocol. (see reliability)

> *Robust(ness):* The insensitivity of test results to departures from the specified test conditions when conducted in different laboratories or over a range of conditions under which the test method might normally be used. If a test is not robust, it will be difficult to use in a reproducible manner within and between laboratories.

> *Transferability*: The ability of a test procedure to be accurately and reliably performed in independent, competent laboratories.

> *Validation*: The process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose.

15.   These concepts represents components of the validation. As regards the mechanistic relevance of biomarkers used in the 21-day Fish Screening Assay, i.e. vitellogenin, secondary sex characteristics, and to a certain extent gonad histopathology, has been described on several occasions. However the established relationship between e.g. vitellogenin induction and adverse health effects in fish is limited. In particular the predictive value of low to moderate vitellogenin induction on the reproductive health of fish is not yet well defined (65); more long term studies will be needed for that in the future but do not form part of the validation programme for this assay. Therefore, for the time being, the purpose of this 21-day fish screening assay is to help prioritise chemicals for further evaluation and is not intended to replace longer-term studies evaluating adverse effects on fish.

16.   In addition to the key goals indicated above for Phase 1B, repeatability of findings will be assessed through the use of 17β-estradiol as a positive control, which was already used in Phase 1A in a series of three concentrations, and in the same laboratories as in Phase 1A.

4. ORGANISATION OF PHASE 1B

4.1   Introduction

17.   For Phase 1B, a technical lead laboratory for each species was designated (LAB 1 for medaka, LAB 7 for fathead minnow and LAB 12 for zebrafish). Each technical lead laboratory was responsible for making standard operating procedures for secondary sex characteristics and vitellogenin measurements in each fish species and for answering questions from other laboratories using the same species. Each participating laboratory, 3 to 4 for each species (see **Annex 3** for laboratories contact details), was asked to conduct experimental work in compliance with the agreed protocol and to submit a study plan for its participation in Phase 1B to the overall lead laboratory, LAB 1, with a copy to the OECD Secretariat, including a schedule of work. A detailed study plan was submitted by 7 (out of 14) participating laboratories.

18.   The main change in the design between Phase 1A and Phase 1B was in holding adult males and females of a narrowly defined age and in spawning conditions, in the same tank. It was considered that these natural conditions might improve the biological relevance of the assay, and also improve the response to chemical exposure by removing potential confounders noted in Phase 1A. Endpoints remained the same as in Phase 1A, except the gonado-somatic index (GSI) which was dropped; the qualitative assessment of spawning (yes/no answer) was included as a new observation. Following recommendations from the EDTA Task Force at its seventh Meeting to use weakly active compounds to appraise the sensitivity of the protocol, the Fish Drafting Group of the VMG-eco made proposals on test substances, already documented in the published literature as weakly active on the fish gonadal axis.

19.   Detailed standard operating procedures were drafted for participating laboratories to follow. With respect to gross morphology, experts advised to provide participating laboratories with a detailed guidance for the measurement of secondary sex characteristics in fathead minnow and in medaka. These standard operating procedures were prepared by LAB 1 and LAB 4 and made available in appendix 6 of the Phase 1B protocol. The development of an extensive draft guidance document for gonad histotechniques and histopathology was coordinated by LAB 4; it was available within appendix 6 of the protocol.

4.2   Overview of the test method

20.   The experimental work was conducted according to the protocol prepared for Phase 1B of the validation of the Fish Screening Assay for Endocrine Active Substances (**Annex 1** to this report). A summary of noteworthy aspects of the protocol is provided below.

21.   The protocol was designed to detect endocrine active chemicals in sexually dimorphic fish. The assay was initiated with fish sampled from populations that were intended to be in spawning condition. The assay was conducted using three chemical exposure concentrations for each test substance, as well as a water control. The use of a solvent carrier was not needed in principle. However, when solvent was used, a solvent control was added. One concentration of a defined positive control substance was included for both 4-*tert*-pentylphenol and prochloraz studies. Two vessels (replicates) per treatment were used (each vessel containing 5 males and 5 females). The exposure was conducted for 21 days, at the end of which fish were sampled. Daily qualitative observations of the spawning status in each test vessel were recorded (yes/no). A spawning substrate was placed in the test chamber for the fathead minnow and zebrafish to enable fish to spawn in normal conditions; eggs were removed daily from the test chamber.

22.   After humane killing of the 20 fish (10 males and 10 females) per treatment level, blood samples were collected for determination of vitellogenin (note - liver was sampled for VTG analysis in medaka).

Secondary sex characteristics in fathead minnow and medaka were quantitatively evaluated. For gonad histology in medaka and zebrafish, animals were fixed and embedded directly, whereas for fathead minnow gonads were fixed *in situ*, excised from the body cavity and embedded, with one exception. In one labloratory (LAB 8) with fathead minnow whole body fixation were made after ventral incision of the body. The concept for this fish assay is derived from work on the fathead minnow *(Pimephales promelas)* (13)(14)(15)(9), the Japanese medaka (*Oryzias latipes*) (16)(17)(18)(19)(20) and the zebrafish (*Danio rerio*) (21)(22)(23)(24).

4.3   Test fish

23.   Fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) were used in the Phase 1B validation work. These are commonly used species for regulatory work in OECD member countries. The fish strain was not defined by the protocol. For medaka, LAB 1, LAB 2, Lab 3, LAB 4, but not LAB 6, used the orange-red strain.

24.   The exposure phase was started with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals (namely, with clear secondary sexual characteristics visible) and actively spawning. It was recommended that fathead minnow be approximately 20 (± 2) weeks of age (assuming they had been cultured at $25 \pm 2°C$ throughout their lifespan), medaka be approximately 16 (± 2) weeks of age (assuming they had been cultured at $25 \pm 2°C$ throughout their lifespan) and zebrafish be approximately 15 (± 2) weeks of age (assuming they had been cultured at $25 \pm 2°C$ throughout their lifespan). The age of the test fish is listed in Table 1. Several studies deviated from the protocol because of the delay in receiving test chemicals or ELISA kits for VTG measurement.

Table 1: Age of the test fish used in each laboratory.

Laboratory's name	Age of the test fish (weeks)		
	4-tert pentylphenol	Prochloraz	Flutamide
Medaka			
LAB 1	16	24	-
LAB 3	17	-	18
LAB 2	16	18	-
LAB 5	16	-	16
LAB 4	-	16	16
LAB 6	-	22	22
Fathead minnow			
LAB 7	approximately 18	-	24
LAB 8	18	22	-
LAB 9	24	24	-
LAB 10	-	-	22
LAB 11	-	52	Invalid study
LAB 4	-	18	18
Zebrafish			
LAB 12	12	24	12
LAB 13	20-24	20-24	-
LAB 14	8	-	12
LAB 6	-	17	17

25.   Following a 48-hour settling-in period, mortalities were recorded and the following criteria applied:

- mortalities of greater than 10% of population in seven days: reject the entire batch;
- mortalities of between 5% and 10% of population: acclimation for seven additional days; if more than 5% mortality during second seven days, reject the entire batch;

26.   The animals did not receive any treatment for disease in the 2-week acclimation period preceeding the test, or during the exposure period. In a sub-sample of the whole batch, animals were individually

weighed at the start of the test. If possible, it was recommended to remain within a range of 20% of the arithmetic mean weight.

4.4   Test chemicals and concentrations

27.   The Fish Drafting Group followed the recommendation from the EDTA Task Force to use weak substances. Three chemicals representing different modes of action were proposed:

Table 2: Test substances used in Phase 1B.

Test substance	CAS number	Lot no., purity	Supplier	Mode of action
4-*tert*-pentylphenol	80-46-6	13625LB, 99.9 %	Sigma Aldrich, US	Estrogen
prochloraz	67747-09-5	2226X, 99.5 %	Sigma Aldrich, US	Aromatase inhibitor
flutamide	1331-84-7	073K1004, >99 %	Sigma Aldrich, US	Anti-androgen

Positive controls substances were used for the estrogenic and aromatase inhibitor modes of action:

Table 3: Positive controls used in Phase 1B.

Positive control	CAS number	Lot no., purity	Supplier	Mode of action
17β-estradiol	50-28-2	103K1117, 100 %	Sigma Aldrich, US	Estrogen
fadrozole	102676-47-1	-	Novartis Pharma, Switzerland	Aromatase inhibitor

28.   All substances except fadrozole were managed through a Central Chemical Repository located in the USA: Battelle Marine Sciences Laboratory (WA, USA). The chemical repository allowed a central coordination of the delivery of chemicals to the participating laboratories, with the assurance that chemicals were coming from a unique lot. Fadrozole was provided by Novartis Pharma, Switzerland.

29.   The choice of test substances and test concentrations was extensively discussed by experts of the Fish Drafting Group in October 2003. The selection of 4-*tert*-pentylphenol as a weak estrogen agonist is based on work done in Japan on medaka (25) and in Europe on fathead minnow (26). Concentrations recommended are in line with those used in the published literature for the work on medaka, and one order of magnitude higher compared to the work on fathead minnow. The spacing of concentrations follows a factor 3.2, which enables to cover an order of magnitude when 3 concentrations are used in this type of test. The selection of one concentration of 17β-estradiol to serve as a positive control for estrogenic effects was based on Phase 1A and the top concentration used in Phase 1A was selected.

30.   Prochloraz was used as an aromatase inhibitor (interfering with the conversion of testosterone into 17-β estradiol in females); it has not been used very often in the past in fish and required a range-finding study to be conducted prior to Phase 1B to identify suitable concentrations. The range-finding study started with literature review to identify the water solubility of prochloraz, followed by a search in the AQUIRE database for the LC_{50}. One laboratory in the United States carried out a 7-day study on fathead minnow - one tank per concentration, containing 4 females and two males- taking 3 concentrations, spread on a log-based 10 series. The top concentration was 1/3 LC_{50}. Fish were sampled at termination of the study and blood collected for vitellogenin measurement, to ensure that the dose-response curve was covered in the range of concentrations used. Fadrozole was used as a positive control substance for the aromatase inhibition mode of action. Previous work documented endpoints response in fish and the concentration to be utilize to elicit a response (27)(28).

31.   Flutamide, a known mammalian anti-androgen, also used in work on fathead minnow in the United Kingdom and in the United States, was selected in Phase 1B to represent the anti-androgen mode of action. It is known as a relatively weak substance in fish for this specific mode of action. The three concentrations of flutamide used in Phase 1B are in line with those from the existing literature (27)(29).

32.   Concentrations of the test substances in Phase 1B were as follows:

- 4-*tert*-pentylphenol: 100, 320 and 1000µg/l (+ water control);
- 17β-estradiol: 100 ng/l to be used as positive control;
- Flutamide: 100, 500, 1000µg/l, highest as positive control (+ water control);
- Prochloraz: 20, 100, 300µg/l (+ water control)
- Fadrozole: 100µg/l to be used as positive control (in the prochloraz studies);

4.5   Participating laboratories

33.   Following a call for participation in Phase 1B, fourteen laboratories in 7 countries (Denmark, Germany, Japan, the Netherlands, Switzerland, United States, United Kingdom) expressed interest. The contact details of each participating laboratory are provided in **Annex 3**.

Table 4: Participating laboratories.

Laboratory's name	Test substance		
	4-tert pentylphenol	Prochloraz	Flutamide
	Medaka		
LAB 1	X	X	
LAB 3	X		X
LAB 2	X	X	
LAB 5	X		X
LAB 4		X	X
LAB 6		X	X
	Fathead minnow		
LAB 7	X		X
LAB 8	X	X	
LAB 9	X (partly invalid, see explanation below)	X (VTG results not available)	
LAB 10			X
LAB 11		X	(Invalid study)
LAB 4		X	X
	Zebrafish		
LAB 12	X	X	X
LAB 13	X	X	
LAB 14	X		X
LAB 6		X	X

34.   The distribution of species and test substances was meant to ensure a minimum of three repetitions for inter-laboratory comparisons. Among the problems and changes from the initial plan:

- LAB 9 using fathead minnow encountered problems with the ELISA kit for vitellogenin measurements from the prochloraz study, therefore no VTG results from LAB 9 are presented in the report.
- In LAB 9, fathead minnow in the 4*tert*-pentylphenol study suffered from an overdose in the last day of the experiment in the mid-dose and a lack of test substance delivery in the high dose group in the last 15 hours of the study; vitellogenin measurements for the 4*tert*-pentylphenol study from LAB 9 are not presented in this report either.
- LAB 10 only performed the flutamide study on feathead minnow and not the 4*tert*-pentylphenol study, due to a lack of time within the schedule set for Phase 1B; therefore only results from the flutamide study are presented in this report.
- LAB 11 had very high mortalities in the flutamide study on fathead minnow, making test results invalid.
- LAB 12 used a solvent (acetone), and a solvent control was thus added to the experiment with 4*tert*-pentylphenol.

4.6   Time schedule

35.   The experimental work started in March 2004 after preliminary approval of this proposal by the VMG-eco. All in-life parts of Phase 1B were completed in September 2004. The overall lead laboratory was responsible for the collection of test results, previously checked for accuracy by the laboratories themselves. The overall lead laboratory performed the statistical analysis and prepared the present report in September-October 2004.

36.   A preliminary version of the present report was presented to the Validation Management Group for ecotoxicity testing (VMG-eco) at its meeting in December 2004. The preliminary version only gave a partial view of the test results because the evaluations of gonadal histopathology were not yet included. Additionally, measures of the reproducibility of the assay across laboratories, such as coefficients of variation intra- and inter-laboratory, were not part of the report. The current version, dated July 2005, is inclusive of all data made available by the participating laboratories.

4.7   Preparation of test solutions

37.   Test solutions of the selected concentrations were prepared by dilution of a stock solution. The stock solution was prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns (solubility columns) were used for achieving a suitable concentrated stock solution in some laboratories. Detailed standard operating procedures for each substance were used (Appendix 9 in **Annex 1**). Acetone was used as a solvent and solvent control was set in LAB 12 for the flutamide experiment in zebrafish. LAB 12 encountered difficulties in achieving the solubility of 4*tert*-pentylphenol in the stock solution without solvent; this possibly explains the low measured concentrations of 4*tert*-pentylphenol in LAB 12 (see Table 8).

4.8   Analytical methods for determination of test concentration

Table 5: Test substances and analytical methods.

Test substance (TS) or Positive control (PC)	Analytical method for determination of test concentration	References
4-tert-pentylphenol	HPLC	(25) (26) (30)
Prochloraz	HPLC	Appendix 9 in **Annex 1**
17β-estradiol	LC-MS	(20) (31)
Flutamide	HPLC	(27) 239)(32)
Fadrozole	HPLC	(28)

38.   Analytical chemistry results for each substance and laboratory are presented in Section 5.1. Analysis was performed on a weekly basis in each tank and reported in the spreadsheet.

4.9   Test conditions

39.   A flow-through test system was used. Such a system continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) in order to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water were checked at intervals and were not supposed to vary by more than 10% throughout the 21 days of the test.

Feeding

40.   Further to the recommended food diet and ration, LAB 1 measured the presence of contaminants such as persistent organic pollutants (POPs) in food. Results are presented in ***Section 5.7***, Table 70.

4.10   Test acceptance criteria

41.   For the test results to be acceptable, the following conditions applied:

- mortality in the control(s) did not exceed 10% at the end of the exposure period, and signs of disease visible in less than 10 per cent of control animals during the course of the test.
- dissolved oxygen concentration was at least 60 per cent of the air saturation value (ASV) throughout the exposure period;
- water temperature did not differ by more than ± 1 °C between test vessels at any one time during the exposure period and was maintained within a range of 2°C within the temperature ranges specified for the test species.

42.   In LAB 11, the fathead minnow study using flutamide turned out to be invalid because of high mortality rates. Test results from that study are not presented in this report.

4.11   Endpoints studied

43.   Three core endpoints as indicators of endocrine activity of the test substances were observed during the course of the test or measured at termination of the test, namely:

i) gross morphology (e.g., secondary sexual characteristics such as nuptial tubercles on the head in fathead minnow and papillary processes on the anal fin in medaka were counted according to the standard operating procedures available, Appendix 6 of the protocol),
ii) vitellogenin levels,
iii) gonadal histology.

44.   Additionally, participating laboratories were requested to record daily the presence of eggs (yes/no answer) in treated and control groups. The data is presented in a temporal way in this report. Knowledge of the spawning status was intended as a check on the reproductive maturity of the fish in test in the control tank.

45.   On the one hand, complete cessation of spawning in a group can be a useful piece of information when interpreting the gonadal histopathology; especially when temporally reported, it gives an indication of when the fish stopped spawning and may assist in interpreting some of the pathological findings in females. On the other hand, there are also limitations to interpretation of variations in the spawning status of fish, as reported in Phase 1B:
- Unless there is complete cessation, it is not possible to have information of the spawning status of individual females to aid the gonadal histopathology evaluations;
- A qualitative recording (yes/no) does not reflect variation in the quantity of eggs spawned and only cessation can make a difference for further interpretation. Quantitative evaluation brings far more nuances for interpretation than qualitative evaluation.

46.   Daily egg counts were left as an optional endpoint; LAB 12, LAB 13 and LAB 14 – all using the zebrafish- counted fish eggs daily in a quantitative way (LAB 12 and LAB 14) or semi-quantitative way (LAB 13). All egg counts are reported in this document in graph format.

47.   Retrospectively, laboratories who counted eggs were asked to indicate the time spent daily on this operation for the whole 21-day study (control and 3 concentrations). Responses varied between ¾ hour and 1hour ½ to 2 hours/day.

4.12   Other observations

Behaviour and external abnormality
48.   Fish were examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration, signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding) noted. Mortality was recorded and the dead fish was removed as soon as possible.

4.13   Data collection

49.   Participating laboratories recorded the raw experimental data from their Phase 1B on standardized Excel spreadsheets (**Annex 2**) developed specifically for the validation study. A workbook (collection of Excel spreadsheets for each endpoint) was prepared for each species. In addition to raw data, means and standard deviations were calculated. All completed data sheets with laboratory results are available from the lead laboratory and from the OECD Secretariat.

4.14   Vitellogenin measurement and supply of kits

50.   VTG is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually-active females of all oviparous species; the production of VTG is controlled by interaction of estrogens with the estrogen receptor. Significantly, males maintain the capacity to produce VTG in response to stimulation with estrogen receptor agonists; as such, induction of VTG in males and immature females has been successfully exploited as a biomarker specific for estrogenic compounds in a variety of OECD fish species. Inhibition of VTG in mature females also appears to be a biomarker of exposure to aromatase inhibitor, where the conversion of endogenous androgen to estrogen is blocked, thus stopping estrogen receptor activation, itself controlling VTG production.

51.   With regard to vitellogenin measurement, a number of comparative studies were conducted in parallel with Phase 1A in OECD member countries: United States (33), France and Japan (6). The outcome of each study was reviewed by the Fish Drafting Group at its meeting in October 2003. The important conclusions relevant to the Validation of the Fish Screening Assay were that:

   i) homologous ELISA methods should be used, i.e. using species-specific antibodies and VTG standard;
   ii) the method should have demonstrated that it can detect levels as low as few ng/ml plasma (alternatively liver for medaka), this is to ensure that the assay can differentiate between control males and males producing low levels of VTG after induction by a weak estrogenic compound for example; and finally
   iii) in the validation exercise the same method should be used by all laboratories using the same fish species.

52.   However, an important note should be made here: the choice of a particular method to be used in laboratories in Phase 1B for each species does not imply that it is the only valid method; it only means that from results gleaned in the comparative studies conducted in the United States, in France and in Japan, the method selected has demonstrated to be sensitive enough for the intended purpose and that experts felt confident enough to use it in the validation exercise.

53.   After the Fish Drafting Group meeting in October 2003, LAB 1 in Japan, LAB 4 in the US, and LAB 12 in Germany selected the methods used by laboratories working on medaka, fathead minnow and zebrafish respectively. Standard operating procedures (SOPs) for the sampling procedure (blood or liver collection, pre-treatment and detailed specifications of the method used) have been added as appendices to

the Phase 1B protocol (<u>Appendix 6</u>). The measurement of VTG was based upon validated homologous ELISA methods. VTG measurement kits, produced by EnBioTec for VTG measurements in fathead minnow and medaka were centrally distributed by Amersham/GE Health Science in the United States and Europe at a negotiated rate to the participating laboratories. In zebrafish, VTG measurement was performed according to the method published by Holbech (7).

Fathead minnow:
- Fathead Minnow Vitellogenin ELISA system (EnBioTec Laboratories, Tokyo, Japan) using monoclonal antibodies;
- ELISA method used at US EPA MED, described by Korte *et al*, 2000 (35), using polyclonal antibodies;

Medaka:
- Medaka Vitellogenin ELISA system (EnBioTec Laboratories Co., Ltd., Tokyo, Japan) using monoclonal antibodies (36), and
- Medaka Vitellogenin (VTG) ELISA kit (TRANS GENIC INC., Kumamoto, Japan) using polyclonal antibodies (8);
  The above two kits were selected because the sensitivity and reproducibility of these two kits are comparable, and a good correlation exists between these two kits when a common VTG standard is used (6). Therefore, it was decided to use Battelle VTG standard for the standard curve;

Zebrafish:
- BCA Protein Assay Reagent Kit (Pierce, Rockford, USA) for the measurement of total protein concentration in zebrafish samples;
- ELISA method used at Odenske University, described by Holbech's method (Holbech *et al*, 2001) (28).

54.   VTG standard proteins for each of the three species were prepared centrally in Battelle Marine Science Laboratory in the United States, and delivered to all participating labs for the purpose of Phase 1B. In all VTG assays using medaka and zebrafish the Battelle standard was used for the standard curve in order quantify the specimen. The VTG standard contained in the kit was not basically used. VTG kit and VTG standard protein used in each laboratory are listed in <u>Table 6</u>. Standard operating procedures for blood/liver sampling and pre-treatment, for the purification of vitellogenin-where it was needed-, for the use of BCA Protein Assay Reagent kit and for the use of the VTG ELISA kits were available to the laboratories.

Table 6: VTG kit and VTG standard protein used in each laboratory.

Laboratory's name	VTG kit / VTG protein for standard curve		
	4-tert pentylphenol	Prochloraz	Flutamide
Medaka			
LAB 1	TG-MK[1] / Battelle-VTG standard		
LAB 3	TG-MK / Battelle-VTG standard		
LAB 2	EnBio-MK[2] / Battelle-VTG standard		
LAB 5	EnBio-MK / Battelle-VTG standard		
LAB 4	EnBio-MK / Battelle-VTG standard		
LAB 6	EnBio-MK / Battelle-VTG standard		
Fathead minnow			
LAB 7	EnBio-FHM[3] / Battelle-VTG standard		
LAB 8	EnBio-FHM / Battelle-VTG standard and VTG standard contained in the kit		
LAB 9	EnBio-FHM / Battelle-VTG standard		
LAB 10	EnBio-FHM / Battelle-VTG standard		
LAB 11	EnBio-FHM / VTG standard contained in the kit		
LAB 4	EnBio-FHM / Battelle-VTG standard		
Zebrafish			
LAB 12	Pierce-ZF[4] / Holbech et al, 2001[5]/Battelle-VTG standard		
LAB 13	Pierce-ZF / Holbech et al, 2001/ Battelle-VTG standard		
LAB 14	Pierce-ZF / Holbech et al, 2001/ Battelle-VTG standard		
LAB 6	Pierce-ZF / Holbech et al, 2001/Battelle-VTG standard		

1) : Medaka VTG ELISA kit (TRANS GENIC INC., Kumamoto, Japan)
2) : Medaka VTG ELISA system (EnBioTec Laboratories Co., Ltd., Tokyo, Japan)
3) : Fathead Minnow VTG ELISA system (EnBioTec Laboratories, Tokyo, Japan)
4) : BCA Protein Assay Reagent Kit (Pierce, Rockford, USA) for the measurement of total protein concentration in zebrafish samples
5) : Method used for vitellogenin measurement in zebrafish samples (7)

4.15   Guidance for gonadal histopathology

55.   The experience from Phase 1A had shown that an insufficient level of guidance and standardization of procedure was available to participants to evaluate gonad samples in a harmonized and comparable way. Further work was warranted to develop a consensus guidance document for use in Phase 1B. A group of experienced fish pathologists met after Phase 1A in October 2003, to identify areas where they could contribute further advice regarding the histological procedures and the pathological evaluation.

56.   Considerable efforts were made to draft a comprehensive document, with all necessary standard operating procedures, illustrated with many annotated photos (e.g., dissection procedures, histological slides), providing the diagnostic terminology and a consensus severity scoring system to be applied in Phase 1B. The guidance document was available as Appendix 6 to the Phase 1B protocol. In the future, it will become a stand alone document.

57.   Following Phase 1B, pathologists met and reviewed their findings. They explained that none of them was blind to the treatment of fish when they first evaluated the slides. In their opinion, for studies such as Phase 1B, blind reading is not appropriate for the initial evaluation of sections because it increases the chances that subtle exposure-related effects might be missed (i.e., blind reading primarily guards against false positive results at the expense of false negative results). Conversely, blind reading may be entirely appropriate for future routine assays because the possible results to a set of previously established histologic findings have already been limited (i.e., we are proceeding under the assumption that there are no new types of findings to be discovered). Pathologists recommended that there should be initial knowledge of the control group because some of the diagnoses are expressed as relative increase or decrease of a particular cell type. In Phase 1B, pathologists confirmed that they were blind to the reproductive cycle status of fish.

58.   In order to streamline diagnoses that are directly relevant to treatment, a short list of primary

diagnoses was prepared by pathologists' post-Phase 1B evaluations. These primary diagnoses, when found in the study experiments in a consistent manner through the laboratories have been marked by shaded cells throughout the tables in the following results' sections.

Table 7: Primary gonad histopathological diagnoses

No.	Males:	Females:
1	Increased proportion of spermatogonia	Increased oocyte atresia
2	Presence of testis-ova	Perifollicular cell hyperplasia/hypertrophy
3	Increased testicular degeneration	Decreased vitellogenesis
4	Interstitial (Leydig) cell hyperplasia/hypertrophy	Gonadal staging (based on improved staging criteria)

4.16   Statistical analysis: procedure followed

59.   Statistics were conducted in accordance with the protocol (**Annex 4**).The statistical analysis was performed on secondary sex characteristics of fathead minnow and medaka, and vitellogenin of three fish species. The spawning status and the gonad histology were excluded because these data are qualitative or semi-quantitative.   Statistical analysis was conducted on the basis of individuals rather than unit of replication (tanks) because two replicates were not sufficient for statistics. The statistical analysis of optional data (e.g., egg numbers) has not been performed.

60.   If a solvent control was used for a test, homogeneity of variances between the control and solvent control groups was checked by Levene's test, and then either Student's t-test (parametric data) or Mann-Whitney U-test (non-parametric data) were used before data analysis to determine whether differences exist between the control and solvent control groups. Where necessary, data were log-transformed for normalization and to reduce variance heterogeneity. If no difference was found, these groups were pooled for subsequent analysis. If differences were found, the control group without solvent was excluded from the subsequent analyses.

61.   The experimental data was checked for homogeneity of variances across treatments by Levene's test. When no homogeneity was observed in the data, a log-transformation was performed and the transformed data was checked for homogeneity of variances across treatments again.   When the assumptions were met (with or without transformation), the data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. When no homogeneity was observed even in the transformed data, the nonparametric Kruskal-Wallis test was used, followed by the Mann-Whitney U test with Bonferroni's adjustment. Differences were conclusively determined in Dunnett's multiple comparison test or Mann-Whitney U test with Bonferroni's adjustment. A two-side test was performed because the initial hypothesis was that either an increase or a decrease in endpoints measured could be considered as a damageable outcome. VTG values lower than the determination limit were transformed to half the value of the determination limit for each analysis. Statistical analysis between positive control and control groups was performed by either Student's t-test (parametric data) or Mann-Whitney U test (non-parametric data) after Levene's test. Where necessary, data were log-transformed.

62.   Differences were considered to be significant at $p < 0.05$ in all tests; however, Bonferroni's p value was used in Mann-Whitney U test. All statistical analyses were performed by JMP ver.4.05J produced by SAS Institute Japan.

63.   Test results are presented in the text as graphic figures. Error bars showed in figures represent the standard deviation. Numerical figures are provided in Annex 5 (list of data) and cross-referenced in the text.

5. RESULTS

5.1 Analytical chemistry

5.1.1 Analytical chemistry of 4-tert-pentylphenol studies

64.   Mean concentrations measured in both replicates at weekly intervals (3 weekly measurements in the 21-day studies) are reported in tables below for each test substance.

Table 8: The means of measured 4-*tert*-pentylphenol and positive control concentrations in the test solutions.

Means of measured concentrations (% nominal) in tank 1 and 2				
Nominal values	100 µg/l	320 µg/l	1,000 µg/l	PC[1] (100 ng/l)
LAB 1	91.0(91)	284(89)	882(88)	102(102)
LAB 2	Medaka	86.8(87)	287(90)	959(96)	105(105)
LAB 3	98.6(99)	314(98)	937(94)	103(103)
LAB 5	85.5 (86)	272 (90)	857 (85)	78 (78)
LAB 7	Fathead	80.1(80)	270(85)	862(86)	90.6(91)
LAB 8	minnow	85.7(86)	298(93)	887(89)	No data
LAB 9	81 (81)	277 (80)	820 (82)	No data
LAB 12	Zebrafish	90.0(90)	294(92)	787(79)	No data
LAB 13	76.8(77)	229(72)	721(72)	No data
LAB 14	22.6(23)	69.5(22)	473(47)	No data

[1] Positive control (17β-estradiol)
Shaded cells: low measured concentrations

65.   The means of measured 4-*tert*-pentylphenol and positive control concentrations in the test solutions during the exposure period indicate that the nominal concentrations of these chemicals remained consistent and within the 80%-120% range throughout the exposure period in most of the studies. LAB 13 was slightly below the 80%-120% range; LAB 14 (see shaded cells) was very much below the expected range (23%, 22% and 47% respectively). LAB 14 explained that it had great difficulties preparing the stock solution without using solvent; the low measured concentration may therefore be due to test substance not sufficiently dissolved in the stock solution.

5.1.2 Analytical chemistry of prochloraz studies

66.   The means measured concentrations of prochloraz and of the positive control remained consistent throughout the exposure period in most of the studies. The actual concentrations in a few laboratories tended to vary from the nominal concentrations.

Table 9: The means of measured prochloraz and positive control concentrations in the test solutions.

Means of measured concentrations (% nominal) in tank 1 and 2					
Nominal values		20 µg/l	100 µg/l	300 µg/l	PC[1] (100 µg/l)
LAB 1	Medaka	17.9(89)	92.5(92)	279(93)	94.5(95)
LAB 2		20.4(102)	94.6(95)	284(95)	111(111)
LAB 4		22.9(115)	99.5(100)	296(99)	99.4(99)
LAB 6		6.73(34)	54.0(54)	217(72)	16.7(17)
LAB 8	Fathead minnow	19.8(99)	97.5(98)	299(100)	No data
LAB 9		21.2 (105)	108 (108)	341 (114)	93 (93)
LAB 11		15.3(76)	68.9(69)	275(92)	97.1(97)
LAB 4		24.1(120)	121(121)	382(127)	105(105)
LAB 12	Zebrafish	14.7(73)	67.1(67)	166(55)	135(135)
LAB 13		19.0(95)*	82.7(83)	194(65)	41.6(42)
LAB 6		6.73(34)	54.0(54)	217(72)	16.7(17)

*: data of week 3 was excluded from mean calculation (tank 1: 415 µg/l, tank 2: 112 µg/l).
[1] Positive control (Fadrozole)
Shaded cells: low measured concentrations

67.   In particular, LAB 6 had very low measured concentrations for all the treatments, respectively 34%, 54% and 72% of nominal values for both the medaka and fathead minnow studies. LAB 12 and LAB 13 had low measured concentrations for the high dose group (55% and 65% of nominal values respectively).

5.1.3   Analytical chemistry of flutamide studies

68.   The means of measured flutamide concentrations in the test solutions during the exposure period indicate that the nominal concentrations of these chemicals remained consistent throughout the exposure period in most of the studies.

Table 10: The means of measured flutamide concentrations in the test solutions.

Means of measured concentrations (% nominal) in tank 1 and 2				
Nominal values		100 µg/l	500 µg/l	1,000 µg/l
LAB 3	Medaka	95.5(95)	518(104)	1,060(106)
LAB 5		94.9 (95)	434.5 (87)	880.7 (88)
LAB 4		97.4(97)	501(100)	996(100)
LAB 6		55.8(56)	221(44)	552(55)
LAB 7	Fathead minnow	68.7(69)	354(71)	754(75)
LAB 10		83.5 (83)	445 (89)	875 (87)
LAB 11		Invalid because of high mortality rates		
LAB 4		88.8(89)	464(93)	940(94)
LAB 12	Zebrafish	76.6(77)	250(50)	788(79)
LAB 14		74.7(75)	397(79)	730(73)
LAB 6		55.8(56)	221(44)	552(55)

Shaded cells: low measured concentrations

69.   However, LAB 6 had very low measured concentrations for both the medaka and the zebrafish studies: 54%, 44% and 55% of nominal values respectively.

5.2 Mortality

5.2.1 Mortality of 4-tert-pentylphenol studies

5.2.1.1 Medaka

70.   Mortalities in the 4-*tert*-pentylphenol groups and positive control were ≤ 5% during the exposure period, except in LAB 5, where mortality in the treated groups was relatively high.

Table 11: Mortality (%) in the 4-*tert*-pentylphenol studies with medaka at the end of exposure.

Nominal values	Cont.	100 µg/l	320 µg/l	1,000 µg/l	PC[1] (100 ng/l)
LAB 1	0	0	0	0	0
LAB 3	0	0	0	0	0
LAB 2	0	0	0	5	0
LAB 5	0	0	20	20	35

[1]: PC= positive control (17β-estrdiol)

5.2.1.2 Fathead Minnow

71.   Mortalities in the 4-*tert*-pentylphenol groups and positive control were ≤ 5% in LAB 7, although those increased dose-dependently in 4-*tert*-pentylphenol exposure conducted at LAB 8. In LAB 9, mortalities in the medium concentration were 100% on the last day of the experiment because fish suffered from over-dosage of the test substance – VTG data and secondary sex characteristics for LAB 9 are therefore not reported in this section.

Table 12: Mortality in the 4-*tert*-pentylphenol studies with fathead minnow at the end of exposure.

Laboratories	Nominal concentrations				
	Cont.	100 µg/l	320 µg/l	1,000 µg/l	PC[1] (100 ng/l)
LAB 7	0	0	0	5	5
LAB 8	0	0	15	45	0
LAB 9	0	10	100	30	5

[1]: PC= positive control (17β-estradiol)

5.2.1.3 Zebrafish

72.   Mortalities in the 4-*tert*-pentylphenol groups and positive control in all studies were ≤ 10% during the exposure period.

