Adopted:
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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Fish Sexual Development Test

INTRODUCTION

1. This Test Guideline (TG) is based on a decision from 1998 to develop new or update existing TGs for the screening and testing of potential endocrine disrupters. The Fish Sexual Development Test (FSDT) was identified as a promising test method covering a sensitive fish life stage responsive to both oestrogen and androgen-like chemicals. The test method went through an inter-laboratory validation exercise from 2006 to 2010, where Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and three spined stickleback (*Gasterosteus aculeatus*) were validated and fathead minnow (*Pimephales promelas*) was partially validated (41) (42) (43). This protocol includes Japanese medaka, the three-spined stickleback and zebrafish. The protocol is in principle an enhancement of TG 210: Fish, Early Life Stage Toxicity Test (1), where the exposure is continued until the fish are sexually differentiated, i.e. about 60 days post-hatch (dph) for Japanese medaka, the three-spined stickleback and zebrafish (the exposure period can be shorter or longer for other species that are validated in the future), and endocrine-sensitive endpoints are added. The FSDT assesses early life-stage effects and potential adverse consequences of putative endocrine disrupting chemicals (e.g. oestrogens, androgens and steroidogenesis inhibitors) on sexual development. The combination of the two core endocrine endpoints, vitellogenin (VTG) concentration and phenotypic sex ratio enable the test to indicate the mode of action of the test chemical. Due to the population-relevant change in phenotypic sex ratio, the FSDT can be used for hazard and risk assessment. However, if the test is used for hazard or risk assessment, the stickleback should not be used because the validation data available so far showed that in this species the alterations of phenotypic sex ratio by the test substances were uncommon.

2. The protocol is based on fish exposed via water to chemicals during the sex labile period in which the fish is expected to be most sensitive to the effects of endocrine disrupting chemicals that interfere with sexual development. Two core endpoints are measured as indicators of endocrine-associated developmental aberrations, the VTG concentrations and sex ratios (proportions of sex) determined via gonad histology. Gonadal histopathology (evaluation and staging of oocytes and spermatogenetic cells) is optional. Additionally, the genetic sex is determined whenever possible (e.g. in Japanese medaka and the three spined stickleback). The presence of a genetic sex marker is a considerable advantage as it increases the power of the sex ratio statistics and enables the detection of individual phenotypic sex reversal. Other apical endpoints that should be measured include hatching rate, survival, length and body weight. The test method might be adaptable to other species than those mentioned above provided that the other species undergo a validation equal to the one accomplished for Japanese medaka, the three-spined stickleback and zebrafish, that the control fish are sexually differentiated at the end of the test, that VTG levels are sufficiently high to detect significant chemical-related variations, and that sensitivity of the test system is established using endocrine active reference chemicals ((anti)-oestrogens, (anti)-androgens, aromatase inhibitors etc). In addition, any validation report(s) referring to FSDT data using other species should be reviewed by the OECD, and the validation outcome should be considered as satisfactory**.**

Initial considerations and limitations

3. VTG is normally produced by the liver of female oviparous vertebrates in response to circulating endogenous oestrogen (2). It is a precursor of egg yolk proteins and, once produced in the liver, travels in the bloodstream to the ovary, where it is taken up and modified by developing eggs. The VTG synthesis is very limited, though detectable, in immature fish and adult male fish because they lack sufficient circulating oestrogen. However, the liver is capable of synthesizing and secreting VTG in response to exogenous oestrogen stimulation (3) (4) (5).

4. The measurement of VTG serves for the detection of chemicals with oestrogenic, anti-oestrogenic, androgenic modes of action and chemicals that interfere with steroidogenesis as for example aromatase inhibitors. The detection of oestrogenic chemicals is possible via the measurement of VTG induction in male fish, and it has been abundantly documented in the scientific peer-reviewed literature. VTG induction has also been demonstrated following exposure to aromatisable androgens (6) (7). A reduction in the circulating level of oestrogen in females, for instance through the inhibition of the aromatase converting the endogenous androgen to the natural oestrogen 17β-oestradiol, causes a decrease in the VTG concentration, which is used to detect chemicals having aromatase inhibiting properties or steroidogenesis inhibitors more broadly (33). The biological relevance of the VTG response following oestrogenic/aromatase inhibition is established and has been broadly documented (8) (9). However, it is possible that production of VTG in females can also be affected by general toxicity and non-endocrine toxic modes of action.

5. Several measurement methods have been successfully developed and standardised for routine use to quantify VTG in blood, liver, whole body or head/tail homogenate samples collected from individual fish. This is the case for zebrafish, three-spined stickleback and Japanese medaka and also the partially validated species fathead minnow; species-specific Enzyme-Linked Immunosorbent Assay (ELISA) methods using immunochemistry for the quantification of VTG are available (5) (10) (11) (12) (13) (14) (15) (16) . In Japanese medaka and zebrafish, there is a good correlation between VTG measured from blood plasma, liver and homogenate samples although homogenates tend to show slightly lower values than plasma (17) (18) (19). Annex 5 provides the recommended procedures for sample collection for VTG analysis.

6. Change in the phenotypic sex ratio (proportions of sex) is an endpoint reflecting sex reversal. In principle, oestrogens, anti-oestrogens, androgens, anti-androgens and steroidogenesis inhibiting chemicals can affect the sex ratio of developing fish (20). It has been shown that this sex reversal is partly reversible in zebrafish (21) following oestrogen-like chemical exposure, whereas sex reversal following androgen-like chemical exposure is permanent (30). The sex is defined as female, male, intersex (both oocytes and spermatogenetic cells in one gonad) or undifferentiated, determined in individual fish via histological examination of the gonads. Guidance is given in Annex 7 and in the OECD Guidance Document on the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (22).

7. Genetic sex is examined via genetic markers when they exist in a given fish species. In Japanese medaka the female XX or male XY genes can be detected by Polymerase Chain-Reaction (PCR), or the Y-linked DM domain gene (DMY) can be analysed (DMY negative or positive) as described in (23) (24). In three-spined stickleback, there is an equivalent PCR method for genetic sex determination described in Annex 10. Where the genetic sex can be individually linked to the phenotypic sex, the power of the test is improved and therefore genetic sex should be determined in species with documented genetic sex markers.

8. The two core endocrine endpoints, VTG and sex ratio, can in combination demonstrate the endocrine mode of action (MOA) of the chemical (Table 1). The sex ratio is a population relevant biomarker (25) (26) and for some well defined modes of action, the FSDT results may be used for hazard

and risk assessment purposes when deemed appropriate by the regulatory agency. These modes of action are at present oestrogens, androgens and steroidogenesis inhibitors.

