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VALIDATION REPORT (PHASE 2) FOR THE FISH SEXUAL DEVELOPMENT TEST FOR THE DETECTION OF ENDOCRINE ACTIVE SUBSTANCES (Updated 2012)

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VALIDATION REPORT (PHASE 2) FOR THE FISH SEXUAL DEVELOPMENT TEST FOR THE DETECTION OF ENDOCRINE ACTIVE SUBSTANCES

IOMC INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides and Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/ehs/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. UNDP is an observer. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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FOREWORD

This document presents the validation report (phase 2) of the Fish Sexual Development Test (FSDT). The Fish Sexual Development Test (FSDT) covers a life-stage where sexual development is particularly sensitive to perturbation caused by endocrine active chemicals. The chemical exposure lasts for about 60 days, at the end of which endpoints of ecological relevance like the sex ratio of the exposed fish is calculated and the biomarker endpoint vitellogenin is measured in individual animals.

In 2003, Denmark, on behalf of the European Nordic countries, proposed a new project o develop a Test Guideline on the fish sexual development test to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT). The project was included on the Test Guidelines workplan in 2003, and extensive validation of the test method was carried out until 2009. Two validation studies were performed, including chemicals representing various modes of action (oestrogen, (anti-)androgen, aromatase inhibitor) and negative chemicals. The chemicals tested in the phase 2 validation included the weakly active oestrogenic 4-tert-octylphenol and the androgenic dihydrotestosterone for the species medaka and zebrafish, and the weakly active oestrogenic 4-tert-pentylphenol, the anti-androgenic flutamide and the oestrogenic 17β-estradiol for the stickleback.

The validation has been overseen by a validation management group for Eco-toxicity testing (VMG-eco) and a fish drafting group. A peer-review of the validation has been organised in 2011 and the report is available in the Series on Testing and Assessment as No.143. The draft validation report has been endorsed by the WNT at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 22 June 2011. The document was completed in 2012 with an annex.

The annex presents a summary of the statistical work undertaken in the course of the validation of the Fish Sexual Development Test (FSDT), principles for the analysis of the sex ratio data, and references to a large number of documents presented in Fish Experts meetings and meetings of the Validation Management Group for Ecotoxicity Testing between 2006 and 2011. The statistical analyses referenced in the document were performed by a consultant for the Secretariat, John Green (BIAC), to support the final test design, including animal numbers, in TG 234, and the statistical flowchart for data analysis contained in the Test Guideline.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

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EXECUTIVE SUMMARY

Four small fish species have been exposed to chemicals with different modes of action in two validation phases in a total of 29 FSDT experiments including two negative studies (ammonia and n-octanol). The tested species are zebrafish, Japanese medaka, fathead minnow and three spined stickleback. Zebrafish has been used in 13 experiments, Japanese medaka in seven experiments, three spined stickleback in six experiments and finally fathead minnow in three experiments. The weak estrogens 4-tert-pentylphenol and 4-tert-octylphenol was tested in zebrafish, fathead minnow and medaka, and zebrafish, medaka and stickleback respectively. The aromatase inhibiting fungicide prochloraz was tested in zebrafish and fathead minnow. The androgen dihydrotestosterone was tested in zebrafish, medaka and stickleback.

INTRODUCTION

1. The need to develop and validate fish assays capable of measuring the effects of endocrine disrupting chemicals (or EDCs) originates from concerns that environmental levels of certain chemicals may be causing adverse effects in both humans and wildlife due to the interaction of these chemicals with the endocrine system. Several cases have been reported where exposures to exogenous chemicals have indeed resulted in effects on wildlife, in particular fish [Jensen et al. 2006;Milnes et al. 2006;Orlando et al. 2004]. In 1997, OECD member countries advised that existing test methods were insufficient to identify such substances and characterize their effects. As part of the OECD Test Guidelines Program a Special Activity on the Testing and Assessment of Endocrine Disrupters was therefore initiated to revise existing, and develop new, OECD Test Guidelines for the screening and testing of potential EDCs.

2. The Fish Sexual Development Test (FSDT) fits into the OECD Conceptual Framework (CF) for the Testing and Assessment of Endocrine Disrupters (EDs) at Level 4 or 5 (the CF is under revision). This framework identifies approaches, assays and long-term tests of increasing biological complexity, meant to gather information on potential EDCs. Each of the methods added to the framework requires validation to ensure its relevance and reliability, its two main pillars. OECD Guidance Document 34 on Validation and Acceptance of New and Updated Test Methods for Hazard Assessment [OECD 2005] provides definitions, principles and concrete examples of validation, applied in different areas of hazard assessment. The validation of the FSDT was conducted to address these principles and is presented in the Phase 1 and Phase 2 reports.

3. The FSDT is a modified version of OECD Test Guideline 210 [OECD 1992], the Fish Early-Life Stage Toxicity Test with added endpoints for the detection of endocrine disrupters (vitellogenin (VTG) measurement and sex ratio). The idea of the assay is that exposure of fish to certain EDs during the sensitive window for sexual development will alter the VTG concentration and/or the phenotypic sex. The FSDT was initially developed for zebrafish (*Danio rerio*), which possesses a sensitive window of exposure from 20-60 days post hatch (DPH). The window of exposure was chosen to avoid exposure during an oversensitive stage, between 10-20 DPH, when high larval mortality can occur [Andersen et al. 2003]. After discussion of the test at the OECD in 2003, it was decided that other OECD fish species such as Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas),* and in 2006 three spined stickleback (*Gasterosteus aculeatus*), should also be tested in the validation exercise. Since the precise duration of sexual differentiation and the sensitive window of exposure in species other than zebrafish had not been fully explored, the test is started with newly fertilized eggs (instead of larvae) for all species, including zebrafish. Chemical exposure was extended until 60 days post hatch, when fish are normally sexually differentiated. At the end of the exposure period, animals are terminated and sampled for vitellogenin measurement and sex determination via histological examination of the gonads.

4. During autumn 2005 and early spring 2006 the test proposal was subjected to in-depth statistical evaluation, based on existing data from earlier experiments conducted on zebrafish. The first round (Phase 1) of this validation exercise took place from mid-2006 until mid-2007. Zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) were used in the Phase 1. The substances tested were 4-tert-pentylphenol, an estrogenic chemical, and prochloraz, an aromatase inhibitor. Five European laboratories participated in Phase 1 of the validation. Results from Phase 1 are available in a separate report. The

outcome of Phase 1 demonstrated that: 1) the fathead minnow sexual differentiation was longer than the exposure duration of the test (i.e. 60 days), and 2) the test design allowing an analysis of the variance should be preferred over a test design meant to determine an effect concentration (ECx) through a regression analysis. Thus for Phas2 of the validation, the fathead minnow was not maintained as a test species, but the zebrafish, the Japanese medaka (*Oryzias latipes*) and the three spined stickleback (*Gasterosteus aculeatus*) were used. The latter two species have the advantage to possess a genetic sex marker. Further, the test design used in Phase 2 was standardized with three test concentrations, 4 replicates per concentration and 40 eggs per replicate at the start of the test.

5. The second round (Phase 2) of the validation exercise took place from spring 2009 to summer 2010. The test substances were 4-tert-octylphenol and dihydrotestosterone. Ten laboratories took part in Phase 2 of the validation. The results of Phase 2 are reported here.

SCIENTIFIC RATIONALE FOR THE CORE ENDPOINTS

Vitellogenin

6. Vitellogenin (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 estrogenic hormones, predominantly 17β-estradiol, with the estrogen receptor [Jobling et al. 1996]. Males retain the capacity to produce VTG in response to stimulation with estrogen receptor agonists; as such, induction of VTG in males has been successfully exploited as a biomarker specific for (anti-)estrogenic compounds in a variety of fish species, including fathead minnow, Japanese medaka, three spined stickleback and zebrafish. A number of VTG measurement methods have been developed and standardized for each of these species [Kunz et al. 2006;Lange et al. 2001;Panter et al. 2002;Panter et al. 2006;Hahlbeck et al. 2004b]. The criteria for selecting the methods used in this validation program are described further in the report.

Sex ratio

7. When fish are exposed to EDs during the sensitive window of their sexual development (approximately from 0 to 60 days post hatch for the species in the FSDT validation), the phenotypic sex is influenced by this chemical exposure. Certain sex-reversed fish may maintain a certain reproductive capacity; however, skewed sex ratio in a fish population exposed to EDs can impact its sustainability [Kidd et al. 2007]. Phenotypic sex ratio (proportions of males, females etc) is determined via histological examination of the gonads. The sex is defined as female, male, intersex or undifferentiated. It is possible to determine the genetic sex in some species and this has been done for stickleback and medaka in the present validation.

OBJECTIVES OF PHASE 2

8. The objectives of a validation program are to establish that a test method is relevant - *meaningful for the intended purpose* - and reliable – *reproducible over time and across laboratories*. Phase 1 of the validation exercise was the occasion to test two chemicals with different modes of action, an estrogen and an aromatase inhibitor, in a limited number of laboratories (n=5), and to demonstrate that the test actually *works* and produces meaningful results. A number of other aspects were also investigated in Phase 1, including the test design and two test species, zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*).

9. In Phase 2 of the validation, the focus was to investigate the reproducibility of results across several participating laboratories using the same fish species, and the same test chemicals at the same concentrations. The objective was to have a minimum of three repetitions for each experiment [chemical x fish species]. Two chemicals were used: 4-tert-octylphenol, another estrogen, and dihydrotestosterone, an androgen. Participating laboratories used zebrafish (LAB 1, LAB 2, LAB 3, LAB 4), the medaka (LAB 4, LAB 5, LAB 9 and LAB 10), and the stickleback (LAB 6, LAB 8). LAB 7 did not submit test results. In most cases, an inter-laboratory comparison of test results was possible. To complete the dataset for the stickleback, LAB 6 conducted additional experiments on flutamide and 17ß-estradiol, and LAB 9 and LAB 10 conducted experiments using 4-tert-pentylphenol, the estrogenic chemical used in Phase 1 on zebrafish and fathead minnow, but not yet on medaka.

10. It is also the purpose of a validation program to understand and define the area of application of the test and any limitations to its use. Such limitations are presented in the test results and discussed further in the report. Beside the mandatory endpoints, the Kidney Epithelium Hight (KEH) was reported for stickleback as an endpoint reflecting the induction of the androgen related protein spiggin. These results are not discussed because the method is not validated. The figures are placed in the Appendix.

ORGANISATION OF PHASE 2

11. The Danish lead laboratory that coordinated Phase 1 also acted as the lead for Phase 2 of the validation. The lead laboratory was responsible for:

- developing the test protocol, including standard operating procedures and distributing it to the participating laboratories;
- preparing a harmonized template for the collection of test results;
- centralizing the distribution of test chemicals;
- collecting all test results;
- preparing the draft report.

12. Phase 2 started in early 2009 and was completed mid-2010. Ten laboratories from Europe and Japan took part in the experimental work, as described in Table 1.

Table 1: Participation in Phase 2, fish species and chemicals tested.

Participating laboratory	Fish species used	Chemicals tested
LAB 1	Zebrafish (ZF)	4-tert-octylphenol, dihydrotestosterone
LAB 2	Zebrafish (ZF)	4-tert-octylphenol, dihydrotestosterone
LAB 3	Zebrafish (ZF)	dihydrotestosterone
LAB 4	Zebrafish (ZF)	4-tert-octylphenol
	Medaka (MK)	4-tert-octylphenol
LAB 5	Medaka (MK)	4-tert-octylphenol, dihydrotestosterone
LAB 6	Stickleback (STK)	4-tert-octylphenol, dihydrotestosterone, flutamide, 17beta-estradiol
LAB 8	Stickleback (STK)	4-tert-octylphenol, dihydrotestosterone
LAB 9	Medaka (MK)	4-tert-octylphenol, dihydrotestosterone, 4-tert-pentylphenol
LAB 10	Medaka (MK)	4-tert-pentylphenol

Overview of the test protocol

13. The experimental work was conducted according to the protocol prepared for Phase 2 of the validation of the Fish Sexual Development Test for Endocrine Active Substances. A summary of the protocol is provided below.

14. Newly fertilized eggs were exposed, 40 per replicate, 4 replicates per treatment level, three treatment levels and appropriate controls (including solvent controls if needed). The chemical exposure was flow-through and lasted until 60 days post hatch, the presumed completion of sexual differentiation in the following fish species: medaka, stickleback and zebrafish. The protocol was designed to detect the effects of EDCs in fish exposed during their sex differentiation period.

15. Exposure to the test chemical was aqueous, with or without carrier solvent. Monitoring continued for up to 60 days post hatch (dph) and included hatching rate, development, survival, growth (total length and body weight), sexual differentiation, and VTG concentrations in individual fish.

Test chemicals and concentrations

16. The test chemicals and test concentrations were discussed and agreed by the Validation Management Group for Ecotoxicity Testing ahead of the study. Dihydrotestosterone is an androgen and 4-tert-octylphenol is a weak estrogen. Nominal concentrations of the test substances were as follows:

- 4-tert-octylphenol: 10, 32 and 100 µg/l (+ water control) for medaka and stickleback Lab 6
  (6.25, 12.5, 25, 50 and 100 µg/l for medaka LAB 9)
  32, 100 and 320 µg/l for stickleback Lab 8 and zebrafish;

- Dihydrotestosterone: 100, 320 and 1000 ng/l (+ water control).

Analytical determination of test concentrations

17. Water samples were collected on a weekly basis in each tank and reported in a spreadsheet. The analytical methods used were GC-MS for 4-tert-octylphenol and LC-MSMS or RIA for dihydrotestosterone.

Test acceptability criteria

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

- The dissolved oxygen concentration was between 60 and 100 per cent of the air saturation value (ASV) throughout the exposure period.

- The water temperature did not differ by more than ± 2.0°C between test vessels at any one time during the exposure period.

Collection of data and statistical analysis:

19. All test results were collected and centrally analysed. Unless otherwise stated, a two-sided hypothesis testing was used.

The statistical analysis of vitellogenin measurements were performed by John W Green (DuPont Applied Statistics) following a defined protocol (Figure 1)

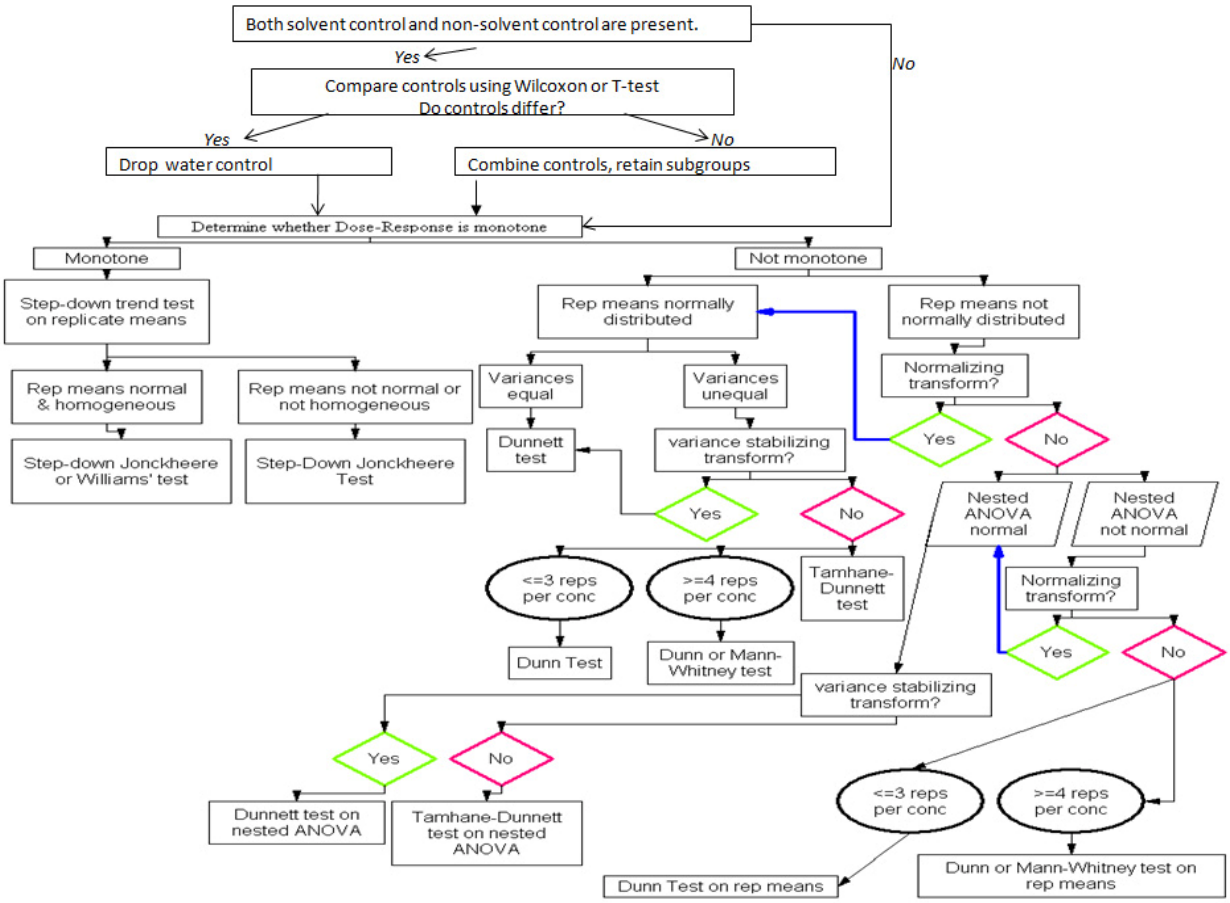


Figure 1: Protocol for guidance in statistical analysis of VTG data

20. The statistical analysis of phenotypic sex was also performed by John W Green following a defined protocol (Figure 2).

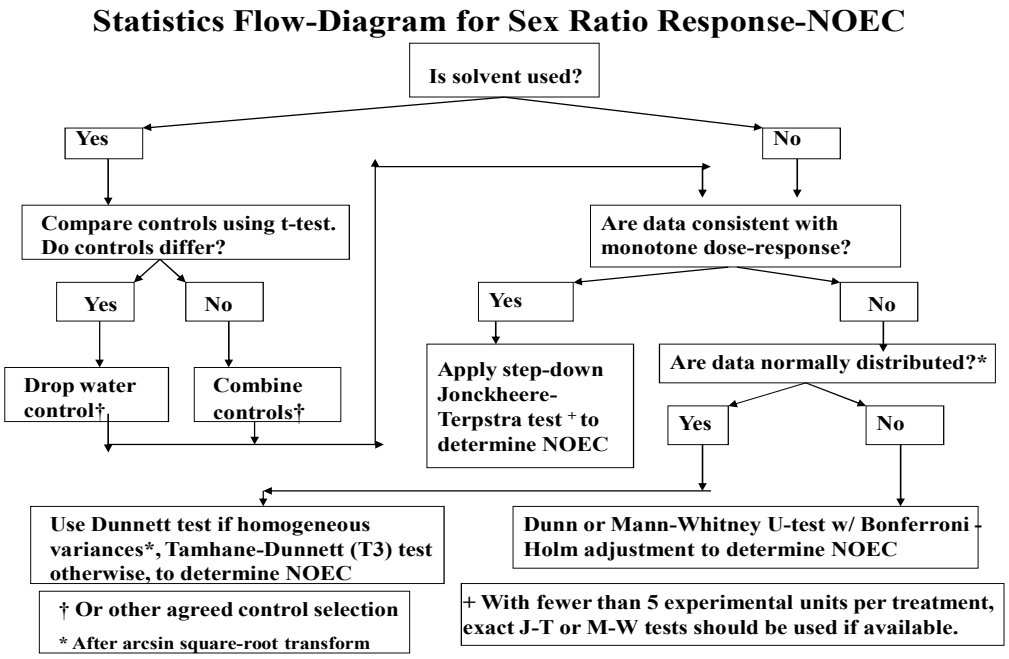


Figure 2: Protocol for guidance in statistical analysis of sex ratio response

RESULTS

Analytical chemistry

4-tert-octylphenol

Table 2: Measured concentrations of 4-tert-octylphe nol; mean concentrations in µg/l ± the standard deviation (SD). Numbers in brackets are N samplings. Lines in Bold are means and SD of all treatment samples. Highlighted lines are outliers from the mean treatment concentration ± 20%. *Lab 9 had nominal concentrations of 6.25, 12.5, 25, 50 and 100 µg/l 4-tert octylphenol.*

nominal conc.	replicate	LAB 1 zebrafish mean ± SD (n)	LAB 2 zebrafish mean ± SD (n)	LAB 4 medaka mean ± SD (n)	LAB 4 zebrafish mean ± SD (n)	LAB 5 medaka mean ± SD (n)	LAB 6 stickleback mean ± SD (n)	LAB 8 stickleback mean ± SD (n)	LAB 9 medaka mean ± SD (n)
control	1	<LOQ (9)	<LOQ (3)	<LOQ (9)	<LOQ (3)	0.0 ± 0.0 (4)	<1 µg/l (9)	<1 µg/l (10)	<LOQ (5)
	2	<LOQ (9)	<LOQ (3)	<LOQ (9)	<LOQ (3)	0.0 ± 0.0 (3)	<1 µg/l (9)	<1 µg/l (10)	<LOQ (4)
	3	<LOQ (9)	<LOQ (3)	<LOQ (9)	<LOQ (3)	0.0 ± 0.0 (3)	<1 µg/l (9)	<1 µg/l (10)	<LOQ (3)
	4	<LOQ (9)	<LOQ (3)	<LOQ (9)	<LOQ (1)	0.0 ± 0.0 (3)	<1 µg/l (9)	<1 µg/l (10)	-
	mean								-
solvent	1		10.1± 15.6 (3)	<LOQ (5)			<1 µg/l (9)	<1 µg/l (10)	
	2		0.3 ± 0.6 (3)	<LOQ (5)			1.4 ± 2.6 (9)	<1 µg/l (10)	
	3	-	0.5 ± 0.9 (3)	<LOQ (5)	-	-	<1 µg/l (9)	<1 µg/l (10)	-
	4		2.1 ± 2.4 (3)	<LOQ (5)			1.3 ± 2.3 (9)	<1 µg/l (10)	
	mean		**3.3 ± 4.9 (12)**				**0.44 ± 0.8 (36)**		
10 µg/l	1			10.8 ± 3,9 (5)	10.2 ± 2.8 (6)	15.0 ± 10.0 (4)	11.2 ± 3.3 (9)		6.0 ± 0.8 (4)
	2			10.5 ± 2.4 (5)	11.1 ± 3.4 (5)	13.3 ± 5.8 (3)	12.5 ± 2.3 (8)		6.8 ± 0.6 (4)
	3	-	-	11.6 ± 2.0 (5)	8.58 ± 1.6 (6)	10.0 ± 0.0 (3)	11.3 ± 1.2 (8)	-	5.7 ± 0.4 (4)
	4			11.8 ± 2.3 (4)	7.93 ± 1.7 (5)	10.0 ± 0.0 (3)	14.1 ± 1.9 (9)		-
	mean			**11.2 ± 2.6 (19)**	**9.5 ± 2.5 (22)**	**12.1 ± 3.9 (13)**	**12.2 ± 2.6 (34)**		**6.2 ± 0.8 (12)**
32 µg/l	1		6.9 ± 4.6 (3)	28.9 ± 6.3 (5)	27.7 ± 5.3 (6)	32.5 ± 12.6 (4)	21.7 ± 4.3 (9)	42.8 ± 17.0 (9)	12.6 ± 1.4 (4)
	2		3.0 ± 5.2 (3)	29.6 ± 7.6 (5)	27.5 ± 6.4 (5)	26.7 ± 5.8 (3)	22.7 ± 6.5 (9)	37.5 ± 10.9 (8)	11.5 ± 0.9 (4)
	3		5.6 ± 8.1 (3)	34.4 ± 10.2 (5)	27.1 ± 6.0 (6)	33.3 ± 15.3 (3)	pooled with rep 2	44.5 ± 17.2 (8)	12.7 ± 2.5 (4)
	4		7.4 ± 5.7 (3)	33.8 ± 10.5 (5)	21.6 ± 4.3 (5)	30.0 ± 0.0 (3)	pooled with rep 2	42.8 ± 17.6 (9)	-
	mean	**13.8 ± 8.0 (12)**	**5.7 ± 5.9 (12)**	**31.7 ± 8.5 (20)**	**26.0 ± 5.7 (22)**	**30.6 ± 8.4 (13)**	**22.2 ± 5.4 (18)**	**41.9 ± 15.4 (34)**	**12.3 ± 1.7 (12)**

	1		12.5± 19.1 (3)	98.7 ± 21.2 (5)	87,6 ± 23,6 (6)	105.0 ± 23.8 (4)	66.9 ± 11.7 (9)	128.8 ± 17.8 (4)	25.2 ± 4.5 (4)
	2		33.9 ± 22.2 (3)	102.2 ± 23.3 (5)	93,2 ± 12,5 (4)	86.7 ± 32.1 (3)	pooled with rep 1	126.5 ± 13.9 (4)	21.1 ± 1.1 (4)
100 µg/l	3		10.9 ± 18.8 (3)	116.0 ± 18.0 (5)	95,6 ± 12,0 (6)	103.3 ± 25.2 (3)	pooled with rep 1	133.0 ± 115.3 (4)	24.4 ± 4.8 (4)
	4		13.3 ± 18.2 (3)	102.0 ± 27.8 (5)	89,6 ±10,1 (4)	63.3 ± 41.6 (3)	pooled with rep 1	134.3 ± 16.3 (4)	-
	mean	**40.6 ± 8.0 (12)**	**17.6 ± 19.4 (12)**	**104.7 ± 22.1 (20)**	**91.5 ± 15.4 (20)**	**89.6 ± 30.7 (13)**	**66.9 ± 11.7 (9)**	**130.6 ± 14.6 (16)**	**23.6 ± 4.0 (12)**
	1		37.3± 64.5 (3)*		342,0 ± 41.2 (2)			481.0 ± 195.2 (2)	52.3 ± 0.7 (4)
*200 µg/l	2		44.9 ± 41.3 (3)*		231,9 ± 0 (1)			450.0 ± 159.8 (2)	48.7 ± 5.5 (4)
320 µg/l	3		39.6 ± 68.7 (3)*	-	323,3 ± 0.78 (2)	-	-	510.0 ± 241.8 (2)	50.1 ± 6.1 (4)
	4		48.1 ± 39.5 (3)*		295,3 ± 0 (1)			514.5 ± 228.4 (2)	-
	mean	**73.1 ± 8.0 (12)**	**42.5 ± 47.2 (12)***		**298.1 ± 45.6 (6)**			**488.9 ± 160.2 (8)**	**50.4 ± 4.6 (12)**
									89.1 ± 7.1 (4)
									105.9 ± 13.2 (4)
									106.9 ± 9.9 (4)
									-
									100.6 ± 12.7 (12)

Measured concentrations were generally close to nominal concentrations, except in LAB 1 and LAB 2 testing zebrafish, where measured concentrations were much lower than expected.