Table 13: Mortality (%) in the 4-*tert*-pentylphenol studies with zebrafish at the end of exposure.

Laboratories	Nominal concentrations					
	Cont.	SC[1]	100 µg/l	320 µg/l	1,000 µg/l	PC[2] (100 ng/l)
LAB 12	5	0	5	0	10	0
LAB 13	0	-	0	0	0	0
LAB 14	0	-	0	5	5	5

[1] : SC=solvent control (acetone); [2]: PC= positive control (17β-estradiol)

5.2.2   *Mortality of prochloraz studies*

5.2.2.1 Medaka

73.   Mortalities in the prochloraz groups and positive control in all studies were ≤ 10% during the exposure period.

Table 14: Mortality (%) in the prochloraz studies with medaka at the end of exposure.

Laboratories					

Laboratories	Cont.	20 μg/l	100 μg/l	300 μg/l	PC[1] (100 μg/l)
		Nominal concentrations			
LAB 1	0	0	0	5	0
LAB 2	0	0	0	5	10
LAB 4	0	0	0	0	5
LAB 6	0	0	0	0	0

[1]: PC= positive control (fadrozole)

5.2.2.2 Fathead Minnow

74.   Mortalities in the prochloraz groups and positive control in the two submitted studies were ≤ 10% during the exposure period.

Table 15 Mortality in the prochloraz studies with fathead minnow at the end of exposure.

Laboratories	Cont.	20 μg/l	100 μg/l	300 μg/l	PC[1] (100 μg/l)
		Nominal concentrations			
LAB 4	5	0	0	0	5
LAB 8	0	0	5	0	0
LAB 9	0	0	0	0	0
LAB 11	5	0	10	0	0

[1]: PC=positive control (fadrozole)

5.2.2.3 Zebrafish

75.   No fish died during the exposure period in two studies; 10-20 % mortalities in prochloraz treatment groups were found in LAB 6 (measured concentrations in LAB 6 were 34%, 54% and 72% of nominal).

Table 16: Mortality (%) in the prochloraz studies with zebrafish at the end of exposure.

Laboratories	Cont.	20 μg/l	100 μg/l	300 μg/l	PC (100 μg/l)
		Nominal concentrations			
LAB 12	0	0	0	0	0
LAB 13	0	0	0	0	0
LAB 6	5	10	10	20	0

[1]: PC=positive control (fadrozole)

5.2.3 Mortality of flutamide studies

5.2.3.1 Medaka

76.   Mortalities in the flutamide treatment groups in the four studies were ≤ 5% during the exposure period, except in LAB 5. In LAB 5, the mortalities does not appear to be related to the dose; it might be due to a bacterial infection- the histopathologist saw signs of this in some fish.

Table 17: Mortality (%) in the flutamide studies with medaka at the end of exposure.

Laboratories	Nominal concentrations			
	Cont.	100 µg/l	500 µg/l	1,000 µg/l
LAB 3	5	5	0	0
LAB 5	0	35	45	10
LAB 4	0	0	0	0
LAB 6	0	5	5	0

5.2.3.2 Fathead Minnow

77.   Mortalities in the flutamide treatment groups in three submitted studies were ≤ 5% during the exposure period.

Table 18: Mortality (%) in the flutamide studies with fathead minnow at the end of exposure.

Laboratories	Nominal concentrations			
	Cont.	100 µg/l	500 µg/l	1,000 µg/l
LAB 7	0	0	0	5
LAB 10	0	0	0	5
LAB 11	Invalid because of high mortality rates			
LAB 4	0	0	0	0

5.2.3.3 Zebrafish

78.   Mortalities in the flutamide treatment groups were ≤ 5% during the exposure period in all studies.

Table 19: Mortality (%)in the flutamide studies with zebrafish at the end of exposure.

Laboratories	Nominal concentrations				
	Cont.	SC [1]	100 µg/l	500 µg/l	1,000 µg/l
LAB 12	0	5	0	0	0
LAB 14	5	0	5	0	5
LAB 6	5	-	5	0	5

[1]: SC=solvent control

5.3 Spawning Status

5.3.1 Spawning status of 4-tert-pentylphenol studies

5.3.1.1 Medaka

79. All control groups spawned well, except in LAB 2. No variation in spawning could be observed between the control groups and the 4-*tert*-pentylphenol treated groups and positive control in LAB 1. A dose-dependent decrease in spawning was observed in at least two of the studies (LAB 3 and LAB 5).

Table 20: Spawning status in the 4-*tert*-pentylphenol studies with medaka during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
Nominal values	Cont.	100 µg/l	320 µg/l	1,000 µg/l	PC[1] (100 ng/l)
LAB 1	21/21	21/21	21/21	21/21	21/21
LAB 2	14.5/21	21/21	18/21	16.5/21	19/21
LAB 3	20/21	19/21	20.5/21	13/21	21/21
LAB 5	21/21	18.5/21	14.5/21	10/21	12.5/21

[1]: PC= positive control (17β-estradiol)

80. A temporal recording of the spawning status indicates that when spawning tended to decrease, it was towards the second half of the 21-day study.

Table 21: daily recording of the spawning status in the 4-*tert*-pentylphenol studies with medaka.

DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 1																						
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LAB 2																						
C		½	½	1	1	½	1	1	1	1	1	1	0	0	1	½	½	½	1	½	½	½
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	½	½	0
H		½	½	1	1	1	1	1	1	1	1	1	1	½	½	½	½	½	½	½	½	½
PC		½	½	½	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LAB 3																						
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	½	1
M		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	1	1
H		1	1	1	1	1	1	1	1	1	1	0	1	0	0	½	½	0	½	½	0	0
PC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LAB 5																						
C	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	½	0	½	1
M	½	1	1	1	1	1	1	1	1	1	1	1	½	½	½	½	½	0	½	0	0	½
H	1	1	1	1	1	1	1	1	1	½	1	½	0	0	0	0	0	0	0	0	0	0
PC	1	1	1	1	1	1	1	1	1	1	1	1	1	½	0	0	0	0	0	0	0	0

1: spawning observed in both replicate tanks
½: spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.
C: control; L: low concentration; M: medium concentration; H: high concentration; PC: positive control
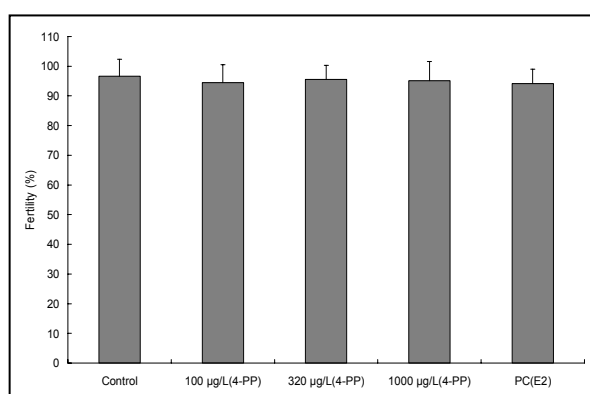
Fig. 1a: The means of fertility in medaka exposed
to 4-*tert*-pentylphenol.
(Optional data of LAB 1)
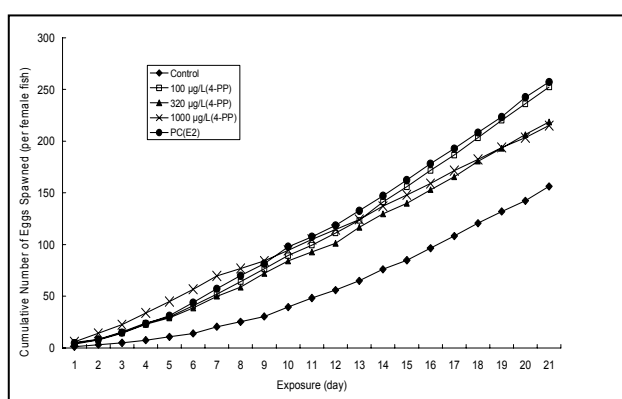
Fig. 1b: The cumulative number of eggs spawned in
medaka 4-*tert*-pentylphenol.
(Optional data of LAB 1)

81. LAB 1 recorded, as optional data, the number of eggs spawned daily by medaka under 4*tert*-pentylphenol treatment (Figure 1a), and the fertility of eggs (Figure 1b). No dose-dependent response was observed for any of the two parameters. Such information was not collected by the other laboratories.

5.3.1.2 Fathead Minnow

82. The control groups did not spawn very well throughout the study, especially in LAB 8 and LAB 9. Fathead minnow are territorial and the group-spawning conditions proposed in Phase 1B were sub-optimal for fathead minnow.

83. Spawning was reduced in a concentration-dependent manner in all studies; especially no eggs were produced in the highest concentration of 4-*tert*-pentylphenol in all three studies.

Table 22: Spawning status of fathead minnow exposed to 4-*tert*-pentylphenol.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
Laboratories	Nominal concentrations				
	Cont.	100 µg/l	320 µg/l	1,000 µg/l	PC[1] (100 ng/l)
LAB 7	17.5/21	14.5/21	13/21	0/21	15/21
LAB 8	4.5/21	3/21	0.5/21	0/21	2/21
LAB 9	7/21	6/21	1/21	0/21	3.5/21

[1]: PC= positive control (17β-estrdiol)

Table 23: Daily recording of the spawning status of fathead minnow exposed to 4-*tert*-pentylphenol.

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 7																						
C	0	½	1	½	1	1	1	1	½	1	1	1	½	½	1	1	1	½	1	1	1	1
L	0	½	½	½	½	½	1	½	1	½	1	½	1	1	1	½	1	½	½	½	1	½
M	0	1	½	1	1	0	½	½	1	½	½	½	½	1	½	½	½	½	1	½	½	½
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PC	0	0	1	½	1	½	1	1	½	½	½	1	½	½	½	1	1	1	½	½	1	1
LAB 8																						
C	0	1	½	0	0	½	½	0	½	0	0	0	0	0	0	½	½	0	0	½	0	0
L	0	½	0	0	0	½	0	0	½	½	0	½	0	0	0	0	0	0	0	0	0	½
M	0	0	0	0	0	0	0	0	0	0	0	½	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PC	0	½	½	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LAB 9																						
C		½	½	½	1	0	0	½	0	½	½	½	0	0	½	0	½	½	½	0	½	0
L		0	0	½	0	½	1	½	½	½	½	0	0	½	0	½	0	0	0	½	0	½
M		0	0	½	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PC		0	0	½	½	0	½	0	0	½	½	0	0	0	½	0	0	½	0	0	0	0

1: spawning observed in both replicate tanks
½: spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.
C: control; L: low concentration; M: medium concentration; H: high concentration; PC: positive control

5.3.1.3 Zebrafish

84. Control groups spawned regularly throughout the 21-day experiment in all three studies. No clear decrease of spawning could be observed following chemical treatment in any of the three studies.

Table 24: Spawning status in the 4-*tert*-pentylphenol studies with zebrafish during the exposure period.

	No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
	Nominal concentrations					
Laboratories	Cont.	SC[1]	100 µg/l	320 µg/l	1,000 µg/l	PC[2] (100 ng/l)
LAB 12	19.5/21	17.5/21	20/21	19/21	16.5/21	20.5/21
LAB 13	6/6(tank 1) 6/6(tank 2)	-	7/7(tank 1) 4/6(tank 2)	7/7(tank 1) 3/6(tank 2)	7/7(tank 1) 3/6(tank 2)	7/7(tank 1) 5/6(tank 2)
LAB 14	18/21	-	18/21	19.5/21	18/21	No data

[1] : SC=solvent control (acetone); [2]: PC= positive control

Table 25: Daily recording of spawning status of zebrafish exposed to 4-*tert*-pentylphenol.

DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 12																						
C		1	½	1	1	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1	½
SC		1	1	1	1	1	1	½	1	1	1	½	1	½	1	1	1	½	½	1	1	½
L		½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1
M		1	½	1	½	1	1	1	1	1	1	11	1	1	1	1	1	1	1	1	½	½
H		½	1	½	½	1	1	½	½	1	1	1	½	1	1	1	1	½	½	1	½	½
PC		1	1	1	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LAB 13																						
C							1		1			1		1		1		1		1		1
L					1	1		1	0		1	1		1	0		1	1		1	1	1
M					1	1		1	0		1	1		1	0		1	0		1	1	1
H					1	1		1	0		1	1		1	1	1	0		1	0	1	
PC					1	1		1	1/2		1	1		1	1	1	1		1	1	1	
LAB 14																						
C	1	1	½	1	½	1	1	1	½	1	1	1	½	1	1	1	1	1	1	1	1	0
L	1	1	1	1	½	1	1	½	½	1	½	1	1	1	½	1	1	½	1	1	1	1
M	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	1	1	½
H	1	1	1	1	1	1	1	1	½	1	1	1	½	1	1	1	1	½	1	0	1	½

1: spawning observed in both replicate tanks

½: spawning observed in one of the replicate tanks

0: no spawning observed in any of the two replicate tanks.

C: control; L: low concentration; M: medium concentration; H: high concentration; PC: positive control

85.   LAB 12, LAB 13 and LAB 14 collected optional data on egg counts. Cumulative numbers of eggs are reported in Figures 2b, 2c and 6e. In LAB 12 and LAB 14, there was no treatment-related response and the tanks where females produced fewer eggs were not necessarily those with treated animals. In LAB 13 (Figure 2e), there was a dose-dependent decrease of spawning, with statistical significance reached at the highest concentration, but not in the positive control, because of high variability.

86.   Fertility was not affected by 4*tert*-pentylphenol treatment in LAB 12 (Figure 2a). In LAB 13 (Figure 2d), although there was a decreasing trend in fertility, statistical significance could not be reached, except in the positive control, because of high variability.
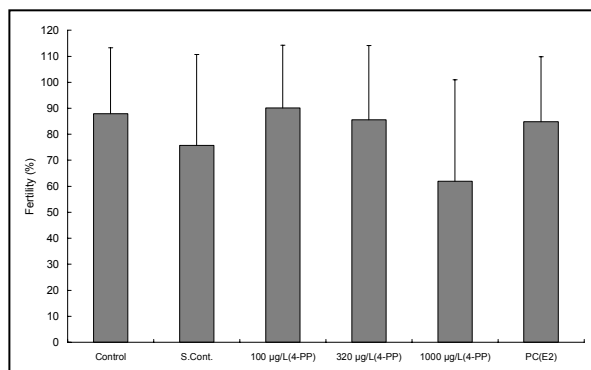
Fig. 2a: The means of fertility in zebrafish
exposed to 4-*tert*-pentylphenol.
(Optional data of LAB 12)
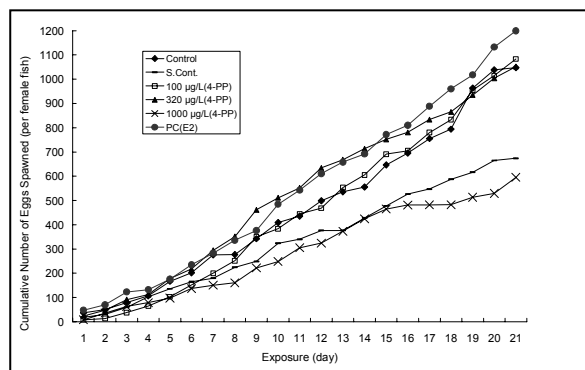
Fig. 2b: The cumulative number of eggs spawned in zebrafish
exposed to 4-*tert*-pentylphenol.
(Optional data of LAB 12)
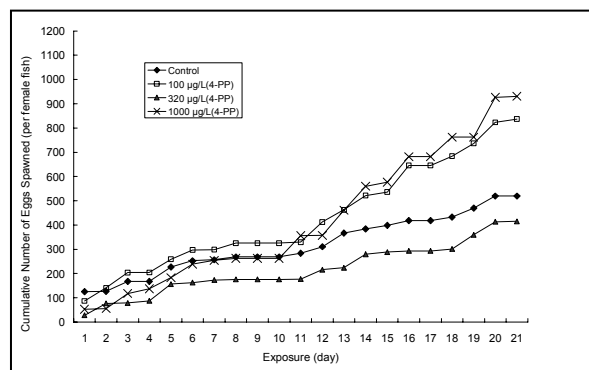
Fig. 2c: The cumulative number of eggs spawned in zebrafish
exposed to 4-*tert*-pentylphenol.
(Optional data of LAB 14)
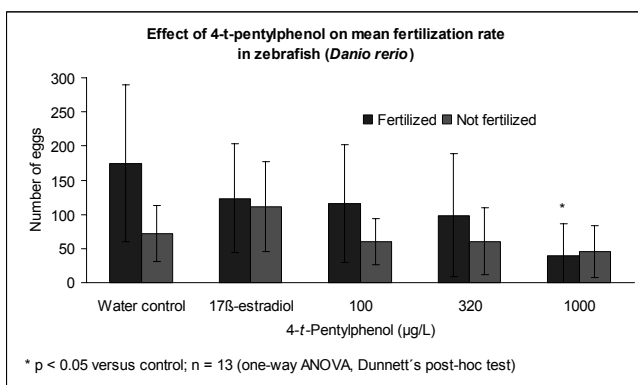
Fig. 2d: Effect of 4-*tert*-pentylphenol on mean
fertilization rate in zebrafish (*Danio rerio*).
(Optional data of LAB 13)
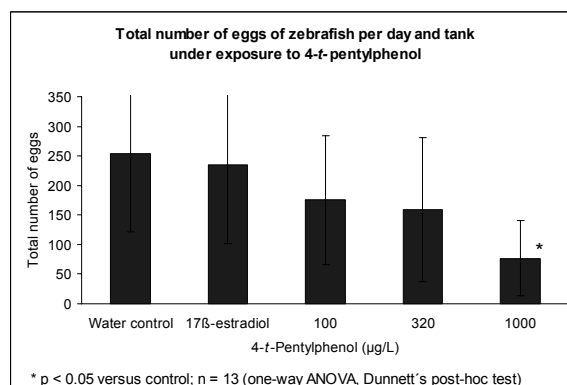
Fig. 2e: Total number of eggs of zebrafish per day
and tank under exposure to 4-*tert*-pentylphenol
(Optional data of LAB 13)

5.3.2 Spawning status of prochloraz studies

5.3.2.1 Medaka

87.   Spawning in all of the control groups was regular and continuous throughout the 21-day study. In the medium (100 μg/l) and high-dose (300 μg/l) groups, females progressively ceased to spawn.

Table 26: Spawning status in the prochloraz studies with medaka during the exposure period.

	No. of days with spawning "yes" (average of tank 1 and 2) / observed days				
	Nominal concentrations				
Laboratories	Cont.	20 μg/l	100 μg/l	300 μg/l	PC[1] (100 μg/l)
LAB 1	21/21	21/21	12/21	3/21	8.5/21
LAB 2	18.5/21	20.5/21	5/21	2/21	1.5/21
LAB 4	21/21	21/21	11/21	2/21	1.5/21
LAB 6	19/20	15.5/20	13/20	1/20	1/20

[1]: PC= Positive control

Table 27: Daily recording of spawning status in the prochloraz studies with medaka exposed to prochloraz.

DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 1																						
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M		1	1	½	½	1	½	0	1	1	1	0	1	½	½	1	½	0	0	½	0	½
H		1	1	½	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PC		1	1	½	1	0	½	½	½	1	½	0	0	0	½	½	½	0	0	0	0	½
LAB 2																						
C		½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	½	½
L		1	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M		1	½	1	0	½	½	1	½	0	0	0	0	0	0	0	0	0	0	0	0	0
H		1	½	0	0	0	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0
PC		1	0	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LAB 4																						
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M		½	½	1	½	0	½	1	1	½	1	0	½	1	0	½	½	0	0	0	0	½
H		0	0	1	½	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0
PC		1	0	0	0	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0
LAB 6																						
C	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
L	0	0	0	½	½	½	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
M	0	0	½	0	½	½	½	1	1	½	0	½	½	1	1	1	1	1	½	1	1	
H	0	0	½	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
PC	0	0	½	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

1: spawning observed in both replicate tanks
½: spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.
C: control; L: low concentration; M: medium concentration; H: high concentration; PC: positive control
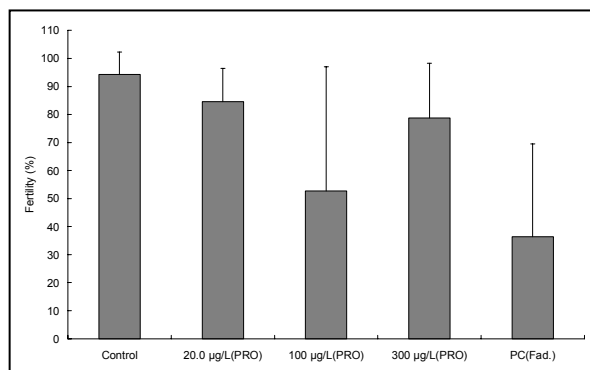
Fig. 3a: The means of fertility in medaka exposed to prochloraz.
(Optional data of LAB 1)
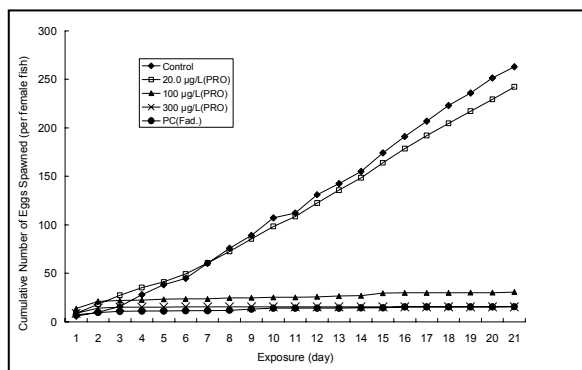
Fig. 3b: The cumulative number of eggs spawned in medaka exposed to prochloraz.
(Optional data of LAB 1)

88.   As optional data, LAB 1 recorded daily the number of eggs spawned and the fertility (Figures 3a and 3b). Fertility was not clearly affected by chemical treatment.

5.3.2.2 Fathead Minnow

89.   Control groups of fathead minnows did not spawn well throughout any of the four studies. Territorial behavior may be the cause of this problem. For this reason, no clear cessation of spawning due to the aromatase inhibitor could be detected. Only in LAB 9 a dose-dependent decrease was visible.

Table 28: Spawning status in the prochloraz studies with fathead minnow during the exposure period.

	No. of days with spawning "yes" (average of tank 1 and 2) / observed days				
	Nominal concentrations				
Laboratories	Cont.	20 µg/l	100 µg/l	300 µg/l	PC[1] (100 µg/l)
LAB 8	0.5/21	2/21	3/21	0.5/21	1.5/21
LAB 9	7.5/21	4.5/21	4.5/21	0/21	-
LAB 11	0.5/5	0/5	0/5	0/5	0.5/5
LAB 4	1.5/21	3/21	0.5/21	0.5/21	1/21

[1]: PC=positive control

Table 29: Spawning status in the prochloraz studies with fathead minnow during the exposure period.

| DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 4																						
C	½	0	0	0	0	½	½	0	0	0	0	0	0	0	0	½	0	0	0	0	0	0
L	0	0	0	0	0	0	0	½	0	0	0	0	0	0	1	0	0	0	½	0	0	½
M	½	0	0	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H	½	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PC	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LAB 8																						
C	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L	0	0	0	0	1	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	½
M	0	0	0	½	½	1	½	0	0	0	0	0	0	0	0	0	½	0	0	0	0	0
H	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PC	0	½	0	0	½	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LAB 9																						
C		0	½	½	1	½	0	½	½	1	0	0	0	½	½	½	1	½	0	0	0	0
L		0	½	0	½	½	½	0	0	½	0	0	½	0	0	0	0	½	0	½	0	½
M		1	0	0	0	½	½	1	0	0	0	0	½	0	0	0	½	0	0	0	0	½
H		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PC		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

1: spawning observed in both replicate tanks
½: spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.
C: control; L: low concentration; M: medium concentration; H: high concentration; PC: positive control

5.3.2.3 Zebrafish

90.   Spawning was not checked strictly everyday of the 21-day study in LAB 12 and LAB 13; however, spawning was regular throughout the study in the control groups. LAB 6 did not provide information on the spawning status of fish.

91.   Prochloraz caused a decrease in egg production at the top-dose and positive control of the study conducted in LAB 12 (more obvious from Figure 4b (quantitative) than from Table 31 (qualitative)). The study in LAB 13 did not indicate any clear effect (this is visible from Tables 30 and 31, and also from Figure 4d).

Table 30: Spawning status in the prochloraz studies with zebrafish during the exposure period.

	No. of days with spawning "yes" (average of tank 1 and 2) / observed days				
	Nominal concentrations				
Laboratories	Cont.	20 µg/l	100 µg/l	300 µg/l	PC (100 µg/l)
LAB 12	14.5/15	14.5/15	13.5/15	10.5/15	10/15
LAB 13	5/6*	6/6	5/6	6/6	2.5/6
LAB 6	No data available				

* in Lab 13, egg counts were done one day in tank A and the next day in tank B, thus the denominator (=6) represents the number of days where egg counts were done in each tank.

Table 31: Daily recording of spawning status of zebrafish exposed to prochloraz.

DAY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 12																					
C	1	1			1	1	1	1	½			1	1	1	1	1			1	1	1
L	1	1			1	1	1	1	1			1	1	1	1	½			1	1	1
M	1	½			1	1	½	1	1			1	1	1	1	1			1	1	½
H	1	1			1	1	0	½	1			1	1	½	1	½			½	½	0
PC	1	0			½	1	1	1	1			1	1	1	0	1			0	½	0
LAB 13																					
C					1			1			½			1		½				1	
L			1	1		1	1		1	1		1	1		1	1		1	1		
M			0	1		1	1		1	1		1	1		1	1		1	1		
H			1	1		1	1		1	1		1	1		1	1		1	1		
PC			0	0		0	½		½	½		0	0		0	½		0	½		

1: spawning observed in both replicate tanks

½: spawning observed in one of the replicate tanks

0: no spawning observed in any of the two replicate tanks.

C: control; L: low concentration; M: medium concentration; H: high concentration; PC: positive control

Note: for LAB 13, "1" represents the presence of eggs in the tank counted on that day (one tank controlled on day n, the other tank controlled on day n+1).

92.   It is worth noting again that measured concentrations of prochloraz in both LAB 12 and LAB 13 were low: 73%, 67%, 55%, and 95%, 83%, 65% respectively for each study. This may explain that the cessation of spawning was not marked, especially at the top-dose.
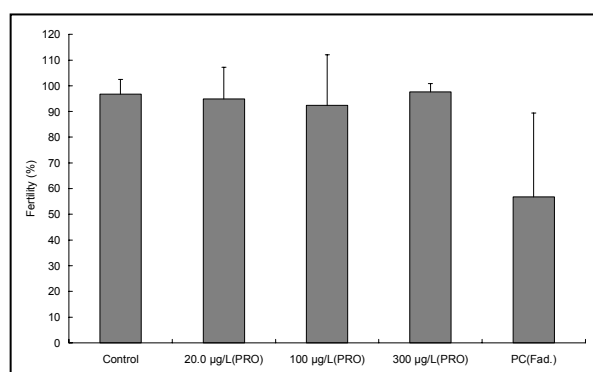
Fig. 4a: The means of fertility in zebrafish exposed to prochloraz.
(Optional data of LAB 12)
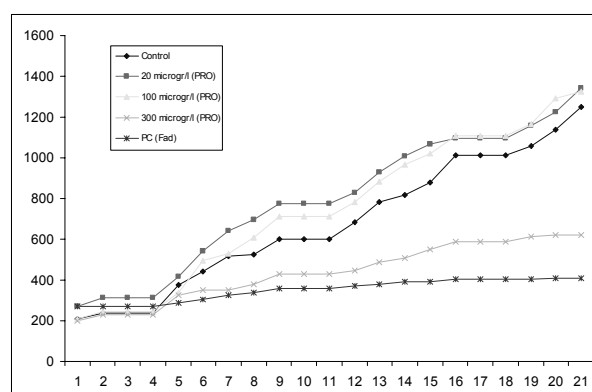
Fig. 4b: The cumulative number of eggs spawned in zebrafish exposed to prochloraz.
(Optional data of LAB 12)
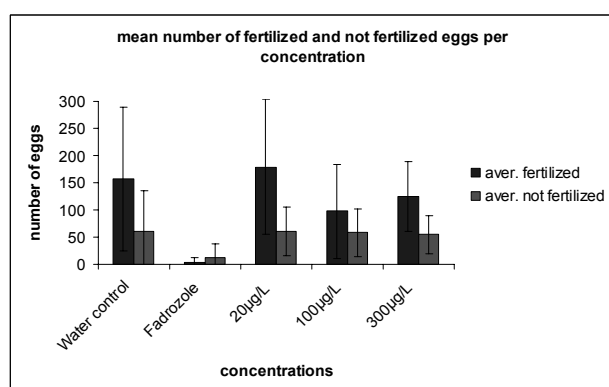
Fig. 4c: Effect of prochloraz on mean fertilization rate in zebrafish.
(Optional data of LAB 13)
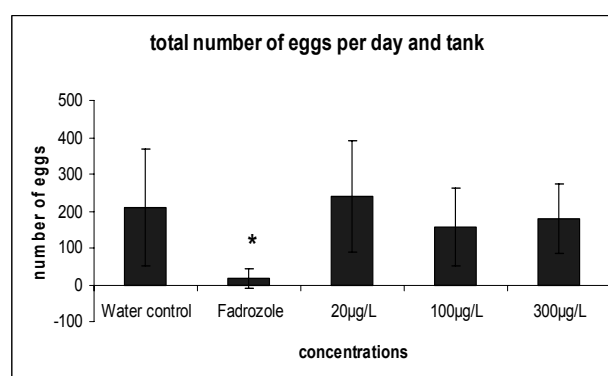
Fig. 4d: Total number of eggs of zebrafish per day and tank under exposure to prochloraz.
(Optional data of LAB 13)

93.   These figures are about optional data collected in LAB 12 and LAB 13. It confirms that the decrease in egg production was visible from the number of days with spawning as well as the number of eggs spawned (LAB 12 for mid- and top-concentrations and positive control and LAB 13 for positive control). When there was no clear decrease in the number of days with spawning (LAB 13 for prochloraz), the decrease in the total number of eggs was not obvious either.

94.   Fertility was not affected in any of the two studies (Figures 4a and 4c).

5.3.3   Spawning status of flutamide studies

5.3.3.1 Medaka

95.   Control groups spawned regularly throughout the 21-day study. Flutamide caused inhibition of egg production in a concentration-dependent manner in one study (LAB 3), whereas no clear treatment-related response could be observed in the other three studies.

Table 32: Spawning status in the flutamide studies with medaka during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days				
	Nominal concentrations			
Laboratories	Cont.	100 µg/l	500 µg/l	1,000 µg/l
LAB 3	15/21	14/21	11.5/21	8.5/21
LAB 5	21/21	13/21	13.5/21	21/21
LAB 4	21/21	20.5/21	21/21	21/21
LAB 6	19/20	17/20	18/20	18.5/20

Table 33: Daily recording of spawning status in medaka exposed to flutamide.

DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 4																						
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LAB 3																						
C		1	1	1	1	1	1	1	½	½	½	½	1	1	0	1	½	½	0	1	½	½
L		1	1	1	1	1	1	1	1	1	1	½	½	0	0	½	½	0	0	½	1	½
M		1	1	1	1	1	1	1	1	1	½	0	½	½	0	0	0	0	0	½	0	½
H		1	1	½	1	½	½	½	½	0	0	0	½	1	0	½	½	0	0	0	0	0
LAB 5																						
C	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	1	1	1	1	1	1	1	1	1	1	1	½	0	½	½	0	0	0	0	½	½	½
M	1	1	1	1	1	1	1	1	1	1	½	½	½	½	½	0	0	0	½	½	½	½
H	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

1: spawning observed in both replicate tanks
½: spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.
C: control; L: low concentration; M: medium concentration; H: high concentration

5.3.3.2 Fathead Minnow

96. Control fish did not spawn well throughout the 21-day study, most likely because of territorial behavior, which disturbed animals. Despite low spawning in the control groups, flutamide exposure inhibited eggs production at the highest concentration in all studies. This is consistent with previous findings (40) reported in the literature.

Table 34: Spawning status in the flutamide studies with fathead minnow during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days				
	Nominal concentrations			
Laboratories	Cont.	100 µg/l	500 µg/l	1,000 µg/l
LAB 7	15.5/21	13.5/21	15.5/21	6/21
LAB 10	5/21	1/21	2.5/21	2.5/21
LAB 4	5.5/21	2/21	0/21	0/21

Table 35: Daily recording of spawning status of fathead minnow exposed to flutamide.

DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 7																						
C	0	½	½	1	½	½	½	½	1	½	½	1	1	½	½	1	1	1	½	1	1	1
L	1	½	0	½	1	½	½	1	1	0	½	1	1	1	1	1	½	½	1	0	½	½
M	0	1	½	1	1	1	1	1	1	0	½	1	1	0	½	1	1	½	½	0	1	1
H	0	0	0	0	0	½	0	½	0	½	½	½	½	½	0	0	1	0	0	0	1	½
LAB 4																						
C	1	½	0	0	½	0	0	0	½	1	1	0	0	½	0	0	½	½	½	0	0	0
L	½	0	0	0	0	½	1	½	0	0	0	0	0	0	0	0	½	0	0	0	0	0
M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LAB 10																						
C	1	0	1	0	½	½	0	0	0	½	0	0	0	½	1	½	½	0	0	0	0	0
L	1	0	½	0	0	0	0	0	0	0	0	0	0	0	½	0	0	0	0	0	0	0
M	½	0	½	0	½	0	0	0	0	0	½	0	0	0	0	0	0	½	0	0	0	0
H	1	0	½	0	0	½	0	0	0	0	½	0	0	0	0	½	0	½	0	0	0	0

1: spawning observed in both replicate tanks

½: spawning observed in one of the replicate tanks

0: no spawning observed in any of the two replicate tanks.

C: control; L: low concentration; M: medium concentration; H: high concentration

5.3.3.3 Zebrafish

97.   Control fish spawned relatively well and regularly throughout the 21-day studies. No clear concentration-dependent response on spawning could be noted in any of the studies.

Table 36: Spawning status in the flutamide studies with zebrafish during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
	Nominal concentrations				
Laboratories	Cont.	SC [1]	100 µg/l	500 µg/l	1,000 µg/l
LAB 12	17.5/20	-	19/20	16.5/20	14.5/20
LAB 14	15.5/21	17.5/21	18.5/21	14/21	12.5/21
LAB 6	No data				

[1]: SC=solvent control

Table 37: Daily recording of spawning status in zebrafish exposed to flutamide.

DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 12																						
C		1	½	1	1	1	1	½	½	1	½	1	1	½	1	1	1	1	1	-	1	1
L		1	1	½	1	1	1	1	1	1	1	1	1	½	1	1	1	1	1	-	1	1
M		1	½	1	½	1	½	½	½	1	1	1	1	1	½	1	1	1	1	-	½	1
H		½	½	½	½	1	1	1	1	1	1	0	1	1	1	0	1	1	0	-	1	½
LAB 14																						
C		½	1	1	1	1	1	1	1	½	½	1	½	1	0	½	1	½	½	1	1	0
L		½	½	1	1	1	1	1	½	½	1	1	1	1	½	½	1	1	1	1	1	0
M		0	1	½	1	½	½	1	1	½	1	1	½	1	0	½	1	½	1	½	1	0
H		0	½	1	½	1	1	½	1	1	½	1	1	1	0	½	0	½	½	0	½	½

1: spawning observed in both replicate tanks

½: spawning observed in one of the replicate tanks

0: no spawning observed in any of the two replicate tanks.

C: control; L: low concentration; M: medium concentration; H: high concentration

LAB 14: semi-quantitative recording of egg counts.

C		0	1	3	4	3	1	2	1	0	0	2	0	2	0	0	2	0	0	3	3	0
		1	2	1	3	1	1	2	1	3	2	4	1	2	0	1	1	2	4	3	3	0
L		2	1	3	1	3	1	1	1	0	1	1	3	2	0	1	1	1	3	1	1	0
		0	0	3	3	4	1	4	0	4	3	3	3	2	3	3	3	3	4	4	3	0
M		0	2	0	1	4	2	2	4	1	3	2	3	1	0	0	3	1	3	2	4	0
		0	2	2	3	0	0	2	3	0	2	2	0	2	0	2	2	0	3	0	4	0
H		0	1	1	0	3	2	1	4	2	2	2	3	2	0	2	0	1	1	0	1	1
		0	0	2	4	3	2	0	3	1	0	2	3	1	0	0	0	0	0	0	0	0

Coding: 0: no egg; 1: <50; 2: 50-100; 3: >100; 4: >>100
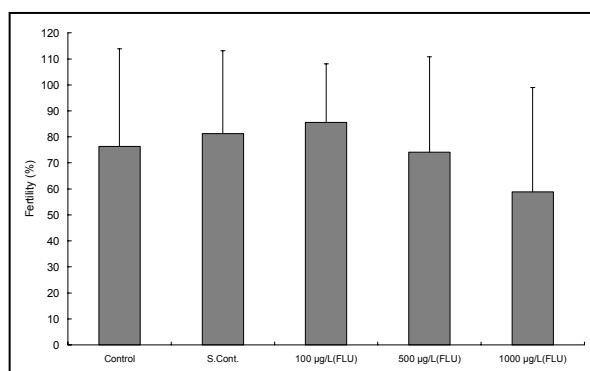
Fig. 5a: The means of fertility in zebrafish exposed to flutamide.
(Optional data of LAB 12)
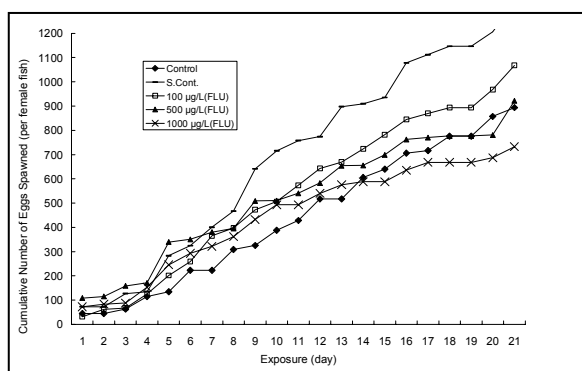
Fig. 5b: The cumulative number of eggs spawned in zebrafish exposed to flutamide.
(Optional data of LAB 12)

98. Under the optional data and further to counting days with spawning, LAB 12 quantitatively evaluated the presence of eggs throughout the study (Figure 5b) and did not report meaningful decrease in any of the treated groups. Only the highest treatment group (1000μg/l) had fewer eggs compared to the control group.
Fertility (Figure 5a) was not affected.

99. In a similar study on adult zebrafish (37) with comparable test concentrations of flutamide, reproductive performance was affected at 1000μg/l. through a significant reduction in the total number of eggs spawned (significant reduction in the number of clutches).

5.4 Secondary Sex Characteristics

5.4.1 Secondary sex characteristics of 4-tert-pentylphenol studies

5.4.1.1 Medaka

100. Secondary sex characteristics in medaka appear in males as papillary processes on the anal fin. They normally respond to androgenic stimulation and can be quantitatively evaluated.

101. No significant difference in the number of joint plate with papillary processes was found in male medaka exposed to 4-tert-pentylphenol compared to control, except in LAB 3 at 1000μg/l, which may be considered to be a false positive. In females, no papillary process was observed in any of the treatment groups, nor in the positive control.
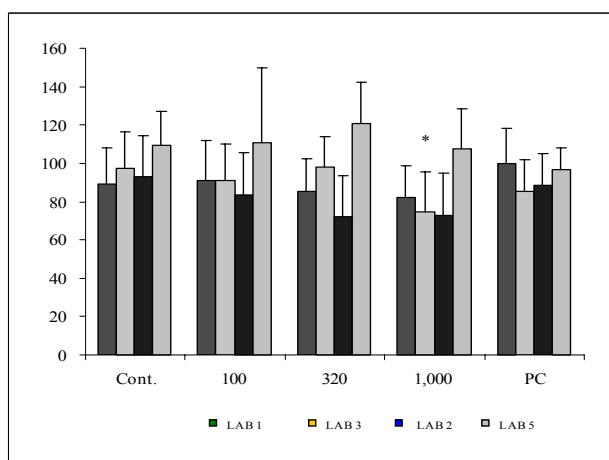
Fig. 6: Number of joint plate with papillary processes in male medaka exposed to 4-*tert*-pentylphenol.

102.   In a study by Seki *et al*. (25), papillary processes in male medaka responded to estrogenic exposure to 4*tert*-pentylphenol in a full life-cycle experiment. At 224 μg/l, males exposed from fertilisation until 101 days post hatch, showed a significant decrease of papillary processes. In Phase 1B, it may be that the assay is either too short to see a response on the secondary sex characteristics, or not including exposure during the life-stage that is most sensitive. The significant finding in LAB 2 may be considered to be a false positive, due to the absence of a dose-response trend.

5.4.1.2 Fathead Minnow

103.   In male fathead minnow the total score of nuptial tubercles decreased with increasing 4-*tert*-pentylphenol concentration, resulting in significant differences at 1,000μg/l in all three studies. LAB 9 appears to have had an absolute decrease that was not statistically significant with the 17β-estradiol; this is a false negative finding and unfortunately, the concentration of the positive control was not measured. No nuptial tubercle was observed in any of the treatment group or positive control in females.
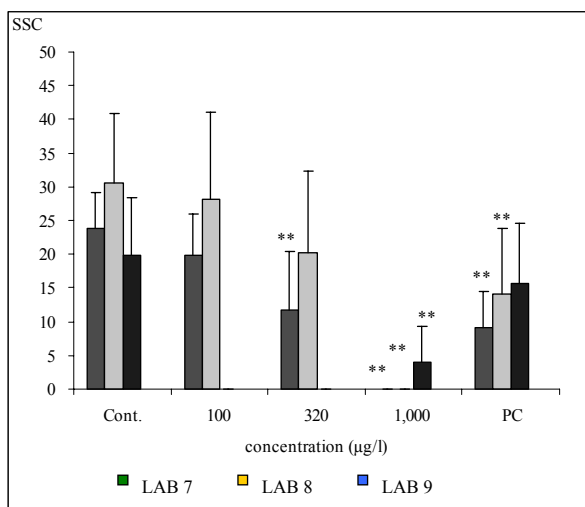
Fig. 7: Total score of the nuptial tubercles in male fathead minnow exposed to 4-*tert*-pentylphenol.

5.4.1.3 Zebrafish – not applicable

5.4.2   Secondary sex characteristics of prochloraz studies

5.4.2.1 Medaka

104.   The number of papillary process in male medaka did not respond to prochloraz exposure, or to fadrozole exposure in any of the studies. No papillary process was observed in any of the treatment groups or positive control in females. This is consistent with findings from the literature on fathead minnow exposed to aromatase inhibitor treatment (27)(28).
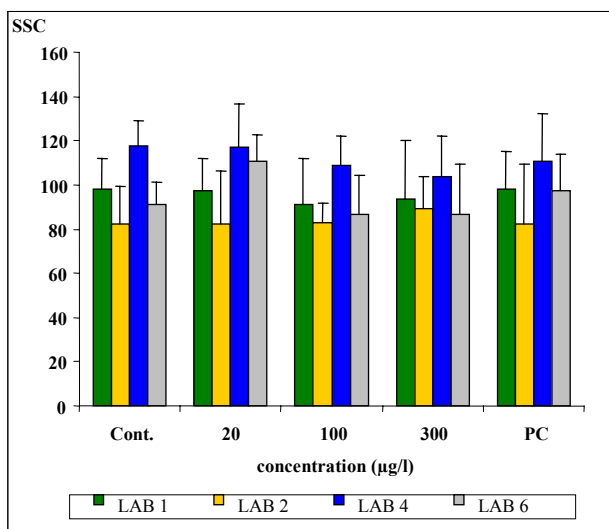
Fig. 8: Number of the joint plate with papillary processes in male medaka exposed to prochloraz.

5.4.2.2 Fathead Minnow

105.   In male fathead minnow exposed to prochloraz no clear response could be observed on the number of nuptial tubercles. This is consistent with previous findings from the literature (18)(19), where alterations in male secondary sex characteristics were not demonstrated following fadrozole exposure. This is because nuptial tubercles are under androgen control and there is no induction of androgen following aromatase inhibition. In females exposed to prochloraz, appearance of one tubercle was found occasionally in two studies.
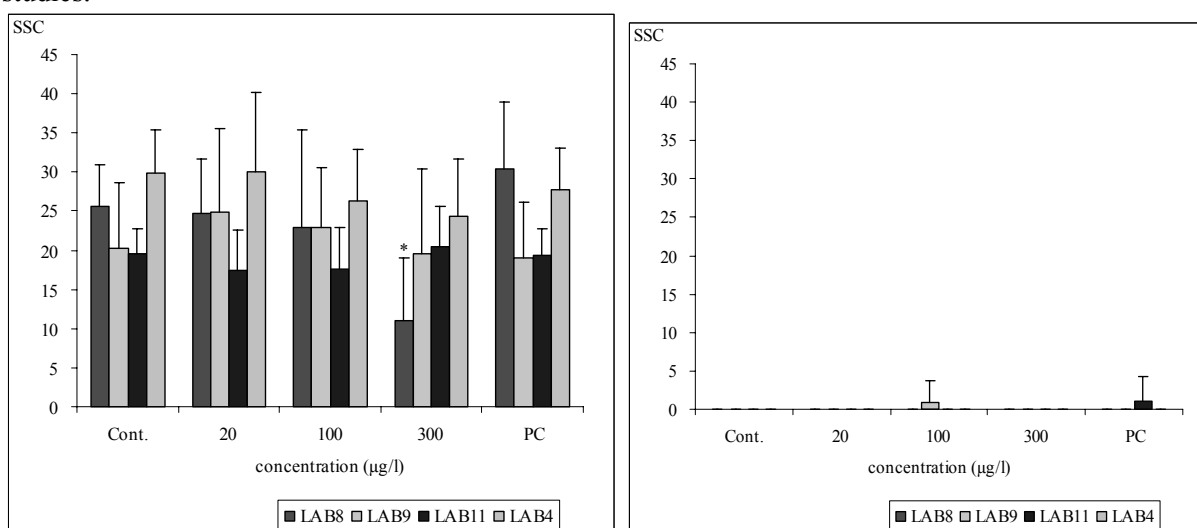
Fig. 9: Total score of the nuptial tubercles in male fathead minnow eposed to prochloraz.

5.4.2.3 Zebrafish – not applicable

5.4.3   Secondary sex characteristics of flutamide studies

5.4.3.1 Medaka

106.   No treatment-related response could be found on male medaka exposed to flutamide. No papillary process was observed in any treatment group or positive control in females.
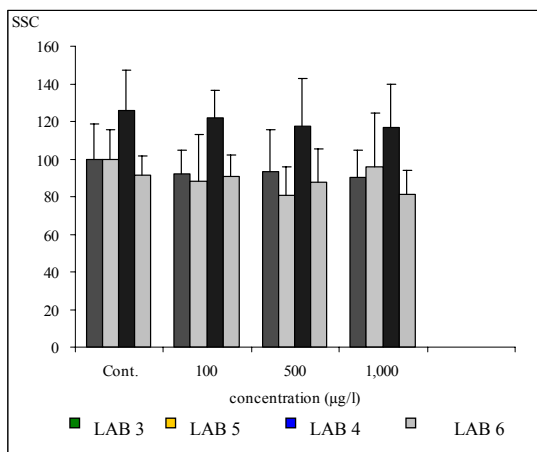
Fig. 10: Number of the joint plate with papillary processes in male medaka exposed to flutamide.

5.4.3.2 Fathead Minnow

107.   In male fathead minnow exposed to flutamide total score of nuptial tubercles significantly decreased at 1000 µg/l in one study, although the other two studies showed no significant difference. Nuptial tubercles were not observed in any of the treated females.
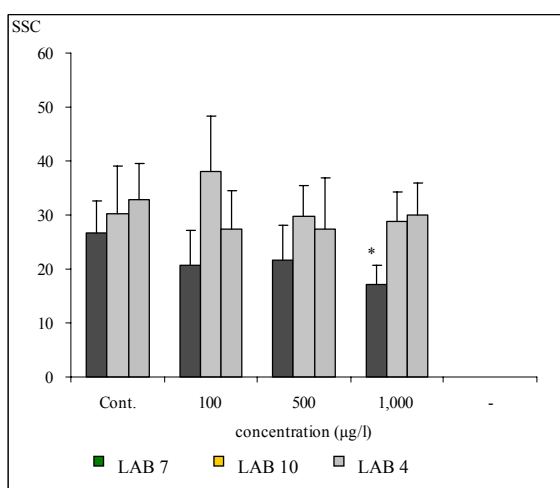
Fig. 11: Total score of the nuptial tubercles in male fathead minnow exposed to flutamide.

108.   Although secondary sex characteristics in fathead minnow are under the control of androgens, one possible reason for not always observing an anti-androgen type of response (e.g. decrease of nuptial tubercles number) was explained by Jensen *et al*. (32) due to the maintenance of peripheral androgen levels

by the males via feedback systems. Another study (27) reported a reduction in the number of nuptial tubercles in male's fathead minnows at 1000μg/l, and also noted that the inhibitory effect was expected to be more pronounced; however there seems to be a lower affinity of flutamide for the androgen receptor in fish than in mammals.

109.   There are at present very few studies with known mammalian anti-androgens, and it remains challenging to interpret study results. One study (38) suggested that the mammalian anti-androgen vinclozolin does not act as anti-androgen in fathead minnow because of low capacity to competitively bind to the androgen receptor. In the case of flutamide, its metabolite hydroxy-flutamide appears to have a greater binding affinity to the androgen receptor than the parent chemical (29).

5.4.3.3 Zebrafish – not applicable

5.5   Vitellogenin Analysis

5.5.1   *Vitellogenin analysis of 4-tert-pentylphenol studies*

5.5.1.1 Medaka

110.   In male and female medaka exposed to 4-*tert*-pentylphenol, hepatic VTG was induced in a concentration-dependent manner in all studies. Significant VTG increases compared to control were found in all exposed groups in both sexes, except in LAB 2 in females at 100 μg/l and in LAB 5 in females at all treatment levels -only the positive control was statistically significant.

111.   In LAB 5 in males, although Figure 12a seems to indicate a large increase of VTG in males at the lowest concentration, it is not statistically significant because only one animal had a high VTG level compared to the others.

112.   In LAB 5 in females, statistical significance was not achieved despite a dose-dependent increase of VTG, probably due to a high variability of measurements within each group, combined with a reduced sample size because of mortality. Evaluation of variability within and across laboratories is provided in Section 6.
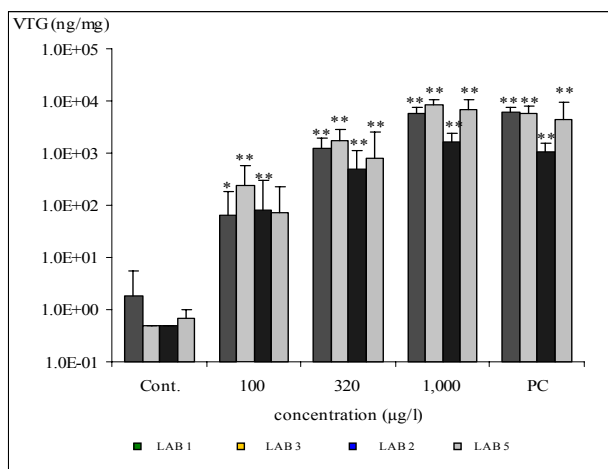
Significant level *:5% **:1%

Fig. 12a: VTG in male medaka exposed to 4-*tert*-pentylphenol.
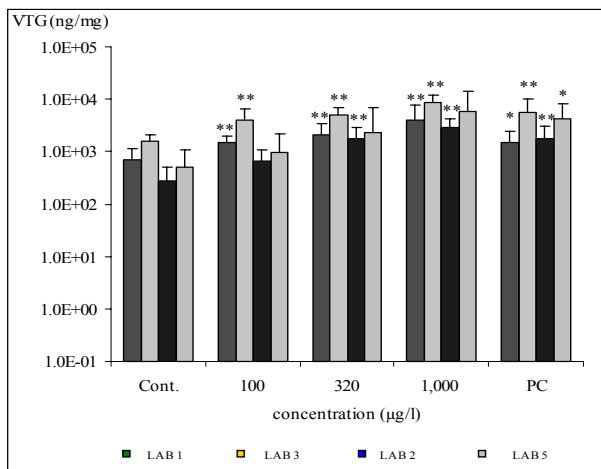
Significant level *:5% **:1%

Fig. 12b: VTG in female medaka exposed to 4-*tert*-pentylphenol.

5.5.1.2 Fathead Minnow

113. Due to a problem in LAB 9 with the test substance dispenser in the last hours of the experiment, resulting in either mortalities on day 20 of the experiment (mid-dose group) , or in non-exposure in the last 15 hours (high dose group), VTG results do not appear in the following graphs.

114. In male fathead minnow exposed to 4-*tert*-pentylphenol serum VTG was induced in a dose dependent manner at ≥ 320 µg/l, resulting in significant differences at this and higher concentrations. In females, VTG levels also increased in the 4-*tert*-pentylphenol the highest treatment group (in one of the two laboratories) and positive control.
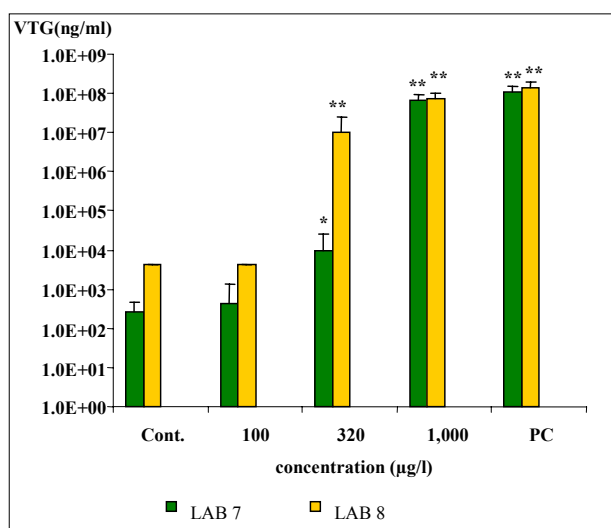
Fig. 13a: VTG in male fathead minnow exposed to 4-*tert*-pentylphenol.
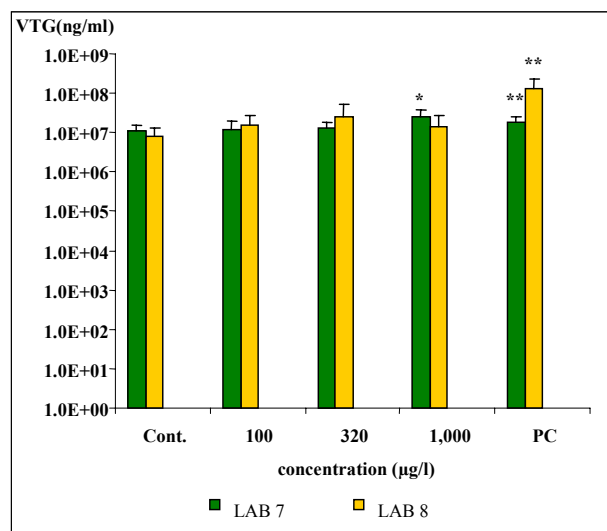
Fig. 13b: VTG in female fathead minnow exposed to 4-*tert*-pentylphenol.

5.5.1.3 Zebrafish

115. In male zebrafish (Figure 14a) serum VTG was induced with increasing 4-*tert*-pentylphenol concentration higher than 320µg/l. Although VTG levels increased in females, it was less pronounced than in males, due to high background level in control animals.

116. It is worth noting that LAB 14 had very low measured concentrations of the test substance: 23%, 22% and 47% of nominal values respectively. The highest measured concentration was 473 µg/l and induced a significant increase of VTG in male zebrafish in LAB 14. These low concentrations combined with a relatively high VTG value in control prevented the detection of statistically significant increase of VTG in at 320µg/l in LAB 14 in male zebrafish exposed to 4-tert-pentylphenol. It should be noted that for the positive control exposure in males, the value produced by LAB 14 can be considered as a false negative.
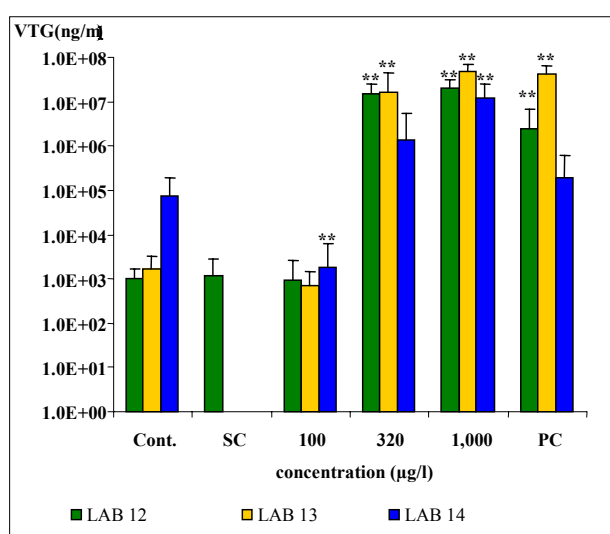
Fig. 14a: VTG in male zebrafish exposed to 4-*tert*-pentylphenol.
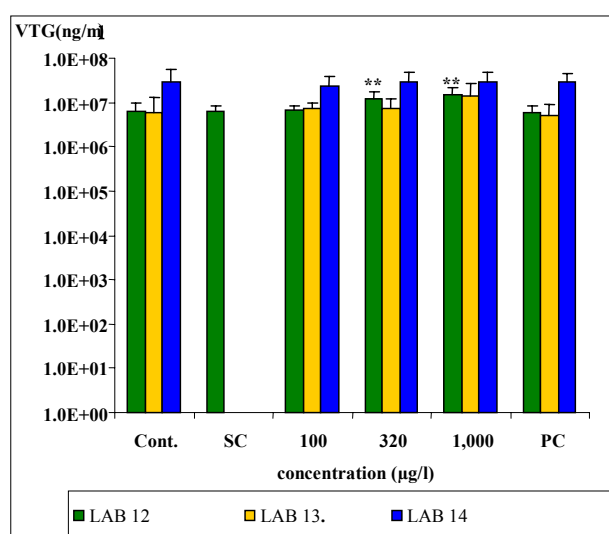
Fig. 14b: VTG in female zebrafish exposed to 4-*tert*-pentylphenol.

5.5.2   *Vitellogenin analysis of prochloraz studies*

5.5.2.1 Medaka
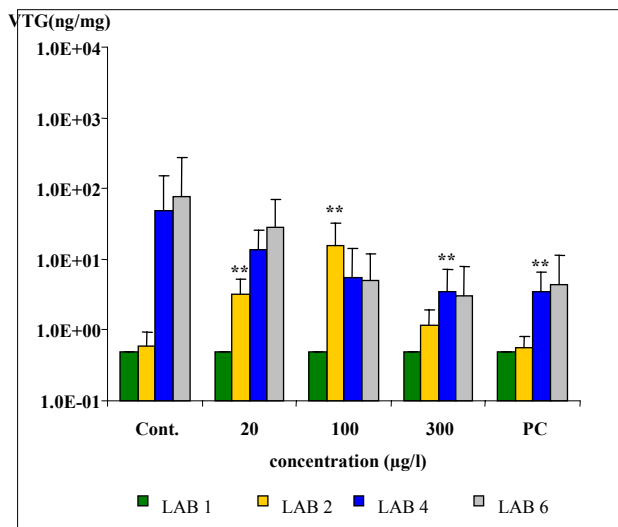
Fig. 15a: VTG in male medaka exposed to prochloraz.
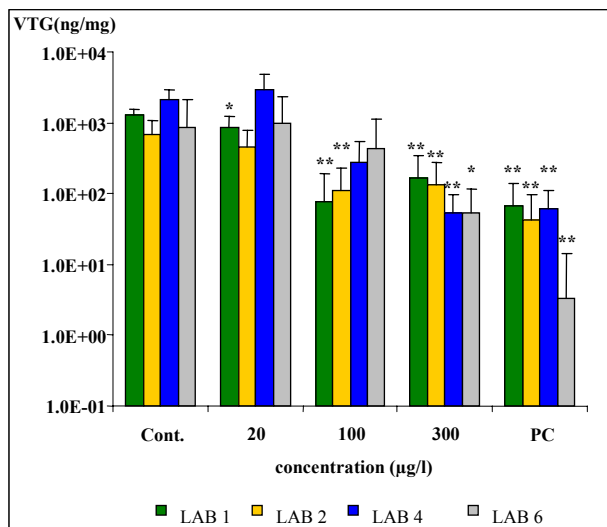
Fig. 15b: VTG in female medaka exposed to prochloraz.

117.   In male medaka exposed to prochloraz no consistent dose-dependent response could be observed on VTG levels across laboratories, although several significant differences were found. In female medaka VTG concentrations decreased dose-dependently in all studies.

118.   Measured concentrations in LAB 6 were only 34%, 54% and 72% of nominal values whereas other laboratories maintained concentrations close to 100% niminal values. These low concentrations in LAB 6 could explain the lack of statistically significant deacrease of VTG in females at 20 μg/l and 100 μg/l prochloraz.

119.   Decrease in vitellogenin level in females and cessation of spawning are consistent with the aromatase inhibition mode of action, where conversion of testosterone to 17beta-estradiol is inhibited, thus preventing vitellogenin production and subsequent egg production.

120.   It is worth noting that for low VTG values (Figure 15a), absolute numbers varied between LAB 1 and LAB 2 (Japanese laboratories) and LAB 4 and LAB 6 (US and European laboratories).

5.5.2.2 Fathead Minnow
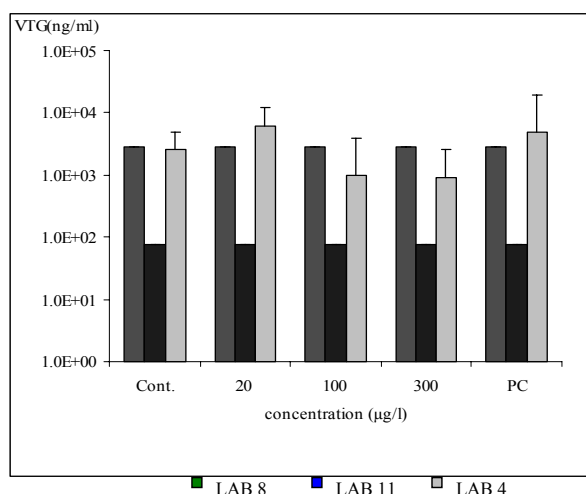
Fig. 16a: VTG in male fathead minnow exposed to prochloraz**.**
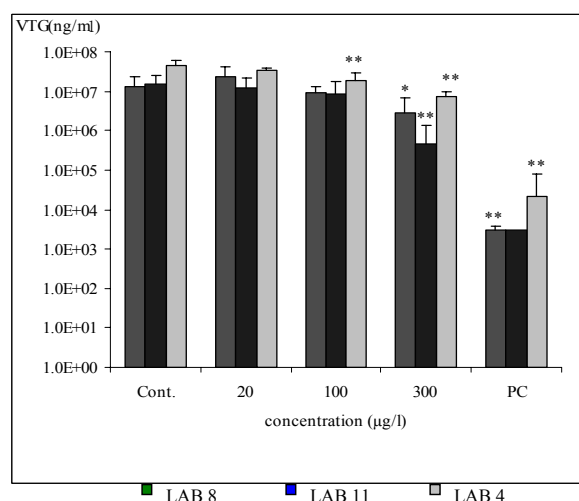
Fig. 16b: VTG in female fathead minnow exposed to prochloraz.

121.   In male fathead minnow exposed to prochloraz no dose-dependent effect was observed. In female fish VTG concentrations decreased dose-dependently in all studies with prochloraz, resulting in significant difference in the highest treatment group in all studies. This is consistent with previous findings on the fathead minnow (18)(19) with fadrozole. Aromatase inhibitors block the conversion of testosterone and to 17beta-estradiol in females, thereby preventing VTG production which is under estrogen control.

122.   In the positive control female group, statistical significance was not achieved in LAB 11 because calculations were made only on one fish; there were problems in determining precisely VTG concentrations in other animals. However, there was a consistent finding with the test compound at the high dose.

5.5.2.3 Zebrafish
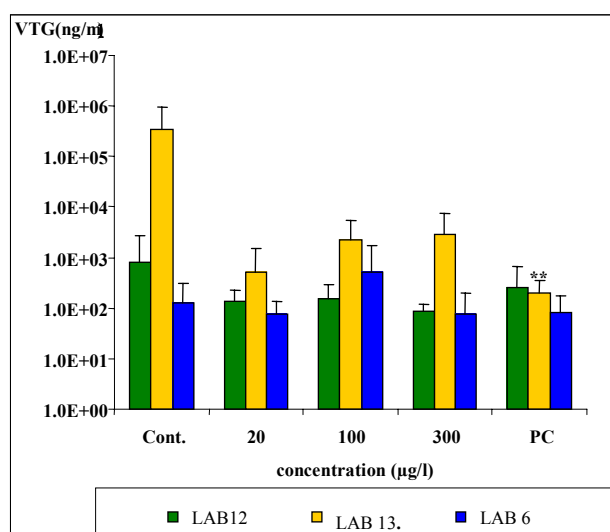
Fig. 17a: VTG in male zebrafish exposed to prochloraz.
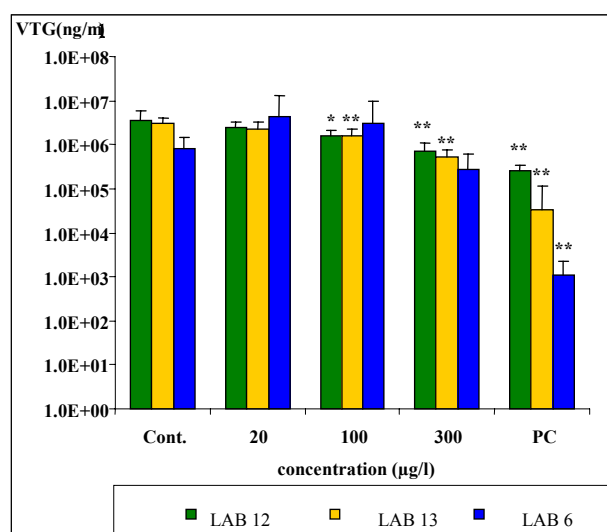
Fig. 17b: VTG in female zebrafish exposed to prochloraz.

123.   In male zebrafish exposed to prochloraz no significant difference could be observed, although high VTG level was detected in the control of one laboratory.

124.   Vitellogenin levels in females' zebrafish decreased dose-dependently. At 100μg/l, 2/3 laboratories detected a significant decrease (LAB 12 and LAB 13). In LAB 6, low measured concentrations, (34%, 54% and 72% of nominal values), combined with a relatively lower mean VTG level in the control group compared to other studies, may have posed a problem for a significant detection of decrease VTG at 100μg/l and 320μg/l. This is a false negative.

125.   In the males, the relative high VTG values in the controls from LAB 13 (possibly due to an outlier) could explain the significant decrease in the positive control; this relates to a false positive, although the trend (decrease) is consistent with the dose-response in females.

5.5.3   Vitellogenin analysis of flutamide studies

5.5.3.1 Medaka

LAB 3   LAB 5   LAB 4   LAB 6   LAB 3   LAB 5   LAB 4   LAB 6
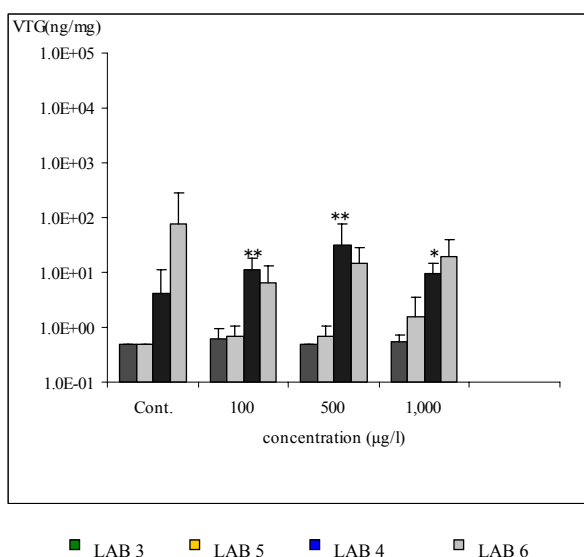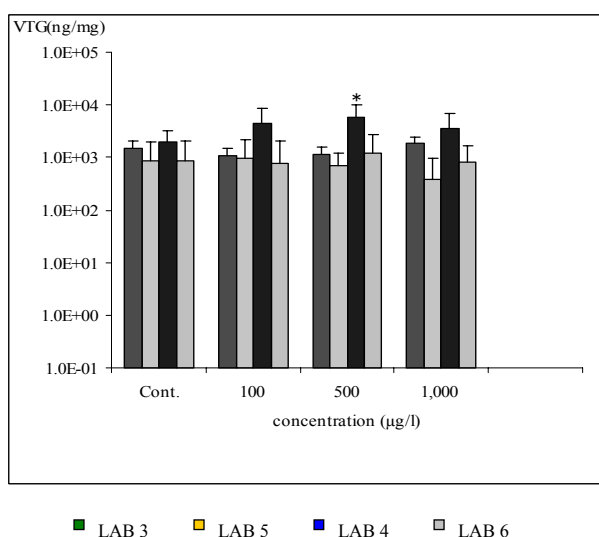
Fig. 18a: VTG in male medaka exposed to flutamide   **Fig. 18a:** VTG in female medaka exposed to flutamide.