Table 1: Reaction of the endocrine endpoints to different modes of action of chemicals:

↑= increasing, ↓=decreasing, - =not investigated

MOA	VTG ♂	VTG ♀	Sex ratio	References
Weak oestrogen agonist	↑	↑	↑♀ or ↑Undiff	(27) (40)
Strong oestrogen agonist	↑	↑	↑♀ or ↑Undiff, No ♂	(28) (40)
Oestrogen antagonist	-	-	↓♀, ↑Undiff.	(29)
Androgen agonist	↓ or -	↓ or -	↑ ♂, No ♀	(28) (30)
Androgen antagonist	-	-	↑♀ ↑Intersex	(31)
Aromatase inhibitor	↓	↓	↓♀	(33)

9. The FSDT does not cover the reproductive life stage of the fish and therefore chemicals that are suspected to affect reproduction at lower concentrations than sexual development should be examined in a test that covers reproduction.

10. Definitions for the purpose of this Test Guideline are given in Annex 1.

11. The *in vivo* FSDT is intended to detect chemicals with androgenic and oestrogenic properties as well as anti-androgenic, anti-oestrogenic and steroidogenesis inhibiting properties. The FSDT validation phases (1 and 2) did cover oestrogenic, androgenic and steroidogenesis inhibiting chemicals. The effects in the FSDT of oestrogen- and androgen antagonists can be seen in

Table 1 but these MOA are less documented at present time.

PRINCIPLE OF THE TEST

12. In the test, fish are exposed, from newly fertilized egg until the completion of sexual differentiation, to at least three concentrations of the test substance dissolved in water. The test conditions should be flow-through unless not possible due to the availability or nature (e.g. limited solubility) of the test substance. The test starts with the placing of newly fertilized eggs (before cleavage of the blastodisc) in the test chambers. The loading of the chambers is described for each species in paragraph 27. For the validated fish species, Japanese medaka, the three-spined stickleback and zebrafish, the test is terminated at 60 dph. At test termination, all fish are euthanized humanely. A biological sample (blood plasma, liver or head/tail homogenate) is collected for VTG analysis from each fish and the remaining part is fixed for histological evaluation of the gonads to determine the phenotypic sex; optionally, histopathology (e.g. staging of gonads, severity of intersex) can be performed. A biological sample (the anal- or the dorsal fin) for the determination of the genetic sex is taken in species possessing appropriate markers (Annexes 9 and 10).

13. An overview of relevant test conditions specific for validated species: Japanese medaka, the three-spined stickleback and zebrafish is provided in Annex 2.

INFORMATION ON THE TEST SUBSTANCE

14. Results from an acute toxicity test or other short-term toxicity assay [e.g. TG 215 (34) and TG 210 (1)], preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapour pressure of the test substance are known and a reliable analytical method for the quantification of the chemical in the test chambers, with known and reported accuracy and limit of detection, is available.

15. Other useful information includes the structural formula, purity of the chemical, stability in water and light, pKa, Pow and results of a test for ready biodegradability (TG 301) (35).

Test acceptance criteria

16. For the test results to be acceptable the following conditions apply:

- The dissolved oxygen concentration should be at least 60 per cent of the air saturation value (ASV) throughout the test;
- The water temperature should not differ by more than ± 1.5 °C between test chambers at any one time during the exposure period and be maintained within the temperature ranges specified for the test species (Annex 2);
- A validated method for analysis of the exposure chemical with a detection limit well below the lowest nominal concentration should be available and evidence should be gathered to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within ±20% of the mean measured values;
- Overall survival of fertilized eggs in the controls and, where relevant, in the solvent controls, should be greater than or equal to the limits defined in Annex 2;

- Acceptance criteria related to growth and proportions of sex at termination of the test are based on data from the control groups (pooled solvent and water control unless they are significantly different, then solvent only):

		Japanese medaka	Zebrafish	Three-spined stickleback
Growth	Fish wet weight, blotted dry	>150 mg	>75 mg	> 120 mg
	Length (standard length)	>20mm	>14 mm	>20 mm
Sex ratio (% males or females)		30-70 %	30-70 %	30-70%

- When a solvent is used it should have no statistical significant effect on survival and should not produce any endocrine disrupting effects or other adverse effects on the early-life stages as revealed by a solvent control.

If a deviation from the test acceptance criteria is observed, the consequences should be considered in relation to the reliability of the test data and these considerations should be included in the reporting.

DESCRIPTION OF THE METHOD

Test chambers

17. Any glass, stainless steel or other chemically inert chambers can be used. The dimensions of the chambers should be large enough to allow compliance with loading rate criteria given below. It is desirable that test chambers be randomly positioned in the test area. A randomized block design with each concentration being present in each block is preferable to a completely randomized design. The test chambers should be shielded from unwanted disturbance.

Selection of species

18. Recommended fish species are given in Annex 2. The procedures for inclusion of new species are given in paragraph 2.

Holding of parental fish

19. Details on holding the parental fish under satisfactory conditions may be found in TG 210 (1). Parental fish should be fed once or twice a day with appropriate food.

Handling of embryos and larvae

20. Initially, embryos and larvae may be exposed within a main chamber in smaller glass or stainless steel chambers, fitted with mesh sides or ends to permit a flow of test substance through the chamber. Non-turbulent flow through these small chambers may be induced by suspending them from an arm arranged to move the chamber up and down but always keeping the organisms submerged.

21. Where egg containers, grids or meshes have been used to hold eggs within the main test chamber, these restraints should be removed after the larvae hatch, except that meshes should be retained to prevent the escape of the fish. If there is a need to transfer the larvae, they should not be exposed to the air and nets should not be used to release fish from egg containers. The timing of this transfer varies with the species and transfer may not always be necessary.

Water

22. Any water in which the test species shows control survival at least as good as in water described in Annex 3 is suitable as test water. It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example by reacting with the test substance) or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Total organic carbon, conductivity, pH and suspended solids should be measured, for example every three months where dilution water is known to be relatively constant in quality. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO4) and pesticides should be done, if water quality is questionable. Details about chemical analysis and water collection can be found in Paragraph 34.

Test solutions

23. Flow-through system should be used if practically possible. For flow-through tests, a system that continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, and saturator system) is necessary to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10% throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable (1). Care should be taken to avoid the use of plastic tubing or other materials, some of which may contain biologically active chemicals or may adsorb the test substance.