Dihydrotestosterone

Table 3: Measured concentrations of DHT; mean concentrations in ng/l ± the standard deviation (SD). Numbers in brackets are N samplings. Lines in Bold are means and SD of all treatment samples. Highlighted lines are outliers from the mean treatment concentration ± 20%.

nominal conc.	replicate	LAB 1 zebrafish mean ± SD (n)	LAB 2 zebrafish mean ± SD (n)	LAB 3 zebrafish mean ± SD (n)	LAB 5 medaka mean ± SD (n)	LAB 6 stickleback mean ± SD (n)	LAB 8 stickleback mean ± SD (n)	LAB 9 medaka mean ± SD (n)
control	1	0.1 ± 0.1 (10)	2.2 ± 1.9 (3)	0.4 ± 1.1 (8)	0.0 ± 0.0 (4)	0.0 ± 0.0 (9)	<LOQ (10)	<LOQ (11)
	2	1.4 ± 4.3 (10)	1.3 ± 1.6 (3)	0.1 ± 0.4 (8)	0.0 ± 0.0 (3)	0.0 ± 0.0 (9)	<LOQ (10)	
	3	9.5 ± 19.6 (10)	6.4 ± 7.2 (3)	0.3 ± 0.7 (7)	0.0 ± 0.0 (3)	0.0 ± 0.0 (9)	-	
	4	35.1 ± 70.8 (10)	1.4 ± 1.6 (3)	0.7 ± 1.8 (7)	0.0 ± 0.0 (3)	0.0 ± 0.0 (9)	-	
	mean	**11.5 ± 38.1 (40)***	**2.8 ± 4.0 (12)**	**0.4 ± 1.1 (30)**				
solvent	1		2.4 ± 2.0 (3)	0.4 ± 1.2 (8)		0.0 ± 0.0 (9)	<LOQ (10)	
	2		3.5 ± 1.8 (3)	1.0 ± 2.8 (8)		0.0 ± 0.0 (9)	<LOQ (10)	
	3	-	3.4 ± 2.5 (3)	1.9 ± 5.1 (7)	-	0.0 ± 0.0 (9)	-	-
	4		4.3 ± 5.4 (3)	2.1 ± 5.7 (7)		0.0 ± 0.0 (9)	-	
	mean		**3.3 ± 2.8 (12)**	**1.3 ± 3.8 (30)**				
100 ng/l	1	42.6 ± 56.0 (10)	4.6 ± 1.0 (3)	20.8 ± 17.2 (8)	77.8 ± 62.9 (4)	123.3 ± 19.7 (9)	186.4 ± 58.3 (9)	94.4 ± 12.4 (11)
	2	43.8 ± 47.4 (10)	2.4 ± 0.7 (3)	16.8 ± 10.8 (8)	40.0 ± 47.8 (3)	125.7 ± 30.3 (8)	209.2 ± 74.4 (9)	-
	3	90.9 ± 198.5 (10)	1.4 ± 1.2 (3)	24.5 ± 10.7 (7)	39.0 ± 28.7 (3)	122.8 ± 24.8 (8)	204.7 ± 55.5 (9)	-
	4	62.8 ± 84.8 (10)	5.0 ± 5.1 (3)	21.0 ± 12.7 (7)	38.3 ± 24.1 (3)	126.3 ± 26.3 (9)	208.9 ± 53.2 (9)	-
	mean	**60.0 ± 111.3 (40)**	**3.3 ± 2.8 (12)**	**20.6 ± 12.8 (30)**	**48.8 ± 40.9 (13)**	**124.6 ± 24.5 (34)**	**202.3 ± 58.3 (36)**	**94.4 ± 12.4 (11)**
320 ng/l	1	142.9 ± 195.5 (10)	3.9 ± 4.6 (3)	17.5 ± 14.5 (8)	188.0 ± 105.4 (4)	263.7 ± 59.6 (9)	352.2 ± 92.5 (9)	314.9 ± 52.1 (11)
	2	204.5 ± 271.4 (10)	2.1 ± 2,9 (3)	14.6 ± 12.2 (8)	150.4 ± 45.1 (3)	273.6 ± 51.1 (9)	339.9 ± 82.9 (9)	-
	3	123.4 ± 145.7 (10)	3.9 ± 6.8 (3)	27.2 ± 15.5 (7)	140.8 ± 63.0 (3)	264.8 ± 45.5 (9)	316.3 ± 82.9 (9)	-
	4	112.3 ± 133.5 (10)	3.5 ± 3.3 (3)	25.1 ± 19.5 (7)	139.2 ± 93.1 (3)	280.2 ± 63.1 (9)	305.7 ± 58.7 (9)	-
	mean	**145.8 ± 190.3 (40)**	**3.3 ± 4.0 (12)**	**20.8 ± 15.6 (30)**	**154.6 ± 76.6 (13)**	**270.6 ± 53.3 (36)**	**328.5 ± 79.8 (36)**	**314.9 ± 52.1 (11)**
1000 ng/l	1	228.2 ± 184.4 (10)	13.2 ± 13,7 (3)	53.1 ± 29.0 (8)	628.8 ± 113.5 (4)	792.4 ± 155.9 (9)	1125.3 ± 263.8 (9)	1003.9 ± 126.6 (11)
	2	303.6 ± 140.6 (10)	16.9 ± 16.2 (3)	83.6 ± 52.7 (8)	765.0 ± 88.5(3)	725.2 ± 125.9 (9)	1089.2 ± 251.8 (9)	-
	3	228.5 ± 154.2 (10)	1.4 ± 0.8 (3)	84.5 ± 87.8 (7)	683.3 ± 161.7 (3)	742.7 ± 138.5 (8)	1116.9 ± 226.4 (9)	-
	4	330.2 ± 250.7 (10)	4.2 ± 2.5 (3)	158.2 ± 200.6 (7)	667.5 ± 66.3 (3)	690.3 ± 131.2 (8)	894.2 ± 482.1 (9)	-
	mean	**272.6 ± 185.7 (40)**	**8.7 ± 11.1 (12)**	**93.1 ± 110.9 (30)**	**711.1 ± 107.5 (13)**	**738.9 ± 137.4 (34)**	**1056.4 ± 324.6 (36)**	**1003.9 ± 126.6 (11)**

In laboratories testing with zebrafish, measured concentrations were generally much lower than nominal concentrations, in particular in LAB 2, the highest concentration tested did not reach 10 ng/L instead of 1000 ng/L. 4-tert-pentylphenol

Table 4: Measured concentrations of 4-tert-pentylphenol; mean concentrations in µg/l ± the standard deviation (SD). Numbers in brackets are N samplings. Lines in Bold are means and SD of all treatment samples.

nominal conc.	replicate	LAB 9 medaka mean ± SD (n)	LAB 10 medaka mean ± SD (n)
control	1	<LOQ (5)	<LOQ (9)
	2	<LOQ (4)	<LOQ (9)
	3	<LOQ (3)	<LOQ (9)
	4	-	<LOQ (9)
solvent	1	-	-
	2		
	3		
	4		
32 µg/l	1	32.8 ± 4.3 (5)	26.8 ± 2,9 (9)
	2	31.5 ± 4.1 (4)	27.0 ± 2.4 (9)
	3	29.7 ± 2.5 (3)	27.0 ± 2.0 (9)
	4	-	27.0 ± 2.3 (9)
	mean	**31.5 ± 3.7 (12)**	**27.0 ± 2.8 (36)**
100 µg/l	1	111.0 ± 10.2 (5)	93.4 ± 6.1 (9)
	2	101.1 ± 15.5 (4)	93.8 ± 6.1 (9)
	3	97.1 ± 14.5 (3)	93.4 ± 7.0 (9)
	4	-	93.7 ± 5.7 (9)
	mean	**104.2 ± 13.4 (12)**	**93.6 ± 5.9 (36)**
320 µg/l	1	324.3 ± 40.6 (5)	295.3 ± 19.7 (9)
	2	324.0 ± 42.3 (4)	294.9 ± 18.2 (9)
	3	298.5 ± 20.3 (3)	293.5 ± 18.3 (9)
	4	-	293.5 ± 17.1 (9)
	mean	**317.7 ± 36.0 (12)**	**294.3 ± 17.6 (36)**

All measured concentrations remained within the +/-20% range of nominal concentrations

Flutamide

Table 5: Measured concentrations of flutamide; mean concentrations in µg/l ± the standard deviation (SD). Numbers in brackets are N samplings. Lines in Bold are means and SD of all treatment samples. Highlighted lines are outliers from the mean treatment concentration ± 20%.

nominal conc.	replicate	LAB 6 stickleback mean ± SD (n)
control	1	<LOQ (5)
	2	<LOQ (1)
	3	<LOQ (1)
	4	<LOQ (5)
solvent	1	<LOQ (5)
	2	<LOQ (1)
	3	<LOQ (5)
	4	<LOQ (1)
32 µg/l	1	42.2 ± 10.7 (5)
	2	17.2 ± (1)
	3	48.3 ± 4.9 (5)
	4	33.0 ± (1)
	mean	**41.9 ± 11.4 (12)**
100 µg/l	1	125.6 ± (1)
	2	145.4 ± 18.0 (5)
	3	135.6 ± (1)
	4	137.1 ± 24.9 (5)
	mean	**139.4 ± 19.5 (12)**
320 µg/l	1	373.2 ± (1)
	2	377.9 ± 42.5 (5)
	3	433.3 ± (1)
	4	388.4 ± 34.0 (5)
	mean	**386.5 ± 36.4 (12)**

Measured test concentrations were generally within an acceptable range of variability around the nominal concentrations.

Table 6: Measured concentrations of E2; mean concentrations in ng/l ± the standard deviation (SD). Numbers in brackets are N samplings. Lines in Bold are means and SD of all treatment samples. Highlighted lines are outliers from the mean treatment concentration ± 20%.

nominal conc.	replicate	Lab 6 stickleback mean ± SD (n)
control	1	<LOQ (5)
	2	<LOQ (1)
	3	<LOQ (1)
	4	<LOQ (5)
solvent	1	<LOQ (5)
	2	<LOQ (1)
	3	1.8 ± 4.1 (5)
	4	<LOQ (1)
32 ng/l	1	29.2 ± (1)
	2	38.0 ± 5.2 (5)
	3	32.1 ± (1)
	4	38.0 ± 6.6 (5)
	mean	**36.8 ± 5.9 (12)**
100 ng/l	1	111.2 ± 31.6 (5)
	2	103.5 ± 37.4 (5)
	3	63.0 ± (1)
	4	47.2 ± (1)
	mean	**98.6 ± 36.2 (12)**
320 ng/l	1	380.1 ± (1)
	2	394.8 ± 67.5 (5)
	3	390.9 ± 63.7 (5)
	4	370.4 ± (1)
	mean	**389.9 ± 56.4 (12)**

Measured test concentrations were generally within an acceptable range of variability around the nominal concentrations.

Hatching rate and survival

4-tert-octylphenol

Table 7: Hatching rate and survival from hatch to end of exposure (4-tert-octylphenol): Survival in percentage per replicate. Lines in Bold are means of all treatment samples. Highlighted lines are outliers from the validation criteria. *Nominal concentrations for LAB 9 were 6.25, 12.5, 25, 50 and 100 µg/l 4-tert-octylphenol*

nominal conc.	replicate	LAB 1 zebrafish		LAB 2 zebrafish		LAB 4 medaka		LAB 4 zebrafish		LAB 5 medaka		LAB 6 stickleback		LAB 8 stickleback		LAB 9 medaka	
		Hatching rate (%)	survival (%) (n)	Hatching rate (%)	survival (%) (n)	Hatching rate (%)	survival (%) (n)	Hatching rate (%)	survival (%) (n)	Hatching rate (%)	survival (%) (n)	Hatching rate (%)	survival (%) (n)	Hatching rate (%)	survival (%) (n)	Hatching rate (%)	survival (%) (n)
control	1	100.0	90.0	100	90.0	70.0	85.7	100.0	80.0	76.0	89.5		95.0	60.0	91.6	80.0	80.0
	2	100.0	95.0	100	88.0	60.0	100.0	100.0	70.0	88.0	90.9		80.0	83.0	95.8	93.3	77.8
	3	100.0	88.0	100	89.0	90.0	83.3	97.5	74.4	84.0	85.7	**77.0**	90.0	90.0	84.3	80.0	80.0
	4	100.0	83.0	100	89.0	90.0	83.3	97.5	66.7	64.0	100		95.0	80.0	100.0	**84.3**	**79.3**
	mean	**100.0**	**89.0**	**100**	**89**.0	**77.5**	**87.1**	**98.8**	**72.8**	**78.0**	**91.5**		**90.0**	**78.3**	**92.9**		
solvent	1	-	-	100	97.0	-	-	-	-	-	-	**77.2**	100.0	53.3	87.5		
	2			100	98.0								90.0	30.0	96.0		
	3			100	100.0								95.0	83.3	88.0		
	4			100	95.0								80.0	80.0	0.0		
	mean			**100**	**97.0**								**91.3**	**61.7**	**67.9**		
10 µg/l	1	-	-	-	-	45.0	88.9	100.0	70.0	100	88.0	**76.4**	90.0			93.3	80.0
	2					45.0	100.0	100.0	72.5	68.0	88.2		69.1			86.7	86.7
	3					35.0	100.0	100.0	75.0	88.0	100		90.0			93.3	93.3
	4					35.0	100.0	100.0	65.0	92.0	95.7		90.0			**91.1**	**86.7**
	mean					**40.0**	**96.9**	**100.0**	**70.6**	**87.0**	**93.0**		**84.8**				
32 µg/l	1	100.0	90.0	100	83.0	-	-	100.0	62.5	76.0	100	**60.2**	75.9	56.6	100.0	90.3	93.3
	2	100.0	100.0	100	79.0	-	-	100.0	52.5	76.0	94.7		80.0	26.6	84.0	100.0	93.3
	3	100.0	85.0	100	80.0	50.0	100.0	97.5	71.8	80.0	100		Pooled	43.3	94.7	80.0	80.0
	4	100.0	90.0	100	95.0	80.0	88.0	95.0	57.9	80.0	100		Pooled	80.0	0.0	**91.1**	**88.9**
	mean	**100.0**	**91.3**	**100**	**84.0**	**65.0**	**92.3**	**98.1**	**61.2**	**78.0**	**98.7**		**78.0**	**51.6**	**69.7**		
100 µg/l	1	100.0	85.0	100	85.0	55.0	90.9	97.5	43.6	68.0	88.2	**17.6**	65.2	60.0	2.7	80.0	73.3
	2	100.0	70.0	100	78.0	70.0	78.6	100.0	17.5	84.0	76.2		Pooled	22.5	0.0	93.3	93.3
	3	100.0	88.0	100	86.0	50.0	90.0	100.0	27.5	84.0	100		Pooled	23.3	0.0	93.3	93.3
	4	100.0	87.0	100	80.0	80.0	100.0	95.0	36.8	96.0	100		Pooled	33.3	0.0	**88.9**	**86.7**

	mean	**100.0**	**82.5**	**100**	**82.0**	**63.8**	**90.2**	**98.1**	**31.4**	**83.0**	**91.1**		**65.2**	**34.8**	**0.7**		
*200 µg/l 320 µg/l	1	100.0	83.0*	100	86.0*			50	0.0					0	0	73.3	73.3
	2	100.0	100.0*	100	79.0*			32.5	0.0					0	0	80.0	80.0
	3	100.0	75.0*	100	79.0*	-	-	32.5	0.0			-	-	0	0	100.0	80.0
	4	100.0	80.0*	100	85.0*			22.5	0.0					0	0	**84.4**	**77.8**
	mean	**100.0**	**84.5***	**100**	**82.0***			**34.4**	**0.0**					**0**	**0**		
																100.0	93.3
																80.0	0
																86.7	73.3
																88.9	**55.6**

Dihydrotestosterone

Table 8: hatching rate and survival from hatch to end of exposure (DHT): Survival in percentage per replicate. Lines in Bold are means of all treatment samples. Highlighted lines are outliers from the validation criteria.

nominal conc.	replicate	LAB 1 zebrafish		LAB 2 zebrafish		LAB 3 zebrafish		LAB 5 medaka		LAB 6 stickleback		LAB 8 stickleback		LAB 9 medaka	
		hatching rate (%)	survival (%) (n)	hatching rate (%)	survival (%) (n)	hatching rate (%)	survival (%) (n)	hatching rate (%)	survival (%) (n)	hatching rate (%)	survival (%) (n)	hatching rate (%)	survival (%) (n)	hatching rate (%)	survival (%) (n)
control	1	100.0	45.0	100.0	85	93.3	78.3	80.0	100		95.0	60.0	91.6	100.0	80.0
	2	100.0	68.0	100.0	61.0	91.7	80.0	72.0	88.9		80.0	83.0	95.8	100.0	85.0
	3	100.0	43.0	100.0	63.0	95.0	83.3	80.0	95.0	**77.0**	90.0	90.0	84.3	95.0	80.8
	4	100.0	50.0	100.0	59.0	90.0	76.7	92.0	100		95.0	80.0	100	100.0	100.0
	mean	**100.0**	**51.5**	**100.0**	**67.0**	**92.5**	**79.6**	**81.0**	**96.0**		**90.0**	**78.3**	**92.9**	**98.8**	**86.4**
solvent	1			100.0	72.0	95.0	88.3				100.0	53.3	87.5		
	2			100.0	74.0	95.0	45.0				90.0	30.0	96.0		
	3	-	-	100.0	76.0	96.7	66.7	-	-	**77.2**	95.0	83.3	88.0	-	-
	4			100.0	58.0	93.3	70.0				80.0	80.0	pooled		
	mean			**100.0**	**70.0**	**95.0**	**67.5**				**91.3**	**61.7**	**90.5**		
100 ng/l	1	100.0	68.0	100.0	82.0	95.0	65.0	96.0	91.7		80.0	83.3	75.4	95.0	85.5
	2	100.0	73.0	100.0	79.0	85.0	65.0	64.0	100	**84.7**	90.0	66.6	90.5	70.0	49.0
	3	100.0	65.0	100.0	65.0	96.7	76,7	64.0	87.5		95.0	43.2	90.5	85.0	68.0
	4	100.0	55.0	100.0	51.0	98.3	80.0	84.0	95.2		90.0	66.6	80.9	80.0	52.0

	mean	**100.0**	**61.5**	**100.0**	**69.0**	**93.8**	**71.7**	**77.0**	**93.6**		**84.8**	**64.9**	**84.3**	**82.5**	**63.6**
320 ng/l	1	100.0	68.0	100.0	45.0	96.7	96.7	88.0	90.9	**84.7**	76.0	25.8	100.0	100.0	95.0
	2	100.0	73.0	100.0	65.0	88.3	83.3	80.0	90.0		80.0	56.6	83.0	85.0	68.0
	3	100.0	73.0	100.0	43.0	95.0	60.0	84.0	100		Pooled	47.0	93.7	95.0	71.3
	4	100.0	55.0	100.0	49.0	96.7	66.7	64.0	100		Pooled	40.0	Pooled	100.0	80.0
	mean	**100.0**	**67.3**	**100.0**	**50.0**	**94.2**	**76.7**	**79.0**	**95.2**		**78.0**	**42.4**	**92.2**	**95.0**	**78.6**
1000 ng/l	1	100.0	85.0	100.0	55.0	93.3	80.0	80.0	100	**85.5**	65.2	78.1	100.0	90.0	63.0
	2	100.0	60.0	100.0	78.0	95.0	81.7	56.0	100		Pooled	56.6	95.8	95.0	80.8
	3	100.0	68.0	100.0	61.0	96.7	63.3	64.0	93.8		Pooled	54.5	90.4	85.0	21.3
	4	100.0	45.0	100.0	79.0	91.7	50.0	80.0	90.0		Pooled	50.0	Pooled	90.0	76.5
	mean	**100.0**	**64.5**	**100.0**	**68.0**	**94.2**	**68.8**	**70.0**	**95.9**		**65.2**	**59.8**	**95.4**	**90.0**	**60.4**

4-tert-pentylphenol

Table 9: hatching rate and survival from hatch to end of exposure (4-tert-pentylphenol): Survival in percentage per replicate. Lines in Bold are means of all treatment samples. Highlighted lines are outliers from the validation criteria.

nominal conc.	replicate	LAB 9 medaka		LAB 10 medaka	
		hatching rate (%)	survival (%) (n)	hatching rate (%)	survival (%) (n)
control	1	86.7	69.3	90.0	90.0
	2	93.3	80.9	95.0	95.0
	3	93.3	68.5	95.0	95.0
	4	-	-	95.0	85.0
	mean	**91.1**	**72.9**	**93.8**	**91.25**
solvent	1				
	2				
	3	-	-	-	-
	4				
	mean				
32 µg/l	1	80.0	64.0	100.0	95.0
	2	93.3	87.1	100.0	95.0
	3	80.0	53.3	100.0	100.0

	4	-	-	100.0	100.0
	mean	**84.4**	**68.2**	**100.0**	**97.5**
100 µg/l	1	93.3	74.7	100.0	95.0
	2	93.3	80.9	100.0	90.0
	3	93.3	80.9	100.0	100.0
	4	-	-	100.0	90.0
	mean	**93.3**	**78.8**	**100.0**	**93.8**
320 µg/l	1	100.0	100.0	100.0	95.0
	2	73.3	53.8	100.0	85.0
	3	86.7	69.3	100.0	100.0
	4	-	-	100.0	95.0
	mean	**86.7**	**74.4**	**100.0**	**93.8**

Flutamide

Table 10: Hatching rate and survival from hatch to end of exposure (flutamide): Survival in percentage per replicate. Lines in Bold are means of all treatment samples. Highlighted lines are outliers from the validation criteria.

nominal conc.	replicate	LAB 6 stickleback	
		hatching rate (%)	survival (%) (n)
control	1	50.0	100.0
	2	71.4	Pooled
	3	75.0	Pooled
	4	87.5	92.3
	mean	**71.0**	**96.2**
solvent	1	75.0	100.0
	2	85.7	Pooled
	3	87.5	100.0
	4	75.0	Pooled
	mean	**80.8**	**100.0**
32 µg/l	1	83.3	100.0
	2	71.4	Pooled
	3	90.0	89.5

	4	66.7	Pooled
	mean	**77.9**	**94.7**
100 µg/l	1	81.8	pooled
	2	80.0	95.8
	3	85.7	Pooled
	4	86.7	90.9
	mean	**83.6**	**93.4**
320 µg/l	1	81.8	Pooled
	2	90.0	94.4
	3	66.7	Pooled
	4	83.3	100.0
	mean	**80.5**	**97.2**

17β-estradiol (E2)

Table 11: hatching rate and survival from hatch to end of exposure (E2): Survival in percentage per replicate. Lines in Bold are means of all treatment samples. Highlighted lines are outliers from the validation criteria.

nominal conc.	replicate	LAB 6 stickleback	
		hatching rate (%)	survival (%) (n)
control	1	50.0	100.0
	2	71.4	Pooled
	3	75.0	Pooled
	4	87.5	92.3
	mean	**71.0**	**96.2**
solvent	1	75.0	100.0
	2	85.7	Pooled
	3	87.5	100.0
	4	75.0	Pooled
	mean	**80.8**	**100.0**
32 ng/l	1	71.4	Pooled
	2	83.3	100.0
	3	50.0	Pooled

	4	89.5	100.0
	mean	**73.6**	**100.0**
100 ng/l	1	88.9	100.0
	2	90.9	100.0
	3	80.0	pooled
	4	80.0	Pooled
	mean	**85.0**	**100.0**
320 ng/l	1	100.0	Pooled
	2	90.9	94.1
	3	71.4	100.0
	4	83.3	Pooled
	mean	**86.4**	**97.1**

Abnormal appearance and behaviour

No dose related abnormal appearance or behaviour was reported from the laboratories except for LAB 6 where male aggressive behaviour was registered (as number of nips) and a 4 fold reduction was observed at 739 ng/l DHT. A 1½ fold reduction was observed from 66.9 µg/l 4-tert-octylphenol.