126.   One study (LAB 4) out of four detected a significant increase of vitellogenin in both males and females. No dose-related trend in VTG response could be observed in any of the three other studies. It is not clear whether the significant findings from LAB 4 can or cannot be considered as false positives. The mechanistic basis for vitellogenin induction is not under direct control of the androgen receptor; flutamide might act via indirect endocrine pathways, thereby creating subtle effects not always detectable. The analytical chemistry showed measured concentrations close to nominal ones, except in LAB 6 (56%, 44% and 55% of nominal values).

5.5.3.2 Fathead Minnow
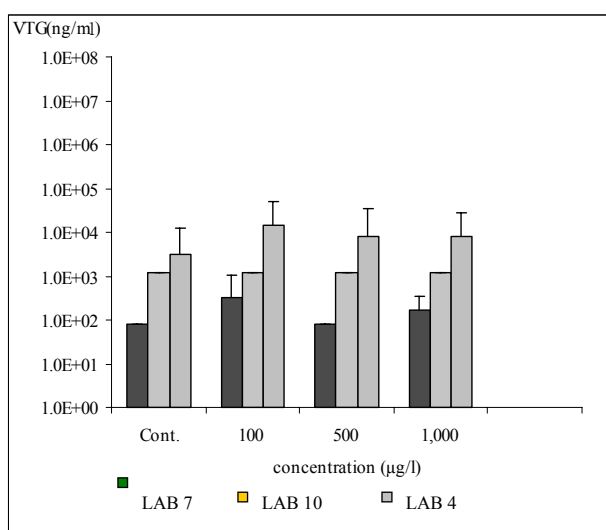
Fig. 19a: VTG in male fathead minnow exposed to flutamide
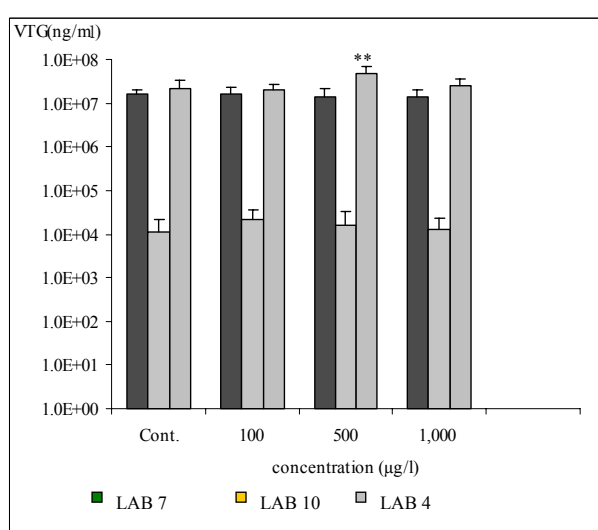
Fig. 19a: VTG in female fathead minnow exposed to flutamide

127.   No dose-dependent effect could be found in either male or female fathead minnows exposed to flutamide conducted in two submitted studies. Only one study reported a significant increase of VTG in females at the mid-concentration, but because it was not reproduced at the top-dose, it can hardly be taken as a really meaningful result. This isolated significant finding might be considered as a false positive finding; however, current knowledge on anti-androgenic effects in fish from the literature do not allow at this stage to make firm conclusions. All measured concentrations were within the 80%-120% range.

5.5.3.3 Zebrafish
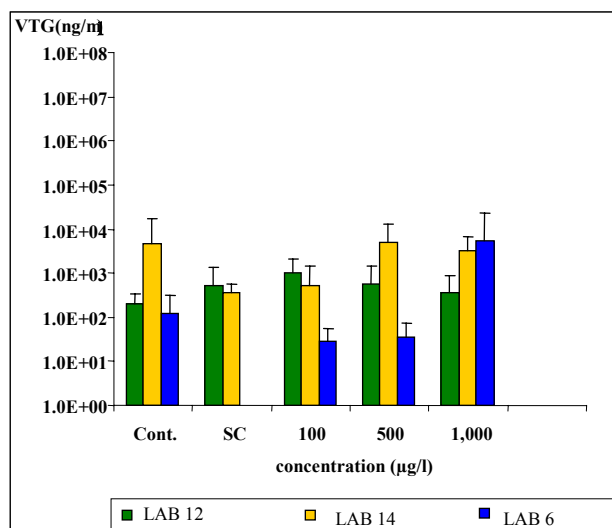
Fig. 20a: VTG in male zebrafish exposed to flutamide
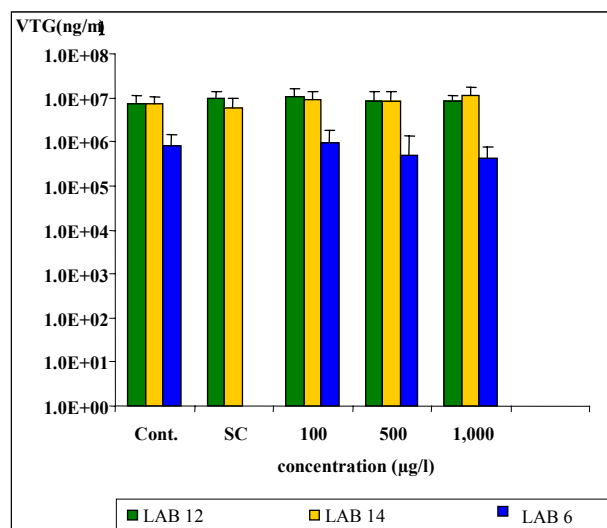
Fig. 20b: VTG in female zebrafish exposed to flutamide

128.   In all studies, neither significant difference nor concentration-dependent response on VTG level could be observed in either male or female of zebrafish exposed to flutamide.

5.6   Histopathology

5.6.1   *Histopathology introduction*

129.   The purpose of histopathology is to identify morphologic differences between unexposed (negative control) and compound-exposed animals, and to determine, to the extent possible, if such differences can be attributed to the test compound. As a bioassay endpoint, histopathology offers several important advantages. Perhaps most significantly, it provides a unique opportunity to evaluate changes, some of which may be subtle, directly within the tissues of interest. Histopathology employs established methodology for the generation of microtomed sections on glass slides, the data is reviewable due to the permanent nature of these slides, and the results of histopathologic examinations are routinely accepted as prima facie evidence of toxicological effect by regulatory agencies. An underappreciated benefit of histopathology is the ability to assess causes of inadvertent morbidity and mortality.

130.   As mentioned in Section 4.15, a workshop-type meeting was held in Heidelberg, Germany in November, 2004, to evaluate the histopathology results. Among others, the meeting participants included ten of the eleven pathologists who performed or supervised the microscopic examinations for these studies (pathologist "E" was not present). Objectives of this meeting were: 1) to determine whether histopathology can be a sensitive, discriminating, reliable, and cost-effective endpoint as part of a reproductive screening assay in fish; 2) to establish consensus findings for each combination of chemical, species, and sex; and 3) to further refine histopathological procedures within the context of the screening assay, primarily via future modifications to the draft guidance document. The agenda of the Heidelberg meeting included: 1) PowerPoint presentations by eight of the pathologists, representing all fourteen laboratories, in which exposure-related findings and other selected findings were described and illustrated; 2) presentation and discussion of the Phase 1B results as collected in Excel spreadsheets; 3) a discussion of deficiencies in, and suggested improvements for, the guidance document; 4) a limited glass slide review of pertinent histopathologic findings; 5) the formation of consensus opinions and recommendations for the VMG-eco group.

131.   In order to assess the reliability of histopathology, the Heidelberg pathologists endeavored to identify similarities and discrepancies among the Phase 1B results as generated by the various laboratories and pathologists. During the meeting it was recognized that interstudy discrepancies could either be "genuine" or "artificial". Genuine discrepancies are those in which the test subjects from different studies react differently when exposed to the same chemical. Genuine discrepancies might occur, for example, due to interstudy differences between nominal and actual dosages of the test compound, differences in the sources and/or ages of the test fish, differences in early husbandry, reproductive cycle variability, or general biological variability. Artificial discrepancies are those in which the test subjects from different studies react similarly when exposed to the same chemical, but the findings are assessed differently by different pathologists. Artificial discrepancies can occur due to interstudy differences in histologic slide quality, diagnostic terminology, lesion observation, or lesion interpretation. Although it may not be possible to assign every inconsistent result to one of these two categories, this goal should be achievable in many instances.

5.6.2   *Some comments pertaining to the histopathological results*

132.   To promote transparency, and a universal understanding of the criteria that the pathologists used to form their consensus findings and conclusions, the results in this report will be presented as "Pre-Heidelberg" and "Post-Heidelberg". The post-Heidelberg results will also contain discussions of the individual findings, whereas general aspects of the histopathology endpoint will be addressed in the Discussion section.

133. For the purpose of results reporting, the term "exposure-related" will be used to signify a finding in which there is a substantial difference in the incidence of this finding in one or more compound-exposed groups as compared to the negative control group; however, findings will not be reported as exposure-related if this difference in incidence only occurs in the low and/or mid dose groups compared to controls, but not in the high dose group. As used in the context of this report, the term "exposure-related" will not automatically indicate that a "cause-and-effect relationship" exists between test compound activity and a specific finding.

134. By convention, the term "control", when used alone, will be used as an abbreviation for the non-exposed control group.

135. Concerning the gonad staging data, only results that are ≥ 1 whole unit will be reported as a significant change (increased or decreased).

136. To facilitate a comparison of the results as observed by the different pathologists, Tables 38 and 39 link the various pathologists (designated symbolically as A through K) with the fish species and chemical(s) that they evaluated, and with their laboratory affiliations for this project (designated numerically as 1 through 14).

Table 38. Phase 1B pathologist assignments for various studies.

Laboratory	Test substance		
	4-tert pentylphenol	Prochloraz	Flutamide
Medaka			
LAB 1	Pathologist H	Pathologist H	
LAB 3	Pathologist E		Pathologist E
LAB 2	Pathologist B	Pathologist B	
LAB 5	Pathologist K		Pathologist K
LAB 4		Pathologist K	Pathologist K
LAB 6		Pathologist I	Pathologist I
Fathead minnow			
LAB 7	Pathologist C		Pathologist C
LAB 8	Pathologist F	Pathologist F	
LAB 9	Pathologist K	Pathologist K	
LAB 10			Pathologist K
LAB 11		Pathologist J	(Invalid study)
LAB 4		Pathologist K	Pathologist K
Zebrafish			
LAB 12	Pathologist G	Pathologist G	Pathologist G
LAB 13	Pathologist A	Pathologist A	
LAB 14	Pathologist D		Pathologist D
LAB 6		Pathologist I	Pathologist I

Table 39. Types and numbers of studies evaluated by different Phase 1B pathologists.

PATHOLOGIST	LABORATORIES	SPECIES	CHEMICALS	NUMBER OF STUDIES
A	13	zbf	4tPP, Prochloraz	2
B	2	jmd	4tPP, Prochloraz	2
C	7	fhm	4tPP, Flutamide	2
D	14	zbf	4tPP, Flutamide	2
E	3	jmd	4tPP, Flutamide	2
F	8	fhm	4tPP, Prochloraz	2
G	12	zbf	4tPP, Prochloraz, Flutamide	3
H	1	jmd	4tPP, Prochloraz	2
I	6	jmd, zbf	Prochloraz, Flutamide	4
J	11	fhm	Prochloraz	1
K	4, 5, 9, 10	jmd, fhm	4tPP, Prochloraz, Flutamide	9

5.6.3   Histopathology results and discussion of findings

5.6.3.1 4tPP (with 17β-estradiol positive control)

<u>4tPP and Medaka</u>

137.   4tPP (and 17β-estradiol) studies in medaka were performed in four laboratories (Labs 1, 2, 3, and 5) and read by four pathologists (Pathologists H, B, E, and K, respectively). "Pre-Heidelberg" results are presented in Tables 40-42.

Table 40: Staging data for male and female medaka exposed to 4-tert-pentylphenol

Dose	MDK-Male							
	LAB 1		LAB 2		LAB 3		LAB 5	
	Median of staging	N	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	3	10	2	9
L	1	10	2	10	2	10	2	98
M	1	10	2	10	2	10	2	9
H	2	10	2	10	2	9	3	8
P	1	10	2	10	2	10	3	6
Dose	MDK-Female							
	LAB 1		LAB 2		LAB 3		LAB 5	
	Median of staging	N	Median of staging	N	Median of staging	N	Median of staging	N
C	2	9	3	10	0.5	10	2	11
L	2	10	3	10	1	10	2	7
M	2	10	2.5	10	1.5	10	2	7
H	2	10	1	10	0.5	10	1	7
P	2	10	3	10	1.5	10	1	7

C: water control; L: low concentration (100 µg/l); M: medium concentration (320 µg/l);
H: high concentration (1000 µg/l); PC: positive control (100 ng/l).

Table 41: Histopathological findings in male medaka exposed to 4-tert-pentylphenol.

Diagnosis	Dose	LAB 1			LAB 3			LAB 2			LAB 5		
		Average of Grade	N	Obser.	Average of Grade	N	Obser.	Average of Grade	N	Obser.	Average of Grade	N	Obser.
Increased cells - ISC	C												
	L										1	1	9
	M										1	1	9
	H												
	P												
Increased cells - SPA	C										2	2	9
	L										3	1	9
	M										2.7	3	9
	H	1	1	10	1.8	4	10				2.7	3	8
	P				1.5	4	10				2.5	4	6
Increased cells - SPZ	C										1.5	2	9
	L										1	1	9
	M										1	2	9
	H										1.3	6	8
	P										1.7	6	6
Decreased cells - SPA	C												
	L												
	M				1	1	10						
	H				1.5	2	10						
	P												
Decreased cells - SPC	C												
	L												
	M												
	H				1.8	4	10						
	P	3	1	10	1.8	4	10						
Decreased cells - SPT	C												
	L												
	M												
	H				1.8	4	10						
	P	3	1	10	1.8	4	10						
Decreased cells - SPZ	C												
	L												
	M										2	1	9
	H				1	1	10						
	P	3	1	10	1.8	4	10						
Testis-ova	C												
	L												
	M												
	H	1	1	10	1	2	10						
	P	3.3	6	10	2.8	4	10	1	1	10			
Testicular degeneration	C				1	1	10						
	L				1	2	10				2	2	9
	M				1	5	10				1.7	3	9
	H	1.4	7	10	1.9	9	10				1.8	6	8
	P	1.5	8	10	2	9	10				1.8	4	6
Asynchronous development, gonad	C												
	L												
	M										1	1	9
	H										1	1	8
	P										1.5	2	6
Asynchronous development, spermatocyte	P										2	1	6
Proteinaceous fluid(intravascular)	C				1	3	10				1	2	9
	L												
	M				1	3	10				1	2	9
	H	2	2	10	1.9	10	10				1.5	4	8
	P	1.5	2	10	1.8	10	10						
Proteinaceous fluid(interstitial)	C												
	L												
	M												

Finding													
	H				1.7	7	10						
	P	1.5	2	10	1.1	9	10						
Nephropathy	C												0	5
	L												0	2
	M										1	2	2
	H										1.8	5	6
	P										2.5	4	4
Interstitial fibrosis	C							2.5	2	10			
	L							2.3	3	10			
	M	1.5	2	10	1	1	10	2	1	10			
	H	1.8	5	10	2	4	10	3.3	3	9			
	P	1.9	7	10	1.7	6	10	3	4	10	1	5	6
Granulomatous inflammation	C										2	1	9
	L												
	M												
	H												
	P										1	1	6
histiocytic cells(intraluminal)	C												
	L	1	1	10	1.5	2	10						
	M				1.3	4	10				1	1	9
	H	1.3	6	10	1.2	5	10				1	1	8
	P	1.9	7	10	2.1	9	10						

C= control; L= low concentration; M=medium concentration; H= high concentration; P= positive control; ISC: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Shaded cells: exposure- related findings.

<u>4tPP and Male Medaka – "Pre-Heidelberg</u>

138.   There were no exposure-related findings that were consistent among all four laboratories. A finding that was exposure-related in the high dose and positive control groups in three of four laboratories (Labs 1, 3, and 5) was testicular degeneration. Two findings that were exposure-related in the high-dose and positive control groups in two of four laboratories (Labs 1 and 3) and these were interstitial fibrosis and intraluminal histiocytic cells. Interstitial fibrosis was also observed as an exposure-related finding in the positive control group of Lab 5. Testis-ova were exposure-related findings in the positive control groups in Labs 1 and 3, and were present as low level occurrences (i.e., non-exposure-related findings) in the high dose groups of Labs 1 and 3, and in the positive control group of Lab 2. Findings that were exposure-related in a single laboratory included: increased spermatogonia, decreased spermatocytes, and decreased spermatids in the high dose and positive control groups (Lab 3); decreased spermatozoa in the positive control group (Lab 3); proteinaceous intravascular and interstitial fluid in the testis (Lab 3); increased spermatozoa in the high dose and positive control group (Lab 5); and nephropathy and increased renal hematopoietic tissue in the high dose group (Lab 5). . Average severity scores of exposure-related findings were generally low (i.e., ≤ 2.0); exceptions included testis-ova, interstitial fibrosis (Lab 5 only), nephropathy (positive control group only) and increased renal hematopoietic tissue. Median testicular staging scores were generally increased (Lab 5), decreased (Lab 3), or stayed approximately the same (Labs 1 and 2) in the exposed animal groups as compared to the control group.

<u>4tPP and Male Medaka – "Post-Heidelberg</u>

139.   Testicular degeneration was a solid exposure-related finding for three of the four laboratories, occurring in both the high dose and positive control groups. Interstitial fibrosis was also exposure-related in those same three laboratories (albeit only in the positive control group for Lab 5). The fourth laboratory (Lab 2) generated relatively few findings for male medaka in either of the two experiments in which they participated. Testicular degeneration was also an exposure-related finding in male fathead minnow, but not zebrafish. Despite numerous studies of estrogenic substances in medaka, it is difficult to find previous histopathologic descriptions pertaining to adult fish. Most experiments are concerned with developing juvenile medaka, and the overwhelming reported effects of estrogenic agonists in developing male medaka are alterations in sex ratio and testis-ova (25).

140.   Testis-ova were observed in the high dose and/or positive control groups from three of four laboratories Labs 1, 2, and 3, but not Lab 5), but only as an exposure-related finding (based on incidence criteria) in two laboratories (Labs 1 and 3). There is anecdotal evidence to suggest that the propensity for testis-ova formation (spontaneous or induced) in adult males can vary from laboratory to laboratory, possibly due to differences in the source of the fish and/or husbandry practices. To a limited extent, this hypothesis is supported by the overall Phase 1B data; testis-ova were found as spontaneous and/or induced findings in only 50% of the laboratories: Labs 1 (medaka), 2 (medaka), 3 (medaka), 4 (medaka), 6 (medaka, zebrafish), 8 (fathead minnow), and 12 (zebrafish). These differences did not appear to be pathologist-dependent: for example, Pathologist K did not diagnose testis-ova in any of the male medaka from Lab 5; however, that same pathologist did report spontaneous testis-ova formation in male medaka in two studies from another laboratory (Lab 4).

141.   One laboratory (Lab 5) reported two exposure-related findings in the kidneys of male medaka exposed to 4tPP: nephropathy and increased renal hematopoietic tissue. Due to the sectioning method, kidney tissue was not available for evaluation for every male fish; the actual incidence of nephropathy in Lab 5 male medaka was 8/9 (89%) of examined kidneys from fish of the mid dose, high dose and positive control groups combined (100% in the high dose and positive control groups combined), whereas the incidence in the control and low dose groups was 0/5 (0%). At the Heidelberg meeting, it became apparent that some pathologists had focused their attention principally on the gonads during their initial examinations, and had not specifically evaluated non-gonadal tissues such as the kidneys. In fact, during the limited slide review conducted at the meeting, it was observed that qualitatively similar kidney findings were qualitatively present in at least the high dose group males from laboratories other than Lab 5. Comparable renal responses to exogenous estrogen exposure have been reported for several species, including fathead minnow (39), sheepshead minnow (40), and rainbow trout (41). A commonly proposed mechanism for these renal changes is that exogenous estrogens induce excessive (for males) vitellogenin production by the liver, and the resulting elevated plasma vitellogenin levels cause a protein overload of the kidney (41). Two results of Lab 3 are supportive of this hypothesis: the high incidence exposure-related findings of increased proteinaceous intravascular and interstitial fluid in the testis.

142.   The results from Lab 3 included simultaneous changes in spermatogonia (increased) and spermatocytes, spermatids, and spermatozoa (all decreased). Because each of these diagnoses is used to indicate a proportional change in the cell type distribution within the germinal epithelium, an increase in spermatogonia would likely be associated with a decrease in at least two of the other three cell types (spermatocytes and spermatids, but not necessarily spermatozoa), and therefore, at least two of the these latter three findings are essentially redundant in this case.

143.   Gonad staging criteria for male medaka, as presented in the guidance document, were found by some pathologists to be unworkable and probably inaccurate, primarily due to microanatomical differences between medaka, which have a "restricted spermatogonial" type of testis, and the testes of the other two species, which are characterized by an "unrestricted spermatogonial" pattern of development. The pathologist associated with Lab 5, Pathologist K, therefore felt obligated to create a novel set of staging criteria for male medaka. This deviation from the standards set forth in the guidance document may have contributed to interlaboratory inconsistency in the staging data for this species and sex.

144.   Intraluminal histiocytic cells was an exposure-related finding for two of four laboratories (Labs 1 and 3). There does not appear to be an immediate, easily confirmed, explanation for a potential relationship between this diagnosis and exposure to an estrogen agonist. Although granulomatous inflammation was not evident in the fish from Labs 1 and 3, it is possible, if not likely, that increased histiocytic cells is a precursor of such inflammation. Granulomatous inflammation was observed as an exposure-related response to 4tPP exposure in male fathead minnow from one laboratory.

145.   The combination of exposure-related findings of increased spermatozoa (Lab 3) and decreased spermatozoa (Lab 5) is one of the few ostensibly contradictory sets of results in the Phase 1B study. Neither of these findings was exposure-related in the other two laboratories (Labs 1 and 2). A similar situation occurred in 4tPP-exposed male fathead minnow. One possible reason for these discrepancies is that in fractional spawners, the amount of spermatozoa present within the testis at any given time is a function of a variety of physiological and behavioral factors that govern sperm production, storage, and release; therefore, the influence of exogenous hormones on sperm concentration is likely to be less predictable than it is on sperm precursors (such as spermatogonia). On the other hand, because some changes in spermatozoa were exposure-related, an even more likely explanation is that certain pathologists gauged spermatozoa numbers according to the amount of area that they occupied (i.e., the relative sizes of tubule lumina and efferent ducts), whereas other pathologists estimated spermatozoa numbers according to sperm density. Concerning 4tPP exposure, one probable scenario is that an increased proportion of spermatogonia resulted in a thickening of the germinal epithelium, which was accompanied by a corresponding decrease in the size of seminiferous tubule lumina or efferent ducts, and an increase in the density of spermatozoa.

Table 42: Histopathological findings in female medaka exposed to 4-tert-pentylphenol.

Diagnosis	Dose	LAB 1			LAB 3			LAB 2			LAB 5		
		Average of Grade	N	Obser.	Average of Grade	N	Obser.	Average of Grade	N	Obser.	Average of Grade	N	Obser. r.
Increased cells - oogonia	C												
	L												
	M												
	H										2	1	7
	P												
Oocyte atresia, increased, immature	C				1	1	10	1	4	10	2	2	11
	L				2.3	4	10	1	1	10	1	2	7
	M	1	2	10	2.3	4	10	1.7	3	10	1.3	6	7
	H	1.67	3	10	2.6	8	10	1	3	10	1.6	7	7
	P	1	1	10	2.3	4	10				1.3	4	7
Oocyte atresia, increased, mature	C										2	1	11
	L	2	1	10	1	2	10						
	M	1	2	10				1	1	10			
	H	1	2	10	1	3	10	2.3	3	10	1.3	3	7
	P				2	1	10				1	1	7
Oocyte atresia, increased, late atretic	C										3	2	11
	L										1	1	7
	M										1	1	7
	H										1	1	7
	P										1	1	7
Proteinaceous fluid, interstitial	C												
	L	1.5	2	10	2	1	10						
	M	2	2	10	1.8	5	10	2	2	10	1.6	5	7
	H	1.8	4	10	2.4	8	10	1.5	2	10	1.8	4	7
	P	2	2	10	1.5	4	10	1	2	10	1.4	5	7
Interstitial fibrosis	C							2	1	10			
	L												
	M												
	H												
	P												
Granulomatous inflammation	C										2.3	6	11
	L										1.5	2	7
	M										2	5	7
	H										1	1	7
	P										2	3	7
Macrophage aggregates, increased	C												
	L				1	1	10						
	M				1.7	3	10						
	H				2.3	3	10						
	P												
Oocyte membrane folding	C												
	L												
	M												
	H							2	1	10			
	P												
Egg debris, oviduct	C												
	L				1	2	10						
	M				1	1	10						
	H				1	1	10						
	P				1	1	10						

C= control; L= low concentration; M=medium concentration; H= high concentration; P= positive control.
Shaded cells: exposure-related findings.

4tPP and Female Medaka – "Pre-Heidelberg

146. There were no exposure-related findings that were consistent among all four laboratories. A finding that was exposure-related in the mid dose, high dose, and positive control groups of two laboratories (Labs 3 and 5), and only the high dose group of a third laboratory (Lab 1), was interstitial proteinaceous fluid in the ovary. A finding that was exposure-related in two of four laboratories in the mid

and high dose groups (Lab 5) and high dose group (Lab 3) was increased atresia of immature oocytes. A finding that was exposure-related in a single laboratory (Lab 5) in the high dose and positive control groups was nephropathy. Average severity scores of exposure-related findings were generally low (i.e., ≤ 2.0); exceptions included oocyte atresia (Lab 3 only) and proteinaceous interstitial fluid in the high dose group (Lab 3 only). Median ovarian staging scores were generally: decreased in the high dose and positive control groups (Lab 5); decreased in the positive control group but not the high dose group (Lab 2); increased in the mid dose and positive control groups but not the high dose group (Lab 3), or stayed the same (Lab 1) in the exposed animal groups as compared to the control group.

4tPP and Female Medaka – "Post-Heidelberg"

147.   Interstitial proteinaceous fluid in the ovary appeared to be a clear exposure-related finding for three of four laboratories (Labs 1, 3, and 5, but not Lab 2).   For Lab 1, this finding was only exposure-related in the high dose group and not the positive control group; additionally, in the other two laboratories, the incidence and/or severity of this finding was lower in the positive control group as compared to the high dose group. This set of results suggests that 4-tPP may be slightly more potent than 17β-estradiol with regard to this effect (at the given exposure concentrations).

148.   Increased atresia of immature oocytes was a relatively strong exposure-related effect of 4tPP for two of four laboratories (Labs 3 and 5), and nearly an exposure-related effect for a third laboratory (Lab 1). Similar to the situation for interstitial proteinaceous fluid, the incidence and severity of increased atresia of immature oocytes were greater in the high dose groups as compared to the positive control groups.

149.   As for male medaka, nephropathy in female medaka was diagnosed only by the Lab 5 pathologist; however, it is highly probable that this finding was present in female medaka from the other laboratories.

150.   During the Heidelberg meeting it was determined that oocyte membrane folding should be considered as one potential component of oocyte atresia rather than as a separate stand-alone diagnosis.

4tPP and Fathead Minnow

151.   4tPP (and 17β-estradiol) studies in fathead minnow were performed in three laboratories (Labs 7, 8, and 9) and read by three pathologists (Pathologists C, F, and K, respectively). "Pre-Heidelberg" results are presented in Tables 43-45.

Table 43: Staging data for male and female fathead minnow exposed to 4-tert-pentylphenol

Dose	FHM-Male					
	LAB 7		LAB 8		LAB 9	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	3	10
L	2	10	2.5	10	-	
M	2	10	2	7	-	
H	3	2*	2	1	3	9
P	2	9	2	10	2	9

Dose	FHM-Female					
	LAB 7		LAB 8		LAB 9	
	Median of staging	N	Median of staging	N	Median of staging	N
C	3	10	3	10	3	10
L	2.5	10	3	10	-	
M	2	10	3	8	-	
H	2	10	4	6	3	7
P	3	10	3	10	4	10

C: water control; L: low concentration (100 µg/l); M: medium concentration (320 µg/l);
H: high concentration (1000 µg/l); PC: positive control (100 ng/l).
*7 additional fish were severely affected and could not be staged

Table 44: Histopathological findings in male fathead minnow following 4-tert-pentylphenol exposure

Diagnosis	Dose	LAB 8			LAB 7			LAB 9		
		Aver. Grade	N	Obser.	Aver. Grade	N	Obser.	Aver. Grade	N	Obser.
Increased cells – SPA	C	1	2	10				1	1	10
	L	1	1	10						
	M	2	2	9						
	H	2.8	5	5	3.3	7	9	2.3	7	9
	P	1.7	7	10				1.7	6	9
Increased cells – SPZ	H				4	6	9			
Decreased cells - SPC	C									
	L									
	M	2.8	4	9						
	H	3.6	5	5	3.4	7	9	2.25	4	9
	P	2.3	7	10				2	2	9
Decreased cells - SPT	C									
	L									
	M	3.4	5	9						
	H	3.8	5	5	3.3	7	9	2.3	4	9
	P	3.5	8	10				2	2	9
Decreased cells - SPZ	C	2	2	10				3	1	10
	L									
	M	2.8	5	9						
	H	2.6	5	5						
	P	2.9	7	10						
Testis-ova	C									
	L									
	M									
	H									
	P									
Testicular degeneration	C							1.5	2	10
	L									
	M	1	1	9						
	H				4	7	9	1.3	6	9
	P	1.3	4	10				1	2	9
Asynchronous development, spermatocyst	C									
	L									
	M									
	H							2	1	9
	P									
Asynchronous development, gonad	C									
	L									
	M									
	H									
	P							2	1	9
Proteinaceous fluid, intravascular	H							1.3	4	9
	P	1.5	2	10				1.4	9	9
Proteinaceous fluid, interstitial	C	1.5	2	10						
	L	1.1	7	10						
	M	1.6	7	9						
	H	2.4	5	5						
	P	3.4	10	10						
Interstitial fibrosis	H							2	2	9
Granulomatous inflammation	C									
	L									
	M									
	H							1	1	9
	P							1	1	9
Histiocytic cells, intraluminal	C							1.8	4	10
	L									
	M									
	H							2.5	6	9
	P							1	1	9
	C							1	3	10

Retained peritoneal attachments	C				1	3	10
	L						
	M						
	H				1.2	6	9
	P				1.4	5	9

C= control; L= low concentration; M=medium concentration; H= high concentration; P= positive control; ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Shaded cells: exposure-related findings.

<u>4tPP and Male Fathead Minnow – "Pre-Heidelberg"</u>

152.   There were three findings that were exposure-related among all three laboratories and these were: increased spermatogonia in the high dose and positive control groups (Labs 8 and 9) or the high dose group alone (Lab 7); decreased spermatocytes in the high dose and positive control groups (Lab 8) or the high dose group alone (Labs 7 and 9); and decreased spermatids in the mid dose, high dose and positive control groups (Lab 8) or the high dose group alone (Labs 7 and 9). A finding that was exposure-related in the high dose group of one laboratory (Lab 7) and the positive control group of another laboratory (Lab 8) was testicular degeneration. Findings that were exposure-related in a single laboratory included: decreased spermatozoa in the mid dose, high dose, and positive control groups (Lab 8); intravascular proteinaceous fluid in the high dose and positive control groups (Lab 9); and interstitial proteinaceous fluid in the low, mid, and high dose, and positive control groups (Lab 8). Average severity scores of exposure-related findings were often moderately high (i.e., > 2.0) and tended to increase with increasing dose. The average severity of exposure-related findings was often greater in the high dose group compared to the positive control group. Median testicular staging scores were: increased in the high dose group (Lab 7, but n = 2); decreased in the positive control group (Lab 9); or generally remained the same (Lab 8) in the exposed animal groups as compared to the control group.

<u>4tPP and Male Fathead Minnow – "Post-Heidelberg"</u>

153.   Comparable changes in the distribution of spermatogenic cell types were observed in 4tPP-exposed male fathead minnow from all three laboratories, and these changes included increased spermatogonia, decreased spermatocytes, and decreased spermatids. Fish from Lab 8 were also identified as having decreased spermatozoa. As previously discussed, a proportional increase in spermatogonia is almost invariably accompanied by relative decreases in spermatocytes and spermatids, therefore, the additional diagnoses are most often redundant. Increased spermatogonia also occurred as an exposure-related finding in male medaka exposed to 4tPP, but only in one out of four laboratories. It may be relevant that a measurably increased proportion of spermatogonia was observed in a study of another estrogenic agonist, bisphenol A, in fathead minnow (42)

154.   Testicular degeneration was exposure-related (high dose or positive control group) in two out of three laboratories, and it was very nearly exposure-related in the third laboratory (high dose group). Testicular degeneration was a more dramatic exposure-related finding in male medaka exposed to 4tPP. Testicular degeneration, characterized by "a loss of germinal cells, degenerate spermatozoa and germ cell syncytia", was also observed in the testes of adult male FHM that were exposed to as little as 0.5 nM E2 for 14 days (43).

155.   Proteinaceous fluid (intravascular or interstitial) was an exposure-related finding for Labs 8 and 9, but not for Lab 7. It should be noted that unlike Labs 8 and 9, Lab 7 did not generate any findings of this sort for either male or female fathead minnow. It is also essential to understand that the precise location of this fluid, i.e., within thin-walled blood vessels or within the adjacent interstitial spaces, is relatively unimportant, and it is likely that this change actually occurred in both compartments to some extent. Thus these two diagnoses could be considered equivalent for the purpose of this assay.   This diagnosis is dependent on a histomorphologic alteration in the color and density of fluid that is presumed to be due to

increased vitellogenin production and secretion by the liver, concomitant with an inability to efficiently utilize or eliminate excess vitellogenin in male fish.

156.   Exposure-related findings in the kidneys of male and female medaka (nephropathy, increased renal hematopoietic tissue) were not reported for fathead minnow. For at least two of the laboratories (Labs 7 and 9), this may have been due to the fact that the kidneys could not be evaluated because the gonads were excised, and the remaining contents of the carcass were not examined histologically. The third laboratory (Lab 8) deviated from the protocol by sectioning the fish transversely; in their experiment, nephropathy was diagnosed in both control and compound-exposed fish, but this finding was not found to be exposure-related.

157.   Unlike the situation for male medaka, interstitial fibrosis was not an effect of 4tPP or 17β-estradiol exposure in male fathead minnow. This may be due, at least in part, to interspecies differences in the testis microanatomy. The interstitium tends to be more pronounced in medaka compared to fathead minnow or zebrafish; therefore, it may be that fibrosis in that location is more readily appreciated in medaka.

