DRAFT REPORT OF PHASE 2 OF THE VALIDATION OF THE FISH SEXUAL DEVELOPMENT TEST

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EXECUTIVE SUMMARY

Will be added later.

INTRODUCTION

1. The need to develop and validate a fish assay 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; Orlando et al. 2004; Milnes et al. 2006). 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 for the Testing and Assessment of Endocrine Disrupters at Level 4 (OECD, 2002). 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, 2004) 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 Guideline 210 adopted in 1992, the Fish Early-Life Stage Toxicity Test with added end-points for the detection of endocrine disrupters (vitellogenin (VTG) measurement and sex ratio). The idea of the assay is that exposure of fish to certain EDCs 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 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 medaka (*Oryzias latipes*) and the stickleback (Gasterosteus *aculeatus*) were used. The latter two species have the great 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 (REF). 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 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]). The criteria for selecting the methods used in this validation programme are described further in the report.

Sex ratio

7. When fish are exposed to EDCs during the sensitive window of their sexual development (approximately from 20 to 60 days post hatch), 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 EDCs impact its sustainability (REF). 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.

OBJECTIVES OF PHASE 2

8. The objectives of a validation programme 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.

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: 4tert-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 17b-estradiol, and LAB 9 and LAB 10 conducted experiments using 4tert-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 programme 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.

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.

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

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

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 continues 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
  32, 100 and 320 µg/l for stickleback 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 XXXXXX for 4tert-pentylphenol and through XXXXXX 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.

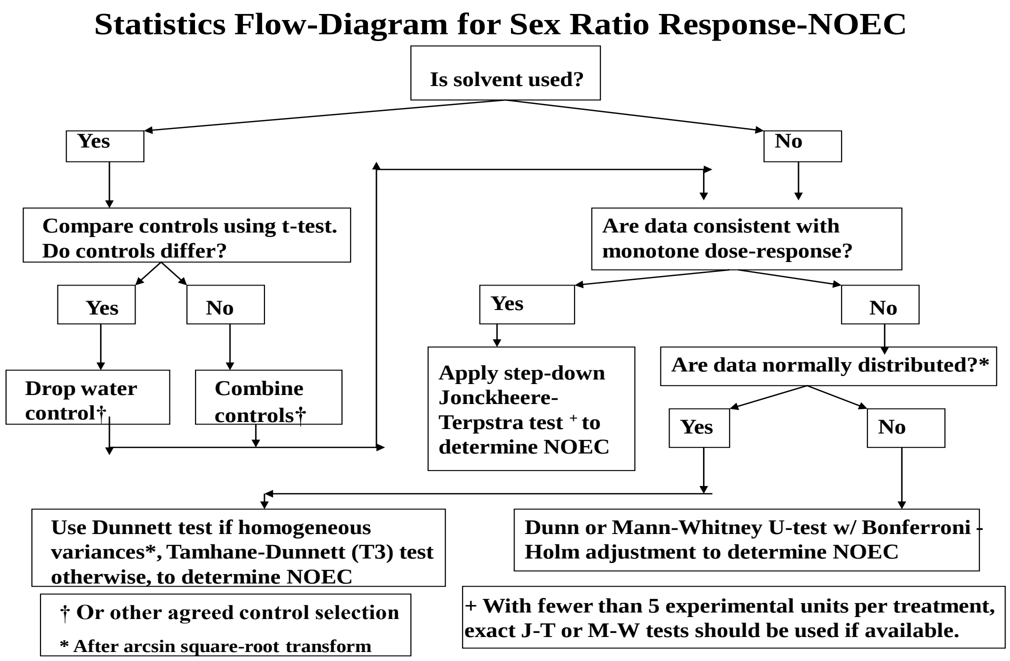
- The water temperature did not differ by more than ± 2.0°C between test vessels at any one time during the exposure period.

Collection of data and statistical analysis:

19. All test results were collected and centrally analysed. The statistical analysis of VTG concentrations was performed following a defined protocol: data were transformed to obtain normality and homogeneity of variances. Results were evaluated using a one-way analysis of variance (ANOVA) followed by a Bonferroni adjusted Fisher's Least Significant Difference test or Kruskal–Wallis One Way Analysis of Variance on Ranks followed by Multiple Comparisons versus Control Group (Dunn's Method).

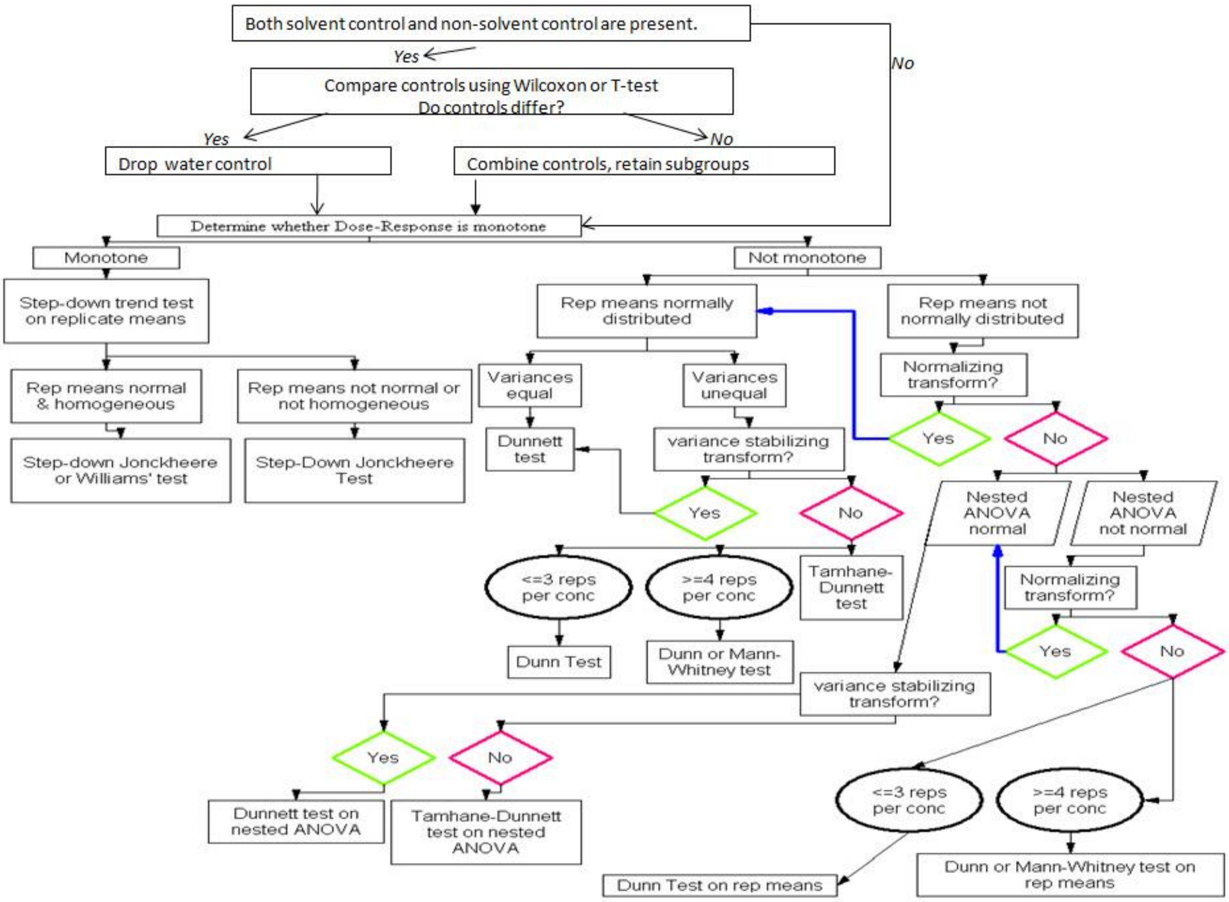
20. The statistical analysis of phenotypic sex was also analysed following a defined protocol (see figure 1 below).

Figure 1:



The statistical analysis of vitellogenin measurements were also analysed following a defined protocol (see figure 2 below).

Figure 2:



RESULTS

Analytical chemistry

4tert-octylphenol

Table 2: Measured concentrations of 4-tert octylphenol; 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 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 med mean ± SD
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 ± 8.0 (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
	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
	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			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 ± 5.6 (13)**	**12.2 ± 2.6 (34)**		**6.2 ± 0.8 (**
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
	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
	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		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 ± 5.4 (12)**	**31.7 ± 8.5 (20)**	**26.0 ± 5.7 (22)**	**30.6 ± 13.4 (13)**	**22.2 ± 5.4 (18)**	**41.9 ± 15.4 (34)**	**12.3 ± 1.7**
100 µg/l	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
	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
	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		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 ± 38.9 (13)**	**66.9 ± 11.7 (9)**	**130.6 ± 14.6 (16)**	**23.6 ± 4.0**
*200 µg/l	1		37.3± 64.5 (3)*	-	-	-	-	481.0 ± 195.2 (2)	52.3 ± 0.7
320 µg/l	2		44.9 ± 41.3 (3)*					450.0 ± 159.8 (2)	48.7 ± 5.5

	3 4 mean	 **73.1 ± 8.0 (12)**	39.6 ± 68.7 (3)* 48.1 ± 39.5 (3)* **42.5 ± 47.2 (12)***					510.0 ± 241.8 (2) 514.5 ± 228.4 (2) **488.9 ± 160.2 (8)**	50.1 ± 6.1 - **50.4 ± 4.6**
									89.1 ± 7.1 105.9 ± 13. 106.9 ± 9.9 - **100.6 ± 12.7**

Measured concentrations were generally closed to nominal concentrations, except in LAB 1 and LAB 2 testing zebrafish and LAB 9 testing medaka, 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)	72.3 ± 20.8 (10)	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)	81.1 ± 28.8 (10)	-
	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)	79.4 ± 21.5 (10)	-
	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)	81.0 ± 20.6 (10)	-
	mean	**60.0 ± 111.3 (40)**	**3.3 ± 2.8 (12)**	**20.6 ± 12.8 (30)**	**51.0 ± 44.1 (13)**	**124.6 ± 24.5 (34)**	**78.4 ± 22.6 (40)**	**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)	205.8 ± 54.1 (10)	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)	198.6 ± 48.4 (10)	-
	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)	184.8 ± 49.6 (10)	-
	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)	178.6 ± 34.3 (10)	-
	mean	**145.8 ± 190.3 (40)**	**3.3 ± 4.0 (12)**	**20.8 ± 15.6 (30)**	**157.2 ± 75.5 (13)**	**270.6 ± 53.3 (36)**	**192 ± 46.6 (40)**	**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)	585.7 ± 137.3 (10)	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)	566.9 ± 131.0 (10)	-
	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)	581.3 ± 117.8 (10)	-
	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)	515.3 ± 206.8 (10)	-
	mean	**272.6 ± 185.7 (40)**	**8.7 ± 11.1 (12)**	**93.1 ± 110.9 (30)**	**704.8 ± 116.4 (13)**	**738.9 ± 137.4 (34)**	**563.5 ± 146.9 (40)**	**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.

4tert-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.

17β-estradiol

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

4tert-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 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)
	1	100.0	90.0			70.0	85.7	100.0	80.0	76.0	80.0	76.0	68.0		95.0	60.0	91.6
	2	100.0	95.0			60.0	100.0	100.0	70.0	88.0	70.0	88.0	80.0		80.0	83.0	95.8
control	3	100.0	88.0	To come	To come	90.0	83.3	97.5	74.4	84.0	74.4	84.0	72.0	**77.0**	90.0	90.0	84.3
	4	100.0	83.0			90.0	83.3	97.5	66.7	64.0	66.7	64.0	64.0		95.0	80.0	100.0
	mean	**100.0**	**89.0**			**77.5**	**87.1**	**98.8**	**72.8**	**78.0**	**72.8**	**78.0**	**71.0**		**90.0**	**78.3**	**92.9**
	1														100.0	53.3	87.5
	2														90.0	30.0	96.0
solvent	3	-	-			-	-	-	-	-	-	-	-	**77.2**	95.0	83.3	88.0
	4														80.0	80.0	0.0
	mean														**91.3**	**61.7**	**67.9**
	1					45.0	88.9	100.0	93.3	80.0	70.0	100.0	88.0		90.0		
	2					45.0	100.0	100.0	86.7	86.7	72.5	68.0	60.0		69.1		
10 µg/l	3	-	-			35.0	100.0	100.0	93.3	93.3	75.0	88.0	88.0	**76.4**	90.0	-	-
	4					35.0	100.0	100.0			65.0	92.0	88.0		90.0		
	mean					**40.0**	**96.9**	**100.0**	**91.1**	**86.7**	**70.6**	**87.0**	**81.0**		**84.8**		
	1	100.0	90.0			-	-	100.0	90.3	93.3	62.5	76.0	76.0		75.9	56.6	100.0
	2	100.0	100.0			-	-	100.0	100.0	93.3	52.5	76.0	72.0		80.0	26.6	84.0
32 µg/l	3	100.0	85.0			50.0	100.0	97.5	80.0	80.0	71.8	80.0	80.0	**60.2**	Pooled	43.3	94.7
	4	100.0	90.0			80.0	88.0	95.0			57.9	80.0	80.0		Pooled	80.0	0.0
	mean	**100.0**	**91.3**			**65.0**	**92.3**	**98.1**	**91.1**	**88.9**	**61.2**	**78.0**	**77.0**		**78.0**	**51.6**	**69.7**
	1	100.0	85.0			55.0	90.9	97.5	80.0	73.3	43.6	68.0	60.0		65.2	60.0	2.7
100 µg/l	2	100.0	70.0			70.0	78.6	100.0	93.3	93.3	17.5	84.0	64.0		Pooled	22.5	0.0
	3	100.0	88.0			50.0	90.0	100.0	93.3	93.3	27.5	84.0	84.0	**17.6**	Pooled	23.3	0.0
	4	100.0	87.0			80.0	100.0	95.0	**88.9**	**86.7**	36.8	96.0	96.0		Pooled	33.3	0.0