Core endpoints

Table 12: Overview of NOEC/LOEC for vitellogenin measurements. *=Jonkheere Terpstra One-sided alternative hypothesis.

Exposure chemical	species	Lab	NOEC VTG (µg/l)	LOEC VTG (µg/l)	comments
4-tert-octylphenol	zebrafish	1	13.8	40.6	increase undifferentiated
4-tert-octylphenol	zebrafish	2	17.6	42.5	increase females and males
4-tert-octylphenol	zebrafish	4	9.5	26.0*	increase males
4-tert-octylphenol	medaka	4	31.7	105	increase females
4-tert-octylphenol	medaka	5	<12.1	12.1	increase undifferentiated
4-tert-octylphenol	medaka	9	6.2	12.3	increase males
4-tert-octylphenol	stickleback	6	22.2	66.9	against solvent control only
4-tert-octylphenol	stickleback	8	>41.9	>41.9	No effect
DHT	zebrafish	1	60.0 ng/l	146 ng/l	decline males
DHT	zebrafish	2	3.1 ng/l	3.3 ng/l	No dose response
DHT	zebrafish	3	20.8 ng/l	93.1* ng/l	increase males
DHT	medaka	5	48.8 ng/l	155 ng/l	decline females
DHT	medaka	9	<94.0 ng/l	94.4 ng/l	increase males
DHT	stickleback	6	739 ng/l	>739 ng/l	no effect
DHT	stickleback	8	<202 ng/l	202 ng/l	decline males, no dose response
4-tert-pentylphenol	medaka	9	31.5	104.2	increase males
4-tert-pentylphenol	medaka	10	<27.0	27.0	increase females
E2	stickleback	6	36.8 ng/l	98.6 ng/l	increase females and undiff.
flutamide	stickleback	6	41.9*	139	increase females
ammonia	zebrafish	1	1.94 mg/l	3.88 mg/l	no dose response
n-octanol	medaka	9	1.0 mg/l	3.2 mg/l	decline females

Table 13: Overview of NOEC/LOEC for sex ratio measurements

Exposure chemical	species	Lab	NOEC sex ratio (µg/l)	LOEC sex ratio (µg/l)	comments
4-tert-octylphenol	zebrafish	1	<13.8	13.8	more females
4-tert-octylphenol	zebrafish	2	5.7	17.6	less males
4-tert-octylphenol	zebrafish	4	9.5	26.0	more females
4-tert-octylphenol	medaka	4	<11.2	11.2	more females
4-tert-octylphenol	medaka	5	<12.1	30.6	more females
4-tert-octylphenol	medaka	9	23.5	50.4	more females
4-tert-octylphenol	stickleback	6	66.0	>66.0	no males
4-tert-octylphenol	stickleback	8	>41.9	>41.9	100% mortality above 41.9 µg/l
DHT	zebrafish	1	<60.0 ng/l	60.0 ng/l	more males
DHT	zebrafish	2	>8.7 ng/l	>8.7 ng/l	low chemical concentration
DHT	zebrafish	3	20.6 ng/l	20.8 ng/l	more males
DHT	medaka	5	<48.8 ng/l	48.8 ng/l	less females
DHT	medaka	9	<94.0 ng/l	94.0 ng/l	genetic females => males
DHT	stickleback	6	271 ng/l	739 ng/l	One intersex fish in middle and two in high exposure conc.
DHT	stickleback	8	202 ng/l	329 ng/l	more intersex, less females
4-tert-pentylphenol	medaka	9	104	318	genetic males => females
4-tert-pentylphenol	medaka	10	27.0	93.6	more intersex
E2	stickleback	6	98.6 ng/l	390 ng/l	more intersex
flutamide	stickleback	6	>383	>383	no males
ammonia	zebrafish	1	>7.75 mg/l	>7.75 mg/l	100% mortality at 15 mg/l
n-octanol	medaka	9	>3.2 mg/l	>3.2 mg/l	no effect

Zebrafish

4-tert-octylphenol

Vitellogenin levels

Table 14: Vitellogenin levels detected in male and female fish following exposure to 4-tert-octylphenol. Standard deviation is indicated in parenthesis. LAB 4 used 10, 32 and 100 µg/L as test concentrations. Shaded cell indicate the statistical significance.

	Sex	Control	32 µg/L	100 µg/L	200 µg/L
LAB 1	Males	4036 (34751)	83 (52)	-	-
	Females	261 915 (226 671)	193 729 (271 226)	162 690 (460 756)	6 606 446 (3 572 973)
	Undiff.	22 402 (66 974)	1 848 (4 535)	158 705 (485 012)	5 716 012 (3561 727)
LAB 2	Males	1129 (2521) [solvent control: 54 (58]	2178 (8510)	678 (3098)	26 797 (39 265)
	Females	352 251 (629 865) [solvent control: 25 101 (56634)]	49 735 (222 099)	13422 (47 939)	354 966 (887 221)
	Undiff	23 038 (54 416) [solvent control: 1395 (4873)]	2544 (10 045)	41 (62)	66 169 (215 054)
LAB 4	Males	11 (6)	11 (5)	22 (19)	-
	Undiff.	12 (7)	10 (5)	694 (1655)	241 925 (270 745)
	Intersex.	10 (6)	13 (11)	1 444 (2 037)	157 801 (159 027)
	Females	18 808 (52 405)	3 751 (7 427)	12 756 (28 091)	184 347 (230 426)

Sex ratio

Table 15: Proportions of each sex determined following exposure to 4-tert-octylphenol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance

	Proportions (SD)	Control	10 µg/L	32 µg/L	100 µg/L	200 µg/L
LAB 1	Males	0.567 (0.06)	----------	0.184 (0.23)	0	0
	Females	0.355 (0.07)	----------	0.762 (0.22)	0.563 (0.12)	0.628 (0.03)
	Intersex	0.014 (0.03)	----------	0	0	0

	Undifferentiated	0.064 (0.03)	----------	0.054 (0.06)	0.438 (0.12)	0.372 (0.03)
LAB 2	Males	0.369 (0.99)	----------	0.285 (0.79)	0.193 (0.46)	0.07 (0.28)
	Females	0.479 (0.46)	----------	0.523 (0.80)	0.496 (0.19)	0.525 (0.56)
	Intersex	None	----------	None	None	None
	Undifferentiated	0.150 (0.79))	----------	0.192 (0.17)	0.309 (0.37)	0.400 (0.31)
LAB 4	Males	0.409 (0.08)	0.274 (0.13)	0.104 (0.09)	0	----------
	Females	0.461 (0.05)	0.619 (0.14)	0.729 (0.06)	0.633 (0.18)	----------
	Not Intersex	0.954 (0.03)	0.938 (0.04)	0.978 (0.02)	0.870 (0.12)	----------
	Not Undifferentiated	0.964 (0.05)	0.973 (0.03)	0.891 (0.02)	0.765 (0.18)	----------

Dihydrotestosterone (DHT)

Vitellogenin levels

Table 16: Vitellogenin levels detected in male and female fish following exposure to dihydrotestosterone. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Sex	Control	100 ng/L	320 ng/L	1000 ng/L
LAB 1	Males	288 (668)	51 814 (314 747)	37 (13)	26 (11)
	Females	1 333 455 (1 671 066)	633 262 (1 478 884)	-	-
LAB 2	Males	363 (1 203)	40 (23)	3545 (17 744)	5 641 (18 302)
	Females	587 960 (1 128 779) [solvent control: 34 497 (66 562)]	1 692 (5 082)	162 958 (467 117)	85 829 (284 038)
LAB 3	Males	35 (36)	53 (43)	78 (111)	61 (39)
	Females	9 076 (32 187)	5 044 (16 518)	15 559 (41 956)	265 (193)

Sex ratio

Table 17: Proportions of each sex determined following exposure to dihydrotestosterone. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Proportions (SD)	Control	100 ng/L	320 ng/L	1000 ng/L
LAB 1	Males	0.561 (0.26)	0.837 (0.80)	1 (0)	0.99 (0.08)
	Females	0.390 (0.22)	0.163 (0.80)	0	0
	Intersex	0	0	0	0.010 (0)
	Undifferentiated	0.049 (0)	0	0	0
LAB 2	Males	0.559 (0.54)	0.50 (0.77)	0.586 (0.41)	0.57 (0.38)
	Females	0.394 (0.58)	0.353 (0.70)	0.363 (0.60)	0.331 (0.27)
	Intersex	None	None	None	None
	Undifferentiated	0.044 (0.05)	0.145 (0.11)	0.061 (0.06)	0.098 (0.02)
LAB 3	*Not* Males	0.400 (0.03)	0.298 (0.16)	0.161 (0.20)	0.087 (0.14)
	Females	0.400 (0.03)	0.287 (0.15)	0.148 (0.17)	0.087 (0.14)
	Intersex	----------	----------	----------	----------
	Undifferentiated	----------	----------	----------	----------

Medaka

4-tert-octylphenol

Vitellogenin levels

Table 18: Vitellogenin levels detected in male and female fish following exposure to 4-tert-octylphenol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Sex	Control	10 µg/L	32 µg/L	100 µg/L
LAB 4	Males	<limit of quantification	<limit of quantification	<limit of quantification	<limit of quantification
	Females	9 (6)	32 (73)	25 (38)	153 (104)
LAB 5	Males	128 (18)	7 165 (36 614)	134 (27)	2629 (6 972)
	Females	6128 (15460)	10 122 (30 584)	27 445	59 599

				(44 327)	(93 915)
LAB 9	Males	0.7 (0.1)	6 (7)	31 (21)	-
	Females	1711 (412)	1170 (415)	1180 (542)	2132 (298)

Sex ratio

Table 19: Proportions of each sex determined following exposure to 4-tert-octylphenol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Proportions (SD)	Control	10 µg/L	32 µg/L	100 µg/L
LAB 4	Males	0.666 (0.57)	0.451 (0.58)	0.296 (0.23)	0.043 (0.15)
	Females	0.203 (0.58)	0.483 (0.60)	0.444 (0.59)	0.478 (0.55)
	Intersex	0	0.032 (0.19)	0.185 (0.48)	0.413 (0.29)
	Undifferentiated	0.129 (0.31)	0.032 (0.19)	0.074 (0.26)	0.065 (0.24)
LAB 5	Males	0.500	0.406	0.452	0.317
	Not Females	0.569	0.440	0.452	0.393
	Intersex	----------	---------	----------	----------
	Undifferentiated	----------	----------	----------	----------
LAB 9	Males	0.519 (0.081)	0.425 (0.115)	0.610 (0.091)	0 (0)
	Females	0.481 (0.081	0.575 (0.115)	0.390 (0.091)	1 (0)

Dihydrotestosterone

Vitellogenin levels

Table 20: Vitellogenin levels detected in male and female fish following exposure to dihydrotestosterone. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Sex	Control	100 ng/L	320 ng/L	1000 ng/L
LAB 5	Males	161 (223)	128 (15)	125 (0.2)	132 (24)
	Females	14 101 (21 365)	13 230 (23 977)	3 919 (7 589)	-
LAB 9	Males	0.5 (0.7)	15 (58)	18 (39)	19 (29)
	Females	329 (320)	402 (195)	179 (125)	-

Sex ratio

Table 21: Proportions of each sex determined following exposure to dihydrotestosterone. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Proportions	Control	100 ng/L	320 ng/L	1000 ng/L
LAB 5	Not Males	0.499	0.394	0.060	0.048
	Females	0.430	0.299	0.060	0
	Intersex	----------	----------	----------	----------
	Undifferentiated	----------	----------	----------	----------
LAB 9	Males	0.486	0.458	0.652	1
	Females	0.514	0.542	0.348	0
	Intersex	----------	----------	----------	----------
	Undifferentiated	----------	----------	----------	----------

4-tert-pentylphenol

Vitellogenin levels

Table 22: Vitellogenin levels detected in male and female fish following exposure to 4-tert-pentylphenol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Sex	Control	32 µg/L	100 µg/L	320 µg/L
LAB 9 (70d)	Males	1.2 (0.3)	1.3 (0.7)	1.7 (0.2)	3 (2)
	Females	882 (513)	613 (172)	767 (417)	-
LAB 10 (60d)	Males	0.68 (0.5)	0.90 (0.88)	2.45 (2.95)	85 (156)
	Females	1019 (736)	1719 (649)	3215 (1480)	5099 (4427)

Sex ratio

Table 23: Proportions of each sex determined following exposure to 4-tert-pentylphenol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance (*=one-sided hypothesis testing).

	Proportion (SD)	Control	10 µg/L	32 µg/L	100 µg/L

LAB 9 (70d)	Males	0.551	0.411	0.403	0.304*
	Females	0.448 (0.08)	0.588 (0.08)	0.596 (0.06)	0.521 (0.07)
	Intersex	----------	----------	----------	----------
	Undifferentiated	----------	----------	----------	----------
LAB 10 (60d)	Males	0.45	0.40	0.35	0.20
	Females	0.55	0.60	0.50	0.70
	Intersex	----------	----------	0.15	0.10
	Undifferentiated	----------	----------	----------	----------

Stickleback

4-tert-octylphenol

Vitellogenin levels

Table 24: Vitellogenin levels detected in males and female fish following exposure to 4-tert-octylphenol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Sex	Control	32 µg/L	100 µg/L	200 µg/L
LAB 6	Males	35 (34) Solvent control: 79 (54)	42 (25)	42 (20)	83 431 (74 047)
	Females	43 (38) Solvent control: 72 (37)	36 (28)	40 (25)	168 842 (192 588)
LAB 8	Males	1632 (566)	2222 (1972)	-	-
	Females	1845 (813)	5724 (7766)	-	-

Sex ratio

Table 25: Proportions of each sex determined following exposure to 4-tert-octylphenol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Proportions (SD)	Control	32 µg/L	100 µg/L	200 µg/L
LAB 6	Males	0.462 (0.30)	0.555 (0.44)	0.372 (0.44)	0.533
	Females	0.537 (0.30)	0.444 (0.44)	0.558 (0.56)	0.466
	Intersex	None	None	0.069 (0.12)	None
	Undifferentiated	None	None	None	None
LAB 8	Males	0.389 (0.17)	0.547 (0.06)	----------	----------
	Females	0.611 (0.17)	0.453 (0.06)	----------	----------
	Intersex	None	None	None	None
	Undifferentiated	None	None	----------	----------

Dihydrotestosterone

Vitellogenin levels

Table 26: Vitellogenin levels detected in male and female fish following exposure to dihydrotestosterone. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Sex	Control	100 ng/L	320 ng/L	1000 ng/L
LAB 6	Males	35 (34) Solvent control:79 (54)	57 (47)	53 (35)	58 (62)
	Females	43 (38) Solvent control:72 (47)	54 (42)	49 (40)	42 (36)
LAB 8	Males	1687 (2129)	621 (441)	815 (586)	1990 (2855)
	Females	1965 (2014)	859 (496)	209 (272)	1283 (644)

Sex ratio

Table 27: Proportions of each sex determined following exposure to dihydrotestosterone. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Proportions (SD)	Control	100 ng/L	320 ng/L	1000 ng/L
LAB 6	Males	0.463 (0.07)	0.428 (0.14)	0.430 (0.11)	0.440 (0.18)
	Females	0.536 (0.07)	0.571 (0.14)	0.555 (0.09)	0.529 (0.12)
	Intersex	----------	----------	0.016 (0.03)	0.031 (0.06)
	Undifferentiated	----------	----------	----------	----------
LAB 8	Males	0.509 (0.80)	0.513 (0.95)	0.612 (0.04)	0.446 (0.17)
	Females	0.490 (0.80)	0.486 (0.95)	0.308 (0.07)	0.412* (0.16)
	Intersex	0	0	0.081* (0.03)	0.143* (0.05)
	Undifferentiated	0	0	0	0

Flutamide

Vitellogenin levels

Table 28: Vitellogenin levels detected in male and female fish following exposure to flutamide for 42 dph. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Sex	Control	45 µg/L	141 µg/L	383 µg/L
LAB 6	Undiff.	95 (212)	54 (13)	57 (8)	52 (7)
	Females	49 (12)	51 (11)	56 (19)	71 (70)

Sex ratio

Table 29: Proportions of each sex determined following exposure to flutamide for 42 dph. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Proportions (SD)	Control	45 µg/L	141 µg/L	383 µg/L
LAB 6	Males	None	None	None	None
	Females	0.608 (0.382)	0.558 (0.02)	0.578 (1.43)	0.583 (0.22)
	Intersex	----------	----------	----------	----------

	Undifferentiated	0.391 (0.38)	0.441 (0.02)	0.421 (1.43)	0.416 (0.22)

17ß-estradiol

Vitellogenin levels

Table 30: Vitellogenin levels detected in male and female fish following exposure to 17ß-estradiol for 42 dph. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

	Sex	Control	100 µg/L	320 µg/L	1000 µg/L
LAB 6	Undiff.	95 (212)	55 (23)	1 059 086 (542 386)	1 314 685 (4 455 324)
	Females	49 (12)	56 (15)	1 143 310 (743 571)	10 167 541 (3 360 089)

Sex ratio

Table 31: Proportions of each sex determined following exposure to 17ß-estradiol for 42 dph.

	Proportions (SD)	Control	32 µg/L	100 µg/L	320 µg/L
LAB 6	Males	None	None	None	None
	Females	0.608 (0.38)	0.464 (0.041)	0.366 (1.14)	0.562 (0)
	Intersex	0	0	0.033 (0.19)	0.156* (0.53)
	Undifferentiated	0.391 (0.38)	0.535 (0.04)	0.600 (0.95)	0.281 (0.53)

DISCUSSION

Purpose of the assay

The purpose of the Fish Sexual Development Test (FSDT) is to assess early life-stage effects and potential adverse consequences of putative endocrine disrupting chemicals (e.g., estrogens, androgens and steroidogenesis inhibitors). The combination of the two core endocrine endpoints, vitellogenin concentration and the population-relevant sex ratio enable the test to be used for hazard and risk assessment when the mode of action of the test substance is already known or identified by the FSDT.

The biological model

The biological model utilized in the Fish Sexual Development Test is the hormone regulated sexual differentiation and development in fish, where substances mimicking, inducing or inhibiting the endogenous hormones can skew the sex ratio of a sexual developing fish population toward more females, more males, more intersex fish or more undifferentiated fish. Besides, the hormone dependant induction or inhibition of the yolk protein vitellogenin (VTG) (see also OECD TG 229 and TG 230) is utilized in combination with the sex ratio to establish an identification of the mode of action (MOA) of the substance as demonstrated in Table 32.

MOA	VTG ♂	VTG ♀	Sex ratio	references
Weak estrogen agonist	↑	↑	↑♀, ↑Undiff	[Panter et al. 2006;Schafers et al. 2007]
Strong estrogen agonist	↑	↑	↑♀ , ↑Undiff, No ♂	[Holbech et al. 2006;Schafers et al. 2007]
Estrogen antagonist	?	↓	↓♀, ↑Undiff.	[Andersen et al. 2004]
Weak androgen agonist	?	?	? ♂	
Strong androgen agonist	↓	↓	↑ ♂, No ♀	[Holbech et al. 2006]
Androgen antagonist	-	↑	↑intersex	[Kiparissis et al. 2003;Panter et al. 2004]
aromatase inhibitor	↓	↓	↓♀	[Kinnberg et al. 2007]

Table 32: Effect response table of VTG and sex ratio to different modes of action of chemicals. ↑=rise in VTG concentration or phenotypic sex. ↓= decline in VTG concentration or phenotypic sex.

Control animal performance: Hatching and Survival, abnormal appearance and behaviour

The Hatching rate in the experiments with 4-tert-octylphenol did fulfil the validity criteria of 80% with exception of four experiments where the hatching rate was 77.5%, 78% and 77.3 and 78% respectively. These small abbreviations from the validity criteria are not expected to influence the results. Non of the laboratories reported any dose related delay in hatching. The survival of larvae and juvenile fish did fulfil the validity criteria of 70% in all experiments with 4-tert-octylphenol. In summary the hatching and survival in the eight experiments with 4-tert-octylphenol were satisfactory.

The hatching rate in all experiments with DHT did fulfil the validity criteria of 80% with exception of two experiments where the hatching rate was 77.0 and 78.3% which did not influence the test results. The survival of larvae and juvenile fish did fulfil the validity criteria of 70% in all experiments with dihydrotestosterone with the exception of two experiments where the survival rate was 51.5% and 67.0% respectively. The results of the first experiment however, were comparable to the results of the other experiments and therefore the aberration from the validity criteria are not expected to influence the results. The second experiment failed in keeping the DHT nominal concentration in acceptable levels but the survival was close to the 70% validity criteria and did not affect the results. In summary the hatching and survival in the eight experiments with dihydrotestosterone were satisfactory.

The Hatching rate in the two experiments with 4-tert-pentylphenol did fulfil the validity criteria of 80%. The survival of larvae and juvenile fish did fulfil the validity criteria of 70% in both experiments with dihydrotestosterone. In summary the hatching and survival in the two experiments with 4-tert-pentylphenol were satisfactory.

The Hatching rate in the experiments with 17ß-estradiol and flutamide (same control groups) did not fulfil the validity criteria of 80% as it was 71%. The survival of larvae and juvenile fish did fulfil the validity criteria of 70% in both experiments. In summary the hatching and survival in the two experiments with 17ß-estradiol and flutamide were satisfactory.

LAB 6 reported a reduction in male aggressive behaviour (registered as number of nips) during the exposures. A 4 fold reduction was observed at 739 ng/l DHT and a 1½ fold reduction was observed at 66.9 µg/l 4-tert-octylphenol**.** The reduced aggressive behaviour during DHT exposure was not expected but might be related to a negative feed back mechanism (on e.g. 11-keto testosterone, that is known to induce this male aggressive behaviour [Bell 2001]) but this has not been confirmed experimentally. The effect of 4-tert-octylphenol is expected because it has previously been observed in stickleback that an estrogen reduces male aggressive behaviour [Bell 2001]. It should though be noted that the reference study was done with adult males in contrast to 0-60 DPH males in the present study. No other laboratories reported any dose related abnormal appearance or behaviour. Single cases of lordosis (curvature of the vertebral column) were observed in some of the laboratories but at a very low rate, unrelated to the test substance and probably something common in different laboratory stocks.