Table 45: Histopathological findings in female fathead minnow following 4-tert-pentylphenol exposure

Diagnosis	Dose	LAB 8			LAB 7			LAB 9		
		Aver. Grade	N	Obser.	Aver. Grade	N	Obser	Aver. Grade	N	Obser
Increased cells - LVO	L	3	1	10						
Increased cells - MSO	C									
	L	2	2	10						
	M									
	H									
	P	3	1	10						
Decreased cells - LVO	C	1.5	2	10						
	L	1.5	2	10						
	M									
	H									
	P	2	1	10						
Oocyte atresia, increased, immature	C							2	2	10
	L									
	M									
	H				3	2	10	1.5	4	7
	P							2	2	10
Oocyte atresia, increased, mature	C	1	1	10						
	L	1.5	4	10						
	M	1	2	8						
	H	2.3	3	6				1	1	7
	P	1.5	2	10						
Asynchronous development, gonad	P							1	1	
Proteinaceous fluid, intravascular	C									
	L									
	M									
	H							2	1	7
	P							1.5	8	10
Proteinaceous fluid, interstitial	C							2	1	10
	L	2	2	10						
	M	1	2	8						
	H	2	2	6				1	2	7
	P	2.2	10	10				2	6	10
Post-ovulatory follicles, increased	C	1.5	4	10						
	L	1.6	5	10						
	M	1.3	7	8						
	H	1	1	6						
	P	2	6	10						
Hepatocyte	H	2	3							

basophilia, decreased										
Granulomatous inflammation	C	1	1	10						
	L				1	2	10			
	M				1	1	10			
	H				3	4	10			
	P	1.5	2	10	1	5	10			
Oocyte membrane folding	C	1.5	2	10						
	L	1.3	3	10						
	M	1	2	8						
	H									
	P	1.2	5	10						
Egg debris, oviduct	C	2.8	4	10						
	L	2.4	5	10						
	M	2.2	6	8						
	H	1	1	6	4	1	10			
	P	2.3	6	10						
Ovarian cyst	C							1.5	2	10
	L									
	M									
	H							1	1	7
	P							1,3	4	10
Oocyte atresia, late, atretic	C							3	1	10
	L									
	M									
	H							3	1	7
	P									

C= control; L= low concentration; M=medium concentration; H= high concentration; P= positive control; MSO: mature/spawning oocytes; LVO: late vitellogenic oocytes.
Shaded cells: exposure-related findings.

4tPP and Female Fathead Minnow – "Pre-Heidelberg"

158.   There were no exposure-related findings that were consistent among all three laboratories. A finding that was exposure-related in the positive control group in two of three laboratories was interstitial proteinaceous fluid (Labs 8 and 9). Intravascular proteinaceous fluid was also exposure-related in the positive control group in one laboratory (Lab 9). Granulomatous inflammation of the ovary was exposure-related in the high dose and positive control groups in one laboratory (Lab 7). . Average severity scores of exposure-related findings were low to moderately high (1.5-3.0). Median ovarian staging scores were: increased in the high dose group only (Lab 8); increased in the positive control group only (Lab 9); or decreased in the mid and high dose groups (Lab 7) as compared to the control group.

4tPP and Female Fathead Minnow – "Post-Heidelberg"

159.   As for male fathead minnow, proteinaceous fluid (interstitial, with or without intravascular) was an exposure-related finding in the females of two laboratories (Labs 8 and 9); however, unlike the males, this was only exposure-related in the positive control groups of both laboratories. Because of the ability of female fathead minnow to utilize vitellogenin for egg production, it might be anticipated that the threshold dose of estrogenic compound required to produce this effect would be higher for females as compared to males.

160.   Granulomatous inflammation in the ovary appeared to be an exposure-related finding in the high dose and positive control groups of Lab 7. There is no evidence in the literature to suggest that this is likely to be a direct estrogenic effect; however, it is possible that 4tPP or 17β-estradiol may have contributed to immunosuppression, as estrogens have been demonstrated to negatively affect cell-mediated immunity in mammals (Luster et al., 1984). It should be noted, however, that the comparatively higher overall incidence of granulomatous inflammation in female medaka from Lab 5 clearly did not appear to be related to either 4tPP or 17β-estradiol exposure.

4tPP and Zebrafish

161.   4tPP (and 17β-estradiol) studies in zebrafish were performed in three laboratories (Labs 12, 13, and 14) and read by three pathologists (Pathologists G, A, and D, respectively). "Pre-Heidelberg" results are presented in Tables 46-48.

Table 46: Staging data for male and female zebrafish exposed to 4-tert-pentylphenol

Dose	ZBR-Male					
	LAB 12		LAB 13		LAB 14	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	8	2	9	2	9
L	1.5	4	2	11	2	8
M	2	5	1	12	2	9
H	1	6	2	11	1.5	10
P	1.5	7	1	10	2	7
Dose	ZBR-Female					
	LAB 12		LAB 13		LAB 14	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	11	3	10	2	9
L	2	15	2	9	3	9
M	2	10	3	10	2	9
H	2	11	2.5	7	3	8
P	2	13	2	8	2	8

C: water control; L: low concentration (100 µg/l); M: medium concentration (320 µg/l); H: high concentration (1000 µg/l); PC: positive control (100 ng/l).

Table 47: Histopathological findings in male zebrafish exposed to 4-tert-pentylphenol

Diagnosis	Dose	LAB 12			LAB 13			LAB 14		
		Average of Grade	N	Obser.	Average of Grade	N	Obser	Average of Grade	N	Obser.
Increased cells - ISC	C									
	L									
	M									
	H							1	2	10
	P							1	5	7
Increased cells - SPA	C									
	L				2	2	11	1	1	8
	M	1.7	3	6	1.9	7	12	1	2	9
	H	2	1	7	2.9	7	11	1	4	10
	P	2	1	7	2	6	10	1	4	7
Increased cells - SPC	H	1	1	7						
Decreased cells - SPT	P	3	1	7						
Decreased cells - SPZ	C									
	L				1.3	3	11			
	M				1.9	8	12			
	H				2.7	9	11			
	P				2.1	9	10			
Testis-ova	C									
	L									
	M									
	H	1	2	7						
	P	3	1	7						
Testicular degeneration	H				3	1	11			
Asynchronous development, spermatocyst	H							1	2	10
Proteinaceous fluid, intravascular	L	1	1	4						
	M	1	4	6						
	H	1.5	6	7						
Interstitial fibrosis	C									
	L				1	3	11			
	M				1.8	5	12			
	H				3.3	3	11	1	1	10
	P				2	2	10			
Sertoli cell hypertrophy	C									
	L	1	1	4						
	M	1.3	4	6						
	H	2	4	7						
	P	1.8	4	7						

C= water control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ISC: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Shaded cells: exposure-related findings.

4tPP and Male Zebrafish – "Pre-Heidelberg"

162.   There were no exposure-related findings that were consistent among all three laboratories. A finding that was exposure-related in the mid dose, high dose and positive control groups of one laboratory (Lab 13) and the high dose and positive control groups of another laboratory (Lab 14) was increased spermatogonia.   Findings that were exposure-related in individual laboratories included: decreased spermatozoa in the mid and high dose groups, and positive control group (Lab 13); intravascular proteinaceous fluid in the mid and high dose groups (Lab 12); and Sertoli cell hypertrophy in the mid and high dose groups, and positive control group (Lab 12). Testis-ova were detected at low occurrence (i.e., not exposure-related) in the high-dose and positive control groups of Lab 12. Average severity scores of exposure-related findings were low to moderately-high (1.0 to 2.9). Median testicular staging scores were: decreased in the high dose group (Lab 12); decreased in the mid dose and positive control groups (Lab 13); or generally remained the same (Lab 14) in the exposed animal groups as compared to the control group.

4tPP and Male Zebrafish – "Post-Heidelberg"

163.   Similar to male medaka and male fathead minnow, increased spermatogonia was an exposure-related finding in male zebrafish, and this occurred in two of three laboratories (Labs 13 and 14, but not 12).   A potentially associated exposure-related finding in Lab 13 was decreased spermatozoa.   In a previous study, an increased proportion of spermatogonia was observed in the testes of adult male zebrafish that were exposed to another exogenous estrogen, ethynylestradiol, for 24 days (44).

164.   In male zebrafish, proteinaceous fluid (intravascular) was only exposure-related in one of three laboratories (Lab 12), in both male and female zebrafish. There were no diagnoses of proteinaceous fluid for male zebrafish from the other two laboratories. The presence of intra- and extravascular proteinaceous fluid has been observed previously in adult male zebrafish exposed to either 1 nM 17β-estradiol for 21 days (37) or to as little as 10 ng/L ethynylestradiol for 3 days (44).

165.   Unlike medaka, there were no findings relative to the kidneys reported for any of the zebrafish experiments involving 4tPP and 17β-estradiol. One possibility for this lack of findings is that kidney tissue was not available for examination due to the frontal (horizontal longitudinal) sectioning approach that was used for zebrafish.

166.   Unlike medaka and fathead minnow, testicular degeneration was not an exposure-related finding in male zebrafish. Nor was interstitial fibrosis exposure-related in male zebrafish (although it was in male medaka).

167.   Testis-ova were observed in male zebrafish from Lab 12 but not from Labs 13 and 14. Although the incidence for this finding was low (3/14 fish in the high dose and positive control groups combined, and none in any of the remaining groups), this finding should probably be considered significant because testis-ova generally occur as "rare events".

168.   Sertoli cell hypertrophy was an exposure-related finding for Lab 12, but this change was not diagnosed in Labs 13 and 14. The lack of similar findings in these latter two laboratories may be due to the relative (albeit minor) difficulty in evaluating this diagnosis as compared to other types of findings. Sertoli cells are small, often requiring high magnification for accurate size assessment, and are rare compared to other testicular cell types.   In fathead minnow, at least, it may be difficult to differentiate some hypertrophic Sertoli cells from spermatogonia (45).

Table 48: Histopathological findings in female zebrafish exposed to 4-tert-pentylphenol

Diagnosis	Dose	LAB 12			LAB 13			LAB 14		
		Average of Grade	N	Obser.	Average of Grade	N	Obser.	Average of Grade	N	Obser.
Increased cells - PNO	C									
	L				3	1	9			
	M				2	3	10			
	H									
	P				1	1	8			
Increased cells - MSO	M				2.5	2	10			
Increased cells - PFC	C							1.3	3	9
	P							3	1	8
Decreased cells-EVO	H				2	1	7			
Oocyte atresia, increased, immature	M				2	1	10			
Oocyte atresia, increased, mature	C	1	3	11				1	3	9
	L	1.5	4	15				1	1	9
	M	1.6	7	10				1.3	3	9
	H	1.8	9	11				1	1	8
	P	1.8	4	13						
Proteinaceous fluid, intravascular	C			11						
	L	1	2	15						
	M	1	6	10						
	H	1.3	6	11						
	P	1	3	13						
Proteinaceous fluid, interstitial	C							1.2	5	9
	L							1	1	9
	M							1	4	9
	H							1	2	8
	P							2.3	3	9
Interstitial fibrosis	C									
	L				1.8	5	9	1	1	9
	M				2	4	10			
	H				1.6	5	7			
	P				1.5	2	8			
Post-ovulatory follicles, increased	C	2	1	11						
	L	2	1	15						
	M				2	1	10			
	H									
	P				1.5	2	8			
Egg debris, oviduct	C	1.7	6	11						
	L	1.8	6	15						
	M	1.8	5	10						
	H	2	3	11						
	P	2.3	6	13						

C= water control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
PNO: perinucleolar oocytes; MSO: mature/spawning oocytes; EVO: early vitellogenic oocytes; PFC: perifollicular oocytes.
Shaded cells: exposure-related findings.

4tPP and Female Zebrafish – "Pre-Heidelberg"

169.   There were no exposure-related findings that were consistent among all three laboratories or even among two laboratories. Findings that were exposure-related in individual laboratories included: increased atresia of mature oocytes in the high dose group only (Lab 12); intravascular proteinaceous fluid in the mid and high dose groups, and the positive control group (lab 12); and interstitial fibrosis in the low, mid, and high dose groups, but not the positive control (Lab 13). Average severity scores of exposure-related findings were generally low (i.e., ≤ 2.0). Median ovarian staging scores were generally unchanged in the exposed animal groups as compared to the control group or did not exhibit an exposure-related pattern of increase or decrease.

4tPP and Female Zebrafish – "Post-Heidelberg"

170.   Findings related to 4tPP exposure in female zebrafish were not highly consistent among the three laboratories, and exposure-related findings tended to be of low severity grade. Qualitatively, two of the three exposure-related findings (increased atresia of immature oocytes and proteinaceous intravascular fluid) were similar to changes observed in compound-exposed female fathead minnow and medaka, whereas a third finding (interstitial fibrosis of the ovary) was not a feature of 4tPP exposure in the other two species. Oocyte atresia was the primary finding in the ovaries of adult female zebrafish that were exposed to ethynylestradiol for 3-24 days (44), and it was observed to a minor degree in adult females exposed to 1 nM 17β-estradiol for 21 days (37).

5.6.3.2 Prochloraz (with fadrozole positive control)

Prochloraz and Medaka

171.   Prochloraz (with fadrozole) studies in medaka were performed in four laboratories (Labs 1, 2, 4, and 6) and read by four pathologists (Pathologists H, B, K, and I, respectively). "Pre-Heidelberg" results are presented in Tables 49-51.

Table 49: Staging data for males and females medaka exposed to prochloraz

Dose	MDK-Male					
	LAB 1		LAB 2		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	2	10
L	2	10	2	10	2	9
M	2	10	2	10	2	10
H	3	10	2	10	3	9
P	2	10	2	10	3	10
Dose	MDK-Female					
	LAB 1		LAB 2		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	2	10
L	2	10	1.5	10	2	10
M	1	10	0	10	1	10
H	1	10	0	10	1	10
P	1	9	0	8	1	9

C: water control; L: low concentration (20 µg/l); M: medium concentration (100 µg/l);
H: high concentration (300 µg/l); PC: positive control (100 µg/l).

Table 50: Histopathological findings for male medaka exposed to prochloraz

Diagnosis	Dose	LAB 1			LAB 2			LAB 4			LAB 6		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - ISC	C												
	L							1	2	10			
	M							1	6	10	2.5	2	10
	H							1.6	9	10	1.7	4	10
	P							1	3	10			
Increased cells - SPZ	C							1	2	10			
	L							1.3	3	10			
	M							1.3	4	10			
	H							1.7	9	10	2	1	10
	P							1.3	6	10			
Decreased cells- SPA	M										2	1	10
	H										2	1	10
Decreased cells- SPZ	L										2	1	10
	M										1.3	3	10
Testis-ova	C										3	1	8
	L										2.6	3	8
	M										2.3	3	10
	H										1.5	2	10
Testicular degeneration	L										4	2	10
Sertoli cell hypertrophy	M										3	1	10
	H										2	1	10
Hepatocyte basophilia, increased	M										3	2	10
Interstitial fibrosis	C				2	1	10						
	L				2	1	10						
	M												
	H												
	P	2	1	10	2	2	10						
histiocytic cells(intraluminal)	C	1	2	10									
	L	1	2	10				1	1	10			
	M	2	1	10									
	H												
	P							1	1	10			

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ISC: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Shaded cells: exposure-related findings.

Prochloraz and Male Medaka – "Pre-Heidelberg"

172.   There were no exposure-related findings that were consistent among all four laboratories, or even three laboratories. A finding that was exposure-related in the mid and high dose groups of one laboratory (Lab 4), and the high dose group of another laboratory (Lab 6), but not in the positive controls of any laboratory, was increased interstitial cells. A finding that was exposure-related in the high dose group (but not positive controls) in a single laboratory (Lab 4) was increased spermatozoa. Testis-ova were observed at moderately-low incidences in the control and all of the prochloraz exposure groups (i.e., unrelated to exposure) except for the positive control group in one laboratory (Lab 6). Average severity scores of exposure-related findings were low to moderately-high (1.0 to 2.6). Average testicular staging scores were: increased in the high dose and positive control groups (Lab 4); increased in the high dose group only (Lab 1); or unchanged (Labs 2) in the exposed animal groups as compared to the control group. There is no staging data available for Lab 6.

Prochloraz and Male Medaka – "Post-Heidelberg"

173.   Although increased interstitial (Leydig) cells was an exposure-related finding in only two of four laboratories (Labs 4 and 6, but not 1 and 2), this finding may yet be relevant because it was also an exposure-related finding in male fathead minnow (but not zebrafish). In rodents, prochloraz has been demonstrated to antagonize peripheral androgen receptors resulting in the reduced growth of androgen-dependent tissues, and to antagonize central androgen receptors leading to an increase in leutinizing hormone via blockade of the negative feed-back mechanism (46). The increase in interstitial cells in the testes of male medaka in the present study is consistent with this latter mode of action, as the lack of negative androgen feedback would likely stimulate enhanced gonadotropic hormone (GtH-I) release (47). On the other hand, increased interstitial cells also occurred in a study in which zebrafish were exposed to the anti-estrogenic substance tamoxifen (37) which may suggest an alternate mode of action.

174.   Increased spermatozoa was an exposure-related finding in male medaka, and to some extent, in both fathead minnow and zebrafish. This increase in spermatozoa is may be at least partially explained by this androgen receptor antagonist mechanism described above. In addition, the increase in spermatozoa may also be related to aromatase inhibition. In a study in which fathead minnows were exposed to fadrozole, aromatase inhibition resulted in a marked accumulation of sperm in the testis, which the authors attributed to increased plasma androgen concentrations (28).

175.   In Lab 6, a moderately-low incidence of testis-ova was observed in all male groups, except for the positive control group, which strongly suggests that these were spontaneous events. Notably, testis-ova were also observed in male medaka from this laboratory's flutamide study, but since they only occurred in the high dose group, the relationship to treatment is less clear.

Table 51: Histopathological findings for female medaka exposed to prochloraz

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
PNO: perinucleolar oocytes; PFC: perifollicular cells.

Diagnosis	Dose	LAB 1			LAB 2			LAB 4			LAB 6		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - PFC	C										1	1	10
	L							1	1	10			
	M							1.4	9	10	1.5	2	10
	H							2	4	10	2.5	10	10
	P							2.1	9	9	2	10	10
Decreased cells - PNO	M							1	3	10			
	H							1	3	10			
	P							1.4	8	9			
Oocyte atresia, increased, immature	C	2	3	10	1	1	10						
	L	2.3	4	10	1	2	10						
	M	3.6	10	10	1	2	10	1.9	9	10	2	1	10
	H	3.4	9	10	1.7	6	10	2.6	10	10	2	10	10
	P	3.7	9	9	1.7	8	8	2	7	9	3.3	10	10
Oocyte atresia, increased, mature	C	1	1	10							1	2	10
	L	2	1	10	1	1	10						
	M	1.6	9	10	1	2	10				2	3	10
	H	1	3	10									
	P	1.7	3	9									
Interstitial fibrosis	C												
	L												
	M				2	1	10						
	H	2	1	9	2	2	10						
	P				1.5	2	8						
Granulomatous inflammation	M							1.1	7	10			
	H							1.8	9	10			
	P							1.3	7	9			
Follicles hypertrophy	L				2	1	10						
	M				2	1	10						
	P				2	1	8						
Lack of vitellogenesis	M										3.3	3	10
	H										3.7	10	10
	P										3.8	10	10
Decreased vitellogenesis	M							3.3	10	10			
	H							4	10	10			
	P							4	9	9			
Increased hypertrophy-PFC	L							1.1	7	10			
	M							2	9	10			
	H							2.3	4	10			
	P							1.9	9	9			

Shaded cells: exposure-related findings.

Prochloraz and Female Medaka – "Pre-Heidelberg"

176.   A finding that was exposure-related in all four laboratories in the mid dose, high dose, and positive control groups (Labs 1 and 4) or in just the high dose and positive control groups (Labs 2 and 6) was increased atresia of immature oocytes. A finding that was exposure-related in one laboratory in the mid dose, high dose, and positive control groups (Labs 4) or in just the high dose and positive control groups (Labs 6) was increased perifollicular cells. Findings that were exposure-related in individual laboratories included: decreased perinucleolar oocytes in the positive control group (Lab 4); lack of vitellogenesis in the high dose and positive control groups (Lab 6); decreased vitellogenesis in the mid dose, high dose, and positive control groups (Lab 4); hypertrophy of perifollicular cells in the low dose, mid dose, high dose, and positive control groups (Lab 4); and granulomatous inflammation in the mid dose, high dose, and positive control groups (Lab 4). Median severity scores of exposure-related findings varied from low (i.e.,

≤ 2.0) to very high (3.3 to 4.0 for decreased vitellogenesis and lack of vitellogenesis, and for immature oocyte atresia in Lab 1). Average ovarian staging scores were decreased in the mid dose, high dose, and positive control groups, as compared to the control group, in all three laboratories (Labs 1, 2, and 4) for which staging data was available.

<u>Prochloraz and Female Medaka – "Post-Heidelberg"</u>

177.   Increased atresia of immature oocytes was a very strong exposure-related finding for female medaka exposed to prochloraz or fadrozole, and overall one of the strongest histopathologic findings in the Phase 1B studies. It also correlates well with the Phase 1B spawning data, in which spawning was clearly inhibited by these substances (for prochloraz, at the 100 mg/L concentration in all four laboratories). Increased oocyte atresia was also exposure-related in fathead minnow, and in zebrafish to a lesser degree. Increased oocyte atresia was also reported in a prior study in which fathead minnow were exposed to fadrozole (28).

178.   The terms "decreased vitellogenesis" and "lack of vitellogenesis" were created independently by two pathologists (Pathologists K and I, representing Labs 4 and 6, respectively) to diagnose what was essentially the same lesion. In medaka, the lesion consisted of vitellogenic-sized oocytes that completely lacked yolk globules and instead contained large, fragmented cortical alveoli. The chorion remained smooth and intact, and perifollicular cells were hyperplastic and hypertrophic rather than vacuolated. Following the Heidelberg meeting it was suggested that the terminology for this entity be changed to the more descriptive designation of "decreased yolk formation". This visually dramatic, exposure-related finding occurred exclusively in the mid dose, high dose, and positive control group female medaka (and fadrozole-exposed female fathead minnow). Decreased yolk formation could be considered a form of oocyte atresia (degeneration), and such oocytes will probably continue to deteriorate; however, this alteration is morphologically quite distinct from standard patterns of oocyte atresia. At the Heidelberg meeting, during the pathologists' presentations and limited slide review, it became apparent that this change was additionally present in experiments performed at Labs 1 and 2, but it had been incorporated into the diagnosis of oocyte atresia. The same explanation applies to the exposure-related increases in the size and number of perifollicular cells (granulosa cells in this case) as reported from Lab 4. Findings such as decreased yolk formation and perifollicular cell hyperplasia / hypertrophy are easily explained as effects of aromatase inhibition. Aromatase is an essential enzyme for estrogen biosynthesis (48). Because estrogen is required for the hepatic production of vitellogenin in fish (47)(28), it is logical to predict that one effect of fadrozole administration would be the disruption of vitellogenesis. Based on ovarian morphology in affected female medaka, it appears that oocytes deprived of estrogenic stimulation fail to progress to the next phase of development, i.e., yolk formation, although they may continue to increase in size to at least to some degree. Because oocyte growth, at least in the later stages, has been attributed to vitellogenin uptake (47), it is possible that the oocytes of prochloraz- or fadrozole-exposed fish continue to sequester vitellogenin, but the processing of vitellogenin to yolk proteins is impaired. Because perifollicular cells (i.e., granulosa cells) are thought to be involved with aromatase production in fish (49)(50), it is possible that the increased number and size of these cells in compound-exposed fish is part of a compensatory mechanism aimed at restoring aromatase to levels required for vitellogenesis.

179.   This was one of the few situations in which gonad staging generated consistent and logical results. The lack of vitellogenesis was responsible for the overall difference in developmental stage between compound-exposed females (mid dose, high-dose group and positive control groups) and females of the control group. This failure of developmental progression (vitellogenesis) may also have been one reason for the increased oocyte atresia.

Prochloraz and Fathead Minnow

180.   Prochloraz (with fadrozole) studies in fathead minnow were performed in four laboratories (Labs 4, 8, 9, and 11) and read by three pathologists (Pathologists K, F, K, and J, respectively). "Pre-Heidelberg" results are presented in Tables 52-54.

Table 52: Staging data for male and female fathead minnow exposed to prochloraz

Dose	FHM-Male							
	LAB 8		LAB 9		LAB 11		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	3	9	2	10	2.5	10
L	2	10	3	10	2.5	10	3	10
M	2	10	3	11	3	10	3	10
H	2	10	3	10	2.5	10	3	10
P	3	10	3	10	2	9	3	10
Dose	FHM-Female							
	LAB 8		LAB 9		LAB 11		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N	Median of staging	N
C	3	10	3	10	3	9	3	9
L	3	10	3	10	3	10	3	9
M	3	9	3	9	4	8	3	10
H	3	10	2	10	3	10	3	10
P	2	10	2	10	4	7	2	9

C: water control; L: low concentration (20 µg/l); M: medium concentration (100 µg/l);
H: high concentration (300 µg/l); PC: positive control (100 µg/l).

Table 53: Histopathological findings in male fathead minnow exposed to prochloraz

Diagnosis	Dose	LAB 11			LAB 8			LAB 4			LAB 9		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - ISC	C												
	L										1	4	10
	M	1	4	10							2	1	11
	H	1	3	10				1	3	10	1	1	10
	P	1.8	5	9				1.7	6	10	1.6	5	10
Increased cells - SPA	C				1.3	3	10	2	1	10	1	1	9
	L				1.5	2	10	2	1	10	1	2	10
	M				2	1	10	1	2	10	1	1	11
	H				1	1	10	1.5	4	10	1	3	10
Increased cells - SPC	C				2	1	10						
Increased cells - SPT	L				1.7	3	10						
	M				2.3	3	10						
	H				1.7	3	10						
	P				2	2	10						
Increased cells - SPZ	C							1	1	10			
	L				1	1	10						
	M				1.8	5	10				1	1	11
	H				1.5	2	10	1	2	10			
	P				1.4	7	10	1	6	10			
Decreased cells - SPC	C				2	1	10						
	L				2	2	10						
	M										1	1	11
	H										1	1	10
	P												
Decreased cells - SPT	C				3	1	10				1	1	9
	L				2	1	10						
	M				1	1	10				1	1	11
Decreased cells - SPZ	C				2	4	10						
	L				1	1	10						
	M				3	1	10						
	H							1	1	10	1	1	10
	P				2	1	10						
Testis-ova	C	-	1	10									
	L	-	3	10									
	M	-	2	10									
	H	-	4	9									
Testicular degeneration	C				1.5	2	10				1	1	9
	L				1	2	10	2	1	10	1	2	10
	M				1	1	10	2	1	10	1	4	11
	H				1.3	3	10				1	2	10
	P				1	2	10						
Asynchronous development, gonad	C												
	L							1	2	10	1	2	10
	M							1	3	10			
	H							1	3	10	1.3	3	10
	P							1.3	3	10	1	3	10
Proteinaceous fluid, intravascular	H				1	2	10						
Proteinaceous fluid, interstitial	C				1.5	2	10						
	L				1.5	2	10						
	M				2	6	10						
	H				1.8	9	10						
	P				1.8	6	10						
Sertoli cell hypertrophy	P	1.3	4	9									
Granulomatous inflammation	C							1.5	2	10			
	L										1	1	10
	M							1	2	10	1	1	11

	H	1	1	10			
	P	1.5	2	10			
Histiocytic cells, intraluminal	C				1	1	9
	L	1	1	10	1.5	2	10
	M	1.3	4	10	1.4	5	11
	H	3	1	10	1	2	10
	P	2	1	10	1	1	10
Mineralization	C	1.2	5	10	1	3	9
	L	1	3	10	1	3	10
	M	1.3	3	10	1	2	11
	H	1	5	10	1	1	10
	P	1	2	10	1	2	10

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ISC: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Shaded cells: exposure-related findings.

Prochloraz and Male Fathead Minnow – "Pre-Heidelberg

181.   There were no exposure-related findings that were consistent among all four laboratories. A finding that was exposure-related in the positive control group in three of four laboratories (Labs 4, 9, and 11) was increased interstitial cells. Interstitial cells were also substantially increased compared to controls in the low dose group of one laboratory (Lab 9) and the mid dose group of another laboratory (Lab 11), but not in the high dose group of either laboratory. A finding that was exposure-related in the positive control group of two laboratories was increased spermatozoa (Labs 4 and 8). Findings that were exposure-related in individual laboratories included: interstitial proteinaceous fluid in the testis in the high dose group (Lab 8); Sertoli cell hypertrophy in the positive control group (Lab 11); and testis-ova in the high dose group (Lab 11). In Lab 11, a low incidence of testis-ova occurred in all of the control and exposed groups, but not in the positive control group. Average severity scores of exposure-related findings were generally low (i.e., ≤ 2.0). Median testicular staging scores were: increased in the positive control group (Lab 8) or essentially the same in the exposed animal groups as compared to the control group (Labs 4, 9, and 11).

Prochloraz and Male Fathead Minnow – "Post-Heidelberg"

182.   Increased interstitial (Leydig) cells was an exposure-related finding in the positive control (fadrozole) groups in three of four laboratories, and in the low dose and mid dose prochloraz groups (but not in the high dose groups) in two laboratories. Although increased interstitial cells was also exposure-related in male medaka (mid and high dose prochloraz groups, but not in males exposed to fadrozole), it was not an exposure-related change in male zebrafish.

183.   A similar argument could be made for the finding of increased spermatozoa. Although this finding occurred in all males of all three species, it was reported by less than half the laboratories overall, and never in both the high dose and positive control groups simultaneously. Interestingly, in fathead minnow and zebrafish, the findings of increased interstitial cells and increased spermatozoa were effects of fadrozole but not prochloraz, whereas the opposite appeared to be true for medaka.

184.   In one of the laboratories (Lab 11), testis-ova were observed in 10/39 control and prochloraz-exposed male fathead minnow. Based on the pathologist's presentation at the Heidelberg meeting, it was determined that the dark purple spherical objects that were initially identified as testis-ova were actually foci of mineralization within the testis and collecting ducts. Such mineralization was also observed as a non-exposure-related finding in two other laboratories (Labs 4 and 9), and there was general agreement among the pathologists that this finding was incidental to the study.

Table 54: Histopathological findings in female fathead minnow exposed to prochloraz

Diagnosis	Dose	LAB 11			LAB 8			LAB 4			LAB 9		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - LVO	M				2	5	9						
	H				2.5	6	10						
Increased cells - EVO	H				2.5	2	10						
	P				3.1	9	10						
Decreased cells - LVO	L				2.5	2	10						
	P				2	1	10						
Oocyte atresia, increased, immature	C							2	2	9	2.5	2	10
	L							1.5	2	9	1.5	2	10
	M							2.3	6	10	2.5	4	9
	H				2	2	10	1.8	8	10	2.4	8	10
	P				1.8	6	10	2.3	8	9	2.2	9	10
Oocyte atresia, increased, mature	C				1	1	10						
	L												
	M	1	2	8	1.7	3	9						
	H	1.8	4	10	2.3	9	10						
	P				1.8	8	10						
Proteinaceous fluid, interstitial	C				1	4	10						
	L				1	1	10						
	H				1.6	5	10						
	P				1.6	7	10						
Post-ovulatory follicles, increased	C				1.9	9	10						
	L				2.4	7	10	1	1	9			
	M				1.5	4	9						
	H				0	0	10						
	P				0	0	10						
Granulomatous inflammation	C				1	1	10	1	3	9	1	4	10
	L							1	1	9	1.5	2	10
	M				1.5	2	9				1	1	9
	H										1	1	10
	P										1	1	10
Oocyte membrane folding	C				1	6	10						
	L				1.3	4	10						
	M				1.6	5	9						
	H				1.6	5	10						
	P				2.8	8	10				2.2	6	10
Egg debris, oviduct	C				2	10	10						
	L				2	6	10						
	M				2	3	9						
	H				2.5	2	10						
	P				1	2	10						
Decreased vitellogenesis	H										1.5	2	10
	P							1.2	4	9	1.6	8	10
Ovarian cyst	C										1	1	10
	L										1	2	10
	M										1	1	9
	H							1	1	10			
	P										1	2	10
Ovarian atresia, increased, late atretic	L							1.5	2	9			
	H										2	1	10

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
LVO: late vitellogenic oocytes; EVO: early vitellogenic oocytes;
Shaded cells: exposure-related findings.

Prochloraz and Female Fathead Minnow – "Pre-Heidelberg"

185.   There were no exposure-related findings that were consistent among all four laboratories. A finding that was exposure-related in three of four laboratories in the high dose and positive control groups (Labs 4 and 9), or just the positive control group (Lab 8), was increased atresia of immature oocytes. There was increased atresia of mature oocytes in two laboratories in the high dose and positive control

groups (Labs 8) or just the positive control group (Lab 11). A finding that was exposure-related in the positive control group in two laboratories (Labs 4 and 9) was decreased vitellogenesis. Findings that were exposure-related in individual laboratories included: increased late vitellogenic oocytes in the mid and high dose groups (Lab 8); increased early vitellogenic oocytes in the positive control group (Lab 8); decreased egg debris in the oviduct in the mid dose, high dose, and positive control groups (Lab 8); and oocyte membrane folding the positive control group (Lab 9). Average severity scores of exposure-related findings were low to moderately-high (1.2 to 3.1). Median ovarian staging scores were: decreased in the high dose and positive control groups (Lab 9) or just the positive control group (Labs 4 and 8), or increased in the mid dose and positive control groups (Lab 11), as compared to the control group.

Prochloraz and Female Fathead Minnow – "Post-Heidelberg

186.   In female fathead minnow, atresia of immature oocytes was a strong response to aromatase inhibitors; this contention is supported by the comparatively high incidences of immature oocyte atresia in the high dose and positive control groups of three of four laboratories and the unambiguous dose-dependent response patterns. The strength of this finding would be further enhanced if the diagnosis of immature oocyte atresia were to be combined with atresia of mature oocytes, the latter of which was reported by Labs 8 and 11. In the fathead minnow especially, there is reasonable justification for this maneuver. Unlike medaka, in which mature spawning oocytes are easily distinguished from earlier stages by the fusion of smaller yolk globules into a central yolk mass, the distinction between these developmental cell types in fathead minnow ovaries is far more subtle. This distinction becomes even cloudier when evaluating atretic (degenerating) follicles. Thus, at the Heidelberg meeting, the pathologists generally agreed that oocyte atresia should be reported as a single diagnosis in the future. It was also decided that oocyte membrane folding should be considered a component of oocyte atresia, and therefore it is not necessary to diagnosis membrane folding as a separate entity.