	mean	**100.0**	**82.5**			**63.8**	**90.2**	**98.1**			**31.4**	**83.0**	**76.0**		**65.2**	**34.8**	**0.7**
*200 µg/l 320 µg/l	1 2 3 4 mean	100.0 100.0 100.0 100.0 **100.0**	83.0* 100.0* 75.0* 80.0* **84.5***			-	-	50 32.5 32.5 22.5 **34.4**	73.3 80.0 100.0 **84.4**	73.3 80.0 80.0 **77.8**	0.0 0.0 0.0 0.0 **0.0**	-	-		-	0.0 0.0 0.0 0.0 **0.0**	0.0 0.0 0.0 0.0 **0.0**
									100.0 80.0 86.7 **88.9**	93.3 0 73.3 **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 medak	
		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 (%)	surv (%)
control	1 2 3 4 mean	100.0 100.0 100.0 100.0 **100.0**	45.0 68.0 43.0 50.0 **51.5**	To come	To come	93.3 91.7 95.0 90.0 **92.5**	78.3 80.0 83.3 76.7 **79.6**	80.0 72.0 80.0 92.0 **86.0**	80.0 64.0 76.0 92.0 **78.0**	**77.0**	95.0 80.0 90.0 95.0 **90.0**	60.0 83.0 90.0 80.0 **78.3**	91.6 95.8 84.3 100 **92.9**	100.0 100.0 95.0 100.0 **98.8**	80 85 80 10 **86**
solvent	1 2 3 4 mean	-	-			95.0 95.0 96.7 93.3 **95.0**	88.3 45.0 66.7 70.0 **67.5**	-	-	**77.2**	100.0 90.0 95.0 80.0 **91.3**	53.3 30.0 83.3 80.0 **61.7**	87.5 96.0 88.0 pooled **90.5**	-	-
100 ng/l	1 2 3 4	100.0 100.0 100.0 100.0	68.0 73.0 65.0 55.0			95.0 85.0 96.7 98.3	65.0 65.0 76,7 80.0	96.0 64.0 64.0 84.0	88.0 64.0 56.0 80.0	**84.7**	80.0 90.0 95.0 90.0	83.3 66.6 43.2 66.6	75.4 90.5 90.5 80.9	95.0 70.0 85.0 80.0	85 49 68 52

	mean	**100.0**	**61.5**			**93.8**	**71.7**	**75.0**	**72.0**		**84.8**	**64.9**	**84.3**	**82.5**	**63**
320 ng/l	1	100.0	68.0			96.7	96.7	88.0	80.0	**84.7**	76.0	25.8	100.0	100.0	95
	2	100.0	73.0			88.3	83.3	80.0	72.0		80.0	56.6	83.0	85.0	68
	3	100.0	73.0			95.0	60.0	84.0	84.0		Pooled	47.0	93.7	95.0	71
	4	100.0	55.0			96.7	66.7	64.0	64.0		Pooled	40.0	Pooled	100.0	80
	mean	**100.0**	**67.3**			**94.2**	**76.7**	**77.0**	**75.0**		**78.0**	**42.4**	**92.2**	**95.0**	**78**
1000 ng/l	1	100.0	85.0			93.3	80.0	80.0	80.0	**85.5**	65.2	78.1	100.0	90.0	63
	2	100.0	60.0			95.0	81.7	56.0	56.0		Pooled	56.6	95.8	95.0	80
	3	100.0	68.0			96.7	63.3	64.0	60.0		Pooled	54.5	90.4	85.0	21
	4	100.0	45.0			91.7	50.0	80.0	72.0		Pooled	50.0	Pooled	90.0	76
	mean	**100.0**	**64.5**			**94.2**	**68.8**	**67.0**	**67.0**		**65.2**	**59.8**	**95.4**	**90.0**	**60**

4tert-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

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**

rate and survival following exposure to 17beta-estradiol.

Core endpoints

Overview of NOEC/LOEC for vitellogenin measurements

Exposure chemical	specie	lab	NOEC VTG (µg/l)	LOEC VTG (µg/l)	Comments
4-tert octylphenol	Zebrafish	1	<13.8	13.8	decline males
4-tert octylphenol	Zebrafish	2	17.6	42.5	against solvent only
4-tert octylphenol	Zebrafish	4	9.5	26.0	decline
4-tert octylphenol	Japanese medaka	4	31.7	104.7	Increase females
4-tert octylphenol	Japanese medaka	5	<12.1	12.1	Increase females
4-tert octylphenol	Japanese medaka	9	6.2	12.3	
4-tert octylphenol	Three spined stickleback	6	26.0	66.0	against solvent only
4-tert octylphenol	Three spined stickleback	8	<41.9	41.9	decline males
DHT	Zebrafish	1	<60.0 ng/l	60.0 ng/l	decline females and males
DHT	Zebrafish	2	>8.7 ng/l	>8.7 ng/l	low chemical concentrations
DHT	Zebrafish	3	<20.6 ng/l	20.6 ng/l	Increase males
DHT	Japanese medaka	5	704.8 ng/l	>704.8 ng/l	No females above 157.2 ng/l
DHT	Japanese medaka	9	94 ng/l	315 ng/l	Increase males
DHT	Three spined stickleback	6	737 ng/l	>737 ng/l	against solvent only
DHT	Three spined stickleback	8	<78.4 ng/l	78.4 ng/l	decline
4-tert pentylphenol	Japanese medaka	9	31.5	104.2	increase males
4-tert pentylphenol	Japanese medaka	10	27.0	93.6	Increase females
E2	Three spined stickleback	6	38 ng/l	107 ng/l	increase
flutamide	Three spined stickleback	6	45	141	Increase
ammonia	zebrafish	1	<1.94 mg/l	1.94 mg/l	no dose response

Table 12: NOEC/LOEC for the vitellogenin measurements in Phase 2 studies.

Overview of NOEC/LOEC for sex ratio measurements

Exposure chemical	specie	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	Japanese medaka	4	<11.2	11.2	more females
4-tert octylphenol	Japanese medaka	5	12.1	30.6	more females
4-tert octylphenol	Japanese medaka	9	23.5	50.4	more females
4-tert octylphenol	Three spined stickleback	6	66.0	>66.0	
4-tert octylphenol	Three spined stickleback	8	<41.9	41.9	
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 concentrations
DHT	Zebrafish	3	20.6 ng/l	20.8 ng/l	more males
DHT	Japanese medaka	5	<51.0 ng/l	51.0 ng/l	less females
DHT	Japanese medaka	9	<94.0 ng/l	94.0 ng/l	genetic females > males
DHT	Three spined stickleback	6	737.0 ng/l	>737.0 ng/l	
DHT	Three spined stickleback	8	78.4 ng/l	192.0 ng/l	
4-tert pentylphenol	Japanese medaka	9	104.2	317.7	genetic males > females
4-tert pentylphenol	Japanese medaka	10	27.0	93.6	more intersex
E2	Three spined stickleback	6	>393 ng/l	> 393 ng/l	
flutamide	Three spined stickleback	6	>383	>383	
ammonia	zebrafish	1	>7.75 mg/l	>7.75 mg/l	100% mortality at 15.5 mg/l

Table 13: NOEC/LOEC for the sex ratio measurements in Phase 2 studies.

Zebrafish

4tert-octylphenol

Vitellogenin levels

	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				
	Females	352 251 (629 865) [solvent control: 25 101 (56634)]	49 735 (222 099)	13422 (47 939)	354 966 (887 221)
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)

Table 14: vitellogenin levels detected in males and female fish following exposure to 4tert-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 ratio

	Proportions (SD)	Control	10 µg/L	32 µg/L	100 µg/L	200 µg/L
LAB 1	Males	0.624 (0.07)	-----------	0.355* (0.17)	0.226* (0.08)	0*
	Females		-----------			
	Intersex	---------------	------------	---------------	---------------	---------------
	Undifferentiated	---------------	------------	---------------	---------------	---------------
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	0.433 (0.08)	0.280 (0.13)	0.116* (0.09)	0*	----------------
	Females	0.514 (0.05)	0.369 (0.14)	0.247* (0.06)	0.363* (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)	----------------

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

Dihydrotestosterone

Vitellogenin levels

	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)

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

Sex ratio

	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	----------------	-----------------	--------------	--------------
	Undifferentiated	----------------	------------------	--------------	--------------
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	------------	------------	------------	------------

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

Medaka

4tert-octylphenol

Vitellogenin levels

	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 (44 327)	59 599 (93 915)

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

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

Sex ratio

	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	----------	----------	-----------	----------

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

Dihydrotestosterone

Vitellogenin levels

	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)	-

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

Sex ratio

	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	*Not* Males	0.517	0.592	0.435	0.316*
	Females	0.502	0.503	0.294*	0*
	Intersex	-----------	------------	------------	-------------
	Undifferentiated	-----------	------------	------------	-------------

Table 21: proportions of each sex determined following exposure to 4tert-octylphenol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

4tert-pentylphenol

Vitellogenin levels

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

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

Sex ratio

	Proportion (SD)	Control	10 µg/L	32 µg/L	100 µg/L
LAB 9 (60d)	Males	0.45	0.40	0.35	0.20*
	Not Females	0.45	0.40	0.50	0.30
	Intersex	-------------	-----------	------------	----------

	Undifferentiated	-------------	-----------	------------	----------
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	0.45	0.40	0.50	0.30
	Intersex	------------	-------------	---------	------------
	Undifferentiated	------------	-------------	-----------	-------------

Table 23: proportions of each sex determined following exposure to 4tert-pentylphenol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

Stickleback

4tert-octylphenol

Vitellogenin levels

	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	1687 (2129)	2338 (8022)	-	-
	Females	1965 (2014)	6278 (22112)	-	-

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

Sex ratio

	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	0.493 (0.82)	0	--------------	--------------
	Females	0.475 (0.78)	0.917* (0.40)	--------------	--------------
	Intersex	None	None	None	None
	Undifferentiated	0.031 (0.20)	0.083 (0.40)	---------------	---------------

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

Dihydrotestosterone

Vitellogenin endpoint

	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)

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

Sex ratio endpoint

	Proportions (SD)	Control	100 ng/L	320 ng/L	1000 ng/L
LAB 6	Males	0.463 (0.07)	0.428 (0.14)	0.424 (0.11)	0.455 (0.14)
	Females	0.536 (0.07)	0.571 (0.14)	0.545 (0.08)	0.544 (0.14)
	Intersex	-------------	-------------	-------------	-------------
	Undifferentiated	-------------	-------------	-------------	-------------
LAB 8	Males	0.509 (0.80)	0.513 (0.95)	0.612 (0.17)	0.442 (0.83)
	Females	0.490 (0.80)	0.486 (0.95)	0.285* (0.18)	0.357* (0.60)
	Intersex	0	0	0.081* (0.12)	0.142* (0.23)
	Undifferentiated	0	0	0.020 (0.14)	0.057* (0.308)

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

Flutamide

Vitellogenin levels

	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)

Table 28: vitellogenin levels detected in males and female fish following exposure to flutamide. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

Sex ratio

	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)

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

17beta-estradiol

Vitellogenin levels

	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)

Table 30: vitellogenin levels detected in males and female fish following exposure to 17beta-estradiol. Standard deviation is indicated in parenthesis. Shaded cell indicate the statistical significance.

Sex ratio

	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)

Table 31: proportions of each sex determined following exposure to 17beta-estradiol.

DISCUSSION

Purpose of the Assay

The purpose of the Fish Sexual Development Test is to serve as an assay for the detection of endocrine disrupting activities of a test substance……..……

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……..

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. Beside, the hormone dependant induction or inhibition of the yolk protein vitellogenin (see also OECD TG 229 and TG 230) is utilized in combination with the sex ratio…….

Control animal performance: Hatching and Survival

The Hatching rate in all experiments with 4-tert octylphenol did fulfil the validity criteria of 80% with exception of two experiments where the hatching rate was 77.5% and 78% respectively. These small abbreviations from the validity criteria are not expected to influence the results. 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 dihydrotestosterone did fulfil the validity criteria of 80% with exception of one experiment where the hatching rate was 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 one experiment where the survival rate was 51.5%. The results of this experiment were though comparable to the results of the other experiments and therefore the abbreviation from the validity criteria are not expected to influence 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.

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. The endpoints responses are though strongly connected to the actual chemical concentrations which can be seen on the NOEC/LOEC values of table 12 and 13 and therefore the experiments are recognised as valid! ………….

Vitellogenin response

The vitellogenin response 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 13 D where the vitellogenin concentration is significant different between genetic males and phenotypically sex reversed females………………………..

Sex ratio response

The skewing of the sex ratio is a population relevant endpoint. ………………………….