Actual chemical water concentrations

The actual chemical water concentration was measured in all experiments. Especially for the experiments with zebrafish, these concentrations were much below the nominal concentrations which is not satisfactory. Especially the exposure concentrations in the Lab 2 experiments are outliers and the results of these two experiments can not be compared to the rest. The endpoint responses are though strongly connected to the actual chemical concentrations which can be seen on the NOEC/LOEC values of Table 12 and Table 13 and therefore the experiments are recognised as valid. The results confirm the necessity of regular chemical analysis of the exposure water and raise the question about exposure system design. A guidance document on aquatic flow through test systems might be a good idea.

Vitellogenin response

Vitellogenin is also a biomarker in TG 229 and TG 230 and the issue of inter laboratory comparison and variation in the response has been addressed in the validation process of these TGs. The vitellogenin response in the FSDT, where hazard and risk assessment could be relevant, should be seen in connection with the sex ratio because the skewing of the sex ratio where genetic sex is changed phenotypically can affect the vitellogenin concentration as seen in Figure 15 D where the vitellogenin concentration is significant different between genetic males and phenotypically sex reversed females. In contrast to TG 229

and TG 230, the FSDT should be able to identify the mode of action of the test substance and in this context, the sex specific changes in the VTG concentrations is a key biomarker as it can be seen from the effect contingency table in the FSDT draft proposal. One should also take into account that the FSDT is designed to be terminated as soon as the fish are sexually differentiated. This means that the size and developmental stages can vary significantly between experiments and therefore the background vitellogenin concentration can also vary between experiments as it is affected by these parameters. It is therefore expected to see higher control variability in the FSDT vitellogenin than in adult assays as TG 229 and TG 230 vitellogenin but this should not affect the FSDT results because induction or reduction in vitellogenin is normally massive in response to chemicals ([Kinnberg et al. 2007;Zerulla et al. 2002], and because the vitellogenin response should always be connected to the sex ratio response as discussed above.

Sex ratio response

The skewing of the sex ratio can be a population relevant endpoint if the skewing causes a reproductive performance decline in the affected fish population. The size of the skewing of the sex ratio that will cause a population relevant effect is dependent on the reproductive strategy of the species. The differences in the sensitivity of species have been demonstrated in a whole lake exposure scenario where EE2 was introduced for three years and different fish populations were followed [Kidd et al. 2007;Palace et al. 2009].

The sex ratio skewing in the present FSDT validation and in published literature, where FSDT-like exposure scenarios were used, demonstrate a very strong response toward both estrogens, androgens and aromatase inhibitors and often a 100% effect on either phenotypic females or males are seen [Holbech et al. 2006;Kinnberg et al. 2007;Lange et al. 2001;Morthorst et al. 2010;Panter et al. 2006]. Due to this strong response scenario a rather marked change in the sex ratio could be defined as the threshold for a population relevant effect on the sex ratio. For example a 50% change in female or male phenotype proportions could be set as the threshold. The size of the population relevant effect threshold should though be discussed and decided in the FDG and/or VMG-Eco. A skewing toward more undifferentiated fish is not necessarily population relevant but can be so if for example the delay in sexual development of one of the sexes causes an asynchronous mating behaviour that affect breeding. The effects of intersex on fish populations are probably very much dependant on the severity of the intersex as well as the percentage of fish with intersex and also whether it is genetic females or males that turn into intersex fish.

It should be noted that in the present validation report and in the FSDT draft proposal, sex ratio is defined as proportions of sex, where sex can be females, males, intersex or undifferentiated. The statistics are made on each of the four sex-definitions based on these proportions. As an example females are tested against all other fish (non-females) and males are tested against all other fish (non-males). Therefore, a significant change related to “more females” does not automatically mean “fewer males” because the proportions of intersex and undifferentiated can also change. The NOEC/LOEC data in Table 13 are based on the most sensitive change in each experiment and this is the cause of the different statements as “more females”, “more intersex” or “less males”.

Genetic sex determination

The determination of the genetic sex of the individual fish is currently possible for Japanese medaka and three spined stickleback. It improves the power of the test because it can identify each single fish that changes phenotypic sex in contrast to gonadal sexing alone where there is a need of a significant skewing of a proportion of sex before phenotypic sex reversal can be addressed. Beside, genetic sex determination clarifies the difference in vitellogenin concentrations between genetic males and sex reversed females and potentially also between genetic females and sex reversed males.

Guidance on the determination of genetic sex in Japanese medaka has been included in the FSDT draft proposal as an appendix.

Performance of endpoints

Hatching and survival

A significant decline in the hatching rate was seen in the experiment with 4-tert-octylphenol and zebrafish in LAB 4 at 298 µg/l. The survival was affected at both 91.5 and 298 µg/l. In LAB 8, 4-tert-octylphenol caused a significant reduction in both the hatching rate and the survival in stickleback at 131 µg/l. at 489 µg/l no surviving larvae were observed. A decline in hatching was also seen in stickleback at 66.9 µg/l in LAB 6. It seems as sticklebacks and zebrafish are slightly more sensitive to 4-tert-octylphenol than Japanese medaka where actual concentrations between 89.6 and 105 µg/l did affect neither hatching nor survival in the three experiments (LAB 4, 5 and 9). The results on medaka are in line with Seki et al., [2003] who found no effect on hatch or survival of 94 µg/l 4-tert-octylphenol.

Vitellogenin:

Vitellogenin was measured in head/tail homogenate or plasma in zebrafish, in head/tail homogenate in stickleback and in liver homogenate or plasma in medaka. Table 12 presents vitellogenin NOEC/LOEC values from all laboratories.

In all experiments with 4-tert-octylphenol, NOECs were equal to or below 41.9 µg/l with the lowest values of 6.2 µg/l for medaka (comparable to a NOEC of 6.9 µg/l in Seki et al. ([2003]), 9.5 µg/l for zebrafish and 22.2 µg/l for stickleback. The mean NOEC from all these experiments was 19.4 µg/l with a standard deviation of 12.1 µg/l. These data are very consistent seen in the context of 8 different experiments with three different species. It is important to underline that the VTG data should not be validated alone but in combination with the sex ratio data.

The NOECs from the experiments with DHT varied from 739 ng/l in stickleback to 20.8 ng/l in zebrafish. Lab 2 was not included in the calculations due to low test concentrations. The mean NOEC from the 6 experiments was 194 ng/l with a standard deviation of 274 ng/l. The high variability was partly caused by large inter species differences and partly by the fact that the female phenotype disappeared in several of the experiments at medium or high exposure concentrations which make a significant decline in VTG difficult to obtain.

Two experiments were performed with medaka and 4-tert-pentylphenol. The NOECs were 31.5 µg/l and 27.0 µg/l respectively, which give a mean of 29.3 µg/l and a standard deviation of 3.2 µg/l. These data are comparable to the validation Phase 1 results of 32 µg/l in zebrafish and 36 µg/l in fathead minnow.

E2 and flutamide was tested in stickleback only but the E2 NOEC of 36.8 ng/l is comparable to the results in zebrafish from a published studies [Holbech et al. 2006].

One negative study with ammonia was performed on zebrafish. No effect was observed on female VTG but a non dose related effect was seen on male VTG with a significant induction in the medium exposure concentration (3.88 mg/l). No effect in the highest exposure concentration was seen and these variable results are probably seen because to few a number of fish were analysed for VTG (<20 for each sex). A negative study with n-octanol was performed on medaka. No effect on male VTG was observed whereas a slight but significant reduction in female VTG was seen at the highest exposure concentration. A reduction in VTG can be caused by a toxic effect on the liver of the fish at his concentration (3.20 mg/l).

Sex ratio

The sexing of fish was performed by histological evaluation of the gonads. Fish were identified asfemales, males, intersex or undifferentiated. The statistics were calculated on basis of the proportions of sex and replicates were taken into account to include between replicate variations.

The six 4-tert-octylphenol experiments with zebrafish and medaka all produced NOECs from 23.5 µg/l or below and LOEC´s from 50.4 µg/l or below (Table 13). The mean NOEC was 12.6 µg/l with a standard deviation of 6.0 µg/l and the mean LOEC was 24.9 µg/l with a standard deviation of 14.5 µg/l. These results are uniform and it can be concluded that zebrafish and medaka are equally sensitive to this weak estrogen and different laboratory populations of the two species perform equally. The medaka results are also comparable to published results with a NOEC/LOEC of 23.7/48.1 µg/l 4-tert-octylphenol [Seki et al. 2003].

The results from the two experiments with stickleback suggest that phenotypic sex reversal in this species is less sensitive to 4-tert-octylphenol than in zebrafish and Japanese medaka because no LOEC was found. A higher systemic toxicity of 4-tert octylphenol to stickleback than to zebrafish and medaka could also be a part of the explanation of lack of effect.

Five experiments were including zebrafish and medaka to DHT exposure. The Lab 2 experiment failed due to a maximum measured test concentration of 8.7 ng/l (nominal 1000 ng/l) and the results from this test can only be used as a "negative" study showing background sex ratio in zebrafish. The remaining four studies had NOECs below 94.4 ng/l for medaka and 60.0 ng/l for zebrafish and LOEC´s from 94.4 ng/l and below for medaka and 60.0 ng/l and below for zebrafish. The mean NOEC was 56.4 ng/l with a standard deviation of 30.2 ng/l and the mean LOEC was 56.5 ng/l with a standard deviation of 30.1 ng/l. These results are also very uniform and it can be concluded that medaka and zebrafish are equally sensitive to the androgen DHT.

Two experiments were including stickleback to DHT exposure. with Lab 6 finding a NOEC of 739 ng/l and no LOEC and Lab 8 finding a NOEC of 202 ng/l and a LOEC of 329 ng/l expressed as intersex fish. The results indicate that in stickleback it is more difficult to reverse the phenotypic sex compared to medaka and zebrafish.

Two studies were performed on medaka with 4-tert-pentylphenol exposure. NOECs were 27.0 µg/l and 104.2 µg/l which can be compared to the NOEC´s of 32 µg/l, 34 µg/l and 36 µg/l in the three valid studies on zebrafish and fathead minnow in the phase 1 validation.

A NOEC of 98.6 ng/l and a LOEC of 390 ng/l (more intersex fish and 13% genetic males changed to phenotypic females) was found in the study on E2 from Lab 6, whereas no LOEC was found in the study on flutamide in stickleback from Lab 6. The NOEC of 98.6 ng/l in the E2 study is higher than published studies on zebrafish and Japanese medaka where NOEC was below 50 ng E2/l, e.g. 24 ng/l [Holbech et al. 2006] and below 33.5 ng/l [Hirai et al. 2006]. Again, it is evident that at least in the populations of stickleback used for the validation work it is difficult to chemically skew in regard to sex ratio. It should be noted that for both experiments (E2 and flutamide) the exposure was terminated at 42 dph, which explain why all undifferentiated fish were genetic males. These two studies were part of investigations aiming to establish if the FSDT could be of shorter duration when using the stickleback based on previous information that sexual differentiation in this species is completed by 42dph [Hahlbeck et al. 2004a]

Two negative studies were performed: One with ammonium in zebrafish and one with n-octanol in Japanese medaka. No effect on sex ratios was seen in the experiments.

Kidney epithelium height (KEH)

The measurement of the height of the stickleback kidney epithelium is a qualitative measurement of the androgen related spiggin induction that should respond to androgens by an increase. It is not a mandatory endpoint in the FSDT. The KEH was analysed in the four experiments conducted in Lab 6: In the experiment with 4-tert octylphenol an increase in KEH was observed for all exposure concentrations. This effect cannot be explained by an androgen mode of action but might indicate some kind of toxic effect of 4-tert octylphenol on the kidneys. In the DHT experiment, an increase was observed at the highest exposure concentration of 739 ng/l. This would be expected to be an androgenic induction. An increase in KEH was also observed after E2 exposure at all concentrations in both males and females. Again this effect cannot be explained by an androgenic effect. No change in KEH was observed after exposure to the anti-androgenic flutamide.

Summary of Phase-1 and Phase-2 validation results

The present validation work is a good example of the importance of analysing the chemical concentrations in the water in aquatic exposure systems regularly. Several of the participating laboratories had difficulties with the acceptability criteria of a maximum deviation of ± 20% of the mean measured concentration. This is not a FSDT specific problem and it should be seen in the context of the diversity of the participating laboratories. Many are university laboratories not used to standardised test protocols. The use of contract- and industrial laboratories would for sure eliminate many of these problems.

The two core endocrine endpoints in the FSDT are sex ratio and vitellogenin concentrations. As described earlier they should be validated in combination because this can give a picture of the MOA of the test substance. It is therefore important that the two endpoints have approximately the same sensitivity to the exposure chemicals. The sensitivity of the endpoints is indeed comparable, which can be seen from Table 12 and 13. For example the mean VTG NOEC from three zebrafish 4-tert-octylphenol experiments is 13.6 µg/l and the corresponding mean sex ratio NOEC is 9.7 µg/l. The corresponding NOECs for medaka are 16.7 µg/l and 15.6 µg/l. For some modes of action as for example strong androgens the skewing of sex toward males is so strong the female VTG measurement is based on very few individuals and therefore not always useful. But in these cases the sex ratio response is sufficient for a risk or hazard assessment of the substance because a “no female” population is achieved.

The validation of zebrafish, Japanese medaka and fathead minnow has identified equally sensitivity to the test substances regardless the strain or population used. A test with an androgen is needed in fathead minnow to complete the validation of this species. The three-spined stickleback showed comparable sensitivity to the zebrafish and the medaka by means of effects on VTG concentrations. However, it was proved less sensitive to phenotypic sex reversal caused by the present exposure chemicals compared to zebrafish and Japanese medaka. In the majority of cases the chemicals did have an effect on stickleback sexual differentiation but this was more by means of inducing a low incidence of intersex rather than causing a dramatic effect in the sex ratio. The reason might be a stronger genetic sex determination mechanism in comparison to the other test species.

APPENDIX

Figures from the FSDT Phase 2 validation

Schematic overview

Table 33: Overview of the Annex 1 figures

Exposure chemical	endpoint	species	LAB	Figure
4-tert-octylphenol	vitellogenin	Zebrafish	1	Figure 3
4-tert-octylphenol	vitellogenin	Zebrafish	2	Figure 4
4-tert-octylphenol	vitellogenin	Zebrafish	4	Figure 5
4-tert-octylphenol	vitellogenin	Japanese medaka	4	Figure 6
4-tert-octylphenol	vitellogenin	Japanese medaka	5	Figure 7
4-tert-octylphenol	vitellogenin	Japanese medaka	9	Figure 8
4-tert-octylphenol	vitellogenin	Three spined stickleback	6	Figure 9
4-tert-octylphenol	vitellogenin	Three spined stickleback	8	Figure 10
Dihydrotestosterone	vitellogenin	Zebrafish	1	Figure 11
Dihydrotestosterone	vitellogenin	Zebrafish	2	Figure 12
Dihydrotestosterone	vitellogenin	Zebrafish	3	Figure 13
Dihydrotestosterone	vitellogenin	Japanese medaka	5	Figure 14
Dihydrotestosterone	vitellogenin	Japanese medaka	9	Figure 15
Dihydrotestosterone	vitellogenin	Three spined stickleback	6	Figure 16
Dihydrotestosterone	vitellogenin	Three spined stickleback	8	Figure 17
4-tert-pentylphenol	vitellogenin	Japanese medaka	9	Figure 18
4-tert-pentylphenol	vitellogenin	Japanese medaka	10	Figure 19
17ß-estradiol	vitellogenin	Three spined stickleback	6	Figure 20
flutamide	vitellogenin	Three spined stickleback	6	Figure 21

ammonium	vitellogenin	zebrafish	1	Figure 22
n-octanol	vitellogenin	Japanese medaka	9	Figure 23
4-tert-octylphenol	sex ratio	Zebrafish	1	Figure 24
4-tert-octylphenol	sex ratio	Zebrafish	2	Figure 25
4-tert-octylphenol	sex ratio	Zebrafish	4	Figure 26
4-tert-octylphenol	sex ratio	Japanese medaka	4	Figure 27
4-tert-octylphenol	sex ratio	Japanese medaka	5	Figure 28
4-tert-octylphenol	sex ratio	Japanese medaka	9	Figure 29
4-tert-octylphenol	sex ratio	Three spined stickleback	6	Figure 30
4-tert-octylphenol	sex ratio	Three spined stickleback	8	Figure 31
Dihydrotestosterone	sex ratio	Zebrafish	1	Figure 32
Dihydrotestosterone	sex ratio	Zebrafish	2	Figure 33
Dihydrotestosterone	sex ratio	Zebrafish	3	Figure 34
Dihydrotestosterone	sex ratio	Japanese medaka	5	Figure 35
Dihydrotestosterone	sex ratio	Japanese medaka	9	Figure 36
Dihydrotestosterone	sex ratio	Three spined stickleback	6	Figure 37
Dihydrotestosterone	sex ratio	Three spined stickleback	8	Figure 38
4-tert-pentylphenol	sex ratio	Japanese medaka	9	Figure 39
4-tert-pentylphenol	sex ratio	Japanese medaka	10	Figure 40
17ß-estradiol	sex ratio	Three spined stickleback	6	Figure 41
flutamide	sex ratio	Three spined stickleback	6	Figure 42
Ammonia	sex ratio	zebrafish	1	Figure 43
n-octanol	sex ratio	Japanese medaka	9	Figure 44
4-tert-octylphenol	KEH	Three spined stickleback	6	Figure 45
Dihydrotestosterone	KEH	Three spined stickleback	6	Figure 46

17ß-estradiol	KEH	Three spined stickleback	6	Figure 47
flutamide	KEH	Three spined stickleback	6	Figure 48

VITELLOGENIN

4-tert-octylphenol

Zebrafish

Lab 1

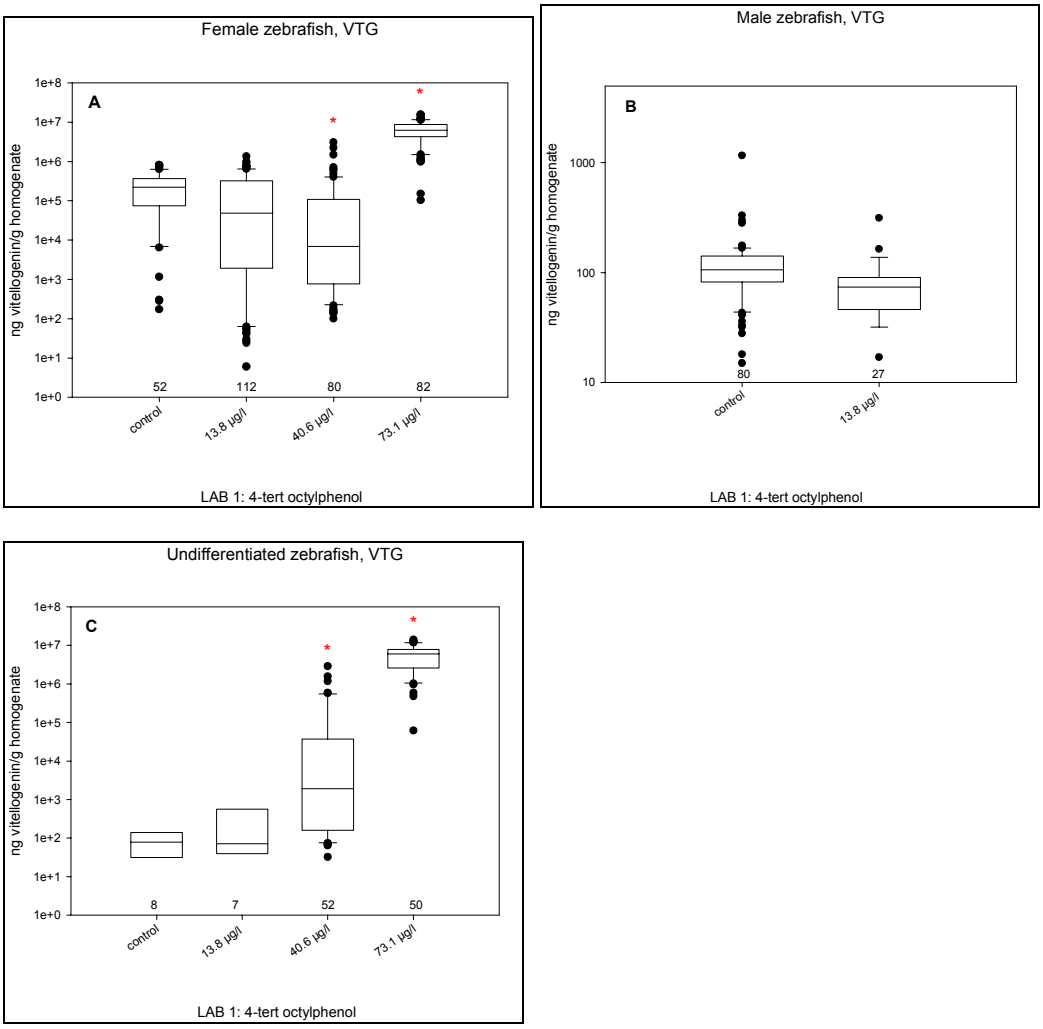


Figure 3: Vitellogenin concentrations in female (A), male (B) and undifferentiated (C) zebrafish after 60 D exposure to 4-tert-octylphenol. Numbers at the bottom are N. P=0.05. *=Significant different from control

Lab 2

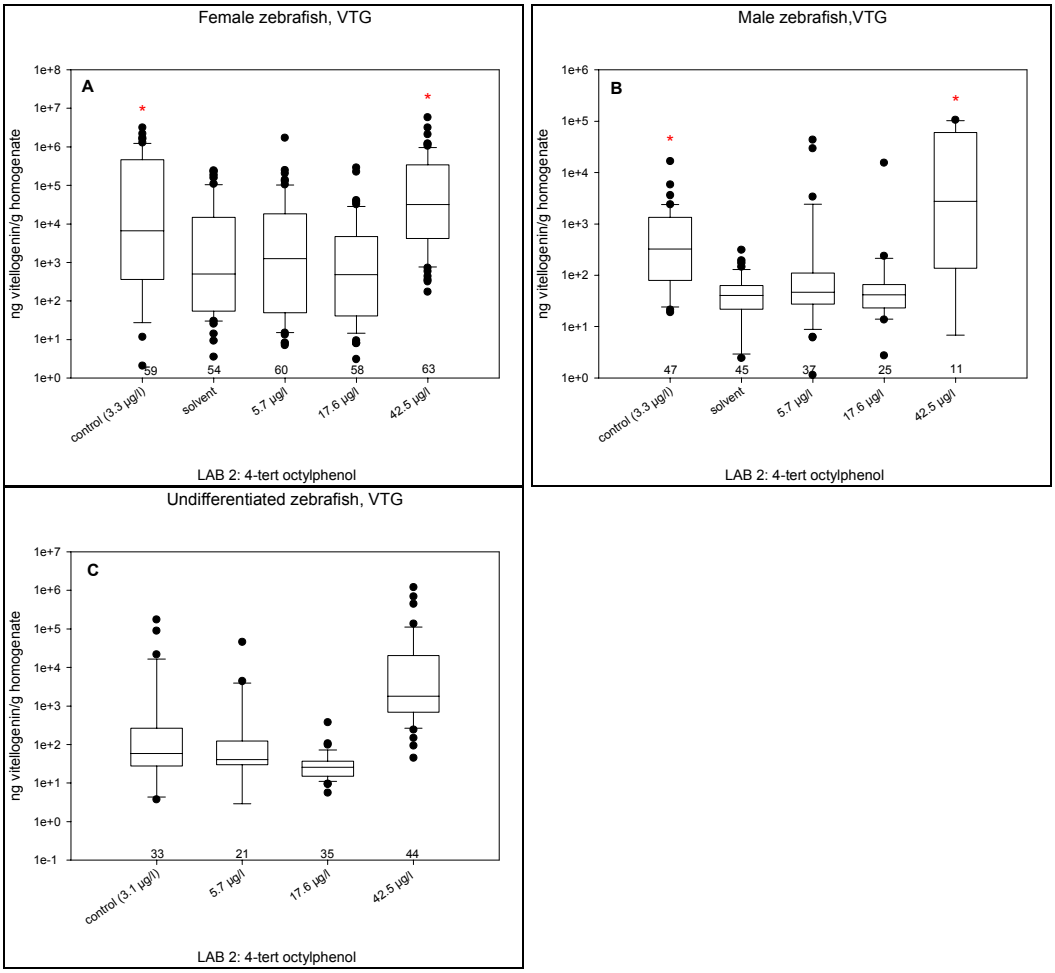
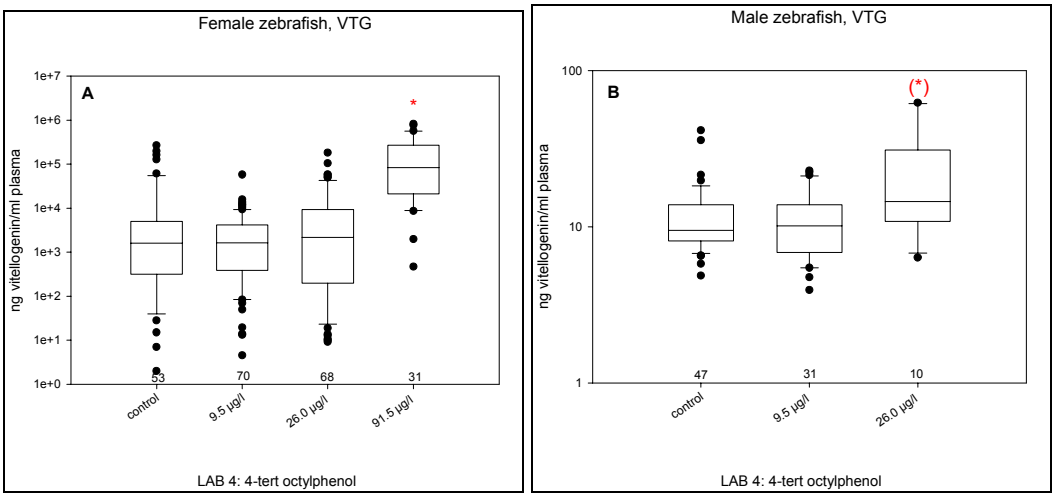