24. The stock solution should preferably be prepared without the use of solvents by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). If the test substance is difficult to dissolve in water, procedures described in the OECD Guidance for handling difficult substances should be followed (36). The use of solvents should be avoided but may be necessary in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are given in (36).

25. Semi-static test conditions should be avoided unless justification is provided on compelling reasons associated with the test substance (e.g. stability, limited availability, high cost or hazard). For the semi-static technique, two different renewal procedures may be followed. Either new test solutions are prepared in clean chambers and surviving eggs and larvae gently transferred into the new chambers, or the test organisms are retained in the test chambers whilst a proportion (at least two thirds) of the test water is changed daily.

PROCEDURE

Conditions of Exposure

Collection of eggs and duration

26. To avoid genetic bias, eggs are collected from a minimum of three breeding pairs or groups, mixed and randomly selected to initiate the test. For the three-spined stickleback, see the description of artificial fertilisation in Annex 11. The test should start as soon as possible after the eggs have been fertilized, the embryos preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage and no later than 12 h post fertilization. The test should continue until sexual differentiation in the control group is completed (60 dph for Japanese medaka, the three-spined stickleback and zebrafish).

Loading

27. The number of fertilized eggs at the start of the test should be at least 120 per concentration divided between a minimum of 4 replicates (square root allocation to control is accepted). The eggs should be randomly distributed (by using statistical tables for randomization) among treatments. The loading rate (for definition, see Annex 1) should be low enough in order that a dissolved oxygen concentration of at least 60% of the ASV can be maintained without direct aeration of the chambers. For flow-through tests, a loading rate not exceeding 0.5 g/l per 24 hours, and not exceeding 5 g/l of solution at any time is recommended. No later than 28 days post fertilisation the number of fish per replicate should be redistributed, so that each replicate contains as equal a number of fish as possible. If exposure related mortality occurs, the number of replicates should be reduced appropriately so that fish density between treatment levels is kept as equal as possible.

Light and temperature

28. The photoperiod and water temperature should be appropriate for the test species (see Annex 2 for experimental conditions for the FSDT).

Feeding

29. Food and feeding are critical, and it is essential that the correct food for each stage is supplied at appropriate time intervals and at a level sufficient to support normal growth. Feeding should be *ad libitum* whilst minimizing the surplus. To obtain a sufficient growth rate, fish should be fed at least twice daily (accepting once daily on weekends), separated by at least three hours between each feed. Surplus food and faeces should be removed, as necessary, to avoid accumulation of waste. As experience is gained, food and feeding regimes are continuously being refined to improve survival and optimize growth. Effort should therefore be made to confirm the proposed regime with acknowledged experts. Feeding should be withheld 24 hours before ending the test. Examples of appropriate food items are listed in Annex 2 (see also the OECD Fish Testing Framework (39).

Test concentrations

30. Test substances should be spaced as described in Annex 4. A minimum of three test concentrations in at least four replicates should be used. The curve relating LC_{50} to period of exposure in the acute studies available should be considered when selecting the range of test concentrations. Five test concentrations are recommended if the data are to be used for risk assessment.

31. Concentrations of the chemical higher than 10% of the acute adult LC_{50} or 10 mg/l, whichever is the lower, need not be tested. The maximum test concentration should be 10% of the LC_{50} on the larval/juvenile life-stage.

Controls

32. A dilution water control (≥ 4 replicates) and, if relevant, a solvent control (≥ 4 replicates) should be run in addition to the test concentrations. Only solvents that have been investigated not to have any statistical significant influence on the test endpoints should be used in the test.

33. Where a solvent is used, its final concentration should not be greater than 0.1 ml/L (36) and it should be the same concentration in all test chambers, except the dilution water control. However, every effort should be made to avoid the use of such solvent or keep solvent's concentrations to a minimum.

Frequency of Analytical Determinations and Measurements

34. Chemical analysis of the test substance concentration should be performed before initiation of the test to check compliance with the acceptance criteria. All replicates should be analyzed individually at the beginning and termination of the test. One replicate per test concentration should be analyzed at least once per week during the test, changing systematically between replicates (1,2,3,4,1,2….). If samples are stored to be analyzed at a later time, the storage method of the samples should be previously validated. Samples should be filtered (e.g. using a 0.45 µ pore size) or centrifuged to ensure that the determinations are made on the chemical in true solution.

35. During the test, dissolved oxygen, pH, total hardness, conductivity, salinity (if relevant), and temperature should be measured in all test chambers. As a minimum dissolved oxygen, salinity (if relevant), and temperature should be measured weekly, and pH, conductivity and hardness at the beginning and at the end of the test. Temperature should preferably be monitored continuously in at least one test chamber.

36. Results should be based on measured concentrations. However, if the concentration of the test substance in solution has been satisfactorily maintained within ±20% of the nominal concentration throughout the test, then the results can either be based on nominal or measured values.

Observations and measurements

Stage of embryonic development

37. The exposure should begin as soon as possible after fertilisation and before cleavage of the blastodisc commences and no later than 12 h post fertilisation to ensure exposure during early embryonic development.

Hatching and survival

38. Observations on hatching and survival should be made at least once daily and numbers recorded. Dead embryos, larvae and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to knock or physically damage adjacent eggs/larvae, these being extremely delicate and sensitive. Criteria for death vary according to life stage:

- for eggs: particularly in the early stages, a marked loss of translucency and change in coloration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance;

- for larvae and juvenile fish: immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque coloration of central nervous system and/or lack of reaction to mechanical stimulus.

Abnormal appearance

39. The number of larvae or fish showing abnormality of body form should be recorded, and the appearance and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several per cent in the control(s) in some species. Abnormal animals should only be removed from the test chambers on death. However, this may be in

conflict with some local regulations, therefore if considerable suffering (very severe and death can be reliably predicted) is observed, animals should be anaesthetized and euthanized according to the description in paragraph 44 and treated as mortality for data analysis.

Abnormal behaviour

40. Abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence and atypical feeding behaviour should be recorded at appearance.

Weight

41. At the end of the test all surviving fish should be euthanized (anaesthetized if blood samples should be taken), and individual wet weight (blotted dry) should be measured.

Length

42. At the end of the test, individual lengths (standard length) should be measured.

43. These observations will result in some or all of the following data being available for reporting:

- cumulative mortality;
- numbers of healthy fish at end of test;
- time to start of hatching and end of hatching;
- length and weight of surviving animals;
- numbers of deformed larvae;
- numbers of fish exhibiting abnormal behaviour.