Genetic sex determination

The determination of the genetic sex of the individual fish as it currently is possible for Japanese medaka and three spined stickleback improves the power of the test and it clarified the difference in vitellogenin concentrations between genetic males and sex reversed females……………………………

Performance of endpoints

To come………………………………

Summary of Phase-1 and Phase-2 Validation Results

To come………………………………

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APPENDIX

Figures from the FDST validation phase 2

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Schematic overview

Table 1: Overview of the Annex 1 figures

Exposure chemical	endpoint	species	LAB	Figure
4-tert octylphenol	vitellogenin	Zebrafish	1	Figure 1
4-tert octylphenol	vitellogenin	Zebrafish	2	Figure 2
4-tert octylphenol	vitellogenin	Zebrafish	4	Figure 3
4-tert octylphenol	vitellogenin	Japanese medaka	4	Figure 4
4-tert octylphenol	vitellogenin	Japanese medaka	5	Figure 5
4-tert octylphenol	vitellogenin	Japanese medaka	9	Figure 6
4-tert octylphenol	vitellogenin	Three spined stickleback	6	Figure 7
4-tert octylphenol	vitellogenin	Three spined stickleback	8	Figure 8
Dihydrotestosterone	vitellogenin	Zebrafish	1	Figure 9
Dihydrotestosterone	vitellogenin	Zebrafish	2	Figure 10
Dihydrotestosterone	vitellogenin	Zebrafish	3	Figure 11
Dihydrotestosterone	vitellogenin	Japanese medaka	5	Figure 12
Dihydrotestosterone	vitellogenin	Japanese medaka	9	Figure 13
Dihydrotestosterone	vitellogenin	Three spined stickleback	6	Figure 14
Dihydrotestosterone	vitellogenin	Three spined stickleback	8	Figure 15
4-tert pentylphenol	vitellogenin	Japanese medaka	9	Figure 16
4-tert pentylphenol	vitellogenin	Japanese medaka	10	Figure 17
17ß-estradiol	vitellogenin	Three spined stickleback	6	Figure 18
flutamide	vitellogenin	Three spined stickleback	6	Figure 19
4-tert octylphenol	sex ratio	Zebrafish	1	Figure 20
4-tert octylphenol	sex ratio	Zebrafish	2	Figure 21
4-tert octylphenol	sex ratio	Zebrafish	4	Figure 22
4-tert octylphenol	sex ratio	Japanese medaka	4	Figure 23

4-tert octylphenol	sex ratio	Japanese medaka	5	Figure 24
4-tert octylphenol	sex ratio	Japanese medaka	9	Figure 25
4-tert octylphenol	sex ratio	Three spined stickleback	6	Figure 26
4-tert octylphenol	sex ratio	Three spined stickleback	8	Figure 27
Dihydrotestosterone	sex ratio	Zebrafish	1	Figure 28
Dihydrotestosterone	sex ratio	Zebrafish	2	Figure 29
Dihydrotestosterone	sex ratio	Zebrafish	3	Figure 30
Dihydrotestosterone	sex ratio	Japanese medaka	5	Figure 31
Dihydrotestosterone	sex ratio	Japanese medaka	9	Figure 32
Dihydrotestosterone	sex ratio	Three spined stickleback	6	Figure 33
Dihydrotestosterone	sex ratio	Three spined stickleback	8	Figure 34
4-tert pentylphenol	sex ratio	Japanese medaka	9	Figure 35
4-tert pentylphenol	sex ratio	Japanese medaka	10	Figure 36
17ß-estradiol	sex ratio	Three spined stickleback	6	Figure 37
flutamide	sex ratio	Three spined stickleback	6	Figure 38
4-tert octylphenol	KEH	Three spined stickleback	6	Figure 39
Dihydrotestosterone	KEH	Three spined stickleback	6	Figure 40
17ß-estradiol	KEH	Three spined stickleback	6	Figure 41
flutamide	KEH	Three spined stickleback	6	Figure 42

VITELLOGENIN

4-tert octylphenol

zebrafish

Lab 1

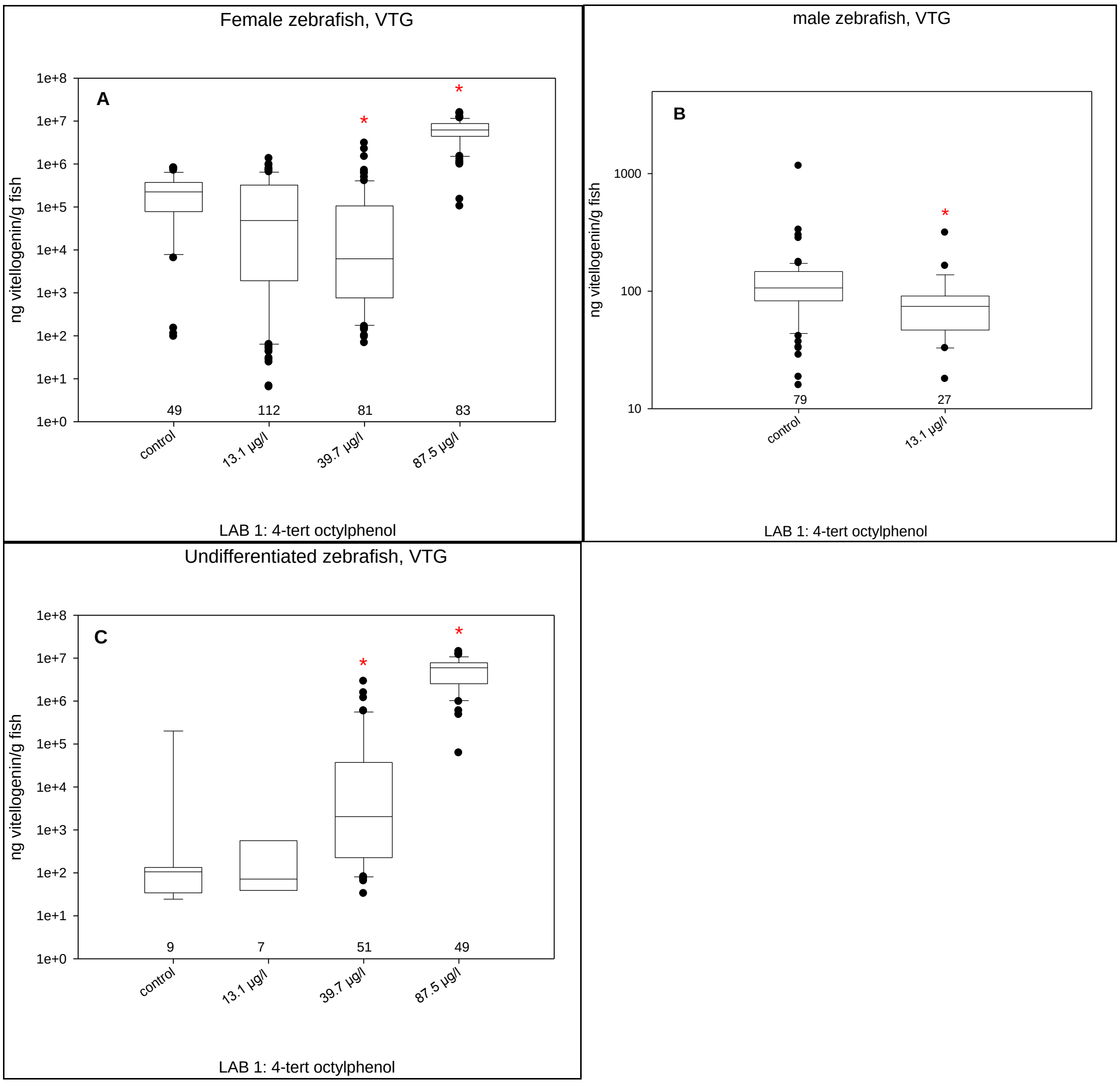


Figure 1: Vitellogenin concentrations in female (A), male (B) and undifferentiated (C) zebrafish after 60 D exposure to 4-tert octylphenol. Numbers at the bottom are N. P=0.05

Lab 2

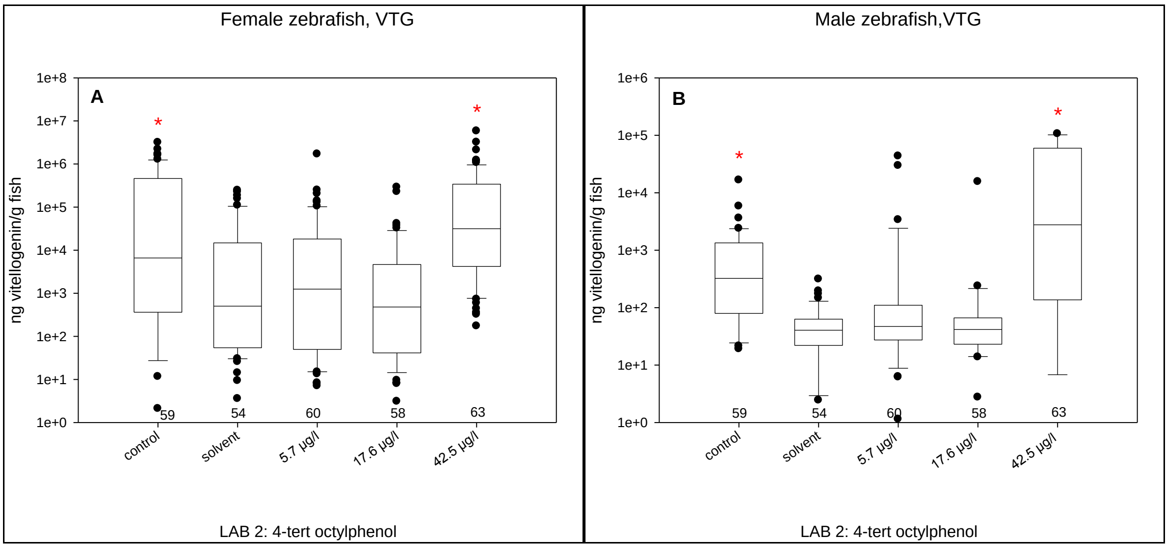


Figure 2: Vitellogenin concentrations in female (A), male (B) zebrafish after 60 D exposure to 4-tert octylphenol. Numbers at the bottom are N. P=0.05

Lab 4

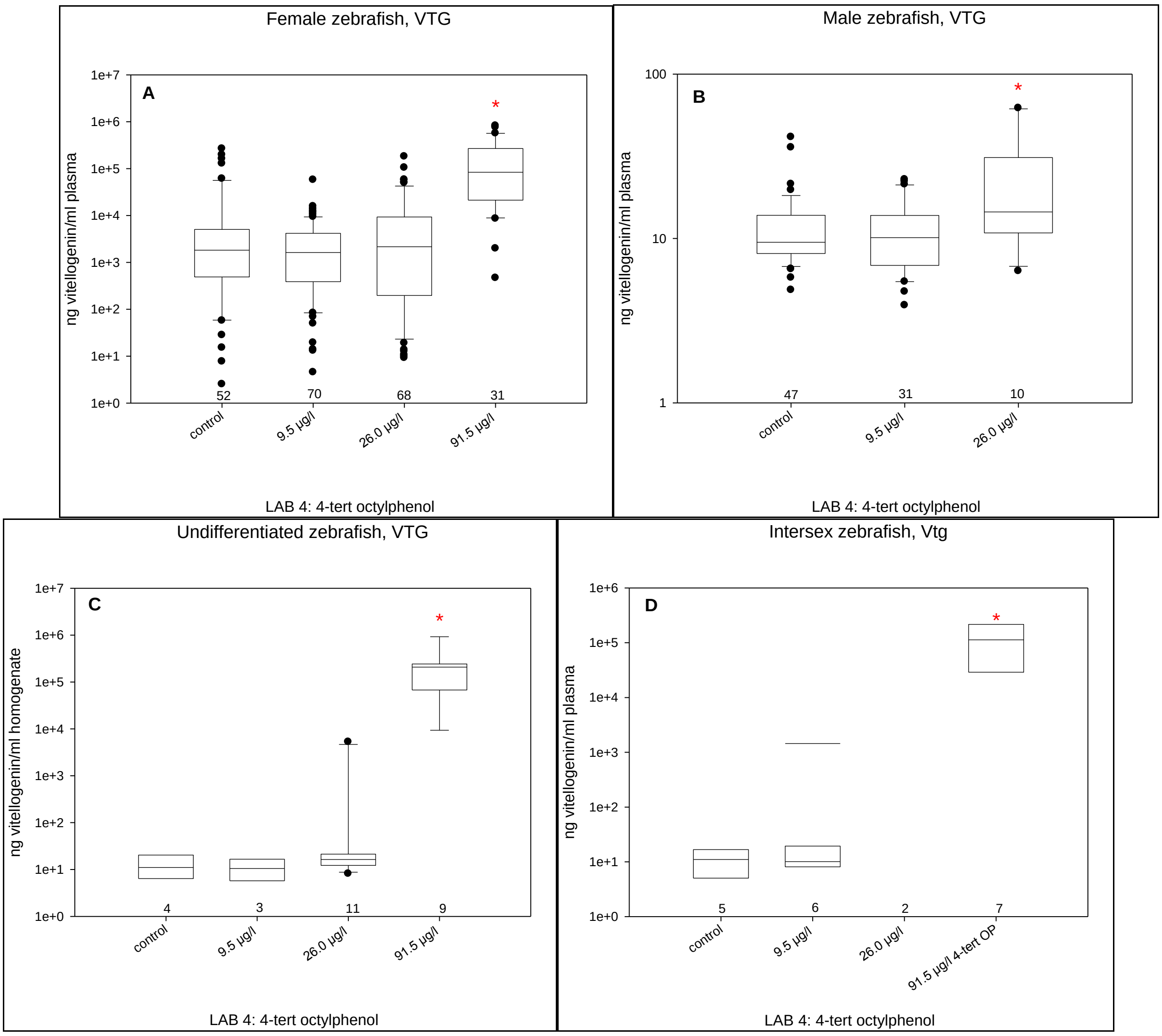


Figure 3: Vitellogenin concentrations in female (A), male (B), undifferentiated (C) and intersex (D) zebrafish after 60 D exposure to 4-tert octylphenol. Numbers at the bottom are N. P=0.05

Japanese medaka

Lab 4

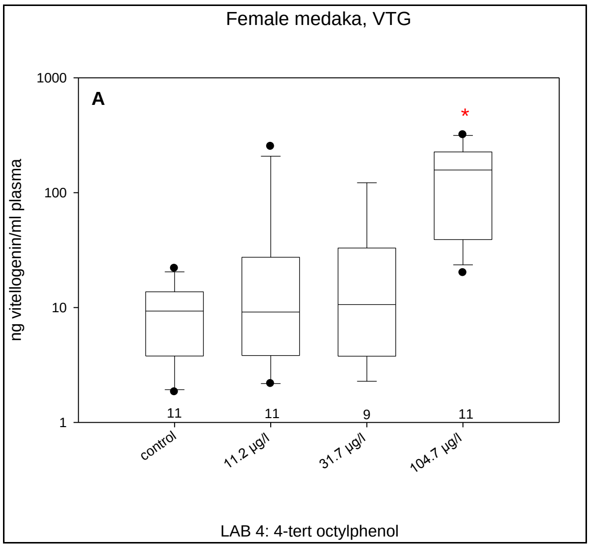


Figure 4: Vitellogenin concentrations in female (A) medaka after 60 D exposure to 4-tert octylphenol. Numbers at the bottom are N. P=0.05