Figure 4: Vitellogenin concentrations in female (A), male (B) and undifferentiated (C) zebrafish after 60 D exposure to 4-tert-octylphenol. Numbers at the bottom are N. P=0.05. *=Significant different from control

Lab 4



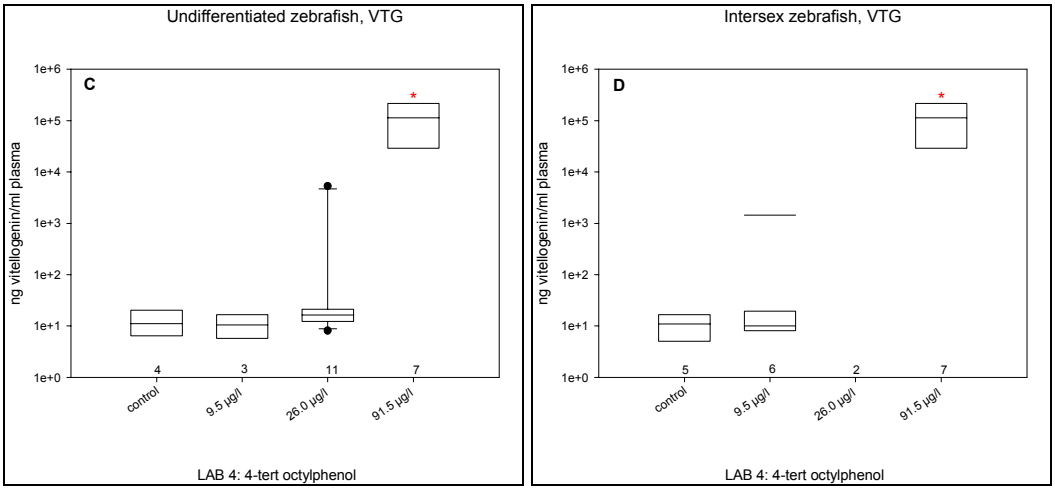


Figure 5: Vitellogenin concentrations in female (A), male (B), undifferentiated (C) and intersex (D) zebrafish after 60 D exposure to 4-tert-octylphenol. Numbers at the bottom are N. P=0.05. *=Significant different from control. (*)=Significant different from control using 1-sided hypothesis testing.

Japanese medaka

Lab 4

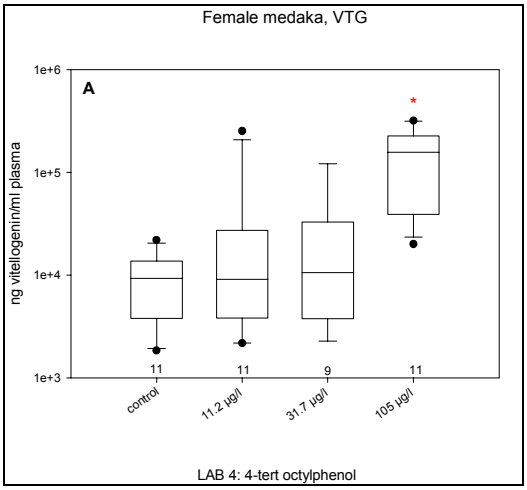


Figure 6: Vitellogenin concentrations in female (A) medaka after 60 D exposure to 4-tert-octylphenol. Numbers at the bottom are N. P=0.05 . *=Significant different from control

Lab 5

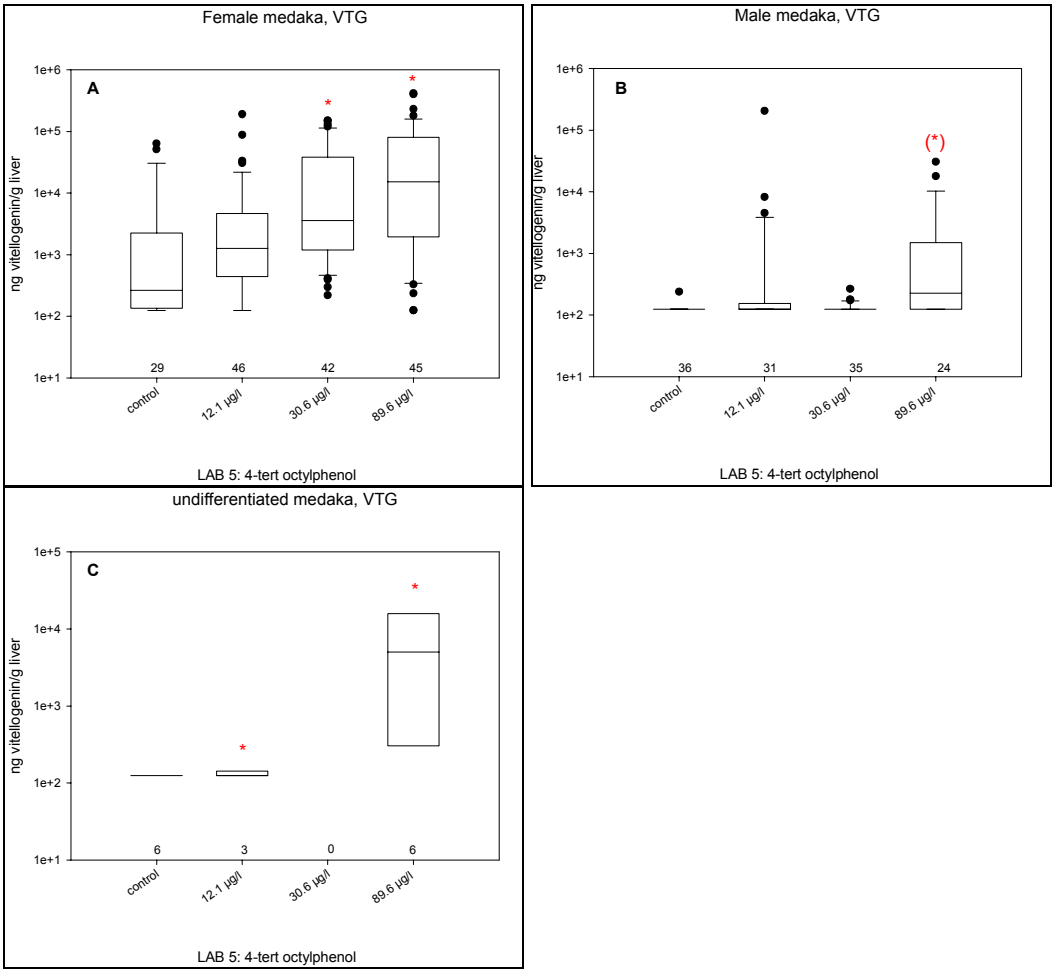
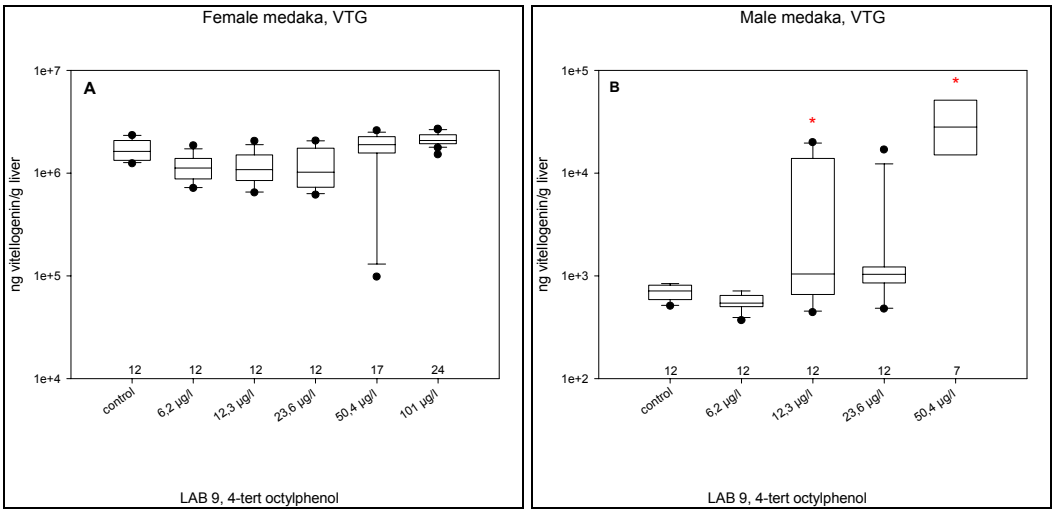


Figure 7: Vitellogenin concentrations in female (A) and male (B) medaka after 60 D exposure to 4-tert-octylphenol. Numbers at the bottom are N. P=0.05. *=Significant different from control. (*)=Significant different from control using 1-sided hypothesis testing.

Lab 9



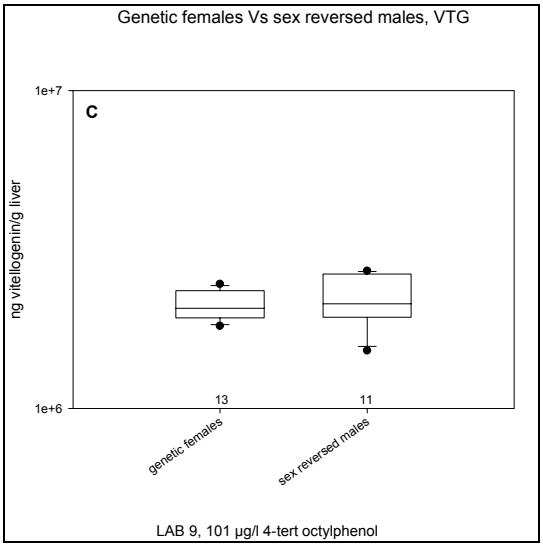


Figure 8: Vitellogenin concentrations in female (A), male (B) and genetic females versus sex reversed males (C) medaka after 60 D exposure to 4-tert-octylphenol. Numbers at the bottom are N. P=0.05. *=Significant different from control

Three spined stickleback

Lab 6

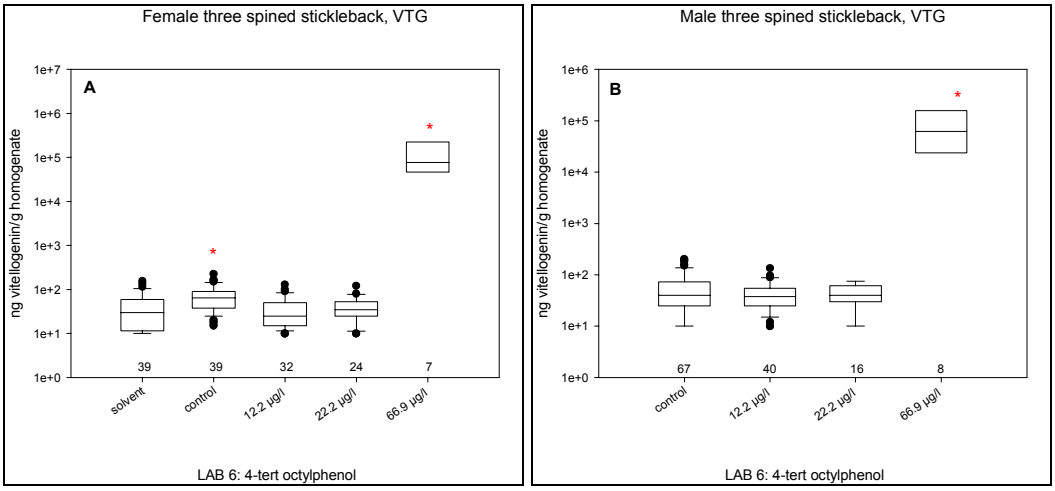


Figure 9: Vitellogenin concentrations in female (A) and male (B) stickleback after 60 D exposure to 4-tert-octylphenol. Numbers at the bottom are N. P=0.05. *=Significant different from control

Lab 8

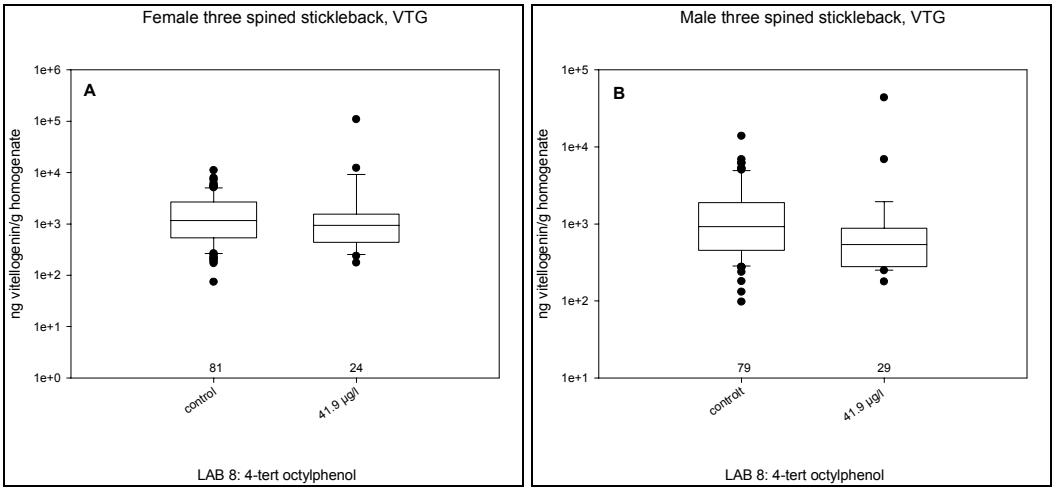


Figure 10: Vitellogenin concentrations in female (A) and male (B) stickleback after 60 D exposure to 4-tert-octylphenol. Numbers at the bottom are N. P=0.05. *=Significant different from control

Dihydrotestosterone

Zebrafish

Lab 1

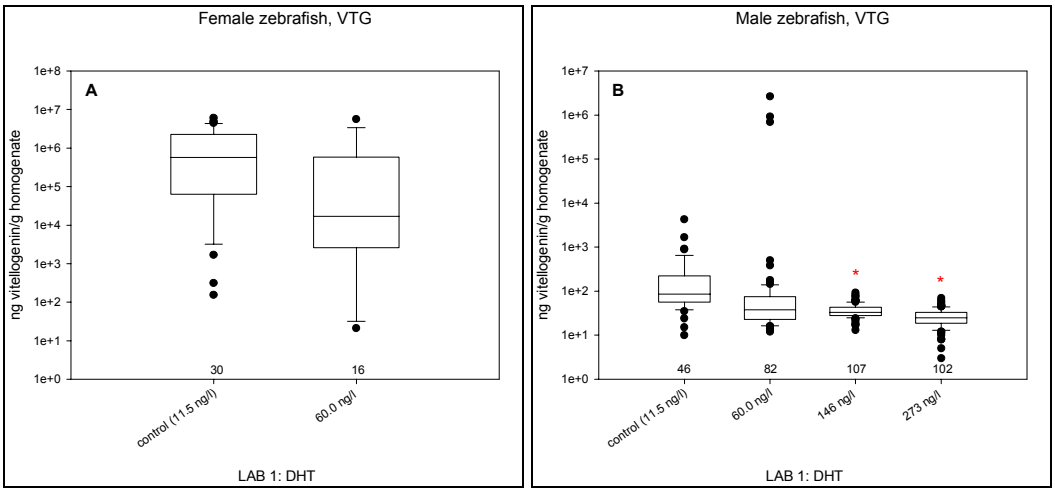


Figure 11: Vitellogenin concentrations in female (A) and male (B) zebrafish after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05. *=Significant different from control

Lab 2

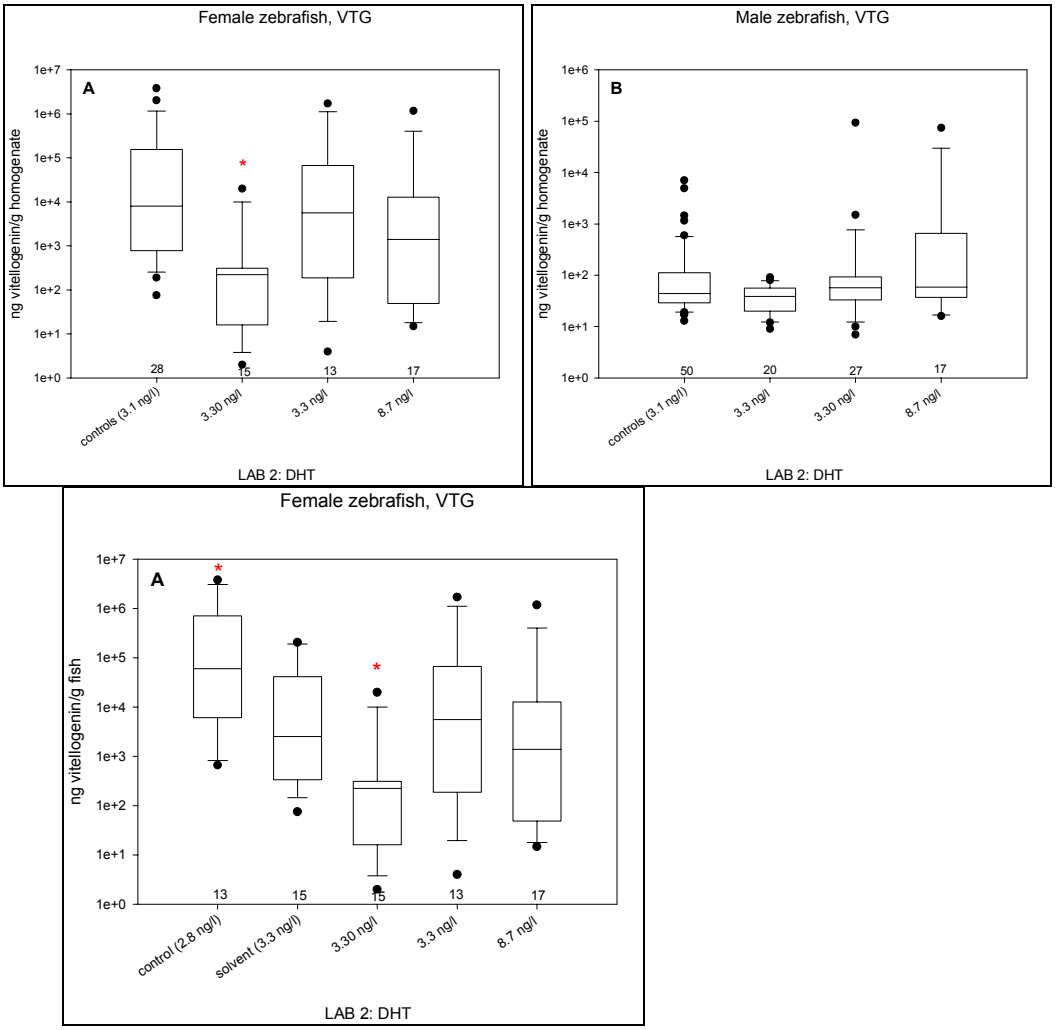


Figure 12: Vitellogenin concentrations in female (A) and male (B) zebrafish after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05. *=Significant different from control

Lab 3

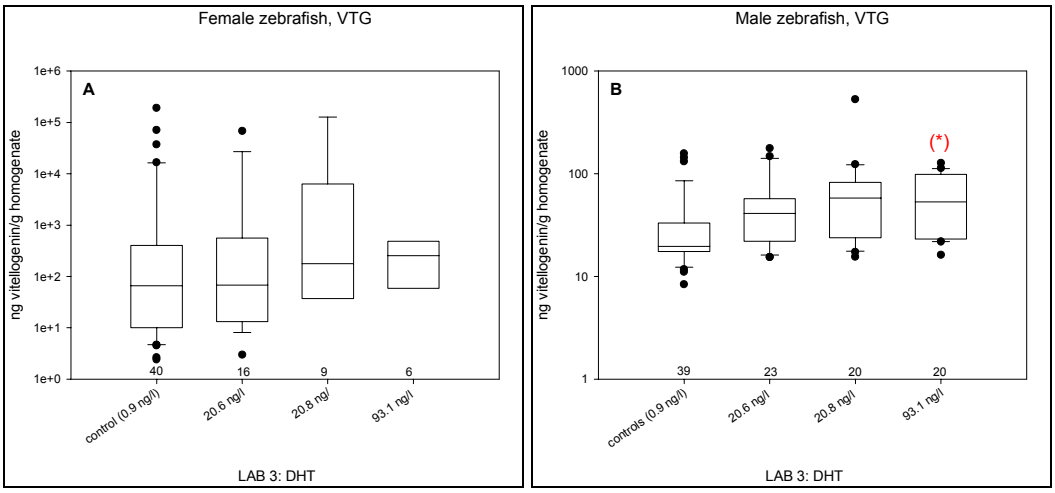


Figure 13: Vitellogenin concentrations in female (A) and male (B) zebrafish after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05. *=Significant different from control. (*)=Significant different from control using 1-sided hypothesis testing.