Sampling of fish

44. Fish sampling is performed at termination of the test. Sampled fish should be euthanized with e.g. MS-222 (100-500 mg per L buffered with 200 mg $NaHCO_3$ per L) or FA-100 (4-allyl-2-methoxyphenol: eugenol) and individually measured and weighed as wet weight (blotted dry) or anaesthetized if a blood sample should be taken (see paragraph 49).

Sampling for VTG analysis and sex determination via histological evaluation

45. All fish should be sampled and prepared for analysis of sex and VTG. All fish should be analyzed histologically to determine sex. For the VTG measurements, a sub-sampling of at least 16 fish from each replicate is accepted. More fish should be analyzed for VTG if the results of the sub-sampling turn out to be unclear.

46. The sampling procedure for VTG and sex determination is dependent on the VTG analysis method:

Head/tail homogenate method for VTG analysis

47. The fish is euthanized. Head and tail of each fish are separated from the body of the fish by cuts made right behind the pectoral fins, and right behind the dorsal fin, using a scalpel (See Figure 1). The head and tail part from each fish are pooled, weighed and individually numbered, frozen in liquid nitrogen and stored at -70º or less for VTG analysis. The body part of the fish is numbered and fixed in an appropriate fixative for histological evaluation (22). By use of this method VTG and histopathology are evaluated on each individual and a possible change in the VTG level can thus be related to the phenotypic sex of the fish or genetic sex (Japanese medaka and the three-spined stickleback) of the fish. For further information see guidance for homogenization (Annex 5) and guidance for VTG quantification (Annex 6).

Liver homogenate method for VTG analysis

48. The fish is euthanized. The liver is dissected out and stored at -70º C or below. Recommended procedures for liver excision and pre-treatment are available in TG 229 (37) or 230 (38). Livers are then individually homogenized as described in TG 229 and 230. The supernatant collected is used for measuring VTG with a homologous ELISA technique (see Annex 6 for an example of quantification in zebrafish or TG 229 for Japanese medaka). Following this approach, it is also possible to have individual fish data on both VTG and gonad histology.

Blood plasma method for VTG analysis

49. Blood is collected from the anaesthetized fish by cardiac puncture, caudal vein or tail cutting, and centrifuged at 4º C for plasma collection. The plasma is stored at -70º C or below until use. The whole fish is euthanized and fixed for histology. Both plasma samples and fish are numbered individually to relate VTG levels to the sex of the fish.

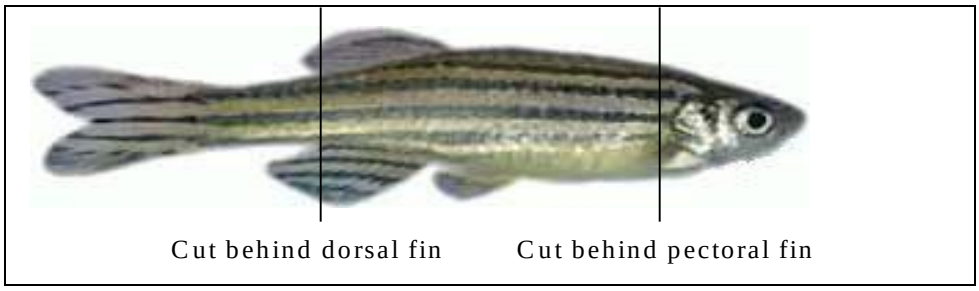


Figure 1: How to cut a fish for measurement of VTG in head/tail homogenate and histological evaluation of the mid section

Genetic sex determination

50. A biological sample for the determination of the genetic sex is taken from individual fish in species possessing appropriate markers. For Japanese medaka, the anal fin or dorsal fin is collected. A detailed description is given in Annex 9 including tissue sampling and sex determination by a PCR-method. Equally, for the three spined stickleback, a description of tissue sampling and a sex determining PCR-method is given in Annex 10.

VTG measurement

51. The measurement of VTG should be based upon a quantitative and analytically validated method. Information should be available upon the intra-assay and inter-assay variability of the method used in a given laboratory. The source of inter- and intra-laboratory variability is (most likely) based on the different developing stages of the fish population. Considering the variability of VTG measurement, NOECs based

on this endpoint alone should be treated with great care. Different methods are available to assess VTG production in the fish species considered in this assay. A measurement technique that is both relatively sensitive and specific is the determination of protein concentrations via enzyme-linked immunosorbent assay (ELISA). Homologous antibodies (raised against VTG of the same species) and most important homologous standards should be used.

Sex determination

52. Dependent on the VTG sampling procedure, whole fish or the remaining mid-section of each fish is placed in a pre-labelled processing cassette and fixed in an appropriate fixative for histological determination of sex (optionally also for evaluation of gonadal staging). Guidance on fixation and embedding is provided in Annex 7 as well as in the OECD Guidance Document on the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (22). After processing, the fish are embedded in paraffin blocks. The individuals should be placed longitudinally in the paraffin block. At least six longitudinal sections (3-5 µm in thickness) in a frontal plane including gonadal tissue from both gonads are taken from each individual. The interval between these sections should be approximately 50 µm for males and 250 µm for females. However, since each block will often contain males and females (if more than one individual are embedded in each block), the interval between sections from these blocks should be approximately 50 µm until at least six sections of the gonads from each male are obtained. Thereafter, the interval between sections can be increased to approximately 250 µm for the females. Sections are stained with haematoxylin and eosin and examined by light-microscopy with focus on sex (male, female, intersex or undifferentiated). Intersex is defined as presence of more than one oocyte in testis per six sections analysed or spermatogenic cells (yes/no) in ovaries. Histopathology and staging of ovaries and testis is optional but if investigated, the results should be statistically analyzed and reported. It should be noted that some fish species naturally lack a fully developed pair of gonads and only one gonad may be present (e.g. Japanese medaka and occasionally zebrafish). All such observations should be recorded.

53. Genetic sex determination in individual Japanese medaka is based on the presence or absence of the medaka male-sex determining gene, DMY, which is located on the Y chromosome. The genotypic sex of medaka can be identified by sequencing the DMY gene from DNA extracted from for instance a piece of anal fin or dorsal fin. The presence of DMY indicates a XY (male) individual regardless of phenotype, while the absence of DMY indicates a XX (female) individual regardless of phenotype (23). Guidance for tissue preparation and PCR method is given in Annex 9. The genetic sex determination in individual three-spined stickleback is also performed via a PCR method, described in Annex 10.