Lab 5

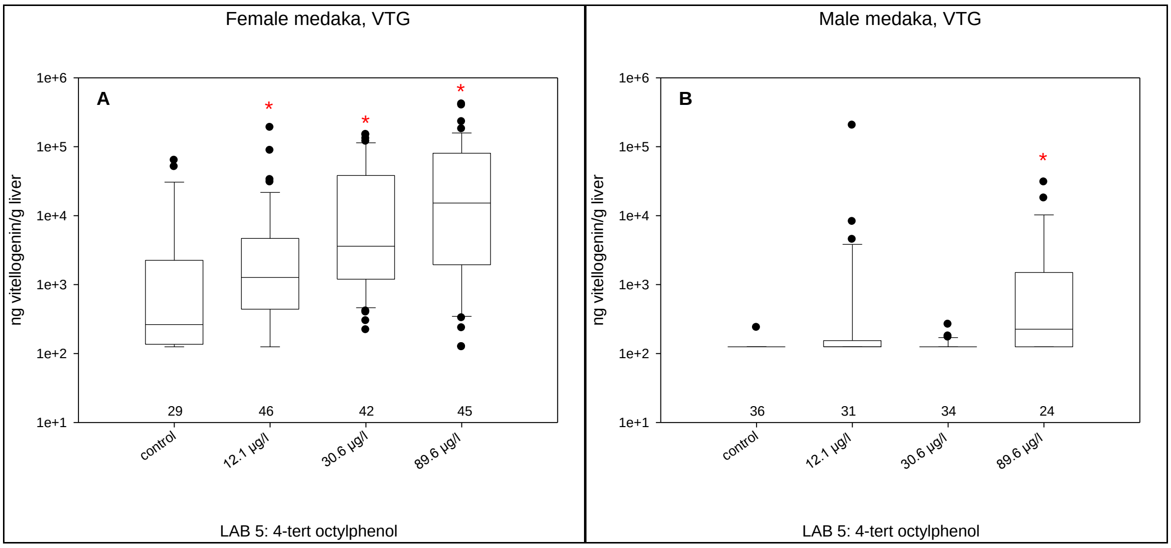


Figure 5: Vitellogenin concentrations in female (A) and male (B) medaka after 60 D exposure to 4-tert octylphenol. Numbers at the bottom are N. P=0.05

Lab 9

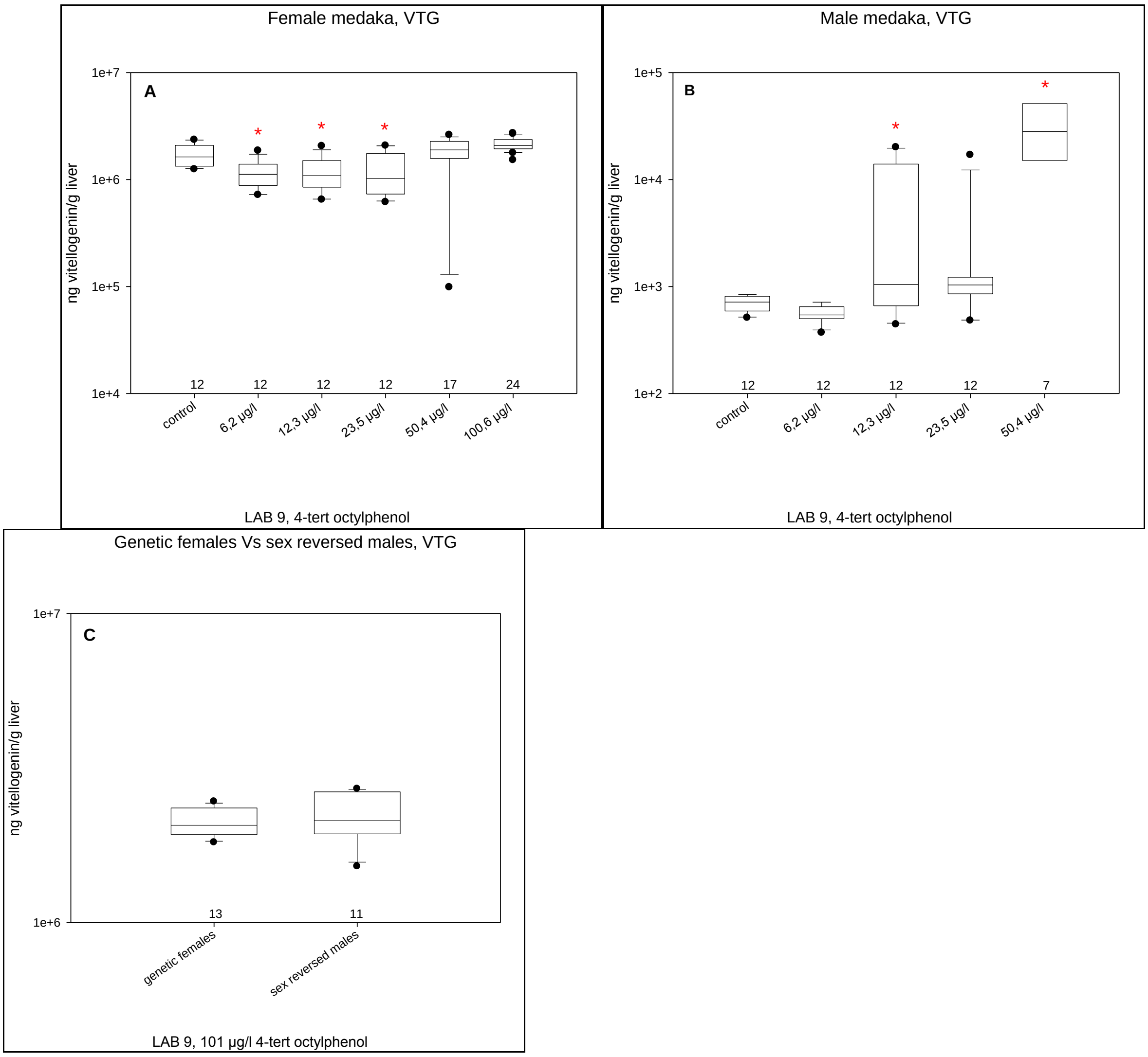


Figure 6: Vitellogenin concentrations in female (A), male (B) and intersex (C) medaka after 60 D exposure to 4-tert octylphenol. Numbers at the bottom are N. P=0.05

Three spined stickleback

Lab 6

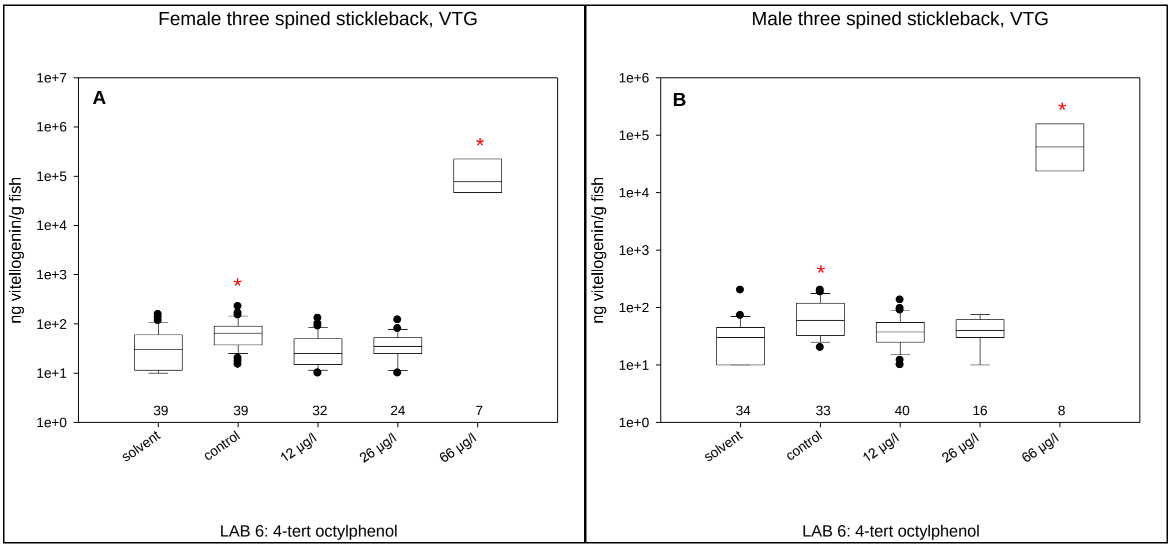


Figure 7: Vitellogenin concentrations in female (A) and male (B) stickleback after 60 D exposure to 4-tert octylphenol. Numbers at the bottom are N. P=0.05

Lab 8

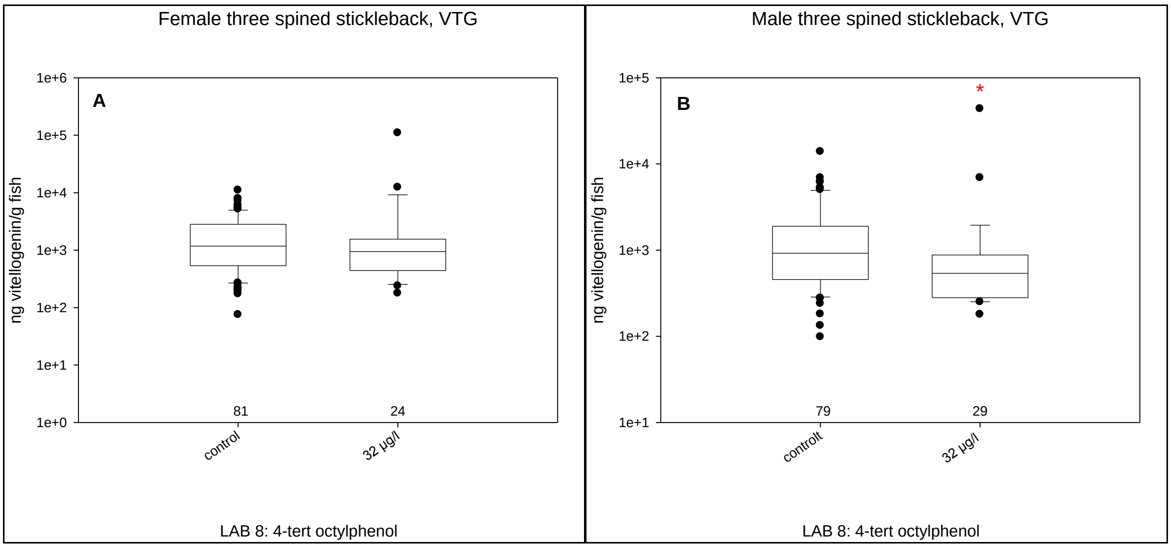


Figure 8: Vitellogenin concentrations in female (A) and male (B) stickleback after 60 D exposure to 4-tert octylphenol. Numbers at the bottom are N. P=0.05

Dihydrotestosterone

Zebrafish

Lab 1

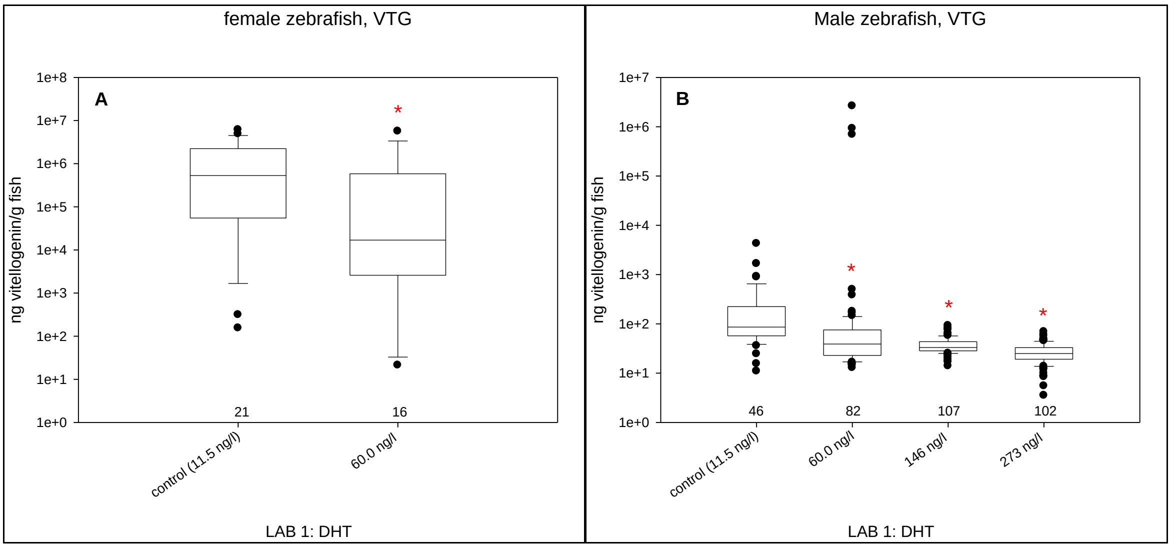


Figure 9: Vitellogenin concentrations in female (A) and male (B) zebrafish after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05