Japanese medaka

Lab 5

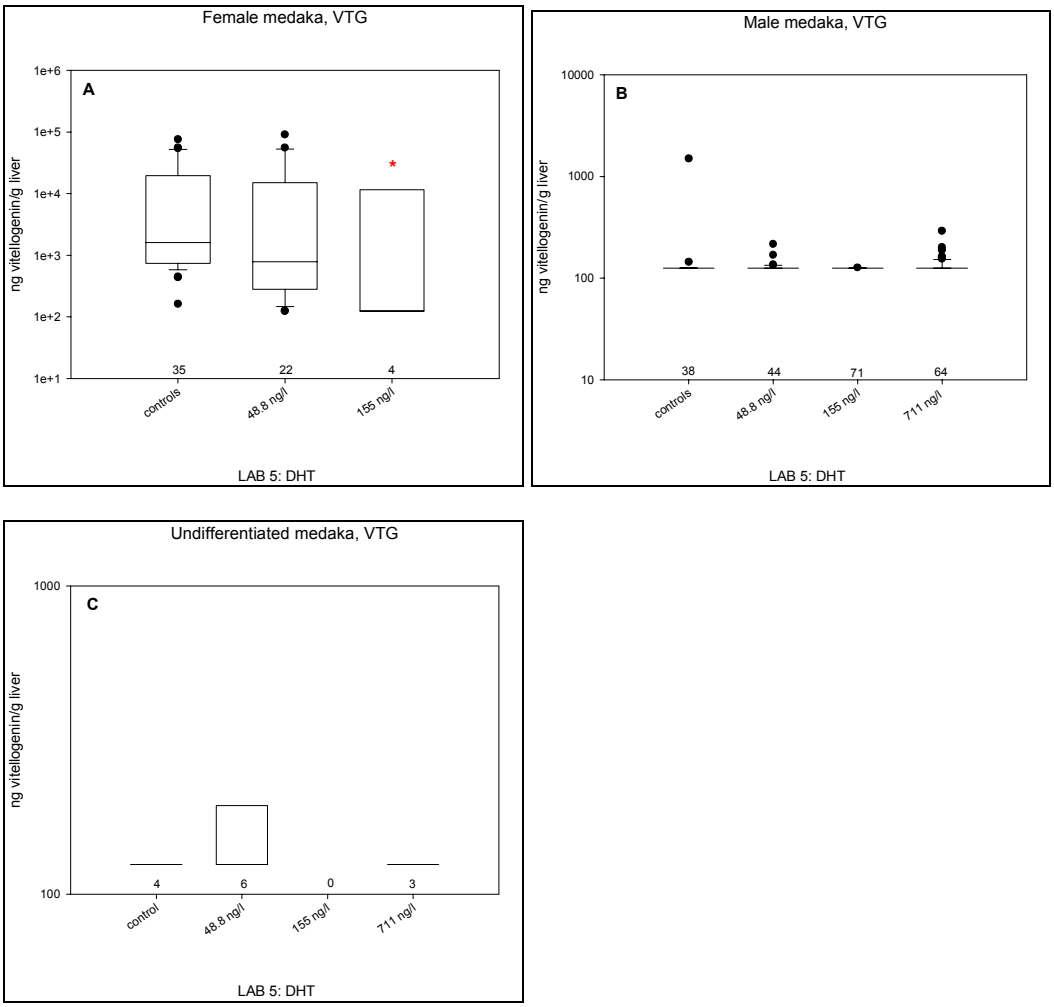


Figure 14: Vitellogenin concentrations in female (A) and male (B) medaka after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05. *=Significant different from control

Lab 9

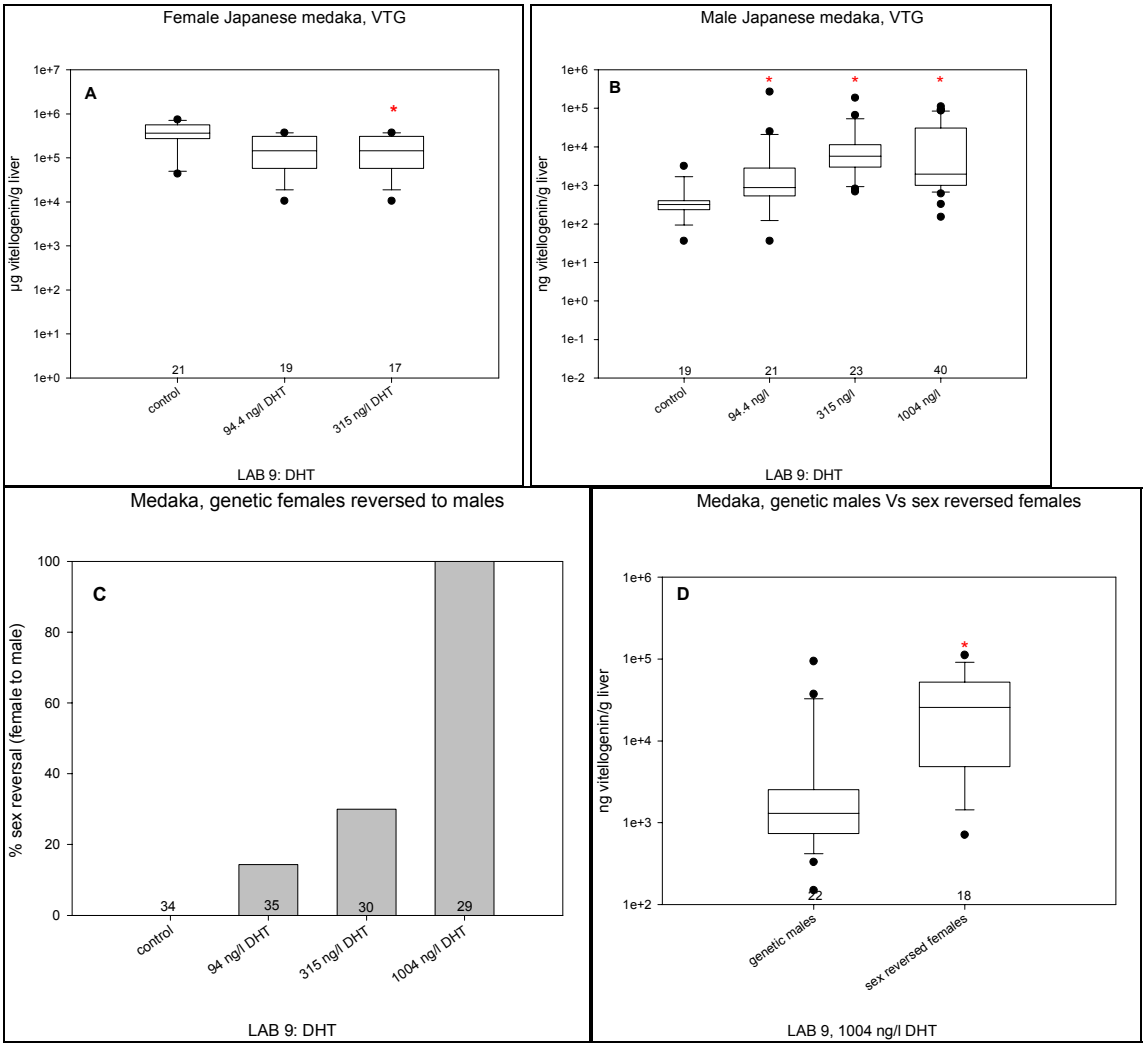


Figure 15: Vitellogenin concentrations in female (A) and male (B) medaka after 60 D exposure to dihydrotestosterone. Percentage sex reversal (C) and vitellogenin of sex reversed fish (D). Numbers at the bottom are N. P=0.05. *=Significant different from control

Three spined stickleback

Lab 6

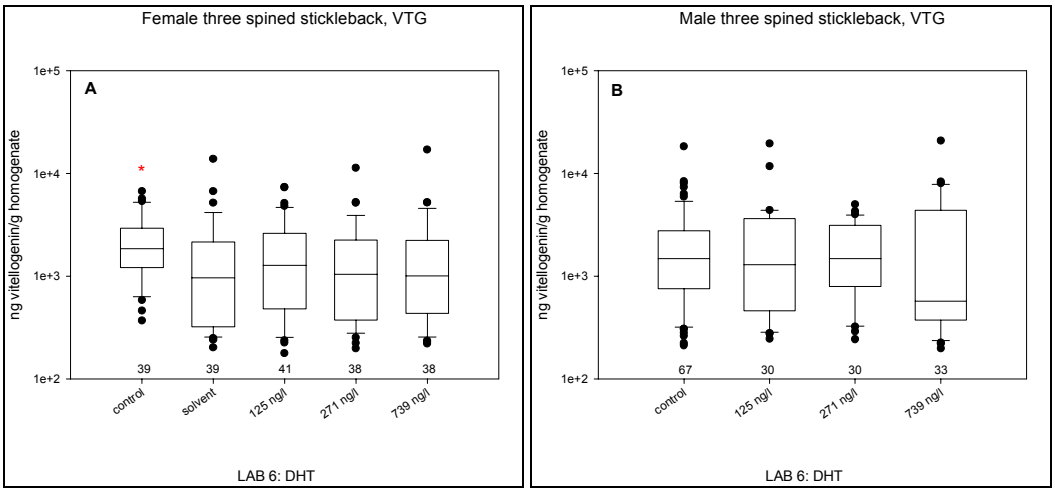


Figure 16: Vitellogenin concentrations in female (A) and male (B) stickleback after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05. *=Significant different from control

Lab 8

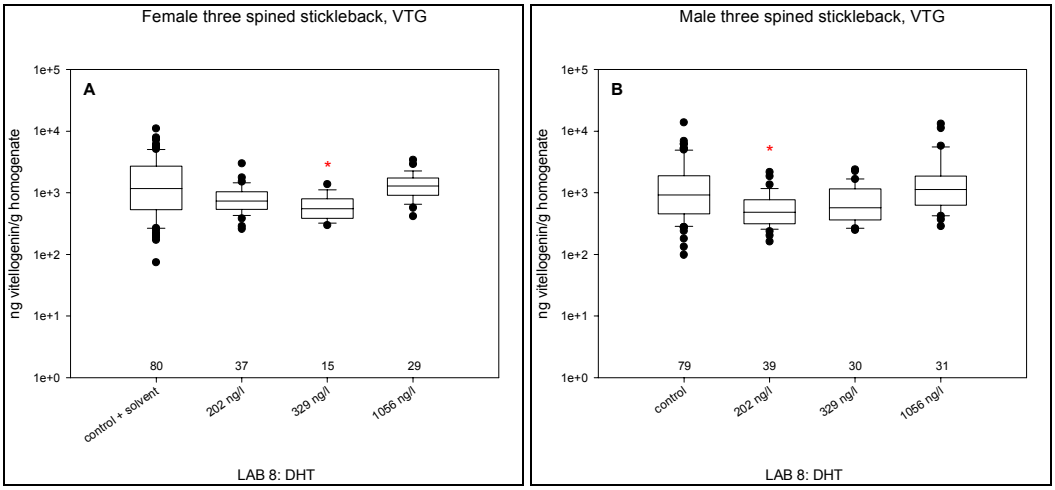


Figure 17: Vitellogenin concentrations in female (A) and male (B) stickleback after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05. *=Significant different from control

4-tert-pentylphenol

Japanese medaka

Lab 9

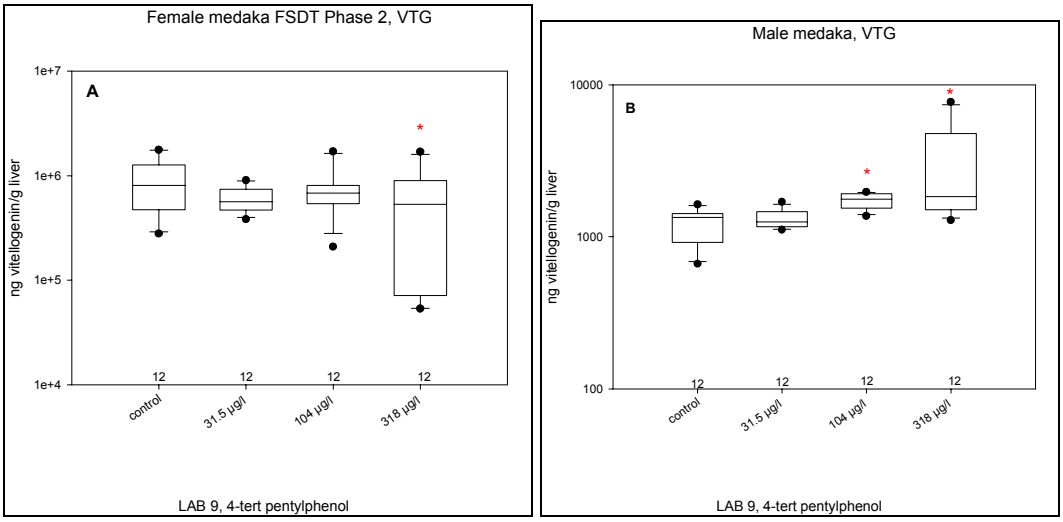


Figure 18: Vitellogenin concentrations in female (A) and male (B) medaka after 70 D exposure to 4-tert-pentylphenol. Numbers at the bottom are N. P=0.05. *=Significant different from control

Lab 10

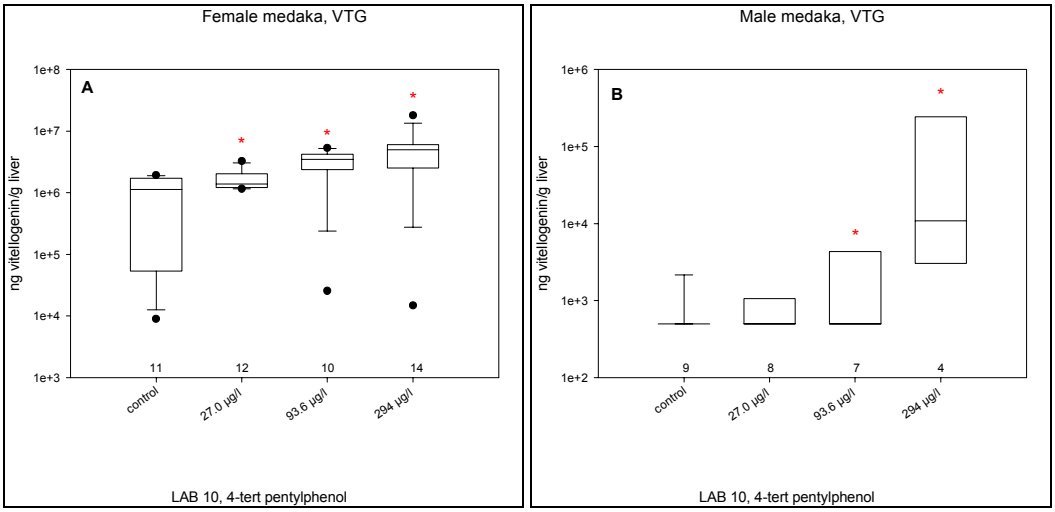


Figure 19: Vitellogenin concentrations in female (A) and male (B) medaka after 60 D exposure to 4-tert-pentylphenol. Numbers at the bottom are N. P=0.05. *=Significant different from control

17ß-estradiol

Three spined stickleback

Lab 6

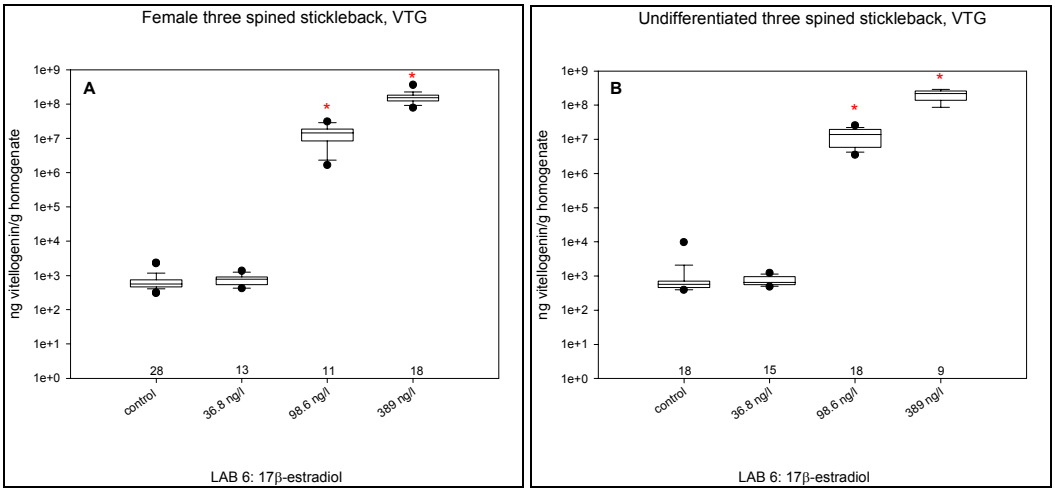


Figure 20: Vitellogenin concentrations in female (A) and undifferentiated (B) stickleback after 37 D exposure to 17ß-estradiol. Numbers at the bottom are N. P=0.05. *=Significant different from control

Flutamide

Three spined stickleback

Lab 6

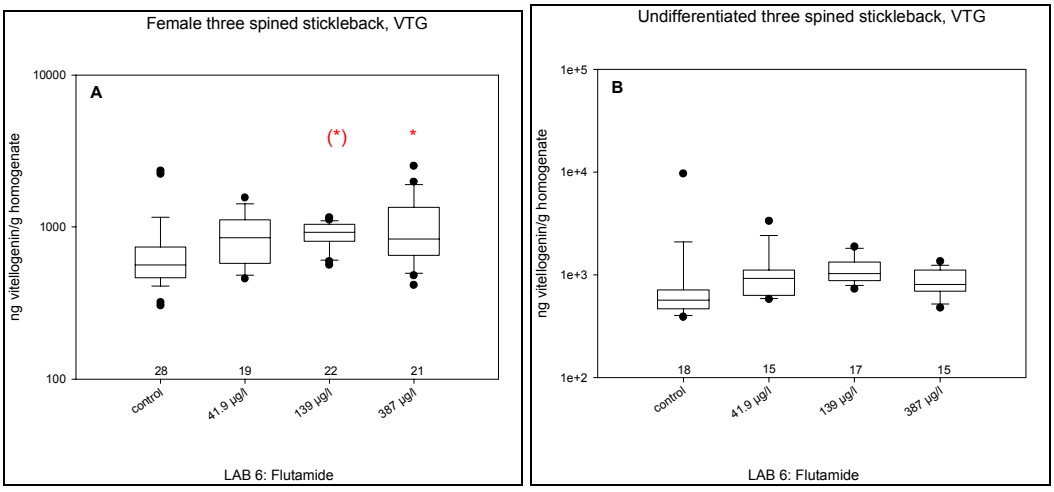


Figure 21: Vitellogenin concentrations in female (A) and undifferentiated (B) stickleback after 41 D exposure to flutamide. Numbers at the bottom are N. P=0.05. *=Significant different from control. (*)=Significant different from control using 1-sided hypothesis testing.

Ammonia

ZebrafishLab 1

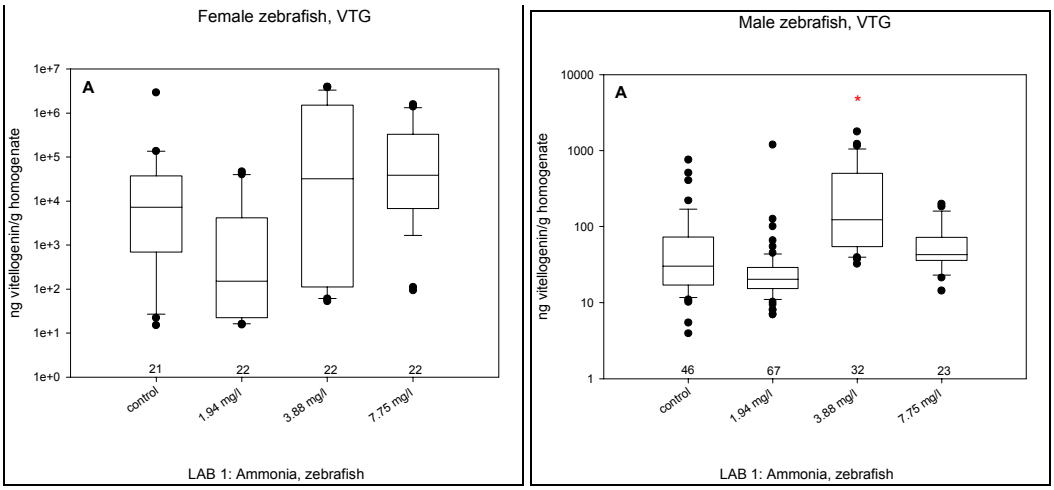


Figure 22: Vitellogenin concentrations in female (a) and male (B) zebrafish after 60 D exposure to ammonium. Numbers at the bottom are N. P=0.05. *=Significant different from control.

N-octanol

Japanese medaka

Lab 9

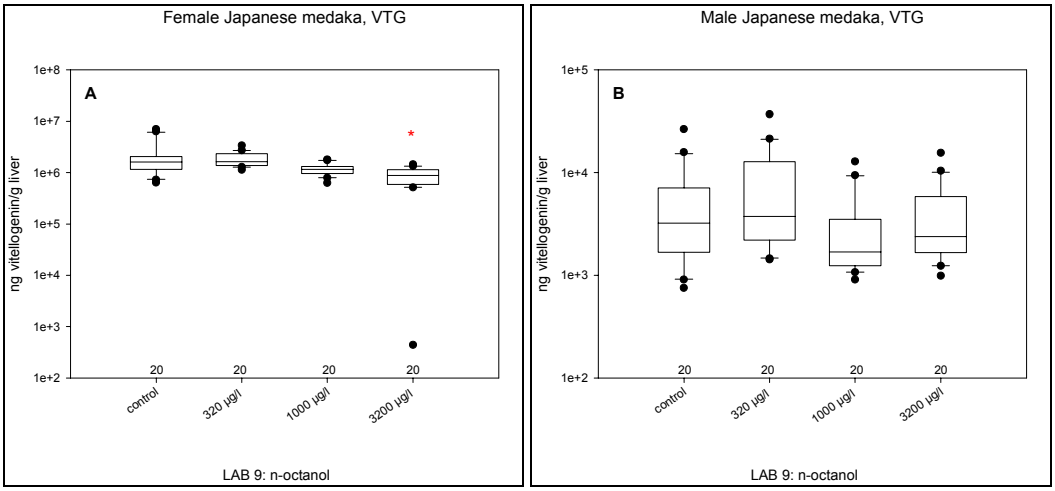


Figure 23: Vitellogenin concentrations in female (A) and male (B) Japanese medaka after 60 D exposure to n-octanol. Numbers at the bottom are N. P=0.05. *=Significant different from control.

SEX RATIO AND GENETIC SEX

4-tert-octylphenol

Zebrafish

Lab 1

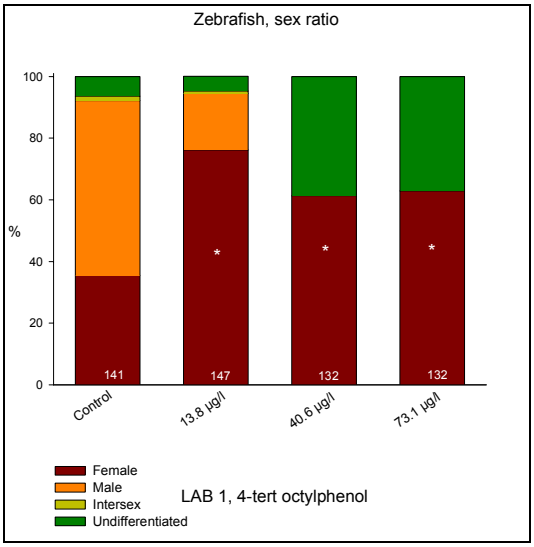


Figure 24: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to 4-tert-octylphenol. Numbers at bottom are N. P=0.05

Lab 2

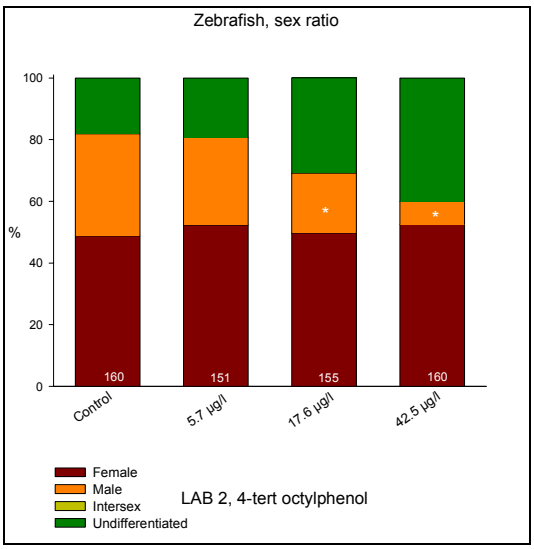


Figure 25: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to 4-tert-octylphenol. Numbers at bottom are N. P=0.05

Lab 4

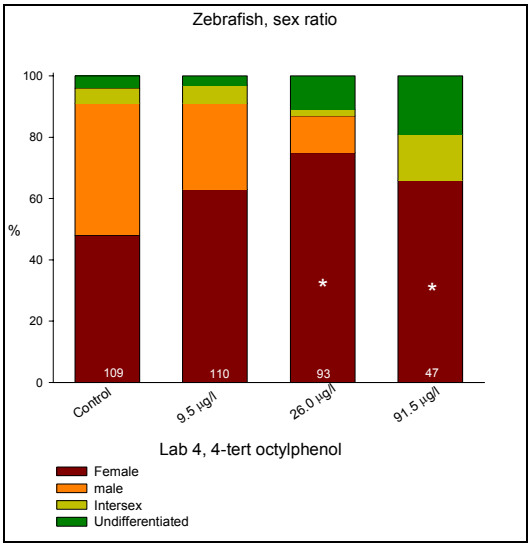


Figure 26: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to 4-tert-octylphenol. Numbers at bottom are N. P=0.05

Japanese medaka

Lab 4

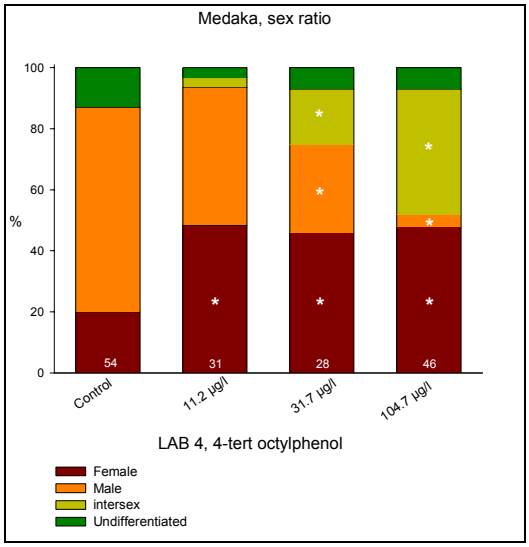


Figure 27: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to 4-tert-octylphenol. Numbers at bottom are N. P=0.05

Lab 5

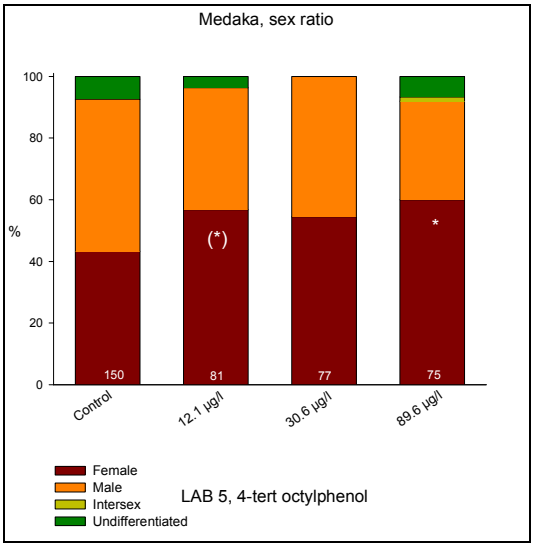


Figure 28: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to 4-tert-octylphenol. Numbers at bottom are N. P=0.05. (*)=one-sided test.