54. The occurrence of intersex (for definition, see Annex 1) should be reported.

Secondary sexual characteristics

55. Secondary sexual characteristics are under endocrine control in species like the Japanese medaka; therefore observations of physical appearance of the fish should if possible be made at the end of the exposure. In the Japanese medaka, the papillary formation on the posterior part of the anal fin in females is androgen sensitive. OECD TG 230 (38) provides relevant photographs of male secondary sex characteristics and androgenised females.

DATA AND REPORTING

Treatment of results

56. It is important that the strongest valid statistical test determine the endpoint. The replicate is the experimental unit but intra-replicate variability should be included in the statistical testing. A decision

flow-chart is available in Annex 8 to help with the most appropriate statistical test to use based on the characteristic of the data obtained from the test. Statistical significance level is 0.05 for all endpoints included.

Proportions of sex and genetic sex

57. The proportions of sex should be analysed for significant effect (NOEC/LOEC approach) of exposure by Jonckheere-Terpstra (Trend test) if a monotone dose-response exists. If non-monotonicity is found then a pair wise test should be applied: Use Dunnett´s test if normality and homogenous variance can be obtained. Use Tamhane-Dunnett if heterogeneous variance is present. Otherwise use exact Mann-Whitney test with Bonferroni-Holm adjustment. A flow chart describing the statistics of the proportions of sex is placed in Annex 8. The proportions of sex should be presented in tables as concentration proportions ± SD of males, females, intersex and undifferentiated. Statistical significance should be highlighted. Examples are presented in the FSDT Phase 2 validation report (42). Genetic sex should be reported as percentage of phenotypic sex reversal of males, females, intersex and undifferentiated.

VTG concentrations

58. VTG concentrations should be analysed for significant effect (NOEC/LOEC approach) of exposure. The Dunnett test is preferable to the t-test with Bonferroni correction. Where a Bonferroni correction is used, the Bonferroni-Holm correction is preferable. Allowance should be made for log-transformation of VTG to achieve normality and variance homogeneity. Next, if the concentration-response is consistent with monotonicity, then the JonckheereTerpstra test is preferable to any of the above. If T-tests or Dunnett's test is used, there is no need for a significant ANOVA F-test in order to proceed. For details see the flow chart in Annex 8. Results should be reported in tables as concentration means ± SD for males, females, intersex and undifferentiated separately. Statistical significance for phenotypic females and phenotypic males should be highlighted. Examples are presented in the FSDT Phase 2 validation report (42).

Test substance actual concentrations

59. The actual chamber concentrations of the test substance should be analysed in frequencies described in paragraph 34. Results should be reported in tables as mean concentration ± SD on replicate basis as well as on concentration basis with information on number of samples and with outliers from the mean treatment concentration ± 20% highlighted. Examples can be found in the FSDT Phase 2 validation report (42).

Interpretation of results

60. The test results should be interpreted with caution where measured test substance concentrations in test solutions occur at levels near the detection limit of the analytical method.

Test report

61. The test report should include the following information:

Test substance: Relevant physical-chemical properties; chemical identification; data including purity and analytical method for quantification of the test substance;

Test conditions: Test procedure used (e.g. flow-through semi-static/renewal); test design including test concentrations, method of preparation of stock solutions (in an Annex),

	frequency of renewal (the solubilising agent and its concentration should be given, when used); the nominal test concentrations, the means of the measured values and their standard deviations in the test chambers and the method by which these were attained (the analytical method used should be presented in an Annex). Evidence that the measurements refer to the concentrations of the test substance in true solution; Water quality within test chambers: pH, hardness, temperature and dissolved oxygen concentration; detailed information on feeding (e.g. type of food(s), source, amount given and frequency and analyses for contaminants (e.g. PCBs, PAHs and organochlorine pesticides) if relevant;
Results:	Evidence that controls met the validity criteria: data on hatching rate should be presented in tables as percentage per replicate and per concentration. Outliers from the acceptance criteria (in controls) should be highlighted. Survival should be presented as percentage per replicate and per concentration. Outliers from the validity criteria (in controls) should be highlighted; Clear indication of the results obtained on the different endpoints observed: embryo survival and hatching success; external abnormalities; length and weight; VTG measurements (ng/g homogenate, ng/mL plasma or ng/mg liver); gonadal histology, sex ratio, genetic sex data; incidence of any unusual reactions by the fish and any visible effects produced by the test substance.

62. The results should be presented as mean values ± standard deviation (SD) or standard error (SE). Statistics should be reported as a minimum as NOEC and LOEC and confidence intervals. The statistical flow chart (Annex 8) should be followed.

References

1. OECD (1992), *Fish, Early Life Stage Toxicity Test*, Test Guideline No. 210, Guidelines for the Testing of Chemicals, OECD, Paris.

2. Jobling, S., D. Sheahan, J.A. Osborne, P. Matthiessen, and J.P. Sumpter, 1996, "Inhibition of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to estrogenic alkylphenolic chemicals", Environmental Toxicology and Chemistry 15, pp. 194-202.

3. Sumpter, J.P. and S. Jobling, 1995, "Vitellogenesis As A Biomarker for Estrogenic Contamination of the Aquatic Environment", Environmental Health Perspectives 103, pp. 173-178.

4. Tyler, C.R., R.van Aerle, T.H. Hutchinson, S. Maddix, and H. Trip (1999), "An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin", Environmental Toxicology and Chemistry 18, pp. 337-347.

5. Holbech, H., L. Andersen, G.I. Petersen, B. Korsgaard, K.L. Pedersen, and P. Bjerregaard (2001a), "Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (Danio rerio)", Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 130, pp. 119-131.

6. Andersen, L., P. Bjerregaard, and B. Korsgaard (2003), "Vitellogenin induction and brain aromatase activity in adult male and female zebrafish exposed to endocrine disrupters", Fish Physiology and Biochemistry 28, pp. 319-321.

7. Orn, S., H. Holbech, T.H. Madsen, L. Norrgren, and G.I. Petersen (2003), "Gonad development and vitellogenin production in zebrafish (Danio rerio) exposed to ethinylestradiol and methyltestosterone", Aquatic Toxicology 65, pp. 397-411.

8. Panter, G.H., T.H. Hutchinson, R. Lange, C.M. Lye, J.P. Sumpter, M. Zerulla, and C.R. Tyler (2002), "Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances", Environmental Toxicology and Chemistry 21, pp**.** 319-326.

9. Sun, L.W., J.M. Zha, P.A. Spear, and Z.J. Wang (2007), "Toxicity of the aromatase inhibitor letrozole to Japanese medaka (Oryzias latipes) eggs, larvae and breeding adults", Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 145, pp. 533-541.