187.   Decreased vitellogenesis (decreased yolk formation) was only reported as an exposure-related finding in two of four laboratories (Labs 4 and 9), and only by a single pathologist (Pathologist K). One possible explanation for this discrepancy is that Pathologist K had a unique opportunity to observe the analogous change in medaka, in which decreased yolk formation was morphologically much more obvious. In fathead minnow, decreased yolk formation was characterized by a variably-sized, central, pale perinuclear zone in which yolk granules were either small and sparse or completely absent. Instead of creating a new diagnosis for these cells, the pathologist from Lab 8 chose to classify such cells as early vitellogenic oocytes (thus the numbers of EVO's were increased as a function of prochloraz exposure) and described the cells as being enlarged in the pathology narrative text. The pathologist from Lab 11 may have grouped these cells with atretic oocytes.

188.   Unlike the situation in female medaka, there were no exposure-related increases in perifollicular size or number associated with prochloraz or fadrozole administration in female fathead minnow (or zebrafish, for that matter). This may very well be due to minor interspecies differences in the microanatomical structure and/or metabolic function of perifollicular cells, evidence for which appears to exist. For example, during early oocyte atresia, fathead minnow granulosa cells typically become enlarged, vacuolated, and have internalized eosinophilic material (Wolf et al., 2004), whereas these types of histomorphologic changes are difficult to appreciate in atretic follicles of medaka.

189.   Decreased egg debris in the oviduct was reported as an exposure-related finding in only one of four laboratories (Lab 8). One likely reason for this discrepancy is that the Lab 8 ovaries were examined histologically in situ rather than as excised tissues (as in the other laboratories), which provided a superior and more consistent view of the oviduct. The Lab 8 pathologist (Pathologist F) reasoned that the decreased egg debris could be associated with the dose-dependent decrease in post-ovulatory follicles, both of which

findings suggested inhibition of spawning. This may also be a reason for the relative increase in late vitellogenic oocytes as reported for Lab 8.

Prochloraz and Zebrafish

190.   Prochloraz (with fadrozole) studies in zebrafish were performed in three laboratories (Labs 6, 12, and 13) and read by three pathologists (Pathologists I, G, and A, respectively). "Pre-Heidelberg" results are presented in Tables 55-57.

Table 55: Staging data for male and female zebrafish exposed to prochloraz

| Dose | ZBR-Male | | | |
| | LAB 12 | | LAB 13 | |
	Median of staging	N	Median of staging	N
C	1	10	2	9
L	2	10	2	8
M	2	10	2	8
H	2.5	10	2	8
PC	2	10	2.5	10

| Dose | ZBR-Female | | | |
| | LAB 12 | | LAB 13 | |
	Median of staging	N	Median of staging	N
C	2	10	2	11
L	2	10	3	9
M	2	10	2	9
H	2	9	3	10
PC	1	8	2	10

C: water control; L: low concentration (20 µg/l); M: medium concentration (100 µg/l);
H: high concentration (300 µg/l); PC: positive control (100 µg/l).

Table 56: Histopathological findings in male zebrafish exposed to prochloraz

Diagnosis	Dose	Lab 6			LAB 12			LAB 13		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - ISC	H				1	3	10			
	P				1	2	10			
Increased cells - SPA	L							3	2	8
	M							2	3	8
	P							2	2	10
Increased cells - SPC	L							2.6	7	8
	M							2.7	7	8
	H							2.9	8	8
	P							2.7	6	10
Increased cells - SPZ	H							2	1	8
	P							1.9	7	10
Decreased cells - SPA	L							2.7	3	8
	M							2.8	4	8
	P							4	3	10
Interstitial fibrosis	L							2.5	8	8
	M							3.1	8	8
	H							3.5	8	8
	P							2.5	10	10
Increased interstitial cells	C	1.5	2	10						
	L	-	-	-						
	M	1	1	10						
	H	-	-	-						
	P	2	3	14						
Sertoli cell hypertrophy	C	2.5	2	10						
	L	2	7	14						
	M	2	4	10						
	H	1.6	5	9						
	P	2	6	14						
Increased Sertoli cells	C	2	1	10						
	L	-	-	-						
	M	-	-	-						
	H	2	3	9						
	P	2	1	14						
Displacement – Spermatogonia	C	-	-	-						
	L	1.5	4	14						
	M	1.5	2	10						
	H	2	1	9						
	P	2	2	14						
Asynchronous development – Spermatogonia	C	-	-	-						
	L	-	-	-						
	M	X	1	10						
	H	-	-	-						
	P	1.5	2	14						
Increased Spermatogonia	C	-	-	-						
	L	2	1	14						
	M	-	-	-						
	H	3	1	9						
	P	-	-	-						
Testis-ova	C	1	1	10						
	L	2	1	14						
	M	-	-	-						
	H	1	3	9						
	P	-	-	-						
Histiocytic cells	C	-	-	-						
	L	-	-	-						
	M	2	1	10						
	H	1	1	9						
	P	-	-	-						

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ISC: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Shaded cells: exposure-related findings.

Prochloraz and Male Zebrafish – "Pre-Heidelberg"

191.   There were no exposure-related findings that were consistent among all three laboratories, or even two of three laboratories. Findings in individual laboratories that were exposure-related all occurred in Lab 13 and included: increased spermatocytes in the low dose, mid dose, high dose, and positive control groups; increased spermatozoa in the positive control group; and interstitial fibrosis in the dose, mid dose, high dose, and positive control groups. A low incidence (not exposure-related) of testis-ova was observed in the control and some compound-exposed groups in Lab 6. Average severity scores of exposure-related findings were generally moderately-high (i.e., > 2.0). Median testicular staging scores were increased in the exposed groups in one laboratory (Lab 12) and essentially unchanged in another laboratory (Lab 13) as compared to the control group. Staging data for Lab 6 was not available.

Prochloraz and Male Zebrafish – "Post-Heidelberg"

192.   As previously mentioned, increased spermatozoa was an exposure-related finding in only one of three laboratories (Lab 13); however, it was also seen as an exposure-related finding, to varying degrees, in the other two fish species. Therefore, increased spermatozoa is probably a true, albeit comparatively weak, response to aromatase-inhibitor exposure in certain male fishes.

193.   The incidences and severities of two findings, increased spermatocytes and interstitial fibrosis, were considerably higher in exposed zebrafish males vs. controls; however, these findings were only reported by one of three laboratories, and neither finding was reported as an exposure effect of prochloraz or fadrozole in the other two species.

Table 57: Histopathological findings in female zebrafish exposed to prochloraz

Diagnosis	Dose	Lab 6			LAB 12			LAB 13		
		Average of grade	N	Obs.	Average of grade	N	Obs.	Average of grade	N	Obs.
Increased cells - PNO	M							2	3	9
Increased cells - LVO	M							3	1	9
	H							3	1	10
	P							3.1	8	10
Increased cells - EVO	P							3.2	5	10
Increased cells - MSO	H							2.5	2	10
Decreased cells - LVO	P							4	1	10
Decreased cells - PNO	P							3.6	7	10
Decreased cells - MSO	P							3.8	8	10
Oocyte atresia, increased, immature	L							1	1	9
	M							1	1	9
	P							2	6	10
Oocyte atresia, increased, mature	C				2	1	10			
	L				1.8	4	10			
	P				1.6	7	9			
Interstitial fibrosis	L							2	7	9
	M							2.7	6	9
	H							2	1	10
Post-ovulatory follicles, increased	C				1.3	3	10			
	L				1.0	2	10	2	1	9
	M						10	2.5	2	9
Hepatocyte basophilia, decreased	P				3.1	8	9			
Oocyte membrane folding	L							2	3	9
	M							2	6	9
	H							1.5	4	10
	P				2.2	9	9	2.4	10	10
Egg debris, oviduct	C				2.5	8	10			
	L				2.8	6	10			
	M				2.3	4	10			
	H				2	2	9			
Hypertrophy perifollicular cells	C	-	-	-						
	L	2	1	3						
	M	-	-	-						
	H	X	1	5						
	P	2	2	5						
Oocyte membrane folding	C	-	-	-						
	L	2	1	3						
	M	-	-	-						
	H	3	1	5						
	P	2	2	5						
Increase perifollicular cells	C	-	-	-						
	L	-	-	-						
	M	-	-	-						
	H	-	-	-						
	P	3	1	5						
Oocyte atresia mature	C	3	1	8						
	L	2	1	3						
	M	2	1	8						
	H	3	2	5						
	P	2	2	5						
Egg debris, oviduct	C	-	-	-						
	L	2	1	3						
	M	-	-	-						
	H	-	-	-						
	P	-	-	-						
Increased interstitial cells	C	-	-	-						
	L	-	-	-						
	M	-	-	-						
	H	-	-	-						
	P	1	1	5						

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.

PNO: perinucleolar oocytes; EVO: early vitellogenic oocytes; LVO: late vitellogenic oocytes; MSO: mature/spawning oocytes. Shaded cells: exposure-related findings.

Prochloraz and Female Zebrafish – "Pre-Heidelberg"

194.	There were no exposure-related findings that were consistent among all three laboratories. A finding that was exposure-related in two laboratories in the mid dose, high dose, and positive control groups (Lab 13) or just the positive control group (Lab 12) was oocyte membrane folding. Findings in individual laboratories that were exposure-related included: increased late vitellogenic oocytes, increased early vitellogenic oocytes, decreased perinucleolar oocytes, and decreased mature spawning oocytes in the positive control group (Lab 13); increased atresia of immature oocytes in the positive control group (Lab 13); increased atresia of mature oocytes in the positive control group (Lab 12); and decreased hepatocyte basophilia (Lab 12). Average severity scores of exposure-related findings ranged from low to high (1.5 to 3.8), but in most cases were at least moderately high (i.e., >2.0). Median ovarian staging scores were decreased in the positive control group (Lab 12) or did not show a dose-dependent pattern of change (Lab 13) in the exposed animal groups as compared to the control group. Staging data is not available for Lab 6.

Prochloraz and Female Zebrafish – "Post-Heidelberg"

195.	As previously discussed, it was generally agreed at the Heidelberg meeting that the diagnoses of immature oocyte atresia and mature oocyte atresia should be combined because the criteria used to distinguish these cell types in fathead minnow and zebrafish were not clear. Additionally, it was concluded that oocyte membrane folding should be considered a component of oocyte atresia. When data from the three fish species is integrated, the evidence suggests that oocyte atresia is a reliable effect of aromatase-inhibitor exposure.

196.	The diagnosis of decreased vitellogenesis (decreased yolk formation) was not specifically reported for compound-exposed female zebrafish. However, if the effect was similar to changes that were observed in fathead minnow, it may have been the case that yolk-deficient oocytes were categorized either as early vitellogenic oocytes or atretic oocytes (relevant glass slides were not available for review at the Heidelberg meeting).

197.	As was the situation for fadrozole-exposed fathead minnow, the increase in late vitellogenic oocytes as reported by Lab 13, accompanied by the decrease in mature spawning oocytes, suggests that there may have been elements of both maturation and spawning arrest in fadrozole-exposed female zebrafish. Overall, there appears to be variation among the three laboratories in the reporting of semi-quantitative changes in the distribution of oogenic cell types. It seems likely that this was due to differences in the diagnostic focus of the various pathologists, as one pathologist (Pathologist A) had a tendency to report oogenic cell type changes in female zebrafish, whereas other pathologists (Pathologists D, G, and I) consistently did not report such findings.

198.	Decreased hepatocyte basophilia was only observed as an exposure-related effect of fadrozole in zebrafish, and then in only one of three laboratories (Lab 12, but not Labs 6 and 10). The lack of this finding in fathead minnow and medaka is readily explained by the fact that the histologic sectioning protocols for those species (as provided by the draft guidance document) do not provide regular (or any) access to liver tissue for microscopic evaluation. Concerning the fact that liver diagnoses were not reported by Labs 6 and 10, it may be the case that Pathologists A and I concentrated their attention on gonad tissue and did not examine the livers (analogous to the inconsistent evaluation of kidney tissue in medaka). Because hepatic production of vitellogenin is dependent on estrogen, and because basophilia is a feature of hepatocytes that have been upregulated for vitellogenin production, decreased hepatocyte basophilia in fadrozole-exposed females could be explained by aromatase inhibition.

5.6.3.3 Flutamide

Flutamide and Medaka

199.   Flutamide studies in medaka were performed in four laboratories (Labs 3, 4, 5, and 6) and read by three pathologists (Pathologists E, K, K, and I, respectively). "Pre-Heidelberg" results are presented in Tables 58-60.

Table 58: Staging data for male and female medaka exposed to flutamide

Dose	MDK-Male					
	LAB 3		LAB 5		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	9	1.5	10
L	2	9	2.5	6	2	10
M	2	10	2.5	6	2	9
H	3	10	2.5	8	2	10
Dose	MDK-Female					
	LAB 3		LAB 5		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	9	1.5	10	2	10
L	2	10	1	7	2	10
M	2	10	2	5	2	10
H	1	10	1	9	2	9

C: water control; L: low concentration (100 µg/l);
M: medium concentration (500 µg/l); H: high concentration (1000 µg/l).

Table 59: Histopathological findings in male medaka exposed to flutamide

Diagnosis	Dose	LAB 3			LAB 5			LAB 4			LAB 6		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells-ISC	C				1	1	9	1	1	10			
	L							1	1	10			
	M							1	2	9	3	2	8
	H				1	2	8	1	3	10	3	1	9
Increased cells-SPA	C				3	1	9						
	L	1	1	9	2.5	2	6	3	1	10			
	M				3.5	3	6						
	H				1	3	8						
Increased cells-SPZ	C				1.5	4	9	1	1	10			
	L				1.4	5	6	1	1	10			
	M				1	3	6	1	2	9			
	H				1.4	5	8	1.7	3	10			
Decreased cells-SPA	M				3	1	6				2	2	8
	H										2	1	9
Testis-ova	C							1	1	10			
	L							3	1	10			
	H							1	1	10	2	1	9
Testicular degeneration	C	1	1	10	2	4	9	1	1	10			
	L	2	1	9	2	1	6	1	2	10			
	M	1	1	10	1.7	3	6	1	1	9			
	H	2	1	10	2	3	8						
Asynchronous dev. spermatocyst	L				1	1	6						
	M				1	1	6						
Asynchronous dev. gonad	M				2	1	6	1	1	9			
	H				1	1	8						
Interstitial fibrosis	H				2	1	8						
Sertoli cell hypertrophy	M										2	2	8
	H										2.3	3	9
Granulomatous inflammation	L				1	1	6						
	H				2	1	8						
Histiocytic cells, intraluminal	L				1	1	6	1	1	10			
	H				1.5	2	8						

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ISC: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Shaded cells: exposure-related findings.

Flutamide and Male Medaka – "Pre-Heidelberg"

200. There were no exposure-related findings for flutamide in male medaka. Median testicular staging scores were increased in the increased in the high dose group in one laboratory (Lab 3). Median testicular staging scores were also increased, albeit not substantially, in the low, mid, and high dose groups of Labs 4 and 5 as compared to the control group.

Flutamide and Male Medaka – "Post-Heidelberg"

201. As stated above, there were no clear exposure-related findings for flutamide in male medaka. There did, however, appear to be a slight general tendency toward increased testicular stage with increased flutamide dose. As increased testicular stage is primarily indicated by thinning of the germinal epithelium, this result would be consistent with a study in which reduced numbers of spermatogenetic cysts were observed in adult male guppies (*Poecilia reticulata*) that were exposed to flutamide at concentrations up to 100 μg/mg of food (51).

202. Although the histopathologic effects of flutamide have been studied in several fishes, including fathead minnow (32), zebrafish (37), guppy (51), and carp (52), similar research in medaka appears to be limited. Apparently, previous histologic studies in medaka have focused primarily on sex-reversal as a potential effect of flutamide; such studies have determined that this phenomenon either does occur (53) or does not occur (54) in male medaka as a consequence of flutamide exposure. Note that in the Phase 1B flutamide/medaka studies, testis-ova were: not observed in two laboratories (Labs 3 and 5); observed at low incidence as a non-exposure-related finding (Lab 4); and observed at low incidence in only the high dose group (Lab 6). In Phase 1B, the relationship between testis-ova formation in medaka and flutamide exposure is equivocal at best.

Table 60: Histopathological findings in female medaka exposed to flutamide

Diagnosis	Dose	LAB 3			LAB 5			LAB 4			LAB 6		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells-OOG	H				1	1	8						
Oocyte atresia, increased, immature	C	1.3	3	9	1.2	6	10						
	L	1.3	6	10	2.3	3	7						
	M	1.6	7	10	1	1	5						
	H	1.9	8	10	1.6	5	9	2	1	9			
Oocyte atresia, increased, mature	C										1	2	10
	L	1	1	10							1	1	10
	M										1	1	10
	H				1	1	9				2	2	9
Proteinaceous fluid	C						8				3	1	10
	M										3	2	10
Post-ovulatory follicle, increased	H				1	1	9						
Granulomatous inflammation	C				1	3	10						
	L				1.7	3	7						
	M				2	1	5						
	H				1	4	9						
Macrophage aggregates, increased	C	1	1	9									
	L	2	1	10									
	M	1.7	3	10									
	H	1	1	10									
Egg debris, oviduct	H	2.7	3	10									
Oocyte atresia, late, atretic	C				1	2	10						
	L				1	2	7						
	H				1.6	3	9						
Post-ovulatory follicle, decreased	L							x	6	10			
	M							x	4	10			
	H							x	6	10			

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
OOG: oogonia.
Shaded cells: exposure-related findings.

Flutamide and Female Medaka – "Pre-Heidelberg"

203. A finding that was exposure-related in the low, mid, and high dose groups in one laboratory (Lab 4) was decreased post-ovulatory follicles. Because the number of post-ovulatory follicles was generally low, even in controls, this particular finding was not graded for severity. Median ovarian staging scores were decreased in the high dose group (Lab 3), were unchanged (Lab 4), or were changed in an irregular pattern that was not exposure-related (Lab 5) in the exposed animal groups as compared to the control group.

Flutamide and Female Medaka – "Post-Heidelberg"

204.   As discussed in regard to male medaka, histopathologic studies of flutamide in this species are limited; therefore there are no previous effects of decreased post-ovulatory follicles as an exposure effect. This effect was not exposure-related in either fathead minnow or zebrafish females in the Phase 1B studies. If this effect were in fact genuine, it would suggest that flutamide either impaired oocyte release or accelerated the regression of post-ovulatory follicles.

Flutamide and Fathead Minnow

205.   Flutamide studies in fathead minnow were performed in three laboratories (Labs 4, 7, and 10) and read by two pathologists (Pathologists K, C, and K, respectively). "Pre-Heidelberg" results are presented in Tables 61-63

Table 61: Staging data for male and female fathead minnow exposed to flutamide

Dose	FHM-Male					
	LAB 7		LAB 10		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	2	9
L	2	10	2	9	2	10
M	2	10	3	10	2	11
H	2.5	10	2	9	3	10
Dose	FHM-Female					
	LAB 7		LAB 10		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	3	10	3	10	2	10
L	3	10	2	10	3	10
M	2	10	3	10	3	9
H	2	9	2	9	2	10

C: water control; L: low concentration (100 µg/l);
M: medium concentration (500 µg/l); H: high concentration (1000 µg/l).

Table 62: Histopathological findings in male fathead minnow exposed to flutamide

Diagnosis	Dose	LAB 7			LAB 10			LAB 4		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - ISC	C				1	1	10			
	L							1	4	10
	M							2	2	10
	H							1	2	9
Increased cells - SPA	C				1	1	10	1	4	10
	L				1	1	10			
	M				1.2	6	11	1	4	10
	H				1.4	10	10	1	3	9
Increased cells - SPZ	M							1	1	10
Decreased cells - SPC	C							2	2	10
	L									
	M							1	2	10
	H				1.3	4	10	1	1	9
Decreased cells - SPZ	L							2	1	10
	H							3	1	9
Testicular degeneration	C				1	2	10			
	L				1	1	10	2	3	10
	M				1	2	11	1.5	2	10
	H							1.3	3	9
Asynchronous development, spermatocyst	C				3	1	10			
Asynchronous development, gonad	C				2	1	10	1	3	10
	L				1	1	10	1	1	10
	M				1	2	11	1.3	3	10
	H							1	4	9
Asynchronous development, right & left gonads	C							2	1	10
Interstitial fibrosis	L							2	1	10
Sertoli cell hypertrophy	H				2	1	10			9
Granulomatous inflammation	C				2	1	10	1.3	3	10
	L				2	1	10			10
	M				2	1	11	1	2	10
	H				1	2	10	1	2	9
Histiocytic cells, intraluminal	C							2	2	10
	L				1.5	2	10			10
	M				1	1	11	1	2	10
	H				2	2	10	1	3	9
Retained peritoneal attachments	C				1	4	10	1	2	10
	L				1.2	5	10	1.5	2	10
	M				1	1	11	1	2	10
	H				1	6	10	1.4	5	9
Mineralization, collecting duct	C				1.4	8	10	1.5	4	10
	L				1.3	10	10	1.5	2	10
	M				2	5	11	2	2	10
	H				1.7	3	10	1.0	3	9

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ISC: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Shaded cells: exposure-related findings.

Flutamide and Male Fathead Minnow – "Pre-Heidelberg"

206.   There were no exposure-related findings that were consistent among all three laboratories, or even two of three laboratories. Findings that were exposure related in individual laboratories included: increased spermatogonia in the mid and high dose groups (Lab 4); decreased spermatocytes in the high dose group (Lab 4); and a decrease in mineralization in the collecting duct the high dose group (Lab 4). Average severity scores of exposure-related findings were generally low (i.e., ≤ 2.0). Median testicular staging scores were increased in the high dose group (Lab 4), or only slightly increased in the high dose group (Lab 7), or increased in the mid dose group only (Lab 10) as compared to the control group.

Flutamide and Male Fathead Minnow – "Post-Heidelberg"

207.   In a previous study of flutamide in fathead minnow (32), the primary histopathologic finding in males was "spermatocyte degeneration and necrosis". Based on the description and photomicrograph in the article, this finding appears to be essentially equivalent to the diagnosis of "testicular degeneration" as described in the draft guidance document for Phase 1B. Testicular degeneration was not observed as an exposure-related finding for flutamide-exposed male fathead minnow in Phase 1B. However, it should be noted that the results of Jensen et al. are not incontrovertible, because the numbers of male fish that they examined histologically were quite small (only 3 males per dose group), and empirical evidence indicates that testicular degeneration can be observed as a spontaneous finding in unexposed fathead minnow males (as occurred in Lab 4).

Table 63: Histopathological findings in female fathead minnow exposed to flutamide

Diagnosis	Dose	LAB 7			LAB 4			LAB 10		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Oocyte atresia, increased, immature	C				1	1	10	1	1	10
	L	2	2	10	1.4	5	10	1.7	3	10
	M				2	9	9	1.7	3	10
	H	3	1	9	1.9	8	10	2	2	9
Oocyte atresia, increased, mature	C									
	L	2	1	10	3.0	2	10			
	M									
	H							1	1	9
Interstitial fibrosis	C	1	1	10						
Post-ovulatory follicles, increased	L							2	1	10
Granulomatous inflammation	C	3	1	10	1	3	10	1.3	4	10
	L	2.5	2	10	1.4	5	10	1	5	10
	M	1.8	4	10	1	1	9	1	6	10
	H	2	3	10	1	3	10	1	5	9
Egg debris, oviduct	H	3.5	2	9				3	1	9
Ovarian cyst	C				1	1	10			
	L							1	1	10
	H							1	1	9
Ovarian mineralization	C				2	1	10			
	L				1	1	10			
	H				1	1	10			
Oocyte atresia, increased, late atretic	C				1	1	10			
	L				3.0	3	10			
	M				1	2	9			
	H				1.6	3	10	3	1	9

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
Shaded cells: exposure-related findings.

Flutamide and Female Fathead Minnow – "Pre-Heidelberg"

208.   There were no exposure-related findings that were consistent among all three laboratories, or even two of three laboratories. A finding that was exposure-related in a single laboratory was increased atresia of immature oocytes in the low, mid, and high dose groups (Lab 4). The average severity scores of this finding were low (i.e., ≤ 2.0). Median ovarian staging scores were decreased in the mid and high dose groups (Lab 7) or were not changed in a pattern that appeared to be exposure-related (Labs 4 and 10).

Flutamide and Female Fathead Minnow – "Post-Heidelberg"

209.   Oocyte atresia was a relatively robust exposure-related finding for Lab 4, but not for the other two laboratories (Labs 7 and 10). A relationship between flutamide exposure and oocyte atresia was also observed in a previous study conducted in fathead minnow (32), in which five females of each dose group

were examined histologically. Although they indicated that oocyte atresia was probably due to androgen receptor blockade, the authors did not offer a mechanistic explanation for this effect. The authors did cite additional studies in which similar histological effects were observed other fishes and rodents.

210.   An additional finding in Jensen et al. (32)was a decrease in mature oocytes relative to immature oocytes. There was no correlate for this finding in the Phase 1B study, as ovarian stage scores were not consistently decreased in flutamide-exposed female fathead minnow, and the pathologists did not report increases or decreases in the various oogenic cell types.

Flutamide and Zebrafish

211.   Flutamide studies in zebrafish were performed in three laboratories (Labs 6, 12, and 14) and read by three pathologists (Pathologists I, G, and D, respectively). "Pre-Heidelberg" results are presented in Tables 64-66.

Table 64: Staging data for male and female zebrafish exposed to flutamide

Dose	ZBR-Male			
	LAB 12		LAB 14	
	Median of staging	N	Median of staging	N
C	2	8	2	7
L	2	10	2	8
M	2	10	2	10
H	1	9	2	9
Dose	ZBR-Female			
	LAB 12		LAB 14	
	Median of staging	N	Median of staging	N
C	2	10	3	10
L	3	10	3	9
M	2	8	3	9
H	2	10	2.5	8

C: water control; L: low concentration (100 µg/l);
M: medium concentration (500 µg/l); H: high concentration (1000 µg/l).

Table 65: Histopathological findings in male zebrafish exposed to flutamide

Diagnosis	Dose	LAB 6			LAB 12			LAB 14		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - ISC	C									
	L							1	1	8
	M				1	5	10	1	5	10
	H				1	6	9	1	4	9
Increased cells - SPA	M				2	1	10	1	3	10
	H				1.8	8	9	1	6	9
Increased cells - SPC	M						10	1	1	10
Decreased cells - SPT	H				1	2	9			9
Asynchronous development, spermatocyst	H						9	1	3	9
Interstitial fibrosis	M						10	1	3	10
Sertoli cell hypertrophy	M				1.6	7	10			10
	H				1	9	9	1	2	9
Increased interstitial cells	C	1.5	2	10						
	L	1.5	2	9						
	M	1.5	2	16						
	H	1.5	2	12						
Increased Sertoli cells	C	2	1	10						
	L	-	-	-						
	M	3	1	16						
	H	-	-	-						
Asynchronous development (right & left gonads)	C	-	-	-						
	L	x	1	9						
	M	-	-	-						
	H	-	-	-						
Histiocytic cells	C	-	-	-						
	L	2	1	9						
	M	-	-	-						
	H	-	-	-						
Sertoli cell hypertrophy	C	2.5	2	10						
	L	2.5	2	9						
	M	1.5	5	16						
	H	1.5	4	12						
Testis-ova	C	1	1	10						
	L	-	-	-						
	M	1	2	16						
	H	1	1	12						
Asynchronous development spermatocysts	C									
	L									
	M									
	H									
Displacement spermatogonia	C	-	-	-						
	L	-	-	-						
	M	2	1	16						
	H	2	1	12						

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ISC: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Shaded cells: exposure-related findings.

Flutamide and Male Zebrafish – "Pre-Heidelberg"

212.   There were no exposure-related findings that were consistent among all three laboratories. There were two findings that were exposure-related in two laboratories (Labs 12 and 14), and these were increased interstitial cells in the mid and high dose groups, and increased spermatogonia in the high dose group. A finding that was exposure-related in an individual laboratory (Lab 12) was Sertoli cell hypertrophy in the high dose group. Average severity scores of exposure-related findings were generally low (i.e., ≤ 2.0). Median testicular staging scores were decreased in the high dose group (Lab 12) or unchanged (Lab 14) in the exposed animal groups as compared to the control group. Staging data is not available for Lab 6.

Flutamide and Male Zebrafish – "Post-Heidelberg"

213.   Increased interstitial cells and increased spermatogonia appeared to be relatively robust, exposure-related findings for male zebrafish exposed to flutamide, as nearly identical, dose-responsive results were present in Labs 12 and 14 (but not Lab 6). Both of these findings were also reported from a previous experiment (37) in which male zebrafish were exposed to flutamide (up to 1000 µg/L in water). In their article, Wester et al. reasoned that the increase in interstitial (Leydig) cells probably occurred as a compensatory mechanism secondary to androgen receptor blockade at the pituitary level. They also attributed the increased proportion of spermatogonia to a lack of androgen-induced spermatogenic progression.

214.   Sertoli cell hypertrophy was an exposure-related finding for Lab 12. Although this finding was diagnosed in Labs 6 and 14, the incidences were low, and the results could not be considered exposure-related in those laboratories. "Nuclear hypertrophy of Sertoli cells" was also reported to be an effect of flutamide exposure by Wester et al. (37).

Table 66: Histopathological findings in female zebrafish exposed to flutamide

Diagnosis	Dose	LAB 12			LAB 14		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - PFC	L				1	1	9
Oocyte atresia, increased, mature	C				1	1	10
	L	2.3	3	10	1	2	9
	M	1	1	8			
	H	1.7	3	10			
Proteinaceous fluid, interstitial	C				2.2	5	10
	L				1	1	9
	M				1	4	9
	H				1.3	4	8
Interstitial fibrosis	H				1	1	8
Post-ovulatory follicles, increased	L	3	1	10			
Egg debris, oviduct	C	1	2	10			
	L	2.7	6	10			
	M	2	3	8			
	H	1.6	5	10			

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
PFC: perifollicular cells;
Shaded cells: exposure-related findings.

Diagnostic	Dose	LAB 6		
		Average grade	N	Observed
Increased perifollicular cells	C	-	-	-
	L	2	1	10
	M	3	1	4
	H	1	1	7
Oocyte atresia, mature	C	3	1	8
	L	3	2	10
	M	-	-	-
	H	2	2	7
Oocyte membrane folding	C	-	-	-
	L	-	-	-
	M	x	1	4
	H	-	-	-
Hypertrophy Perfollicular cells	C	-	-	-
	L	-	-	-
	M	-	-	-
	H	x	1	7
Egg debris, oviduct	C	-	-	-
	L	-	-	-
	M	-	-	-
	H	x	1	7

Flutamide and Female Zebrafish – "Pre-Heidelberg"

215.   There were no exposure-related findings for flutamide in female zebrafish. Median ovarian staging scores did not change in any exposure-related pattern in the exposed animal groups as compared to the control group.

Flutamide and Female Zebrafish – "Post-Heidelberg"

216.   There were no histopathologic findings for flutamide-exposed adult female zebrafish in a previous study (37).

5.6.3.4 Summary of Results

217.   Table 67, which summarizes the exposure-related results by compound, sex, and species, is based on the "Post-Heidelberg" results. In this table, the decision to categorize a finding as positive, negative, or equivocal was based on several factors, including: 1) Consistency of the finding among the relevant laboratories; 2) Average severity of the finding; 3) Precedence for the finding in the scientific literature; 4) Mechanistic logic for the finding based on available knowledge. In Table 67, diagnoses that are shaded represent "primary" diagnoses (to be explained in the Discussion section).

Table 67: Summary of Exposure Related Findings by Compound, Sex, and Species

4-tert-pentylphenol / 17β-Estradiol			
Finding	MALES		
	Medaka	Fathead Minnow	Zebrafish
Decreased Spermatozoa	+/- (P)	+/-	+/-
Histiocytic Cells Intraluminal	+	-	-
Increased Spermatogonia[1]	+/-	+	+
Increased Spermatozoa	+/-	+/-	-
Interstitial Fibrosis	+	-	-
Nephropathy	+	NE	NE
Proteinaceous Fluid [2]	+/-	+	+/-
Sertoli Cell Hypertrophy	-	-	+/-
Testicular Degeneration	+	+	-
Testis-ova[3]	+ (P)	+/-	+/-
Finding	FEMALES		
	Medaka	Fathead Minnow	Zebrafish
Granulomatous Inflammation	-	+/-	-
Increased Oocyte Atresia[4]	+	-	+/-
Interstitial Fibrosis	-	-	+/-
Proteinaceous Fluid[2]	+	+ (P)	-
Prochloraz / Fadrozole			
Finding	MALES		
	Medaka	Fathead Minnow	Zebrafish
Increased Interstitial Cells	+/-	+ (P)	-
Increased Spermatocytes	-	-	+/-
Increased Spermatogonia[1]	-	+/-	-
Increased Spermatozoa	+/-	+ (P)	+/- (P)
Interstitial Fibrosis	-	-	+/-
Proteinaceous Fluid[2]	-	+/-	-
Sertoli Cell Hypertrophy	-	+/- (P)	-
Finding	FEMALES		
	Medaka	Fathead Minnow	Zebrafish
Decreased Mature / Spawning Oocytes	-	-	+/- (P)
Decreased Perinucleolar Oocytes	+/- (P)	-	+/- (P)
Decreased Yolk Formation[5]	+	+ (P)	- (?)
Egg Debris, Oviduct (Decreased)	-	+/- (NE)	+/-
Granulomatous Inflammation	+/-	-	-
Hepatocyte Basophilia, Decreased	NE	NE	+/- (P)
Increased Early Vitellogenic Oocyte	-	+/- (P)	+/- (P)
Increased Late Vitellogenic Oocytes	-	+/-	+/- (P)
Increased Oocyte Atresia[4]	+	+	+ (P)
Ovarian Staging	+	+ (P)	+/- (P)
Perifollicular Cell Hypertrophy / Hyperplasia[6]	+	-	-
Post-ovulatory Follicles, Decreased[7]	-	+/-	-
Flutamide			
Finding	MALES		
	Medaka	Fathead Minnow	Zebrafish
Increased Interstitial Cells	-	-	+
Increased Spermatogonia[1]	-	+/-	+
Sertoli Cell Hypertrophy	-	-	+/-
Testis-ova[3]	+/-	-	-
Finding	FEMALES		
	Medaka	Fathead Minnow	Zebrafish
Post-ovulatory Follicles, Decreased[7]	+/-	-	-
Oocyte Atresia	-	+/-	-

Shaded cells = primary diagnoses; + = exposure-related; - = not exposure-related; +/- = equivocal; NE = not evaluated; (NE) = not evaluated in all laboratories; (P) = positive controls exclusively or primarily affected; ? = result not clearly determined.

[1]This diagnosis may incorporate proportional decreases in other germinal cells (spermatocytes and spermatids).

[2]This diagnosis incorporates both intravascular and interstitial proteinaceous fluid.

[3]Because testis-ova are considered to be a "rare event", this finding may be exposure-related even if there is not a substantially increased incidence in the exposed group(s) compared to the controls.