Lab 9

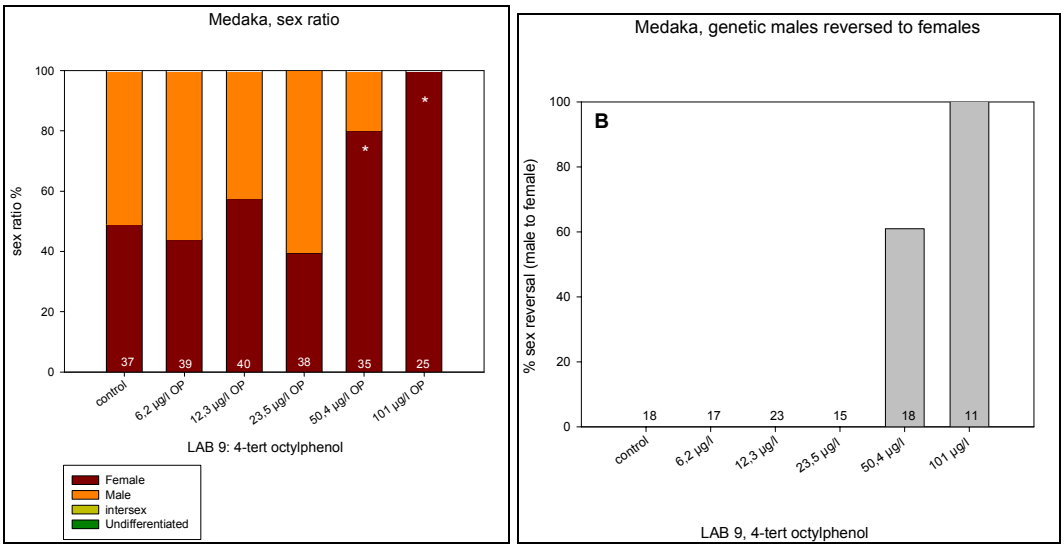


Figure 29: (A) Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to 4-tert-octylphenol. Numbers at bottom are N. P=0.05. (B) Percentage sex reversed males of total number of genetic males.

Three spined stickleback

Lab 6

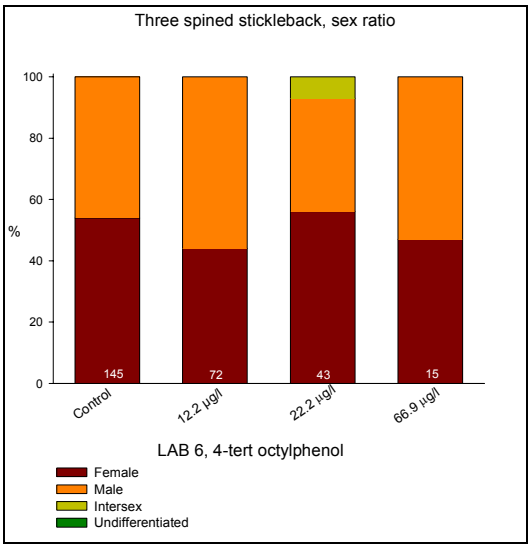


Figure 30: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to 4-tert-octylphenol. Numbers at bottom are N. P=0.05

Lab 8

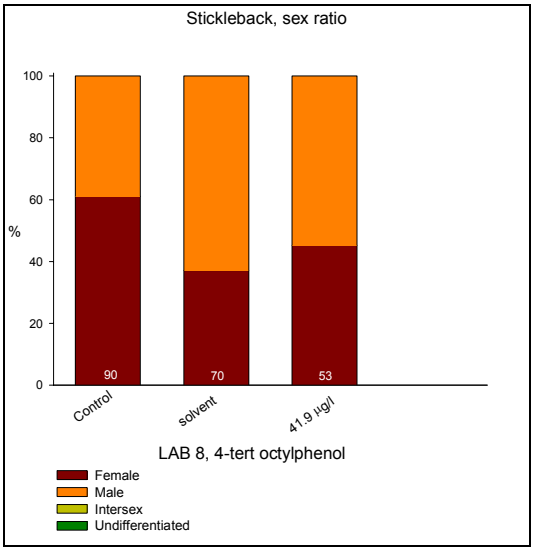


Figure 31: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to 4-tert-octylphenol. Numbers at bottom are N. P=0.05

Dihydrotestosterone

Zebrafish

Lab 1

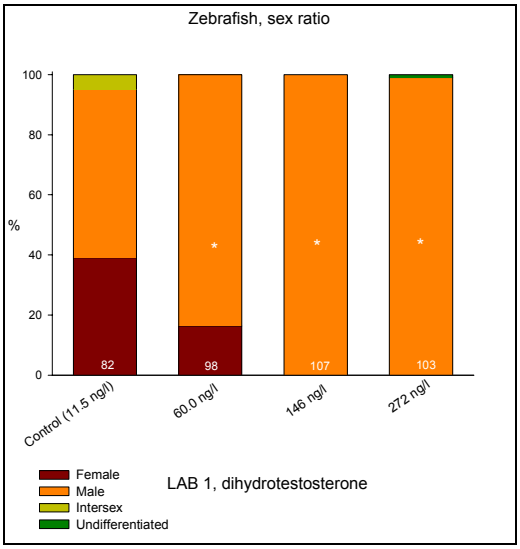


Figure 32: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Lab 2

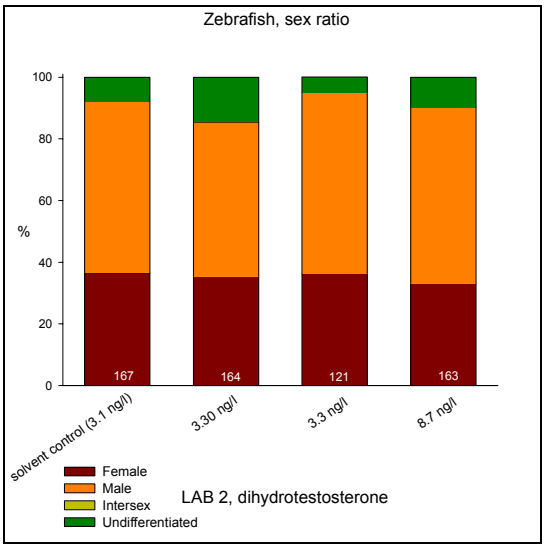


Figure 33: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Lab 3

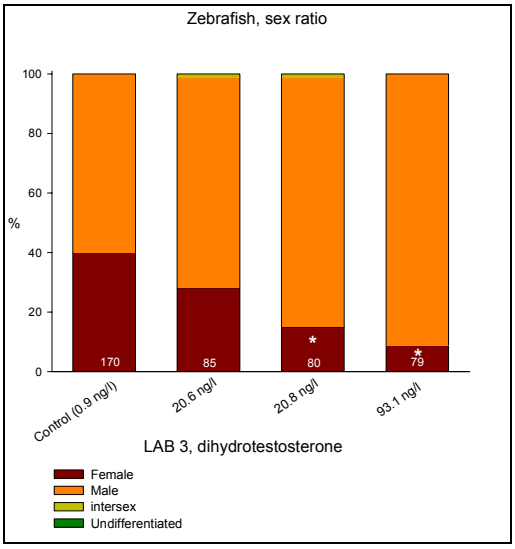


Figure 34: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Japanese medaka

Lab 5

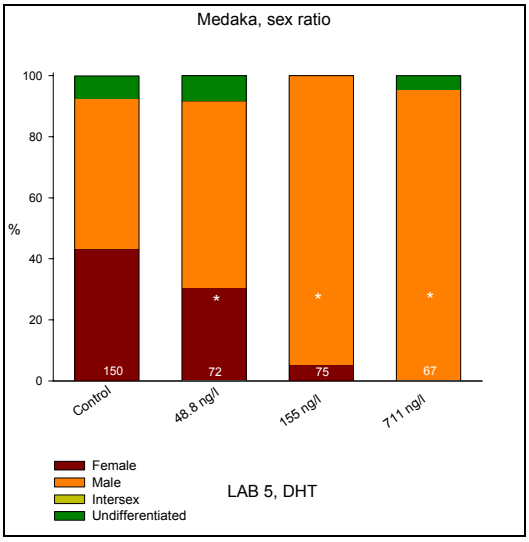


Figure 35: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Lab 9

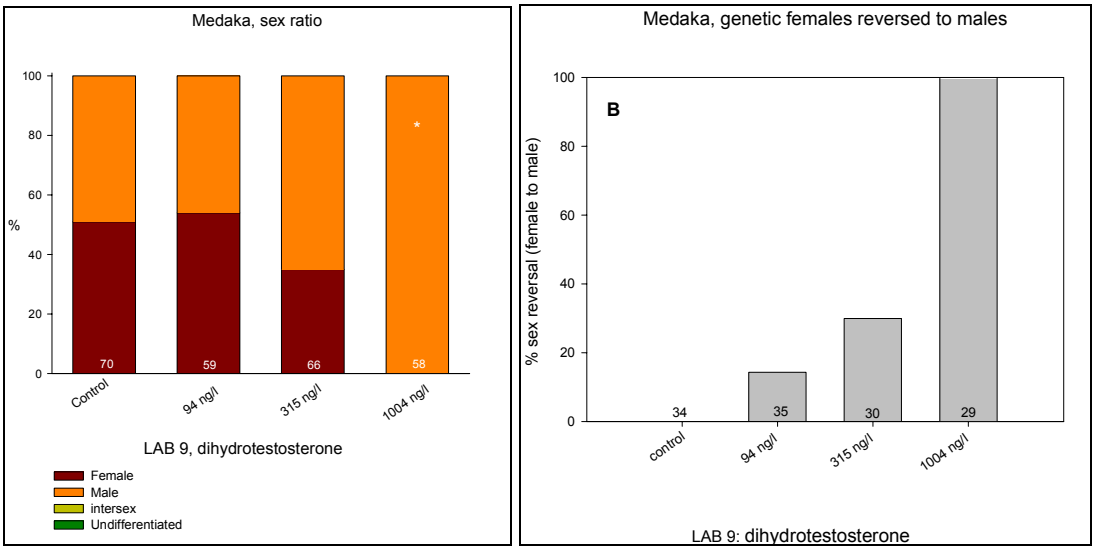


Figure 36: (A) Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05. (B) Percentage sex reversed females of total number of genetic females.

Three spined stickleback

Lab 6

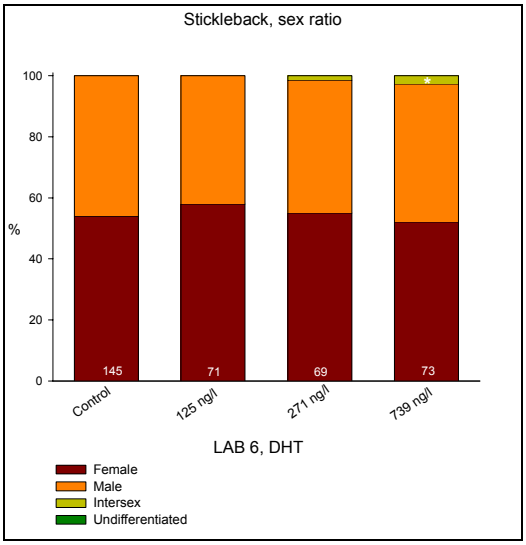


Figure 37: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Lab 8

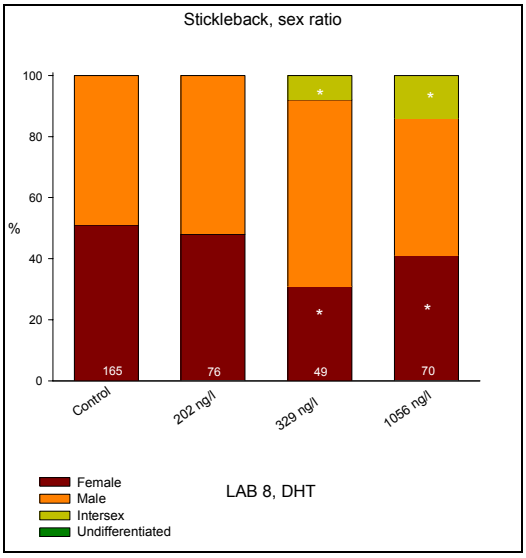


Figure 38: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

4-tert-pentylphenol

Japanese medaka

Lab 9

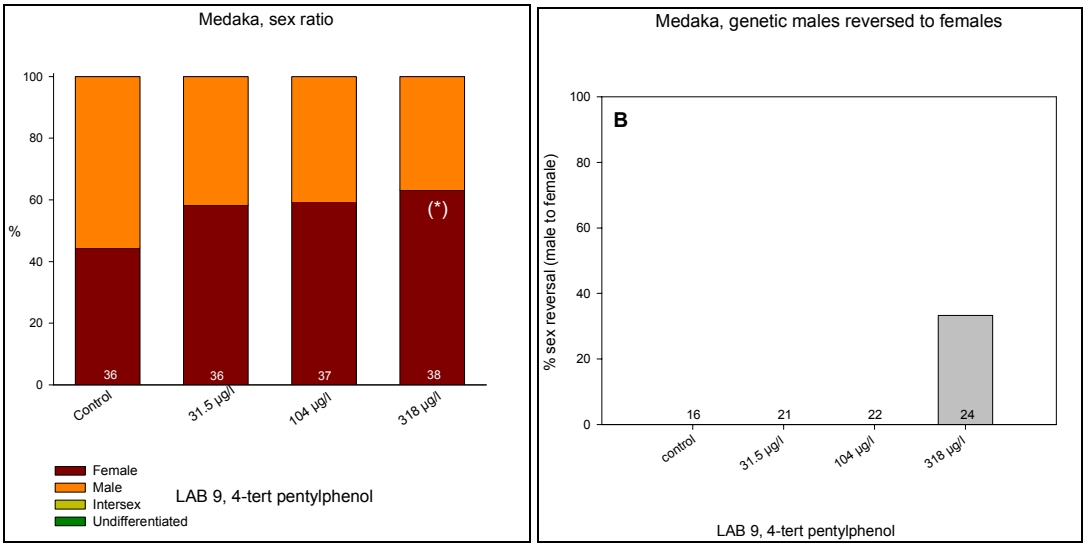


Figure 39: (A) Sex ratio as percentage females, males, undifferentiated and intersex medaka after 70 D exposure to 4-tert-pentylphenol. Numbers at bottom are N. Asterisk in brackets: Genetic males changed to phenotypic females. P=0.05. (B) Percentage sex reversed males of total number of genetic males.

Lab 10

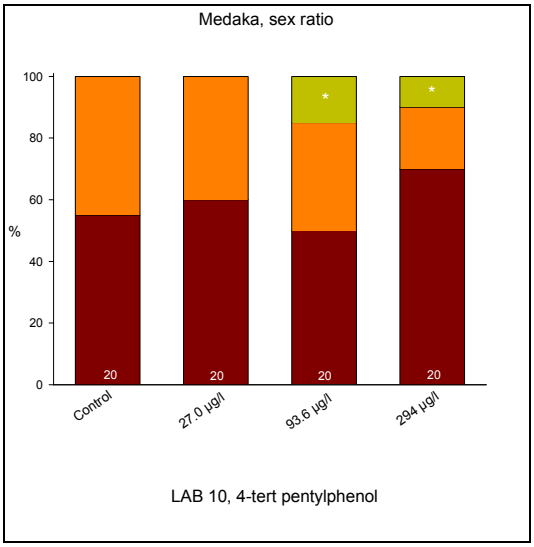


Figure 40: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to 4-tert-pentylphenol. Numbers at bottom are N. P=0.05

17ß-estradiol

Three spined stickleback

Lab 6

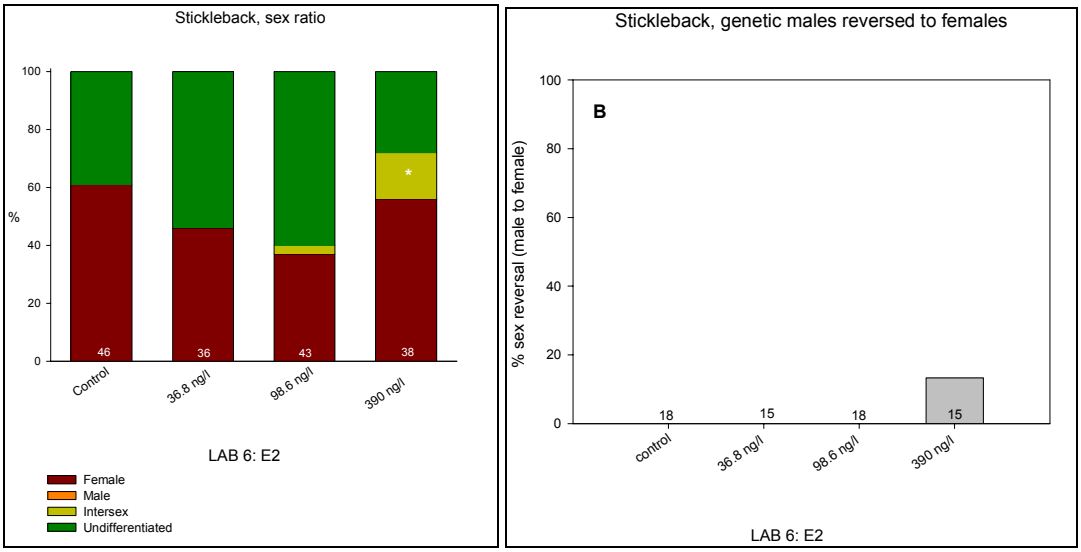


Figure 41: (A) Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 42 D exposure to 17ß-estradiol. Numbers at bottom are N. P=0.05. (B) Percentage sex reversed males of total number of genetic males.

Flutamide

Three spined stickleback

Lab 6

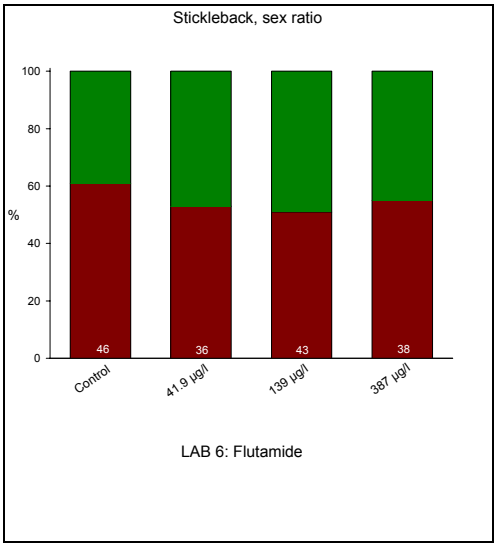


Figure 42: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 42 D exposure to flutamide. Numbers at bottom are N. P=0.05

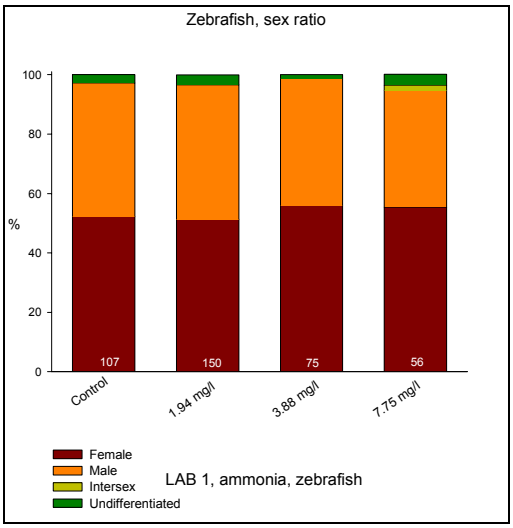


Figure 43: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to ammonium. Numbers at bottom are N. P=0.05

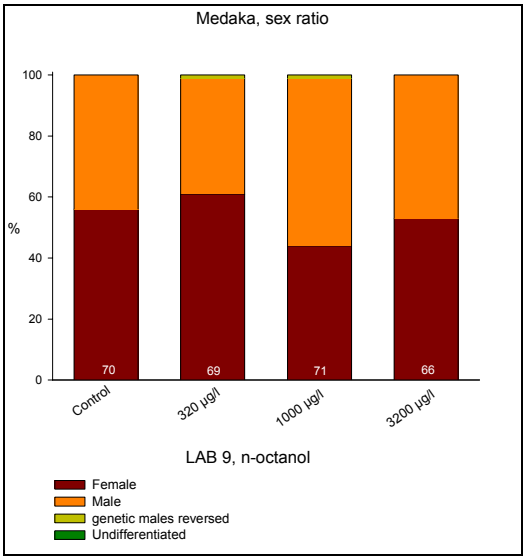


Figure 44: Sex ratio as percentage females, males and sex reversed males. Japanese medaka after 60 D exposure to n-octanol. Numbers at bottom are N. P=0.05

KIDNEY EPITHELIUM HEIGHT (KEH)

The measurement of the stickleback KEH is a qualitative measurement of the androgen related spiggin induction. It is not a mandatory endpoint in the FSDT.