10. Parks, L.G., A.O. Cheek, N.D. Denslow, S.A. Heppell, J.A. McLachlan, G.A. LeBlanc, and C.V.Sullivan (1999), "Fathead minnow (Pimephales promelas) vitellogenin: purification, characterization and quantitative immunoassay for the detection of estrogenic compounds", Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 123, pp. 113-125.

11. Brion, F., B.M. Nilsen, J.K. Eidem, A. Goksoyr, and J.M. Porcher (2002), "Development and validation of an enzyme-linked immunosorbent assay to measure vitellogenin in the zebrafish (Danio rerio)", Environmental Toxicology and Chemistry 21, pp. 1699-1708.

12. Nishi, K., M. Chikae, Y. Hatano, H. Mizukami, M. Yamashita, R. Sakakibara, and E. Tamiya (2002), "Development and application of a monoclonal antibody-based sandwich ELISA for quantification of Japanese medaka (Oryzias latipes) vitellogenin", Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 132, pp. 161-169.

13. Hahlbeck, E., I. Katsiadaki, I. Mayer, M. Adolfsson-Erici, J. James, and B.E. Bengtsson (2004), "The juvenile three-spined stickleback (Gasterosteus aculeatus L.) as a model organism for endocrine disruption - II - kidney hypertrophy, vitellogenin and spiggin induction", Aquatic Toxicology 70, pp. 311-326.

14. Tatarazako, N., M. Koshio, H. Hori, M. Morita, and T. Iguchi (2004), "Validation of an enzyme-linked immunosorbent assay method for vitellogenin in the medaka", Journal of Health Science 50, pp. 301-308.

15. Eidem, J.K., H. Kleivdal, K. Kroll, N. Denslow, R. van Aerle, C. Tyler, G. Panter, T. Hutchinson, and A. Goksoyr (2006), "Development and validation of a direct homologous quantitative sandwich ELISA for fathead minnow (Pimephales promelas) vitellogenin. Aquatic Toxicology", 78, pp. 202-206.

16. Jensen, K.M. and G.T. Ankley (2006), "Evaluation of a commercial kit for measuring vitellogenin in the fathead minnow (Pimephales promelas)", Ecotoxicology and Environmental Safety 64, pp. 101-105.

17. Holbech, H., Petersen, G. I., Norman, A., Örn, S, Norrgren, L., and Bjerregaard, P (2001b), "Suitability of zebrafish as test organism for detection of endocrine disrupting chemicals. Comparison of vitellogenin in plasma and whole body homogenate from zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*)", Nordic Council of Ministers, TemaNord 2001:597, pp. 48-51.

18. Nilsen, B.M., K. Berg, J.K. Eidem, S.I. Kristiansen, F. Brion, J.M. Porcher, and A. Goksoyr (2004), "Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening", Analytical and Bioanalytical Chemistry 378, pp. 621-633.

19. Orn, S., S. Yamani, and L. Norrgren (2006), "Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17 alpha-ethinylestradiol and 17 beta-trenbolone", Archives of Environmental Contamination and Toxicology 51, pp. 237-243.

20. Scholz, S. and N. Kluver (2009), " Effects of Endocrine Disrupters on Sexual, Gonadal Development in Fish, Sexual Development 3, pp. 136-151.

21. Fenske, M., G. Maack, C. Schafers, and H. Segner (2005), " An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, Danio rerio", Environmental Toxicology and Chemistry 24, pp. 1088-1098.

22. OECD (2010), *Guidance Document on the Diagnosis of Endocrine-related Histopathology in Fish Gonads*, Series on Testing and Assessment No. 123, ENV/JM/MONO(2010)14, OECD, Paris.

23. Kobayashi, T., M. Matsuda, H. Kajiura-Kobayashi, A. Suzuki, N. Saito, M. Nakamoto, N. Shibata, and Y. Nagahama (2004), "Two DM domain genes, DMY and DMRT1, involved in testicular

differentiation and development in the medaka, Oryzias latipes", Developmental Dynamics 231, pp. 518-526.

24. Shinomiya, A., H. Otake, K. Togashi, S. Hamaguchi, and M. Sakaizumi (2004), "Field survey of sex-reversals in the medaka, Oryzias latipes: genotypic sexing of wild populations", Zoological Science 21, pp. 613-619.

25. Kidd, K.A., P.J. Blanchfield, K.H. Mills, V.P. Palace, R.E. Evans, J.M. Lazorchak, and R.W. Flick (2007), "Collapse of a fish population after exposure to a synthetic estrogen", Proceedings of the National Academy of Sciences of the United States of America 104, pp. 8897-8901.

26. Palace,V.P., R.E. Evans, K.G. Wautier, K.H. Mills, P.J. Blanchfield, B.J. Park, C.L. Baron, and K.A. Kidd (2009), "Interspecies differences in biochemical, histopathological, and population responses in four wild fish species exposed to ethynylestradiol added to a whole lake", Canadian Journal of Fisheries and Aquatic Sciences 66, pp. 1920-1935.

27. Panter, G.H., T.H. Hutchinson, K.S. Hurd, J. Bamforth, R.D. Stanley, S. Duffell, A. Hargreaves, S. Gimeno, and C.R. Tyler (2006), "Development of chronic tests for endocrine active chemicals - Part 1. An extended fish early-life stage test for oestrogenic active chemicals in the fathead minnow (Pimephales promelas)", Aquatic Toxicology 77, pp. 279-290.

28. Holbech, H., K. Kinnberg, G.I. Petersen, P. Jackson, K. Hylland, L. Norrgren, and P. Bjerregaard (2006), "Detection of endocrine disrupters: Evaluation of a Fish Sexual Development Test (FSDT)", Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 144, pp. 57-66.

29. Andersen, L., K. Kinnberg, H. Holbech, B. Korsgaard, and P. Bjerregaard (2004), "Evaluation of a 40 day assay for testing endocrine disrupters: Effects of an anti-estrogen and an aromatase inhibitor on sex ratio and vitellogenin concentrations in juvenile zebrafish (Danio rerio)", Fish Physiology and Biochemistry 30, pp. 257-266.

30. Morthorst, J.E., H. Holbech, and P. Bjerregaard (2010), "Trenbolone causes irreversible masculinization of zebrafish at environmentally relevant concentrations", Aquatic Toxicology 98, pp. 336-343.

31. Kiparissis,Y., T.L. Metcalfe, G.C. Balch, and C.D. Metcalf (2003), "Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (Oryzias latipes)", Aquatic Toxicology 63, pp. 391-403.