[4]This diagnosis incorporates atresia of both immature and mature oocytes, and also the diagnosis of oocyte membrane folding.

[5]This diagnosis incorporates the diagnoses of "decreased vitellogenesis" and "lack of vitellogenesis". It remains to be seen whether the diagnosis of Increased Early Vitellogenic Oocytes should also be incorporated.
[6]This diagnosis incorporates increased size and numbers of perifollicular cells.
[7]There was a dose-dependent decrease in "Increased Post-ovulatory Follicles".

5.6.4   *General histopathological discussion*

5.6.4.1 Phase 1B Study Consistency

Interlaboratory, Interstudy, and Interpathologist Consistency

Laboratory-Based Sources of Inconsistency

218.   In terms of the histopathology endpoint, laboratory-based issues are more difficult to confirm than are pathologist-based issues as sources of inconsistency.

219.   One laboratory-based issue of concern is the variability in the ages of fish at the onset of experimentation (see Table 1). Fish were outside of the species-specific protocol-recommended age ranges in 14 of 31 experiments. By species, the maximum age differences between experiments were 2 months, 7.5 months and 3.5 months for medaka, fathead minnow, and zebrafish, respectively. The maximum age difference for fathead minnow was disproportionately increased by a single study in which the fish were 12 months old at the start of experimentation (prochloraz study, Lab 11). For adult fish that are reproductively cycling, one might assume that there should be little difference in the performance of the gonads strictly as a function of age. Conversely, it is also reasonable to surmise that certain histopathologic findings, such as the formation of testis-ova, changes in the baseline incidence of oocyte atresia or testicular degeneration, or gametogenic cell-type distribution (especially in underage fish that may not be actively spawning), might be affected by the age of the fish. Scanning the Phase 1B data, it is difficult to find an example in which a laboratory's results were clearly affected by the age of the fish. Although this realization may bode well for the robustness of the assay, it does not help to explain interlaboratory / interstudy variation.

220.   Another laboratory-based issue involves the nominal vs. actual test substance dosages that were used in one experiment versus another. In this case, there are at least two experiments in which an exceptionally low actual concentration of the test substance was empirically associated with a lack or scarcity of exposure-related findings when compared to the same experiment as performed in other laboratories: 1) the relative paucity of findings for male or female zebrafish exposed to 4tPP in Lab 14; and 2) the comparative lack of exposure-related findings for male zebrafish exposed to flutamide in Lab 6.

221.   A third laboratory-based issue that has been identified involves the suitability of the prescribed sex ratio for adult fathead minnow as specified by the study design. A 1:1 ratio was used for the Phase 1B experiments; however, there is evidence to suggest that a ratio of 2 males to 4 females may be more appropriate (15). The primary concern here is that heightened competition for females and territoriality among male fathead minnow may cause stress and altered behavioral patterns that could potentially modify or mute endocrinological responses to chemical exposure. In retrospect, it is difficult to determine the degree to which this factor may have had an effect on the Phase 1B study.

222.   Throughout the Phase 1B studies, there did not appear to be any clear associations between excessive mortality or disease and interlaboratory inconsistencies, other than the reporting of disease-specific diagnoses such as granulomatous inflammation for some laboratories versus others.

223.   Rarely, discrepancies in results could be attributed to study protocol deviations (e.g., egg debris in the oviducts of fathead minnow that were sectioned by the whole-body transverse method versus having their gonads excised).

Pathologist-Based Sources of Inconsistency

224.   There were a number of cases in which interexperiment inconsistencies could be readily attributed to differences in lesion observation or interpretation. Rarely were these discrepancies directly contradictory (the issue of increased vs. decreased spermatozoa in medaka as induced by 4tPP was addressed in Section 5.6.3.1.1). Most of the following examples were identified at the Heidelberg meeting:

 – Two pathologists created a different diagnostic term for a finding for which no previous term existed (e.g., "decreased vitellogenesis" vs. "lack of vitellogenesis").
 – Pathologists occasionally used multiple diagnoses to document what was essentially a single exposure-related effect (e.g., the simultaneous diagnosis of increased spermatogonia with decreased spermatocytes and spermatids).
 – One pathologist (Pathologist K) developed an ad hoc alternative staging system for the medaka testis.
 – Some pathologists did not specifically evaluate extragonadal tissues (e.g., kidney and liver), either due to differences in the prescribed sectioning procedure, or due to a miscommunication of the instructions.
 – In some instances, different pathologists observed the same novel change but interpreted it slightly differently (e.g., increased cortical alveolar oocytes vs. decreased yolk formation).
 – The accuracy of one interpretation was questioned at the Heidelberg meeting (testis-ova vs. mineralization).

225.   A positive aspect of pathologist-based issues is that they are relatively easy to identify and address. There are two general reasons why all pathologists "were not on the same page". First, not every Phase 1B pathologist had the opportunity to attend the highly informative workshops in Bilthoven (6/11 pathologists), Paris (6/11 pathologists), and Heidelberg (10/11 pathologists). Second, although the histopathology guidelines provided valuable information concerning diagnostic criteria and terminology, Phase 1B represented the first trial run for the guidelines, and they were found to be deficient in several aspects, including: insufficient images for all three species; a few redundant diagnoses; some ambiguous criteria; and a lack of diagnoses for changes that were not previously anticipated.

Interspecies Consistency

226.   A casual inspection of Table 67 illustrates a number of interspecies differences in exposure-related results. Although some of these differences are presumed to represent laboratory or tank idiosyncrasies (e.g., granulomatous inflammation may be dependent on exposure to pathogens), this does not help to explain the remaining differences.

227.   It is logical to presume that the mechanisms of action for endocrine active substances are likely to be phylogenetically conserved. Therefore, the tendency for certain histopathologic responses to occur in some fishes, but not in others, might initially appear puzzling. However, it should be remembered that there are approximately 28,900 fish species, and that, these species may be as genetically, anatomically, and functionally different from one another as are mice and elephants. To assume that the responses to endocrine active substances would be more than qualitatively similar, and that the threshold dosages for the elicitation of such responses would be identical, one would have to ignore years of toxicological research in which species-specific results occurred in single-compound comparative bioassays using common laboratory mammals such as mice, rats, rabbits, dogs, cats, and non-human primates. Even if the

general endocrinological mechanisms themselves are held to be comparable, there are a still a great many other interspecies variables that may account for discrepancies in the histologic expression of effects among various fishes, including: 1) kinetic and other mechanistic differences in the absorption, metabolism, utilization, and elimination of hormonal compounds; 2) differences in the density of reproductive hormone receptors in target organs; 3) constituent differences in levels of endogenous hormone antagonists or agonists; 4) inherent differences in spawning cycle, gonochorism, and/or reproductive behavior; 5) structural and functional differences in microscopic gonad anatomy; and 6) protocol-prescribed differences in the sampling and histologic preparation of specimens.

Consistency between Positive Controls and "Weaker" Test Substances

228. In the majority of instances in which exposure-related findings were identified (discounting flutamide for which there were no positive controls), such findings were exposure-related in both the positive control and high dose groups simultaneously (and in some instances, the mid and low dose groups also).

229. Less frequently, findings were exclusively exposure-related in the positive control group (examples include many findings in prochloraz / fadrozole-exposed female zebrafish). This latter result is not surprising, as the positive control agents were selected based on the likelihood that they would generate findings, with anticipation that the test substances themselves might have less potent effects.

230. The situation in which exposure-related findings occurred in test-substance-exposed fish but not in positive controls was the least common outcome. It is unlikely that such results were spurious, because there was often a dose-dependent increase in the incidence and/or severity of these findings (examples include experiments in which female medaka, female zebrafish, or male fathead minnow were exposed to 4tPP). The most plausible explanation for this phenomenon is that exogenously administered analogs, such as 4tPP and 17β-estradiol, do not necessarily maintain the same relative levels of activity in fish that have been demonstrated by in vivo or in vitro experiments involving certain mammals or other fish species. Again, this may be due to interspecies in vivo differences in hormone metabolism.

5.6.4.2 Histopathology as an Endpoint

Sensitivity

231. The relative sensitivities of the various Phase 1B endpoints are presented in Table 68. As is evident from the table, the sensitivity of histopathology was often superior to growth, spawning status, and secondary sex characteristics, and it was often comparable to vitellogenin testing. It is important to note that the only two endpoints that identified flutamide as an endocrine-active agent were spawning status in fathead minnow and histopathology in zebrafish; vitellogenin was not useful for detecting the anti-androgen flutamide.

Table 68: The relative sensitivity of Phase 1B endpoints

Test Substance	Conc. (µg/L)	Species	Growth	Spawning	Secondary Sex Characteristics	Vitellogenin	Histopathology
4-tPP	100, 320, 1000	medaka	-	-	1000 (+/-)	100	320 (+/-) / 1000
		fathead minnow	-	1000	320 (+/-) / 1000	320	100 (+/-) / 1000
		zebrafish	-	-	n/a	100 (+/-) / 320	100 (+/-) / 1000
Prochloraz	20, 100, 300	medaka	-	100	-	20 (+/-) / 300	20 (+/-) / 300
		fathead minnow	-	-	300 (+/-)	100 (+/-) / 300	20 (+/-) / 300
		zebrafish	-	-	100	100	20 (+/-) *
Flutamide	100, 500, 1000	medaka	-	1000 (+/-)	-	100 (+/-) *	100 (+/-) *
		fathead minnow	-	1000	1000 (+/-)	-	100 (+/-) *
		zebrafish	-	-	n/a	-	500

Conc. = concentrations tested; - = no effect; (+/-) = equivocal effect; * equivocal at all higher concentrations also; n.a.: not available.

Specificity

232.   The overall specificity of histopathology as an endpoint could not be fully evaluated because a negative control substance was not tested. Regarding individual diagnoses, there may be concern that certain findings (e.g., increased oocyte atresia or testicular degeneration) might occur secondary to toxic mechanisms that do not involve the reproductive axis. Although theoretically possible, there is no definitive evidence of this to date. In addition, test substance exposure in Phase 1B tended to induce multiple histopathologic changes, that when combined, provided a "weight of evidence" indication of hormonal effect.

Cost-efficiency

233.   At the Heidelberg meeting, there were discussions devoted to options to decrease the cost of histopathology. The following suggestions chiefly involved minimizing the pathologists' time during the microscopic examinations:

−   Allow pathologists to focus their evaluations on a few "primary" or "core" diagnoses, as identified by the Phase 1B assays. A list of such diagnoses is presented in Table 69. In addition to their performance in the Phase 1B assay, diagnoses were selected for this list based on criteria such as ease of use and precedence in the scientific literature. This proposal could accelerate the slide reading process both by decreasing the number of characteristics that would need to be evaluated in a given slide, and by simplifying the recording of diagnoses. During microscopic examinations, pathologists would also be aware of certain "secondary" diagnoses that would be recorded if they appeared as obvious changes (examples include increased proteinaceous fluid in the gonads, and extragonadal findings in the liver or kidney).

Table 69. List of Primary Diagnoses

No.	Males:	Females:
1	Increased proportion of spermatogonia	Increased oocyte atresia
2	Presence of testis-ova	Perifollicular cell hyperplasia/hypertrophy
3	Increased testicular degeneration	Decreased vitellogenesis
4	Interstitial (Leydig) cell hyperplasia/hypertrophy	Gonadal staging (based on improved staging criteria)

−   Reduce or eliminate the narrative section of the pathology report. This time-consuming phase of the evaluation would be less essential if the number of diagnoses was limited as proposed above. In addition, efficient forms for the tabulation of results could be created.

−   Allow the pathologist to be aware of the exposure group status of the test subjects. By directly comparing the high dose group to the negative controls, the pathologist can immediately concentrate his/her attention on significant changes. This topic will be further discussed in Section 5.6.5.3.

−   Have pathologists initially examine the high dose and negative control groups. If exposure-related changes are not evident, the mid and low dose groups may not have to be assessed.

−   The Heidelberg pathologists briefly discussed a proposal to have trained technicians evaluate slides in lieu of pathologists. This suggestion was eventually rejected because it was realized that the

initial cost savings would probably be negated by the expenses involved with technician training, the supervision of technicians by pathologists, and the review of results by pathologists.

5.6.5   Further histopathology recommendations

Statistical analysis

234.   The consensus of the Heidelberg pathologists was that formal statistical analysis was probably not necessary for standard results evaluation.

Inclusion or exclusion of severity grading

235.   In addition to recording the incidence of findings, the Heidelberg pathologists generally agreed that severity grading should be performed routinely (as was the case in Phase 1B). Severity grading results can indicate whether a dose-responsive pattern is present, and can help to support or refute exposure-related findings.

Blind slide reading

236.   As discussed earlier, blind slide evaluation, in which the pathologist is unaware of the treatment-group status of individual animals, generally increases the cost and time incurred in studies. In addition, there is insufficient evidence to support the claim that blind slide reading actually decreases bias, when studies are evaluated by trained histopathologists. There is, however, evidence that a blinded approach may mask subtle or novel findings, and for this reason it has been recommended that the initial slide evaluations in toxicological studies should not be performed in this fashion (55).

237.   Perhaps the most important consideration for the fish screening assay is that, except for testis-ova, all of the core findings involve incremental alterations in the number, size, color, texture and/or shape of organs, cells, interstitial tissues, or fluids; inherently, such changes can only be assessed by a direct and knowledgeable comparison of compound-exposed and unexposed fish (i.e., the identity of the negative control fish needs to be known to the pathologist).

238.   Alternatively, another type of blinding, in which the pathologist is unaware of the name or nature of the test article during the slide evaluation, may have application for the fish screening assay, either as part of a validation exercise or as routine practice.

Improvements to the Guidance Document

239.   As briefly alluded to earlier, the Heidelberg pathologists identified a number of potential areas for improvement of the draft histopathology Guidance Document. Suggestions included:

- Devote a section of the document to the primary core diagnoses as identified during Phase 1B;
- Create a section that lists the anticipated microscopic gonadal effects of exposure to the various compound classes and mechanisms;
- Insert more images, to include images of the same finding for all three species whenever possible;
- Add newly identified diagnostic terms and criteria (related to aromatase inhibitor effects, e.g.);
- Correct a few inaccuracies;
- Streamline the document by excluding ancillary procedures that are not directly related to histopathology.

Pathologist training

240.   It has been suggested that there may benefit in the future by having pathologists attend a workshop that is entirely geared to familiarizing participants with the procedures, with special emphasis on the recognition and documentation of reproductive endocrine effects in the relevant test species.

5.7   Other optional data

241.   Measurement of concentrations of persistent organic pollutants (POPs) in food was conducted at LAB 1. The brine shrimp was from Salt Lake in the U.S.A. The results are shown as follows.

Table 70: Measurements of organochlorine pesticides in the brine shrimp.

Substances	Concentrations of organochlorine pesticides in the brine shrimp (ng/g)
Hexachlorobenzene	1.089
β-Hexachlorocychlohexane	0.098
cis-Chlordane	0.025
trans-Nonachlor	0.068
o,p'-DDT	0.026
p,p'-DDT	N.D.*
p,p'-DDE	0.310
p,p'-DDD	0.072

The determination limit of organochlorine pesticides in the brine shrimp: 0.02 ng/g
*: N.D. < 0.02 ng/g

5.8   Compilation of opinions in participating laboratories

242.   LAB 1 conducted the survey for Phase 1B of the validation of the Fish Screening Assay to collect various opinions from all participating laboratories. Eight out of 14 laboratories joined the survey, and all opinions are listed in **Annex 6**. Summary of the survey is described below.

Protocol instructions

243.   All participating laboratories feel that the present protocol for the Fish Screening Assay is generally sufficient for performing the study.

Selection of test organisms

244.   Laboratories for medaka and fathead minnow think that the selection criteria for test fish are sufficient. Several experts point out that it has to take into account that development time generally depends on holding conditions (biomass loading, food, water renewals per day, etc.) and temperature. Participants for zebrafish feel that 15 (±2) weeks seems not to be sufficient for having actively spawning fish, and suggest that fish size should be more important rather than fish age in this species, e.g., Size/Weight: 0,350 g for males and 0,5 g for females.

Endpoints/Spawning status

245.   All laboratories recognize the potential necessity for measuring spawning status in Fish Screening Assay. Most of the participating laboratories think that the observation of spawning status in the present

protocol is easy and essential. Several experts insist the necessity of quantitative measurement of egg numbers, while another expert for zebrafish suggests that semi-quantitative measurement would be available, because egg counting of zebrafish is time-consuming. However, an expert for medaka points out that spawning status before the exposure should be strictly measured, if the quantitative measurement of the fecundity is adopted in the Fish Screening Assay. An expert suggests the addition of fertility to the Assay. Another expert indicates that senescent or pre-spawning fish should not be selected if this endpoint is used.

Endpoints/Secondary sex characteristics

246.   All laboratories for medaka and fathead minnow feel that the SOPs for the measurement of secondary sex characteristics are generally sufficient. Laboratories for medaka point out a minor problem associated with fixation of the fin rays, i.e., crossed fin rays are sometimes observed in the fixed anal fins, which disturbed the measurement of secondary sex characteristics in medaka. In fathead minnow, one expert feels that the SOPs for this species do not cover the early stages of nuptial tubercle formation (i.e., in some instances tubercles were present as white discs, but this stage did not fit into the three category provided). Another expert for fathead minnow suggests that additional drawings of mapping regions and detailed pictures of tubercle size/structures could be helpful, because tubercles could not always be clearly assigned (transition forms). In addition, a minor problem is indicated that quantitatively ranking of tubercles size was not so easy and observations were mostly not completed within 2 minutes. All participating laboratories feel that there is no problem about Excel reporting template.

Endpoints/VTG measurement

247.   All participants think that the results were satisfactory in terms of reproducibility. A few experts feel that the variation of VTG levels can be reduced. An expert for zebrafish points out the difficulty in measurement of the exact blood volume result in variation of this parameter, suggesting that the variation will be minimised if the same person takes the blood samples from all the exposure groups. Another expert for zebrafish thinks that the more standardized every procedure throughout the experiment is, the lower the observed variations will be.

Other ideas/ observations/comments

248.   Several laboratories indicate the problems associated with preparation for test solution as follow;

1) methods provided need to be suitable for all participants and therefore, avoid the use of specialized equipment,
2) SOP's for the preparation of solvent free dilutions given by LAB 4 were difficult to transform into each laboratory,
3) little or no information on solubility/stability of the test items and no alternatives on preparation for test solutions were available, resulting in deviations from the protocol,
4) the guidance on how to prepare the test stock solutions was given rather late.

249.   Although all participants had no problems regarding analytical chemistry, a few laboratories had difficulties maintaining 4-*tert*-penthyphenol concentrations in the test solutions.

250.   Regarding the design of and reporting into Excel Spreadsheets, several experts suggest the following;

1) redesign the Excel Spreadsheets to enter all biological data in each individual,
2) the size of the boxes could have been larger,
3) the information value of flow-through conditions is not quite clear.

General

251.   Several participants propose that, for any future validation exercises the following suggestions are noted; 1) it is necessary to distribute the test substances, ELISA kits and VTG standard to each participating laboratory without delay, 2) more communication between lead laboratory and participating laboratories, and among lead laboratories, may facilitate the overall exercise, 3) technical SOPs should be provided without delay, and 4) it should be easy to integrate the SOPs in existing systems.

6. REPRODUCIBILITY ASPECTS OF TEST RESULTS

252.   After a detailed presentation and analysis of the results in Section 4, this section is now intended to provide an overview of reproducibility of quantitative endpoints (VTG and secondary sexual characteristics) measured in Phase 1B.

253.   Reproducibility of test results across laboratories using the same fish species, the same protocol and the same chemicals is useful in evaluating the overall reliability of the assay in providing consistent responses when used in different places. A good measure of the reproducibility is the coefficient of variation (CV), a measure of sample variation relative to the mean.

254.   In this specific case, intra-laboratory CV represents the variability of measurements from different individuals (n ≤ 10) within a group (either control or treated group). It is important to note that the intra-laboratory CV does not represent variability of measurements performed n=10 times on the same biological sample. In other words, the intra-laboratory coefficient of variation takes into account the variability of the method used to measure the endpoints, and the inherent variability of individual fish.

255.   The inter-laboratory coefficient of variation is obtained by measuring the variability of same treatment levels (e.g. fathead minnow male VTG values in control groups measured in different laboratories). Coefficients of variation vary substantially according to the treatment level; therefore CV from each treatment level is analyzed separately. This is because the spread or variability of low values of the dose response curve (e.g. VTG in control male fish) is much bigger than the spread of high values (e.g. VTG values in estrogen-induced females).

256.   Very few studies exist in the scientific literature that provides a comparison of performance between ELISA methods used for vitellogenin measurement across laboratories. A comparative study was undertaken in the United States in 2002-2003 (33) to survey several methods available for vitellogenin measurement in fathead minnow, medaka and zebrafish. In this study, the ELISA methods used varied from one laboratory to another, but the vitellogenin concentrations measured were identical. Samples were analyzed in triplicates, which enabled the evaluation of the within-triplicate variability, which is the intra-assay variability based in triplicate mean when the same concentration of vitellogenin is measured. However, the potential sources of variability were not identified. Another important study was conducted in Japan to evaluate and then validate two ELISA methods commercially available for medaka (6). Samples were prepared centrally were then measured in five laboratories using both ELISA kits. Results yielded a measure of variability of the method when used in different places, and a basis for comparison of the two kits. Within-laboratory CVs were calculated on the basis of duplicate samples for each exposure level and were very low.

257.   The vitellogenin method comparison study performed in Japan on medaka (6) appears encouraging as inter-laboratory measurements were closely related, with minimal variability. However, it remains difficult to link the outcome of these studies with the outcome of Phase 1B since intra-laboratory coefficients of variation in Phase 1B are based on biological samples from different fish, and not on replicates of the same samples.

6.1 Reproducibility of results in medaka

6.1.1 Vitellogenin measures in medaka

Intra-laboratory CV

Table 71: Intra-laboratory CV of VTG measurements in medaka

4*tert*-pentylphenol		Concentration (µg/l)				
Sex	Laboratory	Cont.	100	320	1,000	PC
Male	LAB1	197.5	184.0	58.8	37.0	20.7
	LAB 3	0.0	138.5	60.7	23.3	33.1
	LAB 2	0.0	267.9	132.4	44.4	44.2
	LAB 5	42.0	226.6	217.2	61.7	108.4
Female	LAB 1	65.0	35.0	59.8	95.9	56.9
	LAB 3	31.1	66.5	43.3	44.1	83.9
	LAB 2	77.7	65.8	68.5	45.0	77.1
	LAB 5	110.6	121.8	193.3	139.6	98.5
Prochloraz		Concentration (µg/l)				
Sex	Laboratory	Cont.	20	100	300	PC
Male	LAB 1	0.0	0.0	0.0	0.0	0.0
	LAB 2	52.1	66.1	107.1	61.9	41.6
	LAB 4	207.4	83.0	150.5	108.6	85.8
	LAB 6	268.0	143.5	144.0	158.5	149.6
Female	LAB 1	17.6	45.4	143.9	106.4	107.6
	LAB 2	59.1	69.0	105.7	101.2	128.2
	LAB 4	40.5	65.3	100.0	78.7	75.5
	LAB 6	143.5	138.9	165.1	113.7	316.2
Flutamide		Concentration (µg/l)				
Sex	Laboratory	Cont.	100	500	1,000	
Male	LAB 3	0.0	52.3	0.0	30.3	
	LAB 5	0.0	61.2	61.2	127.3	
	LAB 4	166.6	59.5	148.1	57.6	
	LAB 6	268.0	99.6	86.2	105.8	
Female	LAB 3	41.3	33.9	37.9	34.3	
	LAB 5	118.9	125.1	78.2	140.8	
	LAB 4	63.6	92.6	67.5	91.3	
	LAB 6	143.5	154.8	131.9	101.0	

258.   It should be noted that a CV equal to 0 means that all values within a group were all at the lowest detection limit. The highest CVs (>100%) correspond to low VTG levels, usually found in control males or weakly induced males. As VTG levels increase in e.g. 4*tert*-pentylphenol exposures in males, the range of CVs decreases to 30-60% compared to the 100-200% in controls and lower exposure groups.

259.   Interpretation of the CV has to be balanced with the intensity of the effect measured following chemical exposure (VTG increase or decrease). High CV in low parts of the dose-response curve does not mean as such that VTG measurement is not reproducible. As an example, in LAB 1 control males had VTG levels between 0.5 and 12 ng/mg liver (CV=197%). The significance level for 4*tert*-pentylphenol exposure was reached at the lowest concentration when VTG ranged between 9 and 122 ng/mg liver (CV=184%). At the highest concentration, induced males showed VTG levels between 2815 and 8900 ng/mg (CV=37%). The intensity of the response is 1000 time the value in the control group.

260.   These coefficients of variation integrate several sources of variability:

- the variability due to the handling of the method: each laboratory used the same method, but small technique variation can result in differences of measured values;
- the variability between individual fish: measures were performed once only on each liver sample;

261.	Regarding the first point, some laboratories seem to have a better ability to minimize variation; other labs maintain high coefficients of variation even at high VTG levels. For example, LAB 1 and LAB 3 had low coefficients of variation with high VTG values (e.g. 4*tert*-pentylphenol studies), whereas results from LAB 5 and LAB 6 showed a high variability for all VTG values measured. The study conducted in Japan (6), would tend to demonstrate that variability due to handling of the method can be minimized with e.g. experience with the method.

Inter-laboratory CV

Table 72: Inter-laboratory CV of VTG measurements in medaka

4tert-pentylphenol		Concentration (µg/l)				
Sex		Cont.	100	320	1,000	PC
Male	Lab number	4	4	4	4	4
	CV (%)	74.0	73.3	51.6	52.5	53.5
Female	Lab number	4	4	4	4	4
	CV (%)	75.2	83.8	51.3	45.7	59.0
Prochloraz		Concentration (µg/l)				
Sex		Cont.	20	100	300	PC
Male	Lab number	4	4	4	4	4
	CV (%)	118.5	110.3	95.9	70.0	89.6
Female	Lab number	4	4	4	4	4
	CV (%)	51.5	83.9	72.9	56.1	66.4

Flutamide		Concentration (µg/l)			
Sex		Cont.	100	500	1,000
Male	Lab number	4	4	4	4
	CV (%)	183.4	107.9	123.4	112.3
Female	Lab number	4	4	4	4
	CV (%)	40.0	95.7	110.8	85.6

262.	The inter-laboratory coefficient of variation provides an indication of the ability of laboratories to produce similar absolute VTG values with a narrow spread when they are measuring VTG from different fish, exposed to a similar (but not strictly identical) regime and using the same ELISA kit, applied by different technicians. So, it integrates the biological variability of the fish, the variability of the measured concentrations and the variability in the handling of the ELISA kit due to the person using the kit.

263.	To remove variability due to the measured test substance concentration, one should only compare control groups among themselves (i.e. where no variability inherent to treatment exists). When values are close to the detection limit, variability is high (e.g. between 74% and 183% for males). For females, variability of measurements is much less (CV between 40% and 75%).

6.1.2 Secondary sex characteristics in medaka

Intra-laboratory CV

Table 73: Intra-laboratory CV of secondary sex characteristics measurements in medaka

4*tert*-pentylphenol		Concentration (µg/l)				
Sex	Lab	Cont.	100	320	1,000	PC
Male	LAB 1	21.1	22.7	20.1	20.3	18.1
	LAB 3	19.4	21.1	16.1	27.7	19.5
	LAB 2	23.1	27.0	29.5	30.3	17.9
	LAB 5	16.6	35.4	18.0	19.3	11.9
Prochloraz		Concentration (µg/l)				
Sex	Lab	Cont.	20	100	300	PC
	LAB 1	14.1	14.9	23.0	27.9	17.1
	LAB 2	20.7	29.3	10.5	16.2	32.8
	LAB 4	9.5	16.9	11.9	18.1	19.3
Male	LAB 6	11.0	11.1	20.3	26.4	17.2
Flutamide		Concentration (µg/l)				
Sex	Lab	Cont.	100	500	1,000	PC
	LAB 3	18.9	13.6	23.2	16.4	-
	LAB 5	15.8	28.4	19.0	30.0	-
	LAB 4	17.3	11.6	21.5	19.5	-
Male	LAB 6	11.0	12.3	19.9	15.6	-

264.   All laboratories used the same standard operating procedures to measure secondary sex characteristics in medaka. So the CV integrates the biological variability between fish mainly if one compares CV from control groups. The CV in treated groups integrates both the biological variability between fish and the variability of measured concentrations compared to nominal ones.

265.   However, in medaka, none of the chemicals induced a significant response of secondary sex characteristics, so it is difficult to comment on the CV with respect with the effect size expected since there was no effect on papillary processes following exposure. In control animals, the group means ranged between 70 and 120 papillary processes (CV=16.5%).

Inter-laboratory CV

Table 74: Inter-laboratory CV of secondary sex characteristics measurements in medaka

4*tert*-pentylphenol		Concentration (µg/l)				
Sex		Cont.	100	320	1,000	PC
Male	Lab number	4	4	4	4	4
	CV (%)	8.9	12.5	21.8	19.0	7.4
Prochloraz		Concentration (µg/l)				
Sex		Cont.	20	100	300	PC
	Lab number	4	4	4	4	4
Male	CV (%)	15.5	15.1	12.5	8.1	12.0
Flutamide		Concentration (µg/l)				
Sex		Cont.	100	500	1,000	PC
	Lab number	4	4	4	4	-
Male	CV (%)	14.2	16.2	16.9	15.9	-

266.   Inter-laboratory CVs were low in the control groups, between 8.9% and 15.5%.

6.2 Reproducibility of results in fathead minnow

6.2.1 Vitellogenin measures in fathead minnow

Intra-laboratory CV

Table 75: Intra-laboratory CV of VTG measurements in fathead minnow

4*tert*-pentylphenol		Concentration (µg/l)				
Sex	Lab	Cont.	100	320	1,000	PC
Male	LAB 7	74.0	236.8	168.9	37.8	42.0
	LAB 8	0.0	0.0	145.4	43.1	32.0
	LAB 10	-	-	-	-	-
Female	LAB 7	36.9	69.7	39.2	53.4	33.6
	LAB 8	65.5	73.0	108.1	101.7	76.4
	LAB 10	-	-	-	-	-
Prochloraz		Concentration (µg/l)				
Sex	Lab	Cont.	20	100	300	PC
Male	LAB 8	0.0	0.0	0.0	0.0	0.0
	LAB 11	0.0	0.0	0.0	0.0	0.0
	LAB 4	88.1	97.0	278.8	191.6	281.7
Female	LAB 8	85.8	80.4	47.2	144.9	24.9
	LAB 11	58.1	76.5	107.7	216.3	-
	LAB 4	31.5	17.8	52.7	37.0	272.8
Flutamide		Concentration (µg/l)				
Sex	Lab	Cont.	100	500	1,000	PC
Male	LAB 7	0.0	227.2	0.0	107.7	-
	LAB 10	0.0	0.0	0.0	0.0	-
	LAB 4	316.2	246.3	307.2	256.5	-
Female	LAB 7	30.2	47.4	51.2	44.9	-
	LAB 10	95.0	71.6	95.6	74.9	-
	LAB 4	57.6	33.0	38.5	41.0	-

267.   Similar to studies in medaka, the coefficient of variation decreases with increasing VTG values: for example in the 4*tert*-pentylphenol study, for highly induced males and females the CVs vary between 32% and 76% (one CV is outside this range). However, for low and mid-concentration of 4*tert*-pentylphenol, the coefficient of variation of VTG measurements in males remains elevated, this is because VTG induction was not pronounced at the low and mid-concentrations (especially in LAB 7).

Inter-laboratory CV

Table 76: Inter-laboratory CV of VTG measurements in fathead minnow

4*tert*-pentylphenol	Concentration (µg/l)				
Sex		Cont.	100	320	1,000	PC
Male	Lab number	2	2	2	2	2
	CV (%)	124.2	115.5	141.2	6.6	19.8
Female	Lab number	2	2	2	2	2
	CV (%)	27.4	20.9	44.8	38.2	107.2
Prochloraz	Concentration (µg/l)				
Sex		Cont.	20	100	300	PC
Male	Lab number	3	3	3	3	3
	CV (%)	83.1	101.5	106.6	111.2	94.2
Female	Lab number	3	3	3	3	3
	CV (%)	73.1	45.3	47.5	99.8	116.9
Flutamide	Concentration (µg/l)				
Sex		Cont.	100	500	1,000	PC
Male	Lab number	3	3	3	3	-
	CV (%)	105.1	149.5	140.4	136.7	-
Female	Lab number	3	3	3	3	-
	CV (%)	89.6	88.0	119.6	97.2	-

268.   At high VTG induction levels, the inter-laboratory CV can be as low as 6.6%, such low CV can be achieved when there is a good adequacy between absolute VTG values. But the inter-laboratory CV gets large when there are large differences between absolute VTG values between laboratories.

6.2.2   Secondary sex characteristics in fathead minnow

Intra-laboratory CV

Table 77: Intra-laboratory CV of secondary sex characteristics measurements in fathead minnow

4*tert*-pentylphenol		Concentration (µg/l)				
Sex	Lab	Cont.	100	320	1,000	PC
Male	LAB 7	22.3	31.0	74.6	0	59.5
	LAB 8	33.7	46.3	59.1	0	70.7
	LAB 9	42.7	-	-	138.2	56.3
Prochloraz		Concentration (µg/l)				
Sex	Lab	Cont.	20	100	300	PC
Male	LAB 8	21.2	28.0	54.4	71.2	28.3
	LAB 9	41.0	42.6	33.4	54.9	37.5
	LAB 11	16.3	29.2	29.5	24.7	17.6
	LAB 4	19.0	33.7	25.4	30.6	19.1
Flutamide		Concentration (µg/l)				
Sex	Lab	Cont.	100	500	1,000	PC
Male	LAB 7	22.8	32.1	29.9	20.7	
	LAB 10	29.4	26.8	19.4	19.0	
	LAB 4	20.3	25.7	34.3	20.0	

269.   All laboratories working on the fathead minnow had the same standard operating procedures to count the number of nuptial tubercles. In control groups of male fathead minnow, the intra-laboratory variation ranged between 16% and 43%. These CVs integrate the biological variability between fish and the variability of tubercles counts when performed in different laboratories. In the case of secondary sex characteristics, there is no variability due to the technique used.

270.   One observation that can be made when looking at the control groups is that some laboratories seem to constantly have lower CVs (LAB 4 and LAB 7), whereas other have consistently higher CVs (LAB 9).