Lab 2

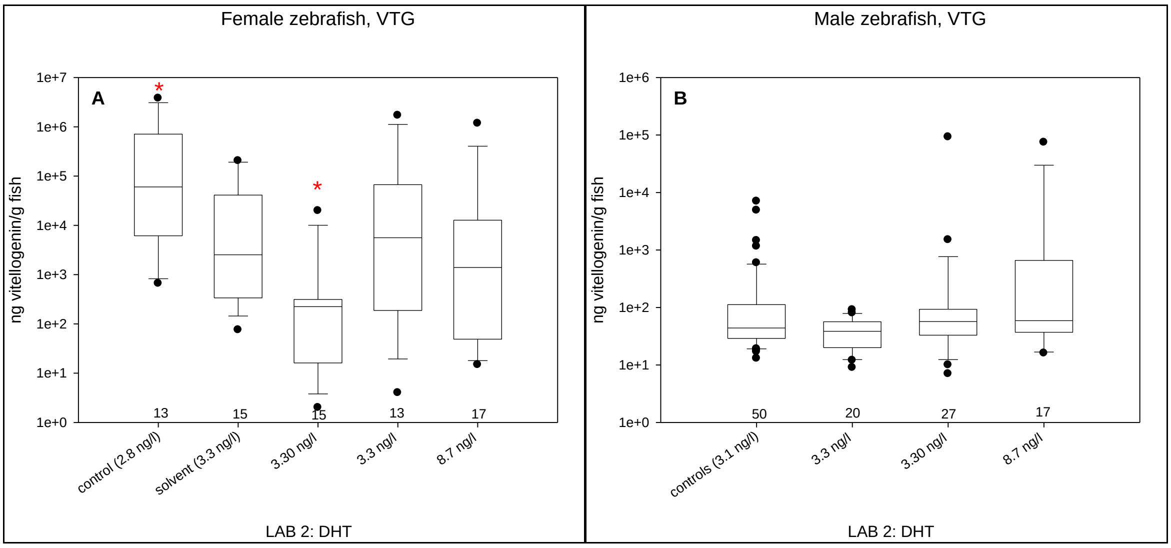


Figure 10: Vitellogenin concentrations in female (A) and male (B) zebrafish after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05

Lab 3

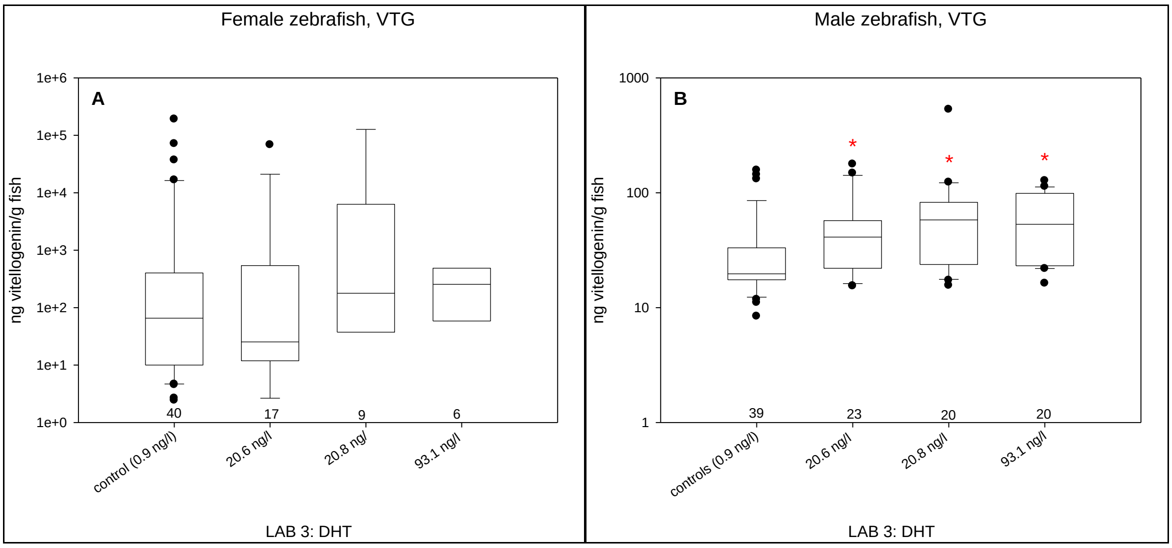


Figure 11: Vitellogenin concentrations in female (A) and male (B) zebrafish after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05

Japanese medaka

Lab 5

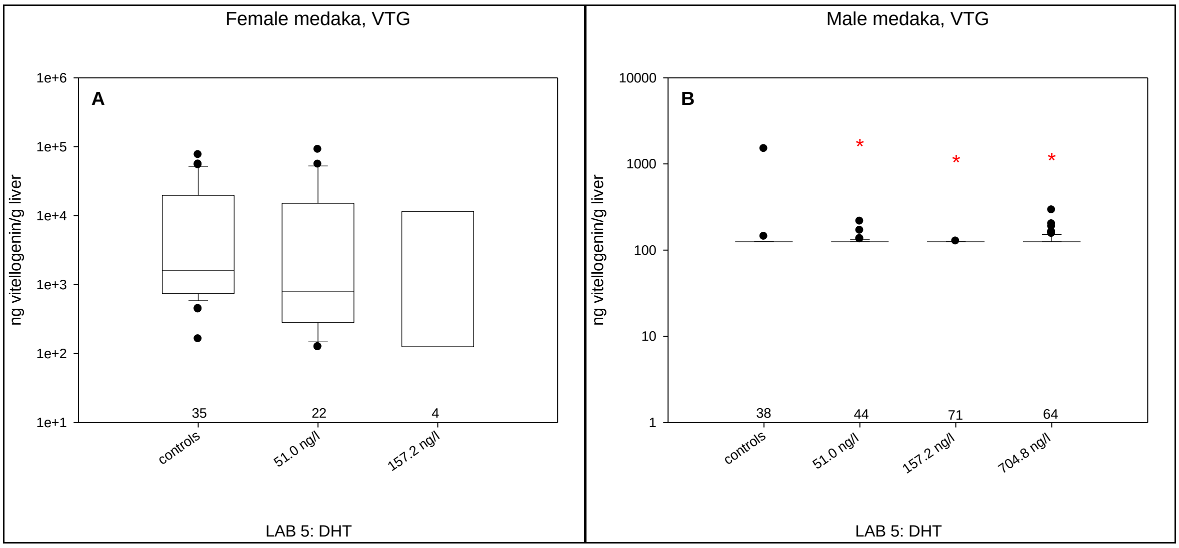


Figure 12: Vitellogenin concentrations in female (A) and male (B) medaka after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05

Lab 9

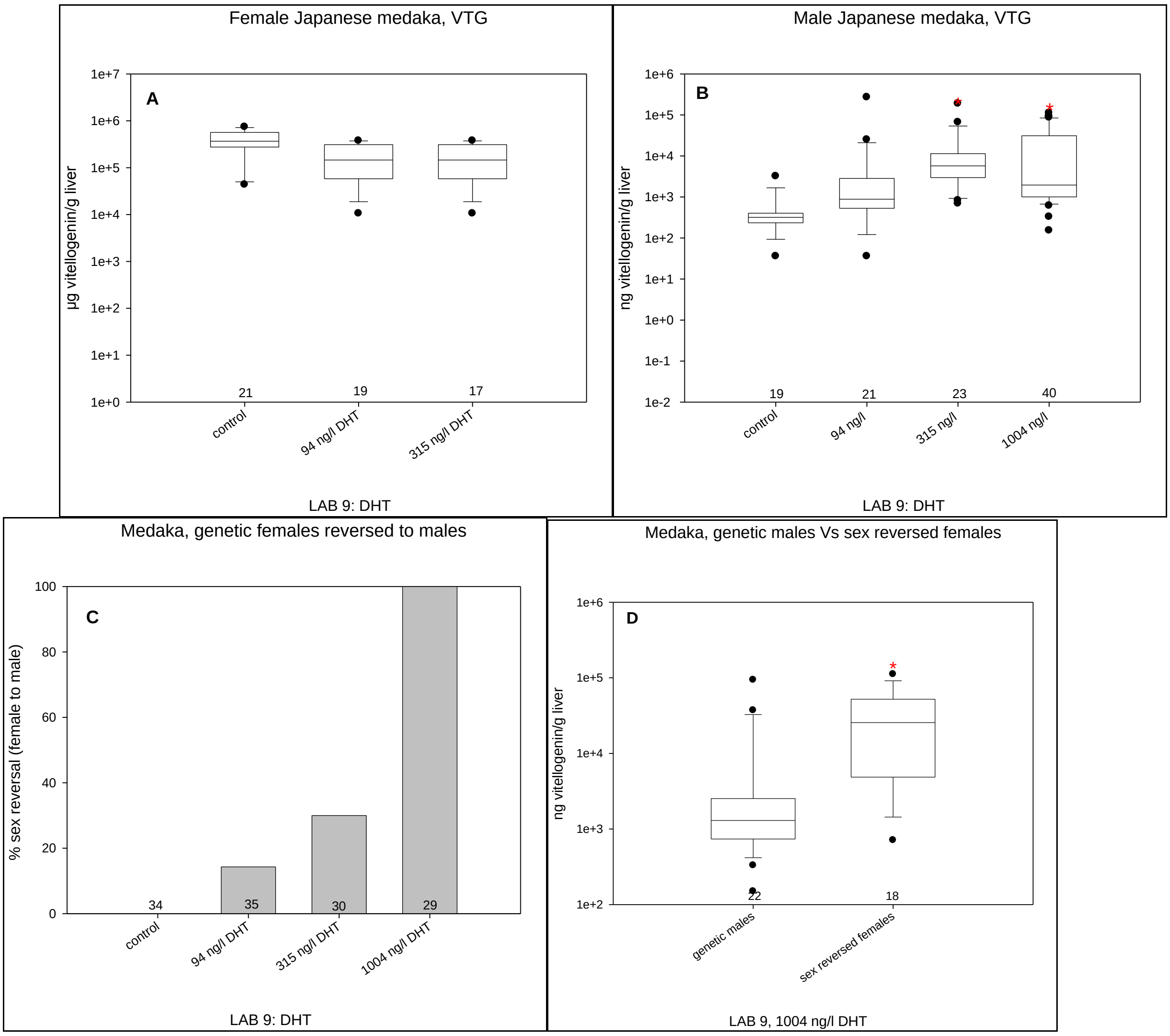


Figure 13: Vitellogenin concentrations in female (A) and male (B) medaka after 60 D exposure to dihydrotestosterone. Percentage sex reversal (C) and vitellogenin of sex reversed fish (D). Numbers at the bottom are N. P=0.05

Three spined stickleback

Lab 6

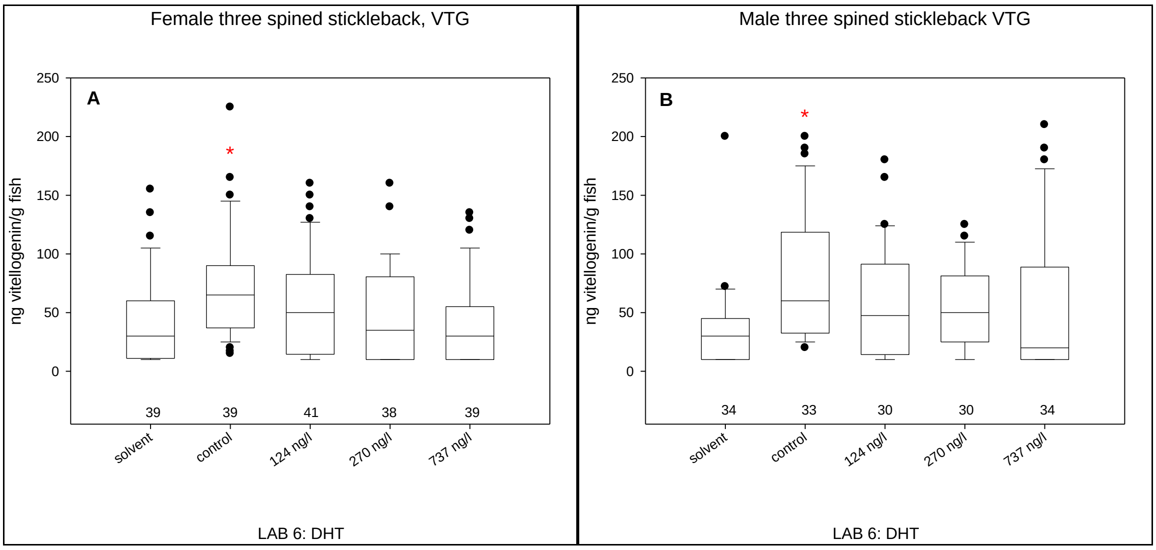


Figure 14: Vitellogenin concentrations in female (A) and male (B) stickleback after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05

Lab 8

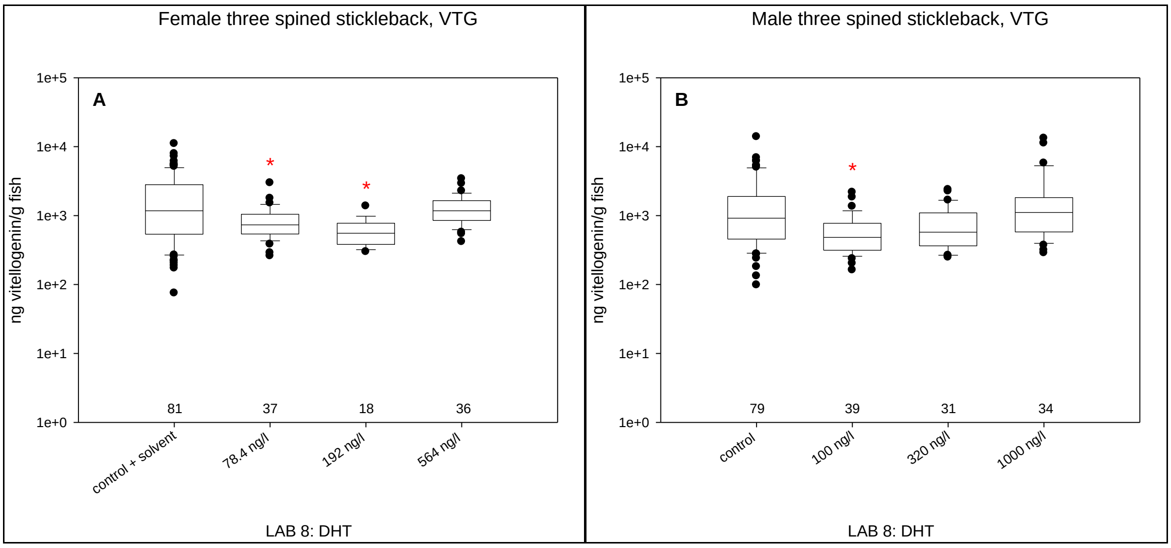


Figure 15: Vitellogenin concentrations in female (A) and male (B) stickleback after 60 D exposure to dihydrotestosterone. Numbers at the bottom are N. P=0.05