32. Panter, G.H., T.H. Hutchinson, K.S. Hurd, A. Sherren, R.D. Stanley, and C.R. Tyler (2004), "Successful detection of (anti-) androgenic and aromatase inhibitors in pre-spawning adult fathead minnows (Pimephales promelas) using easily measured endpoints of sexual development", Aquatic Toxicology 70, pp. 11-21.

33. Kinnberg, K., H. Holbech, G.I. Petersen, and P. Bjerregaard (2007), "Effects of the fungicide prochloraz on the sexual development of zebrafish (Danio rerio)", Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 145, pp. 165-170.

34. OECD (2000), *Fish, Juvenile Growth Test*, Test Guideline No. 215, Guidelines for the Testing of Chemicals, OECD, Paris.

35. OECD (1992), *Ready Biodegradability*, Test Guideline No. 301, Guidelines for the Testing of Chemicals, OECD, Paris.

36. OECD (2000), *Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures*, Series on Testing and Assessment No. 23, ENV/JM/MONO(2000)6, OECD, Paris.

37. OECD (2009), *Fish Short Term Reproduction Assay*, Test Guideline No. 229, Guidelines for the Testing of Chemicals, OECD, Paris.

38. OECD (2009), *21-Day Fish Assay: A Short Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition*, Test Guideline No. 230, Guidelines for the Testing of Chemicals, OECD, Paris.

39 OECD (2011), Fish Testing Framework (draft available at: http://www.oecd.org/document/12/0,3746,en_2649_34377_1898188_1_1_1_1,00.html)

40 Schäfers, C., Teigeler, M., Wenzel, A., Maack, G., Fenske, M., Segner, H (2007), " Concentration- and time-dependent effects of the synthetic estrogen, 17 alpha-ethinylestradiol, on reproductive capabilities of the zebrafish, Danio rerio" Journal of Toxicology and Environmental Health-Part A, 70, 9-10 pp 768-779.

41 OECD (2011), *Validation Report (Phase 1) for the Fish Sexual Development Test*, Series on Testing and Assessment No 141, ENV/JM/MONO(2011)22, OECD, Paris.

42 OECD (2011), *Validation Report (Phase 2) for the Fish Sexual Development Test*, Series on Testing and Assessment No 142, ENV/JM/MONO(2011)23, OECD, Paris.

43 OECD (2011), *Peer Review Report of the validation of the Fish Sexual Development Test*, Series on Testing and Assessment No 143, ENV/JM/MONO(2011)24, OECD, Paris.

ANNEX 1

ABBREVIATIONS & DEFINITIONS

Apical endpoint:	Causing effect at population level
ASV:	Air saturation value
Biomarker:	Causing effect at individual level
Dph:	Days post hatch
DMY:	Y-specific DM-domain gene required for male development in the medaka fish
ELISA:	Enzyme-Linked Immunosorbent Assay
Fish weight:	Fish wet weight (blotted dry)
FSDT:	Fish Sexual Development Test
HPG axis:	Hypothalamic-pituitary-gonadal axis
Intersex fish:	Fish with more than one oocyte in testis per 6 sections analysed or spermatogenetic cells in ovaries (yes/no)
Loading rate:	Wet weight of fish per volume of water
MOA:	Mode of action
RT-PCR:	Reverse Transcriptase Polymerase Chain-Reaction
TG:	Test Guideline
Undifferentiated fish:	Fish with gonads exhibiting no discernible germ cells.
VTG:	Vitellogenin

ANNEX 2

EXPERIMENTAL CONDITIONS FOR THE FSDT (freshwater species)

1. Recommended species	Japanese medaka (*Oryzias latipes*)	Zebrafish (*Danio rerio*)	Three-spined Stickleback (*Gasterostreus aculeatus*)
2. Test type	Flow-through or semi-static	Flow-through or semi-static	Flow-through or semi-static
3. Water temperature	25± 2°C	27 ± 2°C	20± 2°C
4. Illumination quality	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum
5. Light intensity	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
6. Photoperiod	12-16 h light, 8-12 h dark	12-16 h light, 8-12 h dark	16 h light, 8 h dark
7. Minimum chamber size	Individual chambers should contain a minimum of 7 L water volume	Individual chambers should contain a minimum of 7 L water volume	Individual chambers should contain a minimum of 7 L water volume
8. Volume exchanges of test solutions	Minimum of 5 daily	Minimum of 5 daily	Minimum of 5 daily
9. Age of test organisms at start of exposure	Newly fertilised eggs (Early blastula stage)	Newly fertilised eggs (Early blastula stage)	Newly fertilised eggs
10. No. of eggs per treatment	Minimum 120	Minimum 120	Minimum 120
11. No. of treatments	Minimum 3 (plus appropriate controls)	Minimum 3 (plus appropriate controls)	Minimum 3 (plus appropriate controls)
12. No. replicates per treatment	Minimum 4 (unless square root allocation to controls)	Minimum 4 (unless square root allocation to controls)	Minimum 4 (unless square root allocation to controls)

13. Feeding regime	Live *Artemia*, frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily	Special fry food, live *Artemia*, frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily	Live *Artemia*, frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily
14. Aeration	None unless DO concentration falls below 60% saturation	None unless DO concentration falls below 60% saturation	None unless DO concentration falls below 70% saturation
15. Dilution water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water
16. Test substance exposure duration	60-dph	60-dph	60-dph
17. Biological endpoints	Hatching success, Survival Gross-morphology, VTG gonadal histology, Genetic sex Sex ratio	Hatching success, Survival Gross- morphology, VTG gonadal histology, Sex ratio	Hatching success, Survival Gross- morphology, VTG gonadal histology, Sex ratio
18. Test acceptability criteria for pooled replicates of controls	Hatching success > 80% Post hatch survival ≥ 70% growth (Fish wet weight, blotted dry) > 150 mg Length (standard length) >20mm Sex ratio (% males or females) 30%-70%	Hatching success > 80% Post hatch survival ≥ 70% growth (Fish wet weight, blotted dry) > 75 mg Length (standard length) >14 mm Sex ratio (% males or females) 30%-70%	Hatching success > 80% Post hatch survival ≥ 70% growth (Fish wet weight, blotted dry) > 120 mg Length (standard length) >20 mm Sex ratio (% males or females) 30%-70%

ANNEX 3

CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATION
Particular matter	< 20 mg/L
Total organic carbon	< 2 mg/L
Unionised ammonia	< 1 ug/L
Residual chlorine	< 10 ug/L
Total organophosphorus pesticides	< 50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/L
Total organic chlorine	< 25 ng/L

ANNEX 4

FROM TG 215 /GUIDANCE ON TEST CONCENTRATIONS

Column (Number of concentrations between 100 and 10, or between 10 and 1)*						
1	2	3	4	5	6	7
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* A series of three (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage per volume or weight (mg/L or µg/L). Values can be multiplied or divided by any power of 10 as appropriate. Column 1 might be used if there was considerable uncertainty on the toxicity level.