Inter-laboratory CV

Table 78: Inter-laboratory CV of secondary sex characteristics measurements in fathead minnow

4*tert*-pentylphenol		Concentration (µg/l)				
Sex		Cont.	100	320	1,000	PC
Male	Lab number	3	2	2	3	2
	CV (%)	21.9	24.5	38.1	173.2	29.9
Prochloraz		Concentration (µg/l)				
Sex		Cont.	20	100	300	PC
Male	Lab number	4	4	4	4	4
	CV (%)	20.1	21.2	15.9	29.5	24.2
Flutamide		Concentration (µg/l)				
Sex		Cont.	100	500	1,000	PC
Male	Lab number	3	3	3	3	-
	CV (%)	10.4	89.2	88.4	95.9	-

271.   The inter-laboratory CV in the control groups is quite low (from 10% to 22%). High CVs are often contributed by a real difference in the absolute mean number of tubercles between laboratories rather than by high intra-laboratory CV.

6.3   Reproducibility of results in zebrafish

6.3.1   *Vitellogenin measures in zebrafish*

Intra-laboratory CV

Table 79: Intra-laboratory CV of VTG measurements in zebrafish

4*tert*-pentylphenol		Concentration (µg/l)					
Sex	Lab	Cont.	SC	100	320	1000	PC
	LAB 12	71.8	134.9	162.7	63.2	47.0	181.0
	LAB 13	92.2	-	108.5	159.0	42.0	47.9
Male	LAB 14	156.6	-	248.0	299.6	104.5	212.8
	LAB 12	63.7	27.6	24.1	42.6	39.0	41.9
	LAB 13	118.7	-	42.5	65.7	95.7	80.1
Female	LAB 14	96.4	-	73.1	65.3	70.0	65.4
Prochloraz		Concentration (µg/l)					
Sex	Lab		Cont.	20	100	300	PC
	LAB 12		248.2	73.8	97.0	40.9	152.2
Male	LAB 13		186.3	197.2	153.8	163.0	83.9
	LAB 6		138.9	130.8	239.0	181.5	141.6
Female	LAB 12		66.6	35.0	34.7	55.6	26.7
	LAB 13		34.7	43.2	38.4	42.6	247.3
	LAB 6		59.8	137.5	228.1	83.8	96.7
Flutamide		Concentration (µg/l)					
Sex	Lab		Cont.	SC	100	500	1000
	LAB 12		70	145	119	162	137
Male	LAB 14		286	56	194	160	106
	LAB 6		139	-	171	291	333
Female	LAB 12		46	47	55	74	33
	LAB 14		47	71	49	60	64
	LAB 6		60	-	79	120	54

272.   Coefficients of variation in zebrafish are generally higher than for the other two species, and there is much disparity between laboratories and between studies. However, low variability can be achieved for elevated VTG levels (CV= 42%), like in other species. It is difficult to identify specific reasons for the high variability observed in the studies, but again, this needs to be balanced with the size of the effect measured. For example, in LAB 12, VTG values ranged between approximately 900 ng/ml plasma (CV=72%) for control males and 21 millions ng/ml plasma (CV=47%) for highly induced males. Another example is LAB 14 where control males had 76000 ng/ml plasma (CV=156%) and induced males had 12 millions ng/ml plasma (CV=104%).

273.   In zebrafish studies, it is less clear whether some laboratories work in a more reproducible way than others. Low coefficients of variation are not associated with particular laboratory(ies). The ELISA measurements of VTG were performed at a single central laboratory, although the blood sampling was done in each individual laboratory.

Inter-laboratory CV

Table 80: Inter-laboratory CV of VTG measurements in zebrafish

4*tert*-pentylphenol		Concentration (µg/l)					
Sex		Cont.	SC	100	320	1,000	Control
Male	Lab number	3	1	3	3	3	3
	CV (%)	164.4	-	49.8	76.1	69.4	158.6
Female	Lab number	3	1	3	3	3	3
	CV (%)	97.0	-	76.0	73.5	42.9	101.2

Prochloraz		Concentration (µg/l)				
Sex		Cont.	20	100	300	PC
Male	Lab number	3	3	3	3	3
	CV (%)	172.4	72.5	113.5	151.9	34.7
Female	Lab number	3	3	3	3	3
	CV (%)	53.7	80.9	38.5	27.8	144.5

Flutamide		Concentration (µg/l)				
Sex		Cont.	SC	100	500	1,000
Male	Lab number	3	3	3	3	3
	CV (%)	148.0	27.6	79.7	126.1	98.7
Female	Lab number	3	2	3	3	3
	CV (%)	70.3	34.3	73.4	74.4	82.0

7. DISCUSSION

7.1   Overview of the validation work of Phase 1B and core endpoints

274.   Phase 1B of the validation work demonstrated that the protocol for the Fish 21-day assay using any of the three fish species is able to detect substances acting via the endocrine system such as the weak estrogen 4-*tert*-penthyphenol and the aromatase inhibitor prochloraz, via VTG measurement in males and females respectively. For the anti-androgen mode of action, observations were much less consistent across species and across laboratories; an unambiguous explanation for the apparent lack of sensitivity on this latter mechanism can not be provided at this stage, but one speculative explanation is proposed in this section.

275.   Exposure to 4-tert-pentyphenol caused VTG induction in males of the three fish species within the range of concentrations tested. In medaka, a significant induction of VTG was observed at 100 μg/l (*3 labs/4*), in fathead minnow (*2 labs/2*) and zebrafish (*2 labs/3*) at 320 μg/l.

276.   Prochloraz exposure inhibited VTG concentrations dose-dependently in females of the three fish species simultaneous with spawning cessation or decrease within the range of concentrations tested. A significant reduction of VTG was observed at 300 μg/l in medaka (*4 labs/4*), fathead minnow (*3 labs/3*) and zebrafish (*2 labs/3*). Cessation of spawning was clear in medaka at 100 μg/l (*2 labs/3*). In fathead minnow, cessation of spawning was confounded by poor fecundity in control groups. In zebrafish, spawning decreased but a complete cessation was not observed.

277.   Flutamide exposure caused no clear alteration of VTG levels in males or females, no clear alteration of spawning, and no alteration of the secondary sex characteristics. Evaluation of gonadal histology revealed dose-dependent changes occasionally in testis and ovaries of fathead minnow and zebrafish. However, findings were not consistent in males across laboratories using the fathead minnow and medaka; additionally some findings were also observed in control animals.

278.   Vitellogenin measurement was able to correctly detect weak and potent estrogens and aromatase inhibitors in most cases:

- Positive controls:
  - Estrogen: detected in 88% of cases (9 studies)
  - Aromatase inhibitor: detected in 100% of cases (10 studies)
- Test substances:
  - Estrogen: detected in 100% of cases (9 studies)
  - Aromatase inhibitor detected in 90% of cases (10 studies)

279.   Variability was generally higher for vitellogenin measurements in zebrafish. A possible explanation might be the fact that plasma samples were not analyzed by means of a standard kit as was used for medaka and fathead minnow, but were instead sent to the University where the method has been developed, where all zebrafish plasma samples were analyzed. There might be extra handling of samples compared with situations where a kit was used on the site where samples were collected, but this is hypothetical. In general, sources of variability are difficult to identify, due to the limited datasets available. Possibly, the expertise and care within a laboratory might contribute to reduce variability.

280.   In Phase 1B, the evaluation of secondary sex characteristics was responsive to 4-tert-pentylphenol in fathead minnow only. . However, this should not eclipse the value of secondary sex characteristics for the detection of androgen substances in the fathead minnow and the medaka (cf. Phase 1A). Secondary sex

characteristics are directly under control of the androgen receptor. Detailed standard operating procedures have been made available to laboratories to enable measurement of nuptial tubercles in fathead minnow and papillary processes in medaka. Again, training in applying these standard operating procedures will certainly contribute to reduce variability and potential false negatives.

281.   Gonad histology in Phase 1B has benefited from efforts to harmonize the histotechniques to produce standard quality slides. Additionally, efforts have been made by pathologists reading the slides to use the same diagnostic terminology and grading and reporting system. These initiatives were encouraged with the view to facilitate future acceptance of histopathological findings in a regulatory context. Gonadal histopathology was recognized to be a challenging aspect of the validation work, in particular in defining its possible role in the context of a screening assay for endocrine active substances. It is largely acknowledged that gonad histopathology is potentially useful in detecting and characterizing endocrine active substances, since gonads are the target organs for such substances. However, in balancing the discussion in the context of this assay, other aspects also need to be accounted for:

a. Endpoints of diagnostic value are essential to differentiate an endocrine active substance from other substances having an effect on the gonads; the role of gonad histopathology in achieving this goal needs further discussion;
b. Reproducibility of findings across laboratories and across species is not satisfactory at this stage if the assay is intended to be used as a regulatory tool; training of pathologists will be critical to correctly identify and report findings and to improve the overall reproducibility;
c. Cost-efficiency of histology in the context of this assay needs further discussion: a decision path could aid further acceptance of the role of gonad histopathology in this assay.

282.   An observation of spawning status was added in the phase 1B study protocol. Prochloraz exposure caused concentration-dependent decrease of spawning status in medaka and fathead minnow studies, indicating that a daily recording spawning status is useful to detect an effect of aromatase inhibitor. However, lack of, or low, spawning in control groups were reported from most of the fathead minnow studies, except the studies in LAB 7. For medaka and zebrafish, all control animals spawned well throughout the exposure period. This result showed that fathead minnow are territorial and the group-spawning conditions proposed in Phase 1B might be sub-optimal for fathead minnow.

7.2   Application and limitation of the present protocol for the Fish Screening Assay

7.2.1   Weak estrogen

VTG

283.   In this validation work, the VTG levels in exposed males were significantly induced in three fish species. There is a report on VTG induction in the fish exposed to weak estrogen. Seki et al. (25) reported that VTG levels were clearly increased at doses as low as 51.5 µg/l when medaka were exposed to this alkylphenol from embryo to 101 d post hatch. In addition, several studies have reported that weak estrogens induce VTG synthesis in adult fish. Ankley et al. (15) exposed adult fathead minnow to a weak estrogen, metoxychlor, for three weeks and showed that VTG levels increased in male fish. A similar study has been conducted by Kang et al. (20), which demonstrated VTG induction when adult medaka were immersed to weak estrogen, 4-nonylphenol for three weeks. These published reports and this present validation work clearly show that weak estrogen can induce the VTG production of males in adult fish. However, Seki et al. (25) conducted a fish full life cycle test with 4-*tert*-penthyphenol using medaka, suggesting that the weak estrogens may exert the estrogenic effects and lethal and sublethal toxicity at similar concentrations. Caution should be exercised in determining test concentrations of weak estrogen, because general toxicity can obscure the estrogenic effects of the chemical.

284.   As we have seen, the size of the chemical-induced change is usually very large. So, even if high variability exists for low VTG levels (i.e. in the lowest part of the dose-response curve), it should not impede the detection of a significant increase when it is real because the effect is often more than 1000 times the value in the control males.

Secondary sex characteristics

285.   4-*tert*-penthyphenol inhibited secondary sex characteristics of male fathead minnow dose-dependently, resulting in significant differences. Although slight reduction of papillary processes in male medaka was observed at highest concentration, it was less responsive than that in fathead minnow, and there was no significant difference except in one laboratory. A few reports have been published indicating that the number of nuptial tubercles decreased in fathead minnow exposed to weak and strong estrogens (43)(56), while no significant effect was found in the number of papillary processes in male medaka exposed to 4-*tert*-penthyphenol (25). This validation study and published reports suggest that the sensitivity of secondary sex characteristics in fathead minnow is higher than that in medaka when exposed to weak estrogens. Secondary sex characteristics in male fish are controlled by endogenous androgens (57)(58) via the androgen receptor and it is not clear how estrogenic chemicals inhibit the male secondary sex characteristics. Ankley et al. (15) exposed adult fathead minnow to the weak estrogen metoxychlor for three weeks, indicating the decrease of the concentrations of plasma androgens (11-ketotestosterone and testosterone) in males. The author suggested that the inhibition of endogenous androgens might involve the repression of nuptial tubercles in fathead minnow. Although useful as a signal, the biological basis of the response is not established for estrogen. Additionally, it is not known whether all estrogen-like substances will elicit the same profile of response on fathead minnow for this endpoint.

Spawning status

286.   In the observation of spawning status recorded as Yes or No for the presence of eggs, 4-*tert*-penthyphenol caused no clear effect on spawning status of medaka and zebrafish. However, this chemical inhibited the egg production dose-dependently in fathead minnow. In particular, no eggs were produced in the 1,000 μg/l treatment group. The reason for the difference of sensitivity in three fish species is uncertain, but the published reports suggest that medaka may be less sensitive than fathead minnow with regard to fecundity when exposed to estrogens, at the selected range of the present concentrations. Kang et al. (20) reported that exposure of medaka to 463 ng/l 17β-estradiol for three weeks decreased fecundity, but that exposure to 227 ng/l had no effect. When fathead minnows were exposed to 17β-estradiol for 19 d, the 17β-estradiol concentrations expected to cause 50% and 10% inhibition of egg production were 120 and 6.6 ng/l, respectively (58).

287.   Although the mechanism of reproductive impairment of fish exposed to estrogen is unclear, a previous study by Seki et al. (19) showed that the exposure of paired adult medaka to ethinyl estradiol for three weeks induced the development of many previtellogenic oocytes in females, concurrent with decreasing fecundity, suggesting that exposure of estrogens may lead to developmental abnormalities of oocytes, and especially to inhibition of oocyte maturation in the ovary. Another reason for spawning reduction may be the inhibition of sexual behavior (59). However, when comparing substances, the aromatase inhibitors caused cessation of spawning in medaka under the same test conditions. Although fecundity impairment is not specifically diagnostic of the endocrine activity of a given test substance, there may be value in collecting such information, with minimal time and cost implications. This needs to be discussed further, not in isolation, but in considering the regulatory context to the assay.

Gonad histology

288. In male fish, exposure to 4*tert*-pentylphenol caused increased spermatogonia (especially fathead minnow and zebrafish) and testicular degeneration in male fish (especially medaka and fathead minnow). Low incidences of testis-ova were observed in male fish from some laboratories, representing all three species; while these occurred most often in the high dose and positive control groups, testis-ova occasionally also occurred in negative control fish in other Phase 1B experiments. Other prominent findings in male fish included proteinaceous fluid in the testis and nephropathy in the kidneys. There is evidence that nephropathy may have been underreported because the kidneys were not examined in male medaka from some laboratories, and was not consistently available for zebrafish and fathead minnow. The most consistent findings in female fish were oocyte atresia and proteinaceous fluid in the ovary. There are few studies on adult fish exposed to estrogen and where gonadal histology has been evaluated. Previous studies on medaka in a full life-cycle test (25) identified testis-ova as the main response to estrogen induction in males; no histological change was noted in females.

289. If the objective of the assay is only to detect the substance as being an endocrine active one, gonad histopathology does not add substantial weight of evidence compared to vitellogenin measurement for the detection of estrogenic substances.

7.2.2 *Aromatase inhibitor*

VTG

290. In this validation work, the VTG levels in exposed females decreased dose-dependently in three fish species, indicating significant reduction, while those in males were not affected because already very low. Although this phenomenon itself seems to be anti-estrogenic, model study with fish exposed to aromatase inhibitor was conducted and the mechanism of the VTG reduction in femals was discussed by Ankley et al. (28). The authors exposed adult fathead minnow to aromatase inhitor, fadrozole, and indicated dose-dependent supression of VTG in females, accompanied by inhibition of plasma 17β-estradiol levels and brain aromatase activity. The authors suggested that the chemical was likely to inhibit the aromatase activity which directly controls endogenous 17β-estradiol, resulting in reduced VTG concentrations in female fish. Similar findings are reported in a study conducted by the US Environmental Protection Agency (61),where fadrozole inhibited female vitellogenin production in the 14-d and 21-d versions of a related protocol using adult fathead minnows.

291. Therefore, inhibition of VTG in females exposed to prochloraz in this validation work is caused by the reduction of endogenous 17*β*-estradiol which was inhibited by aromatase inhibitor. Ankley et al. (28) reported that VTG and 17β-estradiol concentrations in male fathead minnows were unaffected by exposure to fadrozole. The results of this published report are in agreement with those of the validation work of Phase 1B. In further extrapolating this finding, the assay may be able to detect substances interfering with steroid hormone synthesis and metabolism (e.g. from cholesterol mobilization through the final steroid product, e.g., CYP-scc (cholesterol chain cleavage) through CYP19 (aromatase) for estrogen).

Secondary sex characteristics

292. Prochloraz caused no clear effect on male and female secondary sex characteristics in male medaka and fathead minnow, except one laboratory in fathead minnow. Ankley et al. (28) reported that no significant effects of fadrozole were observed in male and female secondary sex characteristics, although the concentrations of endogenous androgens (11-ketotestosterone and testosterone) were significantly increased in male fish. The reason for the increase of androgen levels in male fish exposed to aromatase inhibitor is uncertain, however, Ankley et al. suggested that inhibition of CYP19 would increase androgen

concentrations in males by blocking conversion of testosterone to 17β-estradiol. In this validation work, nuptial tubercles were observed in female fathead minnow exposed to positive control of aromatase inhibitor in one study. Generally, the present validation work is consistent with the report by Ankley et al., and also with other works on fadrozole (27) (61) suggesting the secondary sex characteristics are poorly responsive to aromatase inhibition.

Spawning status

293.   The aromatase inhibitor clearly inhibited the spawning of three fish species dose-dependently. In addition, the response of spawning status was rapid, because most of the fish in the highest concentration of prochloraz stopped spawning within 2 or 3 days. Furthermore, this cessation of egg production was associated with inhibition of VTG levels. Ankley et al. (28) reported that the exposure of fathead minnow to fadrozole caused concentration-dependent decrease of fecundity as well as reduction in serum estradiol and VTG in females and marked alterations in ovarian histology. The author suggested that these responses provided the linkage of mechanism-specific information (aromatase inhibition) to a cascade of events through the endocrine system (reduction in 17β-estradiol) and target tissues (liver, ovary), to adverse effects in the whole organism (reduced fecundity). The results of this validation work, published works by Ankley et al.(9)(15)(28), and the US EPA multichemical study on fathead minnow (60) show that the observation of the spawning status is a useful piece of information as regards the reproductive status of the fish, despite the lack of diagnostic value for endocrine-mediated response. For fathead minnow, the lack of spawning in control tanks leads to think that crowding had a negative impact. In other studies conducted on fathead minnow (28)(61), two males and 4 females were held in the same tank, and spawning in the control groups was normal.

294.   A question remains on whether egg counts add value to a daily/temporal recording of the spawning status in this type of assay. This needs further discussion on the basis of all information available in Phase 1B and elsewhere in the literature, and considering the scope and practicality of the assay in a regulatory context. Egg production for fathead minnow usually ranges approximately between 20 and 60 eggs/female/day (compilation of studies from the USEPA (61)); this is comparable to egg production in zebrafish, whereas medaka egg production is generally lower (Seki, personal communication). A quick survey indicated that approximately 1 hour every day is necessary to count and remove eggs from the test vessels.

Gonad histology

295.   Among female fish of all three species, the most consistent histopathologic response to prochloraz exposure was increased oocyte atresia. Although less consistently identified, two findings related to oocyte atresia that were reported at high incidence in female medaka from some laboratories were decreased yolk formation and perifollicular cell hypertrophy / hyperplasia. Previous studies on fadrozole (28) had also reported that follicles were undergoing atresia rather than proceeding to maturity. In males, the most reported findings were increased interstitial cells and increased spermatozoa. Ankley et al. found that there was a notable concentration-dependent enlargement of the seminiferous tubules accompanied by an abundant accumulation of sperm in the lumina (28).

7.2.3   *Anti-androgen*

General aspects

296.   Flutamide exposure caused no clear and reproducible alteration in any of the following endpoints: VTG- except at the highest concentration in one fathead minnow study, secondary sex characteristics, spawning status and gonadal histology, . Interpretation of the Phase 1B results regarding the flutamide

study remains challenging. Arguably, it appears that flutamide is a relatively weakly active substance in fish, contrary to expectations from mammalian studies. A couple of studies (27)(29)(37)(61) have documented responses on the core endpoints in fish following flutamide exposure, and not all of them report exactly the same findings. As common features, vitellogenin level in females increased following flutamide exposure (27)(29)(61), without necessarily being significant; fecundity was significantly affected (29)(37)(61); and specific histological changes were noticeable in testis and ovaries (29)(37). In a mechanistic study by Ankley *et al* (29) found that flutamide binds competitively to the androgen receptor *in vitro*, and its metabolite hydroxyl-flutamide binds with a much higher affinity. *In vivo*, Ankley found that concomitant exposure of flutamide and trenbolone (androgen) blocked masculinisation of females through formation of nuptial tubercles, which are under androgen receptor control. Though it is unclear why flutamide, a mammalian anti-androgen apparently active in fish and in particular fathead minnow, could not be detected more clearly and reproducibly in this 21-day assay, there is a speculation which can be made. Flutamide may require metabolic activation which is more pronounced in mammals than fish, and hydroxyl-flutamide is more likely the active form.

VTG

297.   Flutamide had no effect on VTG levels in males and females in three fish species. Nozaka et al. (8) exposed adult medaka to flutamide (90.4-1,470 µg/l, measured concentrations) for 21 days, indicating that no dose-dependent effect was found in male and female fish. The authors suggested that parameters other than VTG might be appropriate to detect anti-androgen. Panter et al. (27) reported that the exposure of pre-spawning adult fathead minnow to flutamide (95.3, 320.4 and 938.6 µg/l, measured concentrations) for 21 days caused no VTG induction in males, which consistent with the results of this validation work. However, the authors showed that significant VTG induction was found in females exposed to this chemical. The reason for this divergence in female VTG responses between this validation work and the published report is uncertain. In their work, Ankley *et al* (29) also found that VTG expression is not directly mediated via the androgen receptor.

Secondary sex characteristics

298.   No clear profile of flutamide could be drawn from Phase 1B experiments. In one study, flutamide inhibited secondary sex characteristics of male fathead minnow at the highest concentraiton, resulting in significant difference in LAB 7, but this finding was not reported in other studies. Panter et al. (27) reported that the flutamide exposure (938.6 µg/l) for three weeks significantly reduced the nuptial tubercles number in male fathead minnow. The finding of this validation work in LAB 7 concurs with the previous study by Panter et al. This reduction of male secondary sex characteristics might be caused by anti-androgenic effect of flutamide, because endogenous androgens control the expression of male sexual characteristics in fish. Other works by the US EPA (61) and by Jensen et al (32) did not demonstrate exposure related reduction of secondary sex characteristics in male fathead minnow. Although there is no published paper on secondary sex characteristics of medaka exposed to an anti-androgen, medaka seems to be responsive to anti-androgen when exposed from embryo to adult. The fish full-life cycle test with flutamide using medaka showed dose-dependent reduction of papillary processes in male fish (Seki et al, personal communication).It remains unclear why secondary sex characteristics, which regulation is mediated via the androgen receptor, were not more responsive to the anti-androgen flutamide. It is hypothesized that this weak activity is the result of limited bioactivation compared with mammals, or an inadequate exposure duration or life-stage.

Spawning status

299.   Flutamide caused no clear effect on spawning status of medaka and zebrafish; however, this chemical inhibited the egg production of fathead minnow in the 1,000 µg/l treatment group; consistent with

other study reports (32)(61). In zebrafish, Wester et al. (37) found that flutamide at 1000 µg/l reduced significantly the number of clutches, thereby reducing the egg number. In the fathead minnow, the spawning ability was likely compromised by the grouping used in the protocol. Fathead minnows are territorial and a more appropriate grouping (e.g. 2males and 4 females per replicate) would likely substantially improve spawning performance which could also improve this species ability to respond to the anti-androgen mode of action.

Gonad histology

300.   The most prominent findings were increased interstitial cells and increased spermatozoa in the testes of male zebrafish. These are consistent with findings reported in the literature (32)(37)(61). Other findings in zebrafish, and the other two fish species, were few and inconsistently reported. Compared to the other endpoints, histopathology appeared to have the foremost ability to detect the endocrine activity of flutamide.

7.3   Outcome of the validation work of Phase 1A and 1B

VTG

301.   Results from Phase 1A and 1B demonstrate that this protocol can detect estrogenic effects of strong and weak estrogens through VTG induction in males in three fish species. Reproducibility between laboratories was generally good, except when the measured concentration of the test substance was outside the expected 80%-120% range. Intra-laboratory coefficients of variation were within ranges reported in other studies (43). VTG also responded to androgenic exposure in Phase 1A: VTG production in females significantly decreased after 17β-trenbolone exposure in the three fish species; however, the mechanism of this reaction is uncertain and not directly mediated via the androgen receptor; other androgens, especially those that undergo aromatization, generally cause other types of response. Aromatase inhibitor also decreased VTG levels in females in three fish species, suggesting that this endpoint may be appropriate to detect the endocrine effects of aromatase inhibitors. VTG poorly responds to anti-androgen; where there was an increase of VTG in females following anti-androgenic exposure, reproducibility across laboratories was not good.

302.   In the Phase 1A of the validation work, extreme variability of VTG levels in control males was noted in zebrafish studies. Therefore, in preparation for Phase 1B participating laboratories agreed to use a single, homologous ELISA, method for each species. Standard operating procedures and a unique VTG standard, both available to each participant enable to minimise variations in methodologies applied for e.g. blood sampling and pretreatment. Results from Phase 1B indicated that the variability of VTG levels in control fish still existed. The reasons of this variability could be:

   i) the determination limit of the VTG levels was not unified in all participating laboratories, because the SOPs for VTG measurement in fathead minnow and zebrafish did not describe the lowest limit of the calibration curve of the VTG standard and the minimal dilution factor of the specimen;
   ii) male zebrafish had a wide range of VTG levels in Phase 1A and 1B, suggesting an intrinsic characteristic of zebrafish;
   *iii)*contamination in food may be of concern in VTG variability, because LAB 1 showed that brine shrimp from Salt Lake contained estrogenic chemicals such as *o,p'*-DDT. The levels of POPs in food seem to be fairly low, however, criteria for food contaminants may be needed.

Secondary sex characteristics

303.   This protocol can detect androgenic effect of the chemicals as masculinization of the secondary sex characteristics in females of medaka and fathead minnow. However, we do not have any parameter to identify the androgenic effect of the chemicals in zebrafish, because this species has no clear secondary sex character that can be quantitatively measured. Weak and strong estrogens inhibit the expression of this parameter in male fathead minnow, although the mechanism of this change is uncertain. Weak and strong estrogens cause no alteration of this parameter in male medaka. Anti-androgen may potentially reduce the secondary sex characteristics in male fish, but the present 21-day fish assay does not detect this type of response. Aromatase inhibitor did not cause alteration of this parameter in medaka and fathead minnow.

304.   In the validation work of Phase 1A, different responses of the secondary sex characteristics was obserbed in a few cases. One of the reason might have come from insufficient experience in measuring this parameter. To minimise the impact of individual laboratory experience in this respect, detailed SOPs for fathead minnow and medaka were prepared by the technical lead laboratories in Phase 1B. As a result, similar outcomes were generally yielded following chemical exposure in Phase 1B studies in both medaka and fathead minnow, suggesting that the level of guidance is now appropriate.

Spawning status

305.   This observation was added to the protocol in Phase 1B of the validation, as it allows a verification of the reproductive activity in control group, as a basic principle of the assay. In addition, daily observation in control and treated groups enables the detection of treatment-related effects on fecundity. For instance, the aromatase inhibitor study indicated that spawning was reduced in three fish species, most probably following a reduction of endogenous estrogen availability. In addition, weak estrogen and anti-androgen exposures inhibited spawning in fathead minnow. Although the spawning status in the fish exposed to strong estrogen and androgen was not validated in phase 1A, some reports on fathead minnow and medaka indicate reproductive impairment when exposed to these chemicals (9)(15)(19)(20)(58).

306.   An important point in this parameter is to select reproductively active fish for exposure. If senescent or pre-spawning fish are selected, the spawning status is not valid to detect the reduction of spawning. Several participating laboratories felt that (semi-)quantitative measurement of eggs would add strength to a mere qualitative observation of spawning; however there may be resource implications in terms of time involved. A rapid survey with participating laboratories shows that egg counts demand between one and two hours a day for a study of this type. Reproduction tests developed in fathead minnow (9)(15) and medaka (19)(20)(62) (63) provide the possibility to extend the present protocol in this respect.

Gonad histology

307.   Results from Phase 1A demonstrated that this endpoint was able to detect endocrine disputing effects in the exposure of strong estrogen and androgen. However, the experience from Phase 1A had shown that an insufficient level of standardization of procedures and guidance for evaluation were available to participants to read gonad samples in a comparable way and with common criteria and diagnoses. Further work was warranted to develop a consensus guidance document for use in Phase 1B. A group of experienced fish pathologists met after Phase 1A in October 2003, to identify areas where they could contribute further advice regarding the histological procedures and the pathological evaluation. Considerable efforts were made to draft a comprehensive document, with all necessary standard operating procedures, illustrated with many annotated photos (e.g., dissection procedures, histological slides), providing the diagnostic terminology and a consensus severity scoring system to be applied in Phase 1B. A meeting with pathologists was held after Phase 1B to review slides and findings. The outcome of this meeting was an agreement on four exposure-related diagnoses in males and four exposure-related

diagnoses in females. This will considerably reduce time involved in gonad evaluation because pathologists will know what diagnoses are meaningful following exposure to an endocrine active substance. The guidance document will be supplemented with androgenic-related examples and will then be made available as an OECD monograph.

Other items

308.   For Phase 1B of the validation work, the test design of the 21-day fish assay was improved to increase its biological relevance and optimize the use of animals. First, sampling of fish was fixed to 10 males and 10 females on day-21 of the experiment from each treatment level in Phase 1B, while sampling was divided between day-14 and day-21 in Phase 1A. This change not only simplified the test design, but also increased the number of fish sampled on day-21, resulting in improvement of the statistical power. Furthermore, a 21-day exposure is more appropriate for weak compounds. Second, the age of the test fish was strictly described in the protocol in Phase 1B, i.e., 20 (+/- 2) weeks in fathead minnow and 16 (+/- 2) weeks in medaka and 15 (+/- 2) weeks in zebrafish to exclude the variability associated with fish maturation. This generally contributed to a reduction in the variability of the results in Phase 1B, but several studies deviated from the protocol because of the delay in receiving test chemicals or ELISA kits for VTG measurement. In addition, a few experts in zebrafish commented that 15 (+/- 2) weeks in zebrafish may be too young. Particularly, it is difficult to distinguish males and females in zebrafish from the external characteristics, meaning that most of the laboratories for zebrafish could not exactly select males and females at the beginning of the exposure. Sexual maturity depends on not only fish age but also environmental conditions in each laboratory. Therefore, it may be needed to revise the age criteria for zebrafish. Third, the gonadosomatic index (GSI) was dropped in Phase 1B because GSI was not responsive to estrogen or androgen exposure in Phase 1A. As a result, this change facilitated the dissection procedure in Phase 1B. Forth, male and female fish were exposed together in Phase 1B because separation of males and females with a mesh in Phase 1A caused confounding in gonad histology evaluation, i.e., marked atresia in control ovaries.

8. RECOMMENDATIONS

309. With the view to ascertain the endocrine specificity of responses on core endpoints, it was recommended by the Validation Management Group for Ecotoxicity tests (VMG-eco) at its meeting in December 2004 to select a substance that will not elicit a response on any of the three core endpoints in the 21-day fish assay. This work will constitute a separate Phase 2 and will be reported separately.

310. It was also suggested that, if the spawning status was to be part of the core endpoints of the assay, an amendment should be made to offer the fathead minnow appropriate reproductive conditions. Currently, the assay proposes a ratio male:female of 1:1; fathead minnow being territorial, spawning conditions are being impeded by the crowding in test vessels. However, such change in the test design potentially impacts on the sample size for other endpoints measured (e.g. VTG, secondary sex characteristics). A comparative assessment of the possible test designs and sex ratios would be necessary to see if they are equivalent in terms of power to detect a significant change. Such change in the test design, if it was decided, would probably not apply to medaka and zebrafish, hence the need to show equivalence between options.

311. It was also agreed at the last meeting of the VMG-eco to collect additional quantitative data on fecundity. At this stage, the purpose of fecundity data in the fish 21-day assay is not entirely clear. On the one hand, members of the VMG-eco agreed that interpretation of fecundity alone could not be used to qualify the endocrine activity of a substance, nor could it be used to aid gonad histopathology because individual female contribution to fecundity of the group can not be known. On the other hand, the EDTA Task Force noted that quantitative data on fecundity could form part of a general Test Guideline on fish reproduction, as an enhanced version of the fish 21-day assay. Further discussion on the matter is warranted in the near future at the EDTA Task Force level, when additional data on fecundity is collected by the US EPA in 2005.

312. While the VMG-eco approved the present report, they recommended adding a reference to Guideance Document 34 on the Validation and International Acceptance of New and Updated Test Methods for Hazard Assessment (64), regarding the OECD principles and criteria for test method validation. Once Phase 2 on the negative substances is completed, these criteria will be reviewed in light of all information available.

Table 81: OECD PRINCIPLES AND CRITERIA FOR TEST METHOD VALIDATION

a) The rationale for the test method should be available.

This should include a clear statement of the scientific basis, regulatory purpose and need for the test.

b) The relationship between the test method's endpoint(s) and the (biological) phenomenon of interest should be described.

This should include a reference to scientific relevance of the effect(s) measured by the test method in terms of their mechanistic (biological) or empirical (correlative) relationship to the specific type of effect/toxicity of interest. Although the relationship may be mechanistic or correlative, test methods with biological relevance to the effect/toxicity being evaluated are preferred.

c) A detailed protocol for the test method should be available.

The protocol should be sufficiently detailed and should include, *e.g.,* a description of the materials needed, such as specific cell types or construct or animal species that could be used for the test (if applicable), a description of what is measured and how it is measured, a description of how data will be analysed, decision criteria for evaluation of data and what are the criteria for acceptable test performance.

d) The intra-, and inter-laboratory reproducibility of the test method should be demonstrated.

Data should be available revealing the level of reproducibility and variability within and among laboratories over time. The degree to which biological variability affects the test method reproducibility should be addressed.

e) Demonstration of the test method's performance should be based on the testing of reference chemicals representative of the types of substances for which the test method will be used.

A sufficient number of the reference chemicals should have been tested under code to exclude bias (see paragraphs on "Coding and Distribution of Test Samples").

f) The performance of the test method should have been evaluated in relation to relevant information from the species of concern, and existing relevant toxicity testing data.

In the case of a substitute test method adequate data should be available to permit a reliable analysis of the performance and comparability of the proposed substitute test method with that of the test it is designed to replace.

g) Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of GLP.

Aspects of data collection not performed according to GLP should be clearly identified and their potential impact on the validation status of the test method should be indicated.

h) All data supporting the assessment of the validity of the test method should be available for expert review.

The detailed test method protocol should be readily available and in the public domain. The data supporting the validity of the test method should be organised and easily accessible to allow for independent review(s), as appropriate. The test method description should be sufficiently detailed to permit an independent laboratory to follow the procedures and generate equivalent data. Benchmarks should be available by which an independent laboratory can itself assess its proper adherence to the protocol.

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