4-tert pentylphenol

Japanese medaka

Lab 9

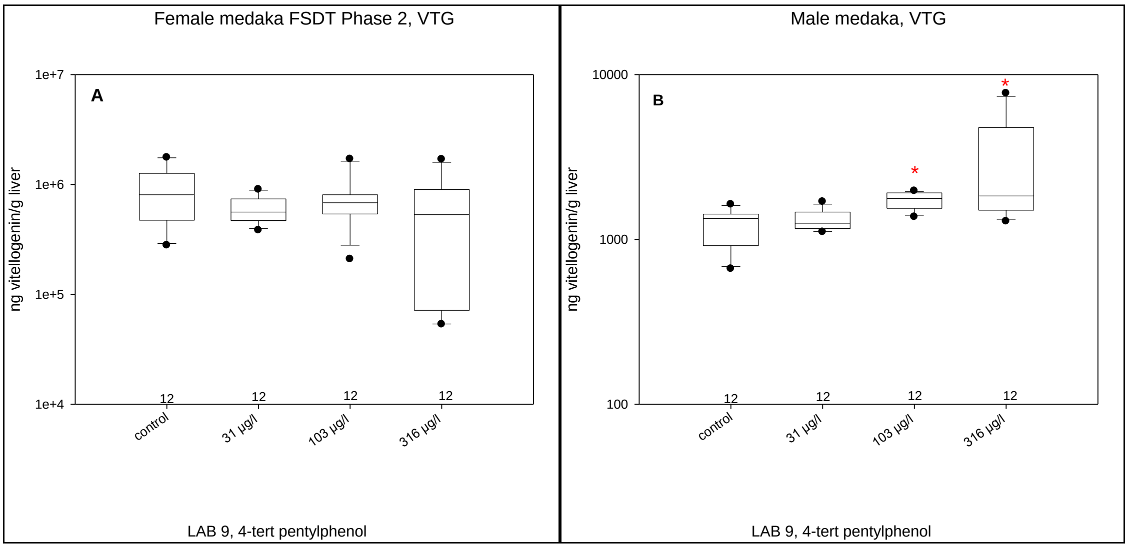


Figure 16: Vitellogenin concentrations in female (A) and male (B) medaka after 70 D exposure to 4-tert pentylphenol. Numbers at the bottom are N. P=0.05

Lab 10

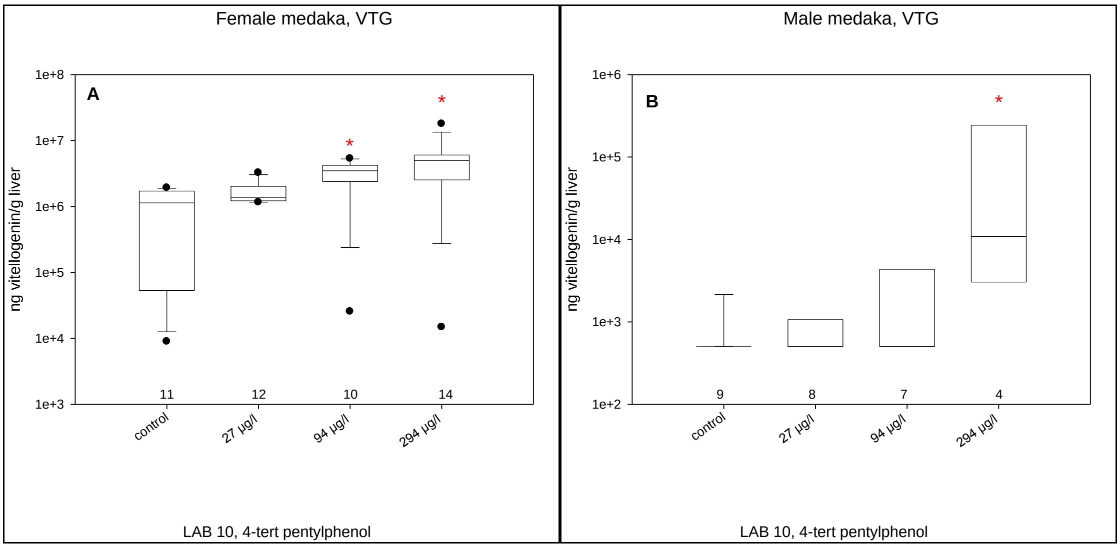


Figure 17: Vitellogenin concentrations in female (A) and male (B) medaka after 60 D exposure to 4-tert pentylphenol. Numbers at the bottom are N. P=0.05

17ß-estradiol

Three spined stickleback

Lab 6

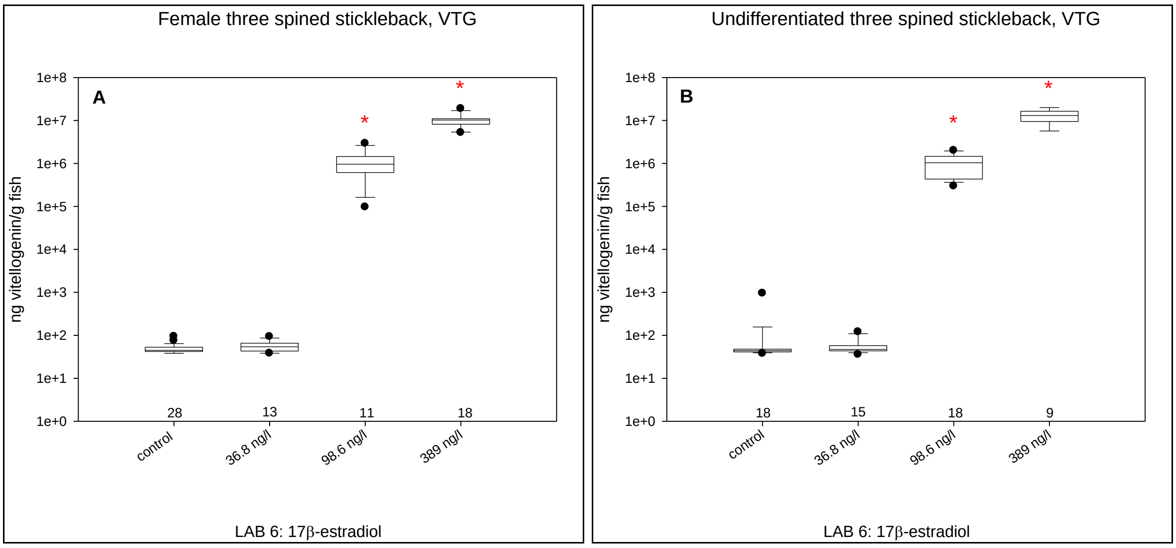


Figure 18: Vitellogenin concentrations in female (A) and undifferentiated (B) stickleback after 60 D exposure to 17ß-estradiol. Numbers at the bottom are N. P=0.05

Flutamide

Three spined stickleback

Lab 6

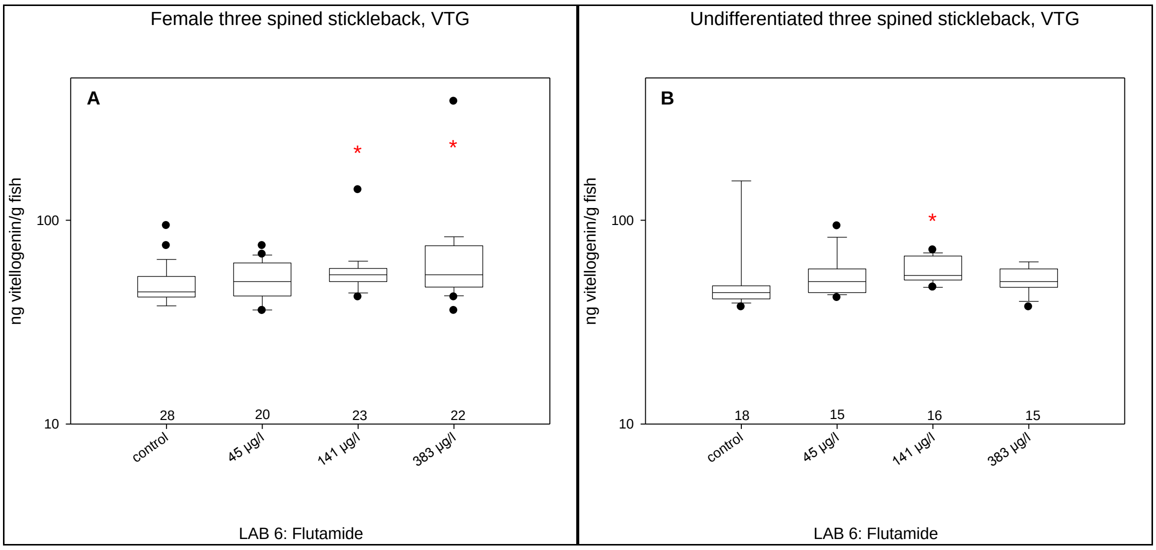


Figure 19: Vitellogenin concentrations in female (A) and undifferentiated (B) stickleback after 60 D exposure to flutamide. Numbers at the bottom are N. P=0.05

SEX RATIO AND GENETIC SEX

4-tert octylphenol

Zebrafish

Lab 1

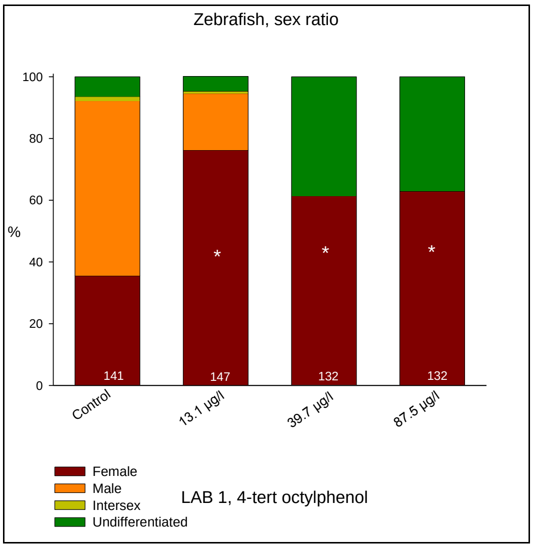


Figure 20: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to 4-tert octylphenol. Numbers at bottom are N. P=0.05

Lab 2

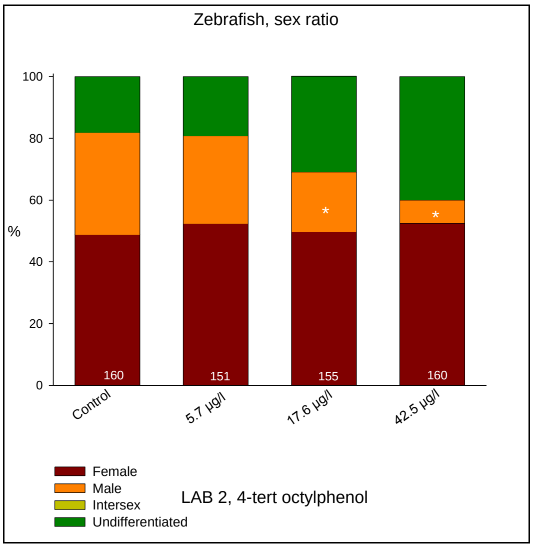


Figure 21: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to 4-tert octylphenol. Numbers at bottom are N. P=0.05

Lab 4

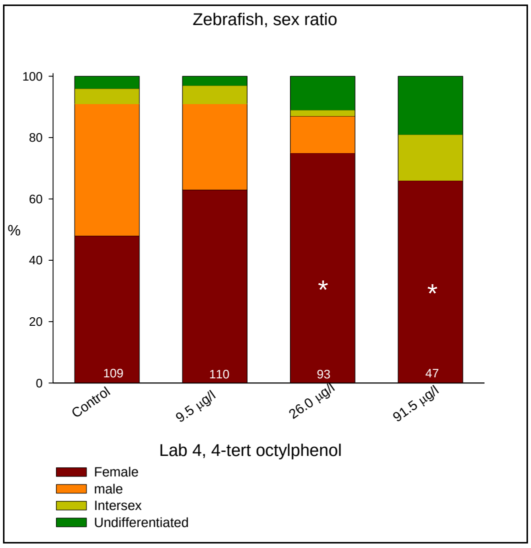


Figure 22: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to 4-tert octylphenol. Numbers at bottom are N. P=0.05

Japanese medaka

Lab 4

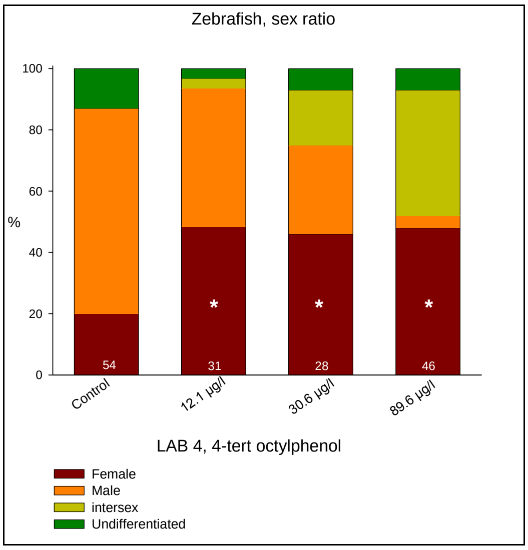


Figure 23: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to 4-tert octylphenol. Numbers at bottom are N. P=0.05