4-tert-octylphenol

Three spined stickleback

Lab 6

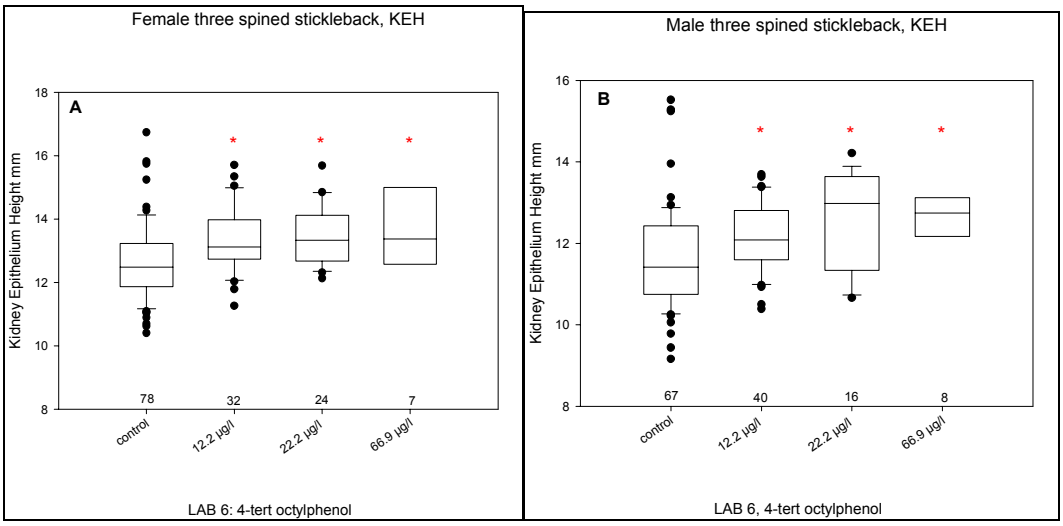


Figure 45: Kidney epithelium height (KEH) in mm after 60 D exposure to 4-tert-octylphenol of female (A) and male (B) stickleback. Numbers at bottom are N. P=0.05

Dihydrotestosterone

Three spined stickleback

Lab 6

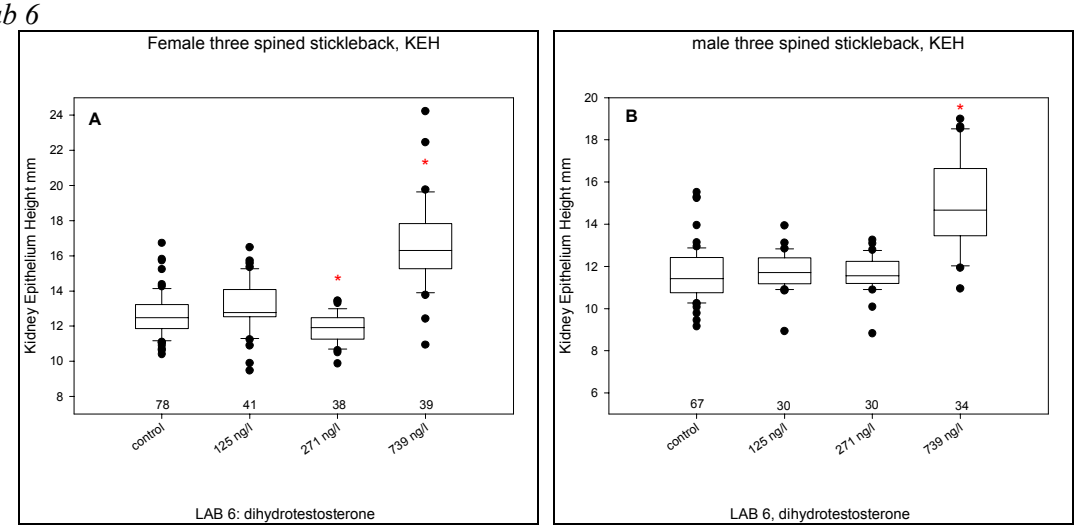


Figure 46: Kidney epithelium height (KEH) in mm after 60 D exposure to dihydrotestosterone of female (A) and male (B) stickleback. Numbers at bottom are N. P=0.05

17ß-estradiol

Three spined stickleback

Lab 6

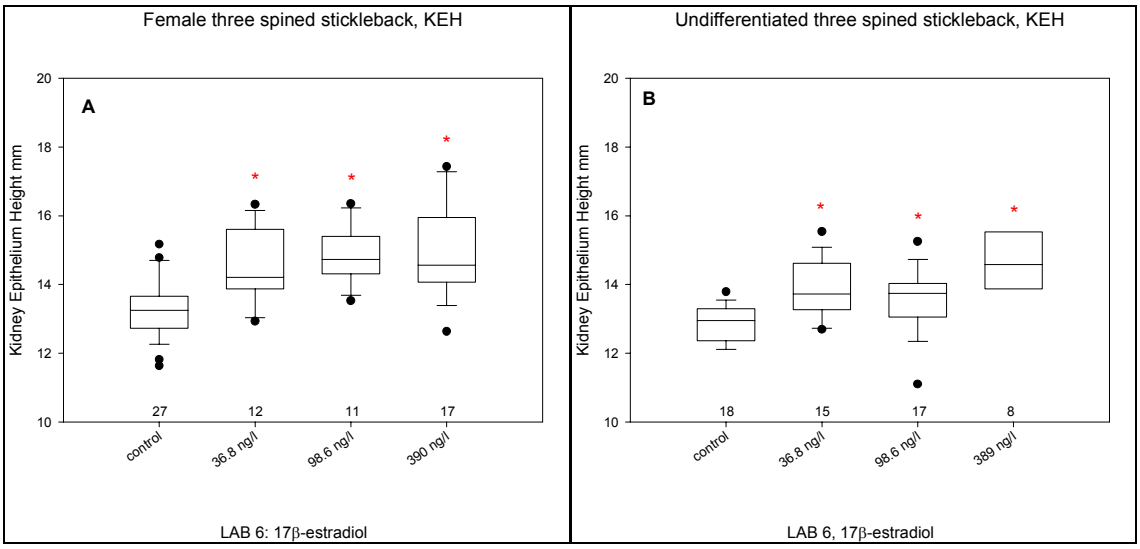


Figure 47: Kidney epithelium height (KEH) in mm after 42 D exposure to 17ß-estradiol of female (A) and undifferentiated (B) stickleback. Numbers at bottom are N. P=0.05

Flutamide

Three spined stickleback

Lab 6

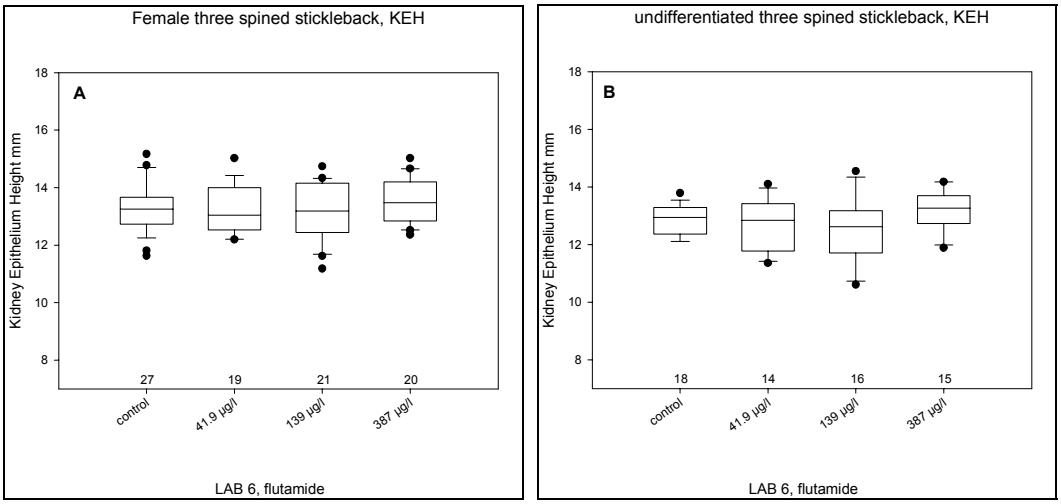


Figure 48: Kidney epithelium height (KEH) in mm after 42 D exposure to flutamide of female (A) and undifferentiated (B) stickleback. Numbers at bottom are N. P=0.05

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ANNEX

Statistical Issues Concerning the Fish Sexual Development Test (FSDT)

John W. Green, Ph.D., Ph.D.
Principal Consultant: Biostatistics
DuPont Applied Statistics Group

OVERVIEW

The primary responses required by the Fish Sexual Development Test (FSDT) are sex ratio (proportions of male, female, intersex, and undifferentiated fish) and vitellogenin (VTG). The species specified for the FSDT are Japanese medaka, zebrafish, and Stickleback. Statistical issues considered in developing the test guideline were general statistical approach, that is, hypothesis testing (NOEC/LOEC) or regression (ECx), experimental design, power or sensitivity to detect effects, and the possible need to transform the data for statistical analysis.

The basic experimental design calls for a water control and several test concentrations, with multiple tanks of multiple fish per tank at each test concentration (including the zero concentration control). If a solvent is used, then there will also be a solvent control. Experimental design then must consider the trade-off between the number of fish per tank and the number of tanks per test concentration, as well as the number and spacing of test concentrations. The unit of analysis is the tank, not the individual fish, since it is well documented that fish in the same tank tend to respond to the chemical differently than fish in different tanks even at the same nominal test concentration.

It is generally necessary to transform proportion data using an arcsine-square-root transform (or something similar, such as the Freeman-Tukey transform) to normalize the data and stabilize the variance and transform VTG data using a log-transform to reduce the very high variability arising from responses that can span several orders of magnitude. The need to transform the data complicates regression modeling, since there is no simple relationship between a 20% change in the proportion males and any specific proportion change in the transformed data, nor is there a simple relationship between the percent effect in VTG and its log-transformed values. In addition, extra-binomial variability is often observed in sex ratio data and this can greatly inflate confidence intervals for ECx estimates leading to EC10 or EC20 concentration estimates at which the estimated effect cannot be distinguished statistically from the zero-effect control or where the confidence interval for the estimate spans several test concentrations, bringing into question the value of the estimate.

In either the NOEC/LOEC or ECx approach, it is necessary to specify the size effect it is important to detect. The reason for that is obvious for ECx, since it is the "x." The experimental design is then driven by the need to estimate or detect this size effect and the statistical model or test employed. For the NOEC/LOEC approach, there should be at least 75-80% power to detect the specified size effect if it exists. For the regression approach, the 95% confidence interval for ECx should not contain zero and should not span several test concentrations and the 95% confidence interval for the mean response at ECx should not contain the control mean response. The Fish Framework Document [ENV/JM/TG(2012)4], Chapter 3, provides additional details on these requirements. There are additional considerations for each approach that are also explored in these same references. The conclusion reached was that for these endpoints, the hypothesis testing (NOEC/LOEC) approach is preferred over regression (ECx).

The preferred statistical test for sex ratio responses is the step-down Jonckheere-Terpstra test where the data are consistent with a monotone concentration-response and for Dunnett's test otherwise. Exact permutation implementation of the Jonckheere-Terpstra test is preferred where the number of replicate tanks per concentration is four or fewer, though standard large sample methods are acceptable. For VTG, the data often do not follow a monotone concentration-response, so Dunnett's test will often be the preferred approach. For VTG, Dunnett's test can be implemented using replicate means, with weights determined by the number of fish per replicate tank, or using a nested variance component approach with tank(concentration) and fish(tank(concentration)) as the two variances. These will give essentially the same results.

All of the statistical tests and all but two of the regression models discussed in the above comments or in the following documents are described in depth in OECD (2006). All of the regression models are described in *Statistical Analysis of Fish Sexual Development Sex Ratio Data.doc, Annex 1.*

DOCUMENTS AND STUDIES SUPPORTING THE FSDT EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

Statistical Analysis of FSDT VTG Validation Data.pdf (available from the Secretariat upon request)

This report, dated March 2011, summarizes the analysis of the VTG data produced from twenty-one studies from ten laboratories in the Phase 2 validation experiments for the Fish Sexual Development Test (FSDT) following the statistical protocol in the Draft FSDT Test Guideline (FSDT TG). Details of the analyses are provided in eight annexes. Two annexes, 4 and 5, were revised to include studies that were inadvertently omitted in the initial report.
Annex 1 Female Medaka.pdf
Annex 2 Female Zebrafish.pdf
Annex 3 Female Stickleback.pdf
Annex 4 Male Medaka.pdf
Annex 4 Male Medaka rev.pdf
Annex 5 Male Zebrafish.pdf
Annex 5 Male Zebrafish rev.pdf
Annex 6 Male Stickleback.pdf
Annex 8 Intersex.pdf

Power Analyses for FSDT.pdf (available from the Secretariat upon request), dated March 2011

Power Analyses for FSDT REV03082011.pdf

These reports describe a power simulation study done to finalize the experimental design for the FSDT. It is shown that under a proposed design of 20 fish per replicate, 4 reps per concentration, three positive concentrations plus control, the likelihood of finding biologically important changes in female VTG for all three species (medaka, zebrafish, and stickleback) is unsatisfactory under the high variance scenario for each species. However, there is not much loss in power for a smaller design with 8-10 fish per replicate. For median or low variance scenarios, 8-10 fish per replicate does provide adequate power. For male medaka, a design of 4 replicate tanks per concentration of 8 fish each provides adequate power to detect biologically important increases in male VTG under all three variance scenarios. For male stickleback, under the maximum variance scenario, this same design provides adequate power to detect a 130-150% increase in VTG. Only one stickleback study found effects that large. Under the median variance scenario, there was adequate power to detect effects of a magnitude observed in the validation studies. For zebrafish, this design provides adequate power to detect biologically important effects only under median or low variance scenarios. Larger designs of practical size (4 reps of 20 or 6 reps of 10) do not provide adequate

power to detect even 10-fold (1000%) increases in male VTG in this species under the maximum variance scenario.

For sex ratio analysis where only phenotypic sex can be determined, then 8 fish of each sex at the end of the experiment are sufficient to give 75% power to detect an increase from 31% change in sex-ratio (i.e., a change from 50% (fe)males to 81% or 19% (fe)males. In the 2008 report, *Power to Detect Sexual Inversion in Fish*, it can be inferred that this design is adequate to detect changes of approximately 15-25% when genetic sex is known. This latter figure includes intersex and undifferentiated sex as well as males and females. Given the true percent of males is likely to range from 30% to 70%, this may be sufficient for regulatory purposes.

Power to Detect Change in Sex-ratio.pdf

This describes a power simulation study to finalize the experimental design for the FSDT with respect to sex ratio endpoints. It is similar in intent to the report *Power Analyses for FSDT REV03082011.pdf*, which was for VTG. It concerns experiments where only phenotypic sex is known.

Power to Detect Sexual Inversion in Fish Reproduction Screens.pdf

This describes a power simulation study to determine the experimental design for the FSDT with respect to sex ratio endpoints where genetic sex is known. It is similar in intent to the report *Power Analyses for FSDT REV03082011.pdf*, which was for VTG.

Power analysis of FSDT sex ratio and VTG March 2010.doc

This is a brief summary of the above three power studies that was used to prepare a paper for publication.

Draft report of Phase 2 of the validation of the Fish Sexual Development Test [ENV/JM/MONO(2011)25]

These two reports describe and summarize the validation studies done on zebrafish, Japanese medaka, fathead minnow and three spined stickleback that have been exposed to chemicals with different modes of action in two validation phases in a total of 29 FSDT experiments including two negative studies (ammonia and n-octanol). Zebrafish has been used in 13 experiments, Japanese medaka in 7 experiments, three spined stickleback in 6 experiments and finally fathead minnow in 3 experiments. The weak estrogens 4-tert-pentylphenol and 4-tert-octylphenol was tested in zebrafish, fathead minnow and medaka and zebrafish, medaka and stickleback respectively. The aromatase inhibiting fungicide prochloraz was tested in zebrafish and fathead minnow. The androgen dihydrotestosterone was tested in zebrafish, medaka and stickleback.

OECD, TG 234 Fish Sexual Development Test, adopted 28 July 2011

These two documents show the final drafts of the TG for the FSDT for zebrafish and Japanese medaka. One shows edits that have been made since the previous version and the other shows only the final draft with no edits. These include the experimental design recommendations from the power analyses discussed above.

Additional Documents on Sex Ratio in the FSDT (available from the Secretariat upon request)

Phase II Sex Ratio Results rev.ppt
This is a presentation of results from *Comments of sex ratio modeling final.doc*, including the statistics flow chart.

Statistical Analysis of Sex Ratio.ppt
This is a presentation of results from *Comments of sex ratio modeling final.doc* that focuses on issues with regression modeling and specifically with analysis of the "normalized" sex ratio proportions. It also includes NOEC results and recommendations.

Additional Sex Ratio Analyses.doc
Since the May 6, 2010 report, Comments of sex ratio modeling final.doc, was issued, six additional datasets including 22 responses to be analyzed were received. The purpose of the current report is (1) to present analyses of these additional data, and (2) update the conclusions reached in the earlier report in view of these new data. The general discussions of hypothesis testing (NOEC) and regression modeling (ECx) given in Part 1 will not be repeated here. The reader is referred to Part 1 of this report for those discussions.

Comments of sex ratio modeling final.doc
This report has two primary focuses. Part 1 concerns (1) the document "Supplement B Trenbolone_SEDC Fish Testing Strategy_revised.doc", (2) "R+D Project No. FKZ 204 67 454/02, Studies to Support the Validation of a Definitive Fish Tests (Full Life-Cycle / annex 1Two-Generation)**".** Part 2 concerns (3) FSDT Phase 2 Preliminary Results, a presentation at the December, 2009 meeting, and data subsequently reported related to that report. The first cited document, investigate both regression modeling, to obtain an ECx, and hypothesis testing, to obtain a NOEC, for several endpoints arising from a 2-generation fish sexual development test. This report will consider only the analysis of sex ratio in the F1 generation. The statistical issues for sex ratio are the same, though clearly the two types of experiments have different overall objectives.

This report contains a careful development of hypothesis testing methods (NOEC/LOEC) and of regression (ECx) methods and summarizes the results from Phase II under both types of analysis. It also details the mathematical and statistical error in the use of "normalized" proportions from sex ratio data.

Stickleback Results.doc
This is a detailed report of the analysis of sex ratio from Phase II medaka experiments that is summarized in the report *Comments of sex ratio modeling final.doc*.

Zebrafish Results.doc
This is a detailed report of the analysis of sex ratio from Phase II zebrafish experiments that is summarized in the report *Comments of sex ratio modeling final.doc*.

Power to Detect Sexual Inversion in Fish Reproduction Screens.doc
The purpose of this report is to determine experimental designs appropriate for Phase 2 of the validation work for the fish sexual development test, with focus on the power to detect sexual inversion. Previous power simulations were presented when only phenotypic sex determination was available and large designs were needed. Recent techniques have been developed to determine the genetic sex of fathead minnow and medaka, so that analysis can now be done on the proportion of fish whose phenotypic sex differs from their genetic sex.

Fish SEXRATIO Power Analyses.doc
This report provides power analyses for the sex ratio endpoint when only phenotypic sex is known. The data source was USEPA using experiments on six chemicals (atrazine, flutamide, trenbolone, E1, E2, and Hospital Waste Water), 4-8 dose levels (plus two experiments with both water and solvent controls), involving one species (zebrafish), with 1, 2, or 4 replicates of 7-96 fish at each dose level.

Analysis of Sex Ratio.pdf – January, 2007
This document discusses two ways to analyze sex ratio: (1) ratio of proportion males to proportion females (or reciprocal), (2) analysis of proportions male, female, intersex, undifferentiated. Both NOEC and ECx are discussed. ECx not recommended for intersex and undifferentiated and discussed as not problematic for males and females. Discusses expected control variance. Data used 4tPP and Prochloraz Phase 1 data.

Statistics for 4tPP and Prochloraz Experiments.ppt
This is a PowerPoint presentation of above report.

Analysis of Sex Ratio Part2.doc
This second analysis was issued primarily to incorporate a new understanding of how the 4tPP and prochloraz experiments were conducted. In the first version of this report, each analysis of either of these chemicals used the controls identified for that chemical. Subsequently, it was learned that the experiments were conducted in such a way that the controls identified for the two chemicals applied equally well to both experiments. Thus, all analyses were redone to reflect the combined controls, keeping replicate vessel information. In addition, some simulation results have been added and some discussions have been expanded or re-organized.

This document discusses the Rao-Scott Cochran-Armitage test, Jonckheere-Terpstra, and Williams tests as being suitable for finding NOECs from proportion male or female data. For ECx, discusses Bruce-Versteeg, logistic, Michaelis-Minton, Brain-Cousins (or Schabenberger) hormetic, and OECD2-5 (exponential and doubly exponential with and without bounds) models.

Use the NOEC approach on the proportions of males and females, and on the proportion intersex and proportion undetermined is recommended.

Zebrafish SexRatio Analysis.doc – dated June, 2007
Three experimental designs were used to explore the sex ratio in zebrafish using both regression (ECx) and hypothesis testing (LOEC/NOEC) approaches. Not all datasets could be fit by a regression model.

Statistical Analysis of Fish Sexual Development Sex Ratio Data.doc
Four laboratories participated in experiments with fish exposed to various concentrations of two chemicals, prochloraz and 4tPP in Phase 1B. Three of the Labs used zebrafish and one lab, Lab 5, used fathead minnows. This report explored the comparative value of NOEC and ECx approaches for these experiments and arrived at the same conclusion as before that for sex ratio, the ECx approach is not recommended. Annex 1 describes the statistical models used in regression analysis of these data. Annex 2 gives the regression analyses for the prochloraz and Annexes 3 and 4 provide the NOEC results for prochloraz and 4tPP, respectively. Regression analysis for the 4tPP results were given in a previous report, *Analysis of Sex Ratio*.doc, dated January, 2007.

Additional Documents on VTG in the FSDT (available from the Secretariat upon request)

Comments on control variability v1.doc
These comments address the variability of VTG values observed in the four phases of experimentation conduction for VMG-eco for fathead minnow, medaka, and zebrafish, including proposed acceptability criteria. These data make very clear that there is great variability between reps within controls in the same experiment, among experiments within the same lab, and among labs. These sources of variability are quantified. It should be clear from these results that developing meaningful acceptance criteria for controls will be very challenging.

Power for VTG and SSC analyses v2.doc
The purpose of this report is to investigate the power of the proposed experimental designs and statistical protocols for 21-day fish screens for fathead minnow, medaka, and zebrafish. The results suggest that the proposed designs are adequate for detecting effects in VTG in females but possibly inadequate for detecting effects on secondary sex characteristics in male fathead minnow and medaka. There is a power comparison of six different statistical tests for determining NOEC/LOEC. The step-down Jonckheere-Terpstra and Williams tests are shown to be very similar in power and superior to all others. The next most powerful test is Dunnett's test.

Data from 4 labs using 3 chemicals in Phase 1B validation work were analyzed and used as a basis for new power simulations for the two proposed designs comprised of three test concentrations plus a control. For fathead minnow (FHM), four reps of 2 males and 4 females are proposed, whereas for medaka and zebrafish, two reps of 5 males and 5 females are proposed. In the Phase 1B experiments, each rep contained five males and 5 females. There were three experiments with two chemicals for FHM and two experiments with two chemicals for each of medaka and zebrafish. Data from 3 labs were available.

Power Analysis of Endocrine Disrupting Chemicals in Fish and Frog Experiments.doc – Dec 2007
The primary objectives of this power simulation study were to determine, within certain constraints, (1) the optimal allocation of tadpoles and certain fish species to replicate tanks and the number of tanks per dose in order to study the effect of certain endocrine disrupting chemicals on key biological endpoints; (2) which statistical tests are appropriate for determining a NOAEL for these key biological endpoints.

The endpoints reported for fish are sex ratio and vitellogenin (VTG) levels. The statistical tests considered were the Dunnett, Dunn, Williams, Jonckheere (both exact and standard) and Mann-Whitney (both exact and standard).

Experimental design issues considered were (1) the number of subjects per replicate, (2) the number of replicates per dose, (3) the allocation rule (i.e., whether equal numbers of replicates are assigned to all groups or more replicates are assigned to the control than to the positive dose groups, (4) the number of dose levels per experiment.

VTG Power Analyses.doc
These power analyses were based on early data and were used to guide the development of statistical protocols for the FSDT. There were two sources of VTG data: (1) Les Touart of USEPA reported on experiments on six chemicals (atrazine, flutamide, trenbolone, E1, E2, and Hospital Waste Water), 4-8 dose levels (plus two experiments with both water and solvent controls), involving one species (zebrafish), three sex categories (male, female, unspecified), with up to four replicates of 20 fish at each dose level. (2) Rodney Johnson of USEPA Duluth reported on experiments with four chemicals (flutamide, trenbolone, methoxychlor, and fadrozole), 3-6 dose levels, one species (FHM), both sexes, up to four replicates of 2-4 fish per replicate.

These results and others were expanded and included in *Power Analysis of Endocrine Disrupting Chemicals in Fish and Frog Experiments.pdf*, cited above.

Comments on meeting document 4.ppt
This discusses power properties of MW, Dunnett, JT, Williams tests for VTG.

Statistical Analysis of Phase 2.doc
VTG responses on zebrafish and Japanese medaka from Phase 2 were analyzed from experiments which used negative substances. One objective was to explore false positive rates. General statistical methodology was discussed for regression (ECx) and hypothesis testing (NOEC) approaches.

General Results Related to FSDT

Effect of Loss of Replicates.ppt
This presentation explores the effect on the power of the step-down Jonckheere-Terpstra test when entire replicates are missing. This is not restricted to sex ratio or VTG, but is of general applicability.

OECD (2006): *Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application*, OECD Series on Testing and Assessment, No. 54, ENV/JM/MONO (2006)18, which can be downloaded at http://www.oecd.org/LongAbstract/0,2546,en_2649_201185_37719579_119669_1_1_1,00.html