Lab 5

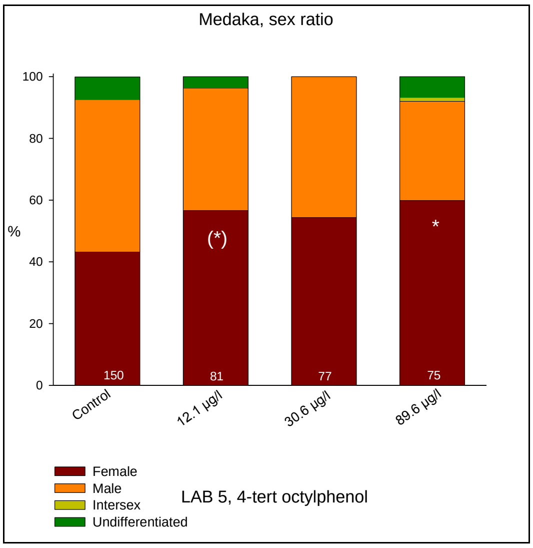


Figure 24: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to 4-tert octylphenol. Numbers at bottom are N. P=0.05

Lab 9

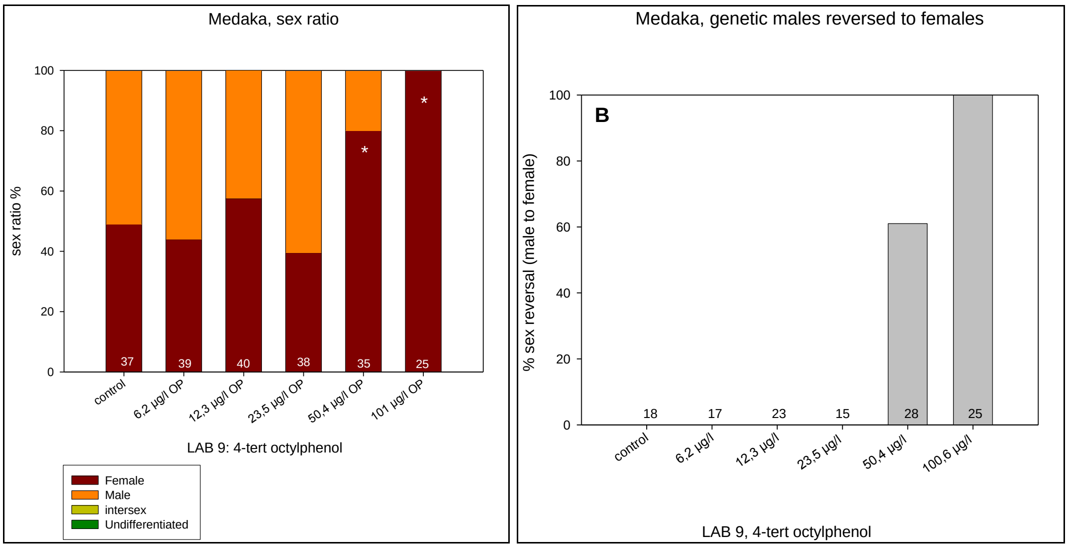


Figure 25: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to 4-tert octylphenol. Numbers at bottom are N. P=0.05. B: Percentage sex reversed males

Three spined stickleback

Lab 6

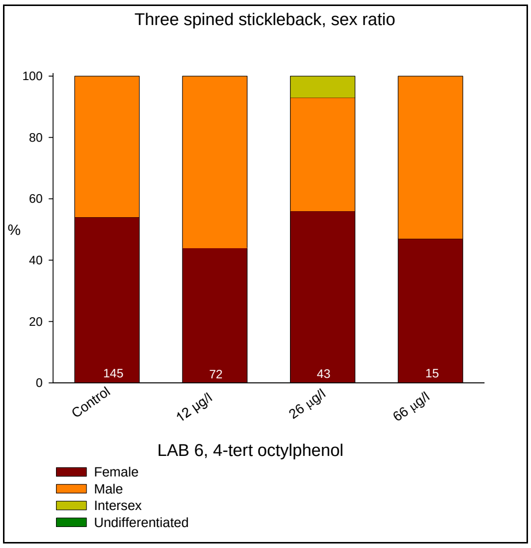


Figure 26: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to 4-tert octylphenol. Numbers at bottom are N. P=0.05

Lab 8

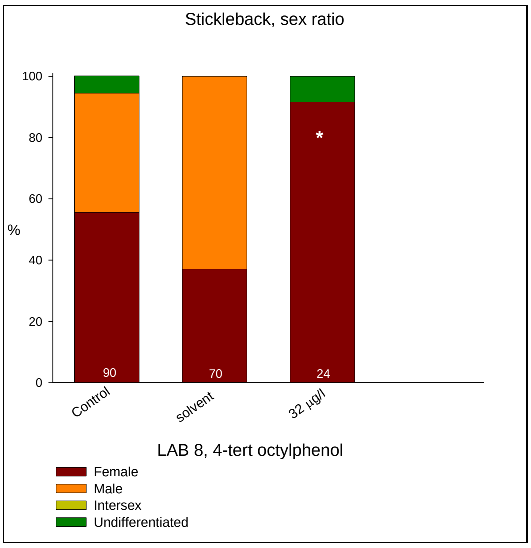


Figure 27: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to 4-tert octylphenol. Numbers at bottom are N. P=0.05

Dihydrotestosterone

Zebrafish

Lab 1

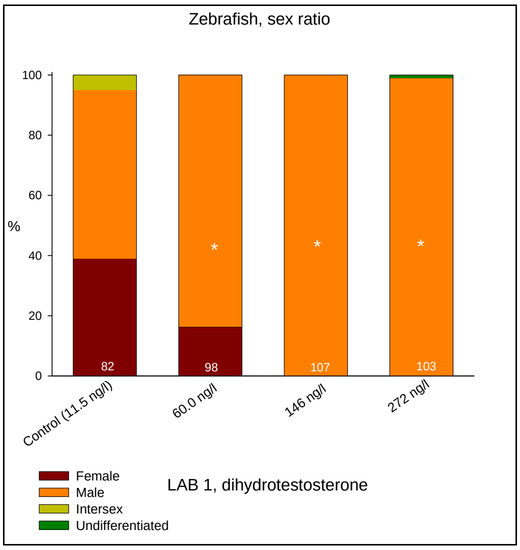


Figure 28: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Lab 2

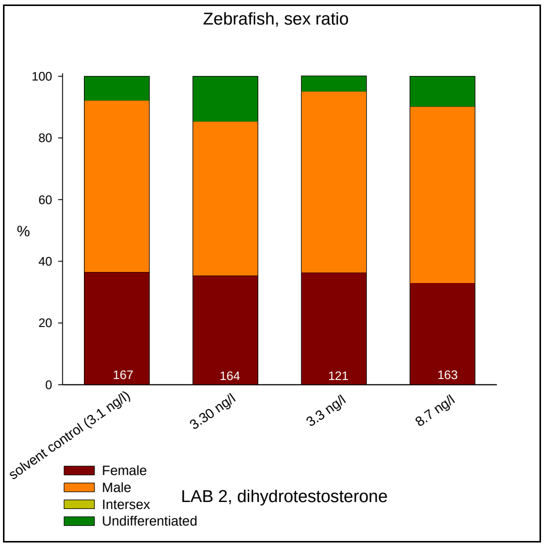


Figure 29: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Lab 3

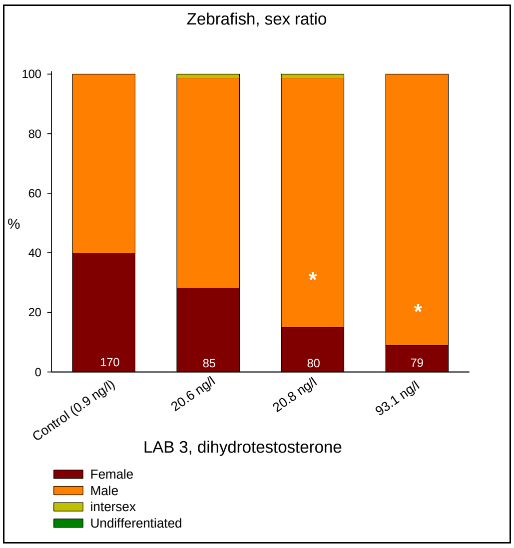


Figure 30: Sex ratio as percentage females, males, undifferentiated and intersex zebrafish after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Japanese medaka

Lab 5

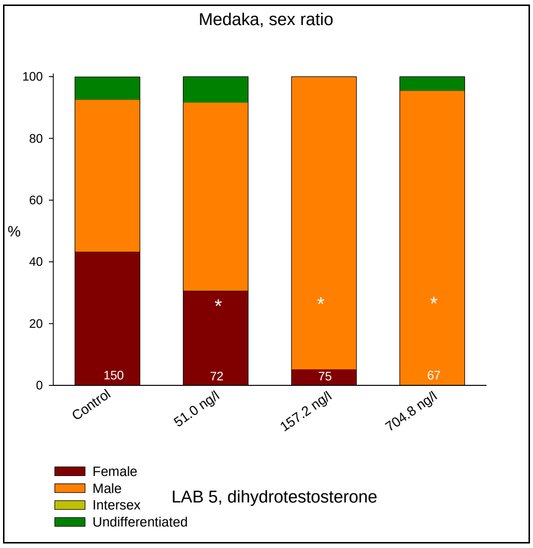
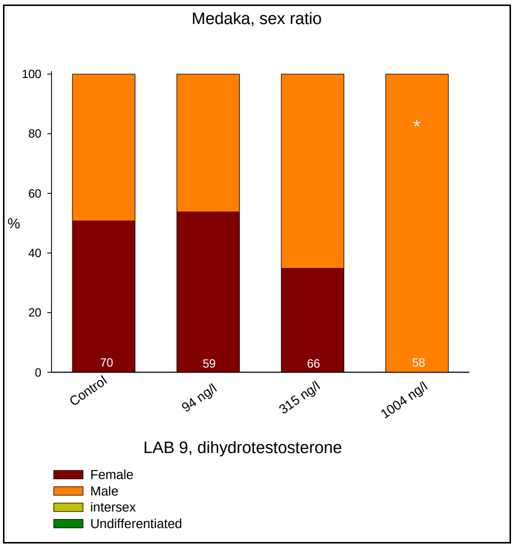


Figure 31: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Lab 9



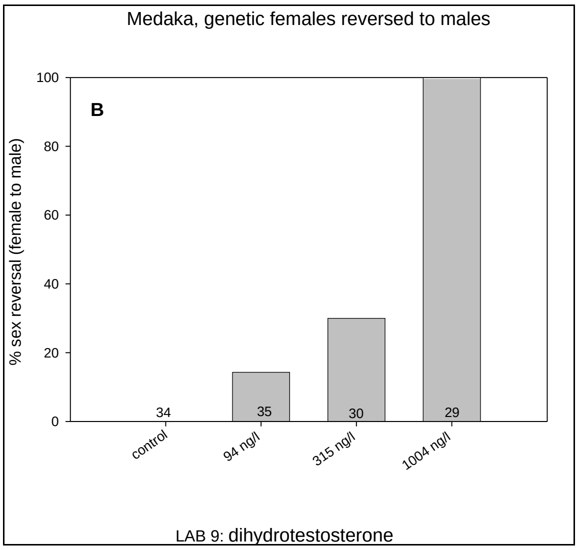


Figure 32: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05. B: Percentage sex reversed females

Three spined stickleback

Lab 6

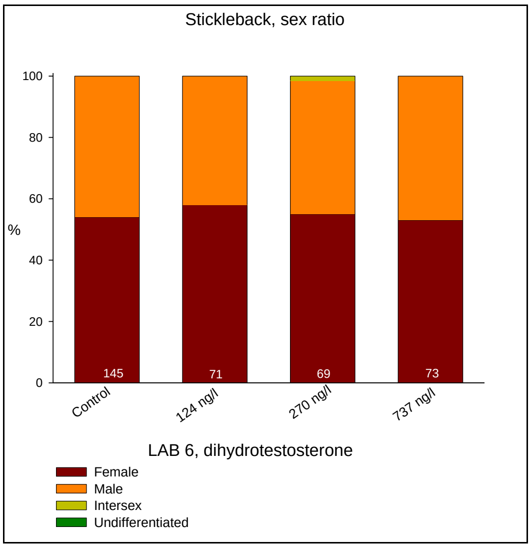


Figure 33: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

Lab 8

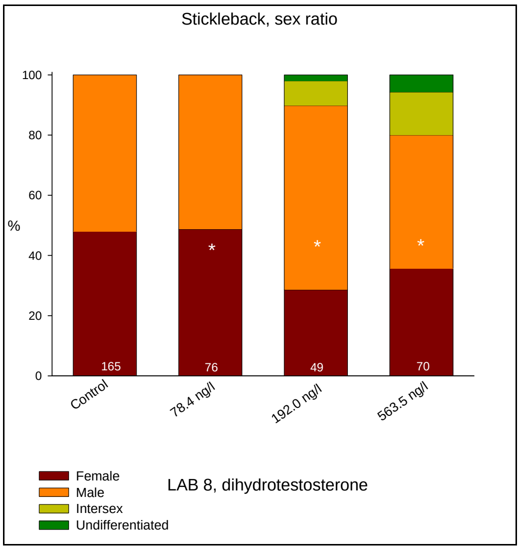


Figure 34: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to dihydrotestosterone. Numbers at bottom are N. P=0.05

4-tert pentylphenol

Japanese medaka

Lab 9

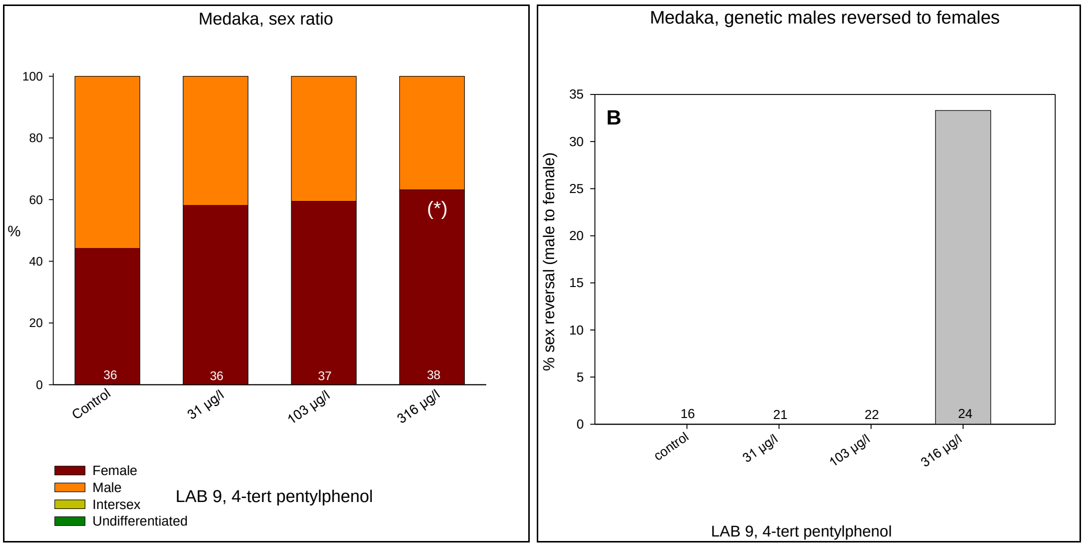


Figure 35: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 70 D exposure to 4-tert pentylphenol. Numbers at bottom are N. Asterisk in brackets: Genetic males changed to phenotypic females. P=0.05. B: Percentage sex reversed males.

Lab 10

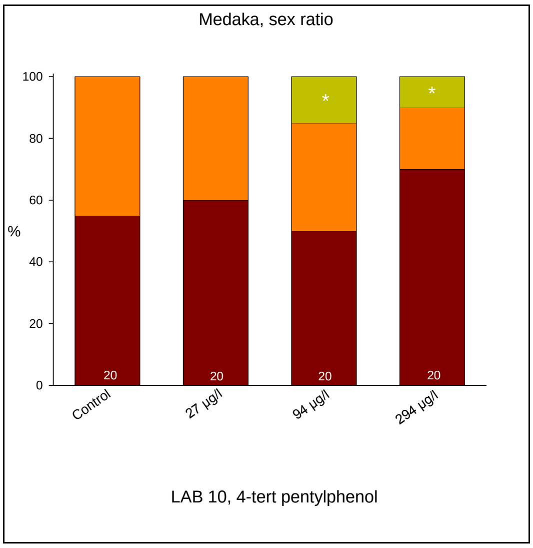


Figure 36: Sex ratio as percentage females, males, undifferentiated and intersex medaka after 70 D exposure to 4-tert pentylphenol. Numbers at bottom are N. P=0.05

17ß-estradiol

Three spined stickleback

Lab 6

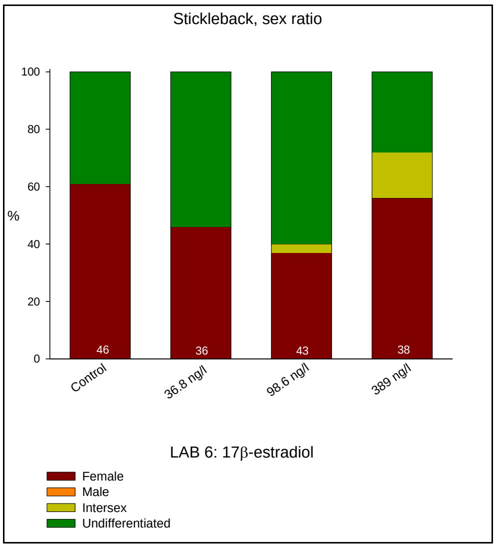


Figure 37: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to 17ß-estradiol. Numbers at bottom are N. P=0.05

Flutamide

Three spined stickleback

Lab 6

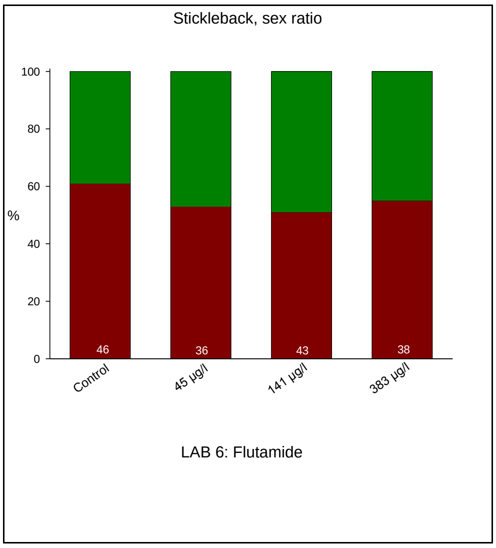


Figure 38: Sex ratio as percentage females, males, undifferentiated and intersex stickleback after 60 D exposure to flutamide. Numbers at bottom are N. P=0.05

KIDNEY EPITHELIUM HEIGHT

4-tert octylphenol

Three spined stickleback

Lab 6

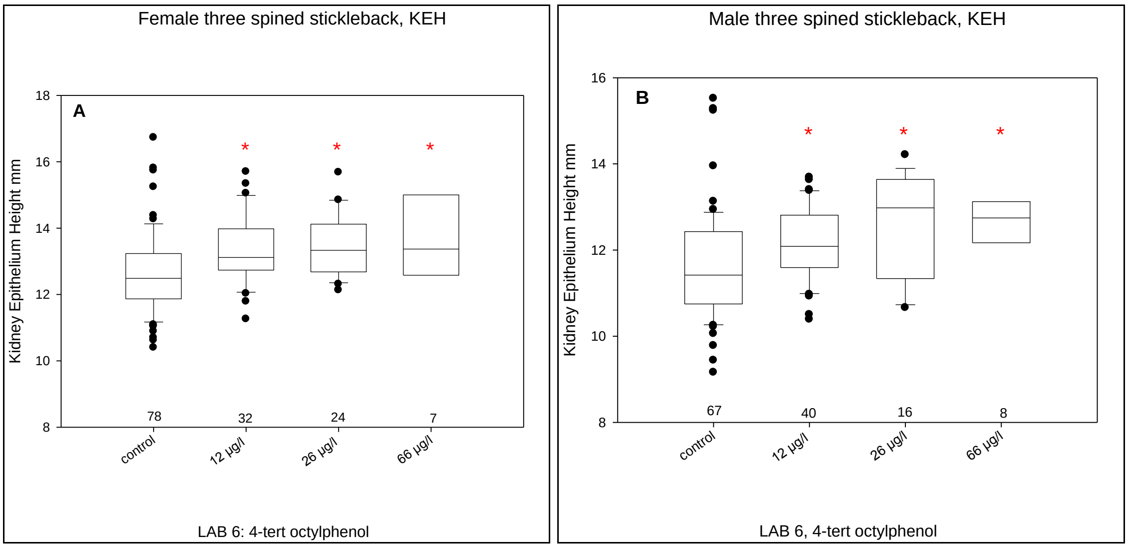


Figure 39: Kidney epithelium height (KEH) in mm after 60 D exposure to 4-tert octylphenol of female (A) and male (B) stickleback. Numbers at bottom are N. P=0.05

Dihydrotestosterone

Three spined stickleback

Lab 6

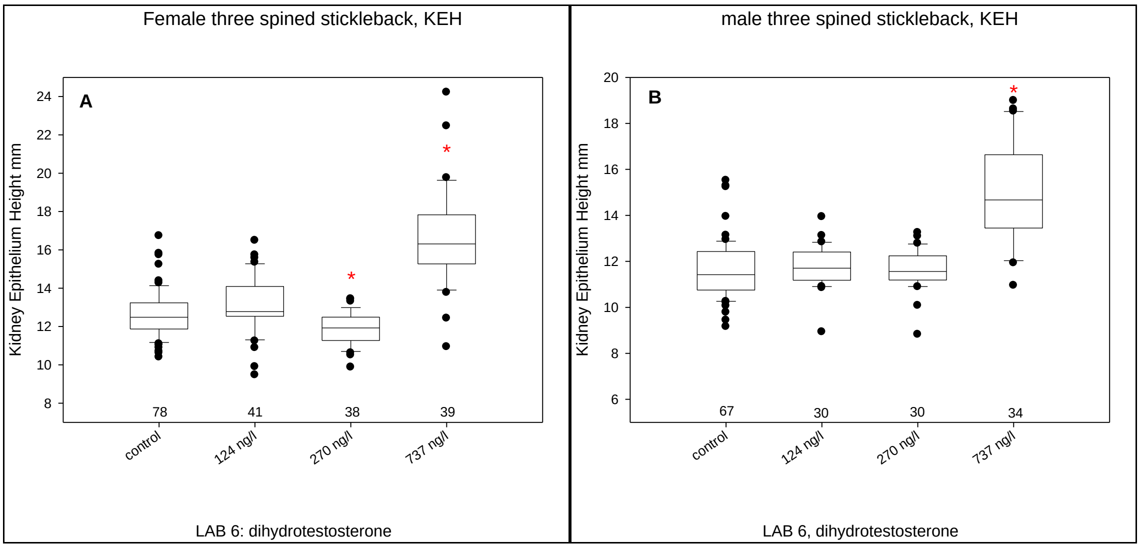


Figure 40: Kidney epithelium height (KEH) in mm after 60 D exposure to dihydrotestosterone of female (A) and male (B) stickleback. Numbers at bottom are N. P=0.05

17ß-estradiol

Three spined stickleback

Lab 6

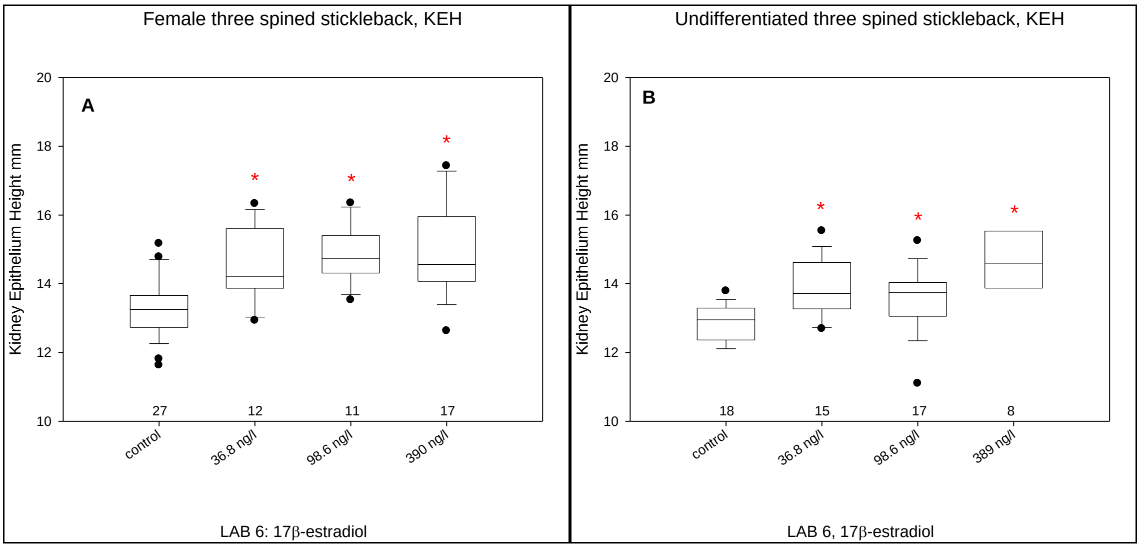


Figure 41: Kidney epithelium height (KEH) in mm after 60 D exposure to 17ß-estradiol of female (A) and undifferentiated (B) stickleback. Numbers at bottom are N. P=0.05

Flutamide

Three spined stickleback

Lab 6

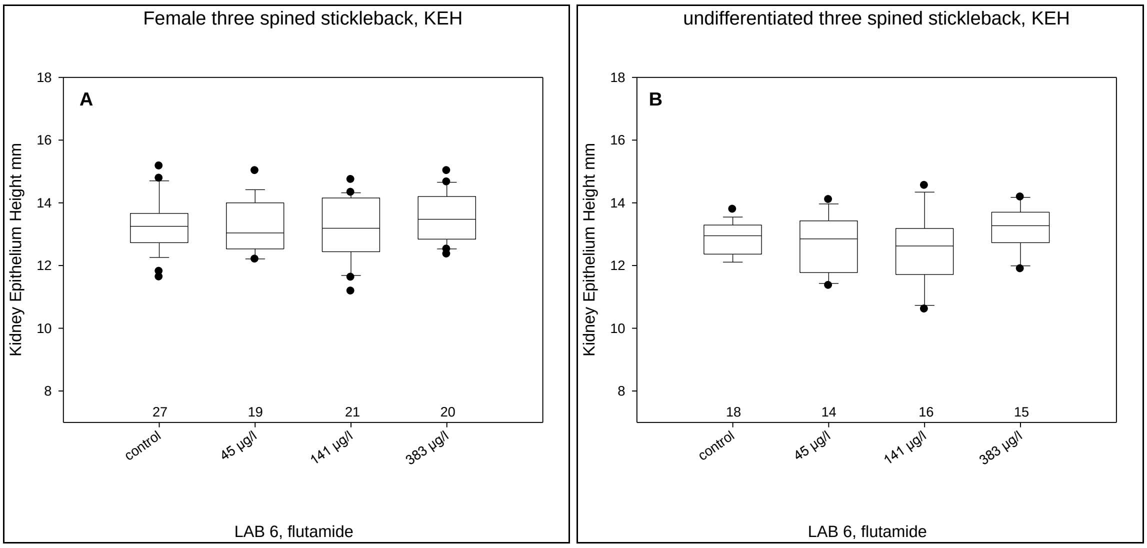


Figure 42: Kidney epithelium height (KEH) in mm after 60 D exposure to flutamide of female (A) and undifferentiated (B) stickleback. Numbers at bottom are N. P=0.05