ANNEX 5

GUIDANCE FOR HOMOGENISATION OF HEAD & TAIL FROM JUVENILE ZEBRAFISH, FATHEAD MINNOW, THREE SPINED STICKLEBACK AND JAPANESE MEDAKA

The purpose of this section is to describe the procedures that occur prior to the quantification of the VTG concentration. Other procedures that result in comparable VTG quantification can be used. It is an option to determine the VTG concentration in blood plasma or liver instead of head/tail homogenate.

Procedure

1. The fish are anaesthetised and euthanized in accordance with the test description.

2. The head and tail are cut of the fish in accordance with the test description. Important: *All dissection instruments, and the cutting board should be rinsed and cleaned properly (e.g. with 96% ethanol) between handling of each single fish to prevent "VTG pollution" from females or induced males to un-induced males.*

3. The weight of the pooled head and tail from each fish is measured to the nearest mg.

4. After being weighed, the parts are placed in appropriate tubes (e.g. 1.5 mL eppendorf) and frozen at –80 ºC until homogenisation or directly homogenised on ice with two plastic pistils. (Other methods can be used if they are performed on ice and the result is a homogenous mass). Important: *The tubes should be numbered properly so that the head and tail from the fish can be related to their respective body-section used for gonad histology.*

5. When a homogenous mass is achieved an amount of 4-10 time the tissue weight of ice-cold **homogenisation buffer*** is added (note the dilution). Keep working with the pistils until the mixture is homogeneous. Important note: *New pistils are used for each fish.*

6. The samples are placed on ice until centrifugation at **4°C** at 50000 g for 30 min.

7. Use a pipette to dispense portions of 20 to 50 µL (note the amount) supernatant into **at least two** tubes by dipping the tip of the pipette below the fat layer on the surface and carefully sucking up the supernatant without fat- or pellet fractions.

8. The tubes are stored at -80°C until use.

**Homogenisation buffer:*

50 mM Tris-HCl pH 7,4; 1% Protease inhibitor cocktail (Sigma): 12 mL Tris-HCl pH 7,4 + 120 µl Protease inhibitor cocktail (or equivalent protease inhibitor cocktails).
TRIS: TRIS-ULTRA PURE (ICN)
Protease inhibitor cocktail: From Sigma (for mammalian tissue) Product number **P 8340.**
NOTE: The homogenisation buffer should be used the same day as manufactured. Place on ice during use

ANNEX 6

GUIDANCE FOR QUANTIFICATION OF HEAD & TAIL HOMOGENATE VITELLOGENIN IN ZEBRAFISH (*DANIO RERIO*) (MODIFIED FROM HOLBECH ET AL., 2001). OTHER PROCEDURES USING HOMOGENOUS ANTIBODIES AND STANDARDS CAN BE USED.

1. Microtiterplates (certified Maxisorp F96, Nunc, Roskilde Denmark) previously coated with 5 μg/mL anti zebrafish lipovitellin-IgG are thawed and washed 3 times with washing buffer*.
2. Battelle zebrafish Standard AP4.6.04 (1.18 mg/mL (AAA)) is serially diluted to 0.2, 0.5, 1, 2, 5, 10 and 20 ng/mL in dilution buffer** and samples are diluted at least 200 times (to prevent matrix effect) in dilution buffer and applied to the plates. An assay control is applied in duplicate. 150 μL are applied to each well. Standards are applied in duplicate and samples in triplicate. Incubate over night at 4°C on a shaker.
3. The plates are washed 5 times with washing buffer*
4. HRP coupled to a dextran chain (e.g. AMDEX A/S, Denmark) and conjugated antibodies are diluted in washing buffer; Actual dilution differs by batch and age. 150 μL are applied to each well and the plates are incubated for 1 hour at room temperature on a shaker.
5. The plates are washed 5 times with washing buffer* and the bottom of the plates is carefully cleaned with ethanol.
6. 150 μL TMB plus*** are applied to each well. Protect the plate against light with tinfoil, and watch the colour development on a shaker.
7. When the standard curve is fully developed the enzyme activity is stopped by adding 150 μL 0.2 M H_2SO_4 to each well.
8. The absorbance is measured at 450 nm (e.g. on a Molecular Devices Thermomax plate reader). Data are analysed on the associated software (e.g. Softmax).

***Washing buffer:**

PBS-stock****	500.0	mL
BSA	5.0	g
Tween 20	5.0	mL

Adjust pH to 7.3 and fill to 5 L with millipore H_2O. Store at 4º C.

****Dilution buffer:**

PBS-Stock****	100.0	mL
BSA	3.0	g
Tween 20	1.0	mL

Adjust pH to 7.3 and fill to 1 L with millipore H_2O. Store at 4º C.

*** TMB plus is a "ready-to-use" substrate produced by KemEnTec (Denmark). It is sensitive to light. Store at 4º C.

*****PBS stock**

NaCl	160.0	g

KH_2PO_4	4.0	g
$Na_2HPO_4,2H_2O$	26.6	g
KCl	4.0	g

Adjust pH to 6.8 and fill with millipore H_2O to 2 L. Store at room temperature.

ANNEX 7

GUIDANCE FOR THE PREPARATION OF TISSUE SECTIONS FOR SEX DETERMINATION AND STAGING OF GONADS

The purpose of this section is to describe the procedures that occur prior to the evaluation of histological sections. Other procedures that result in similar sex determination and gonadal staging can be used. With a few exceptions, these procedures are similar for Japanese medaka (JMD) and zebrafish (ZF).

Euthanasia, Necropsy, and Tissue Fixation

Objectives:

1. Provide for the humane sacrifice of fish.
2. Obtain necessary body weights and measurements.
3. Evaluate secondary sex characteristics.
4. Dissect tissues for VTG analysis.
5. Fixation of the gonads.

Procedures:

1. Fish should be sacrificed immediately prior to necropsy. Therefore, unless multiple prosectors are available, multiple fish should not be sacrificed simultaneously.
2. Using the small dip net, a fish is removed from the experimental chamber and transported to the necropsy area in the transport container.
3. The fish is placed in the euthanasia solution. The fish is removed from the solution when there is cessation of respiration and the fish is unresponsive to external stimuli.
4. The fish is wet weighed.
5. For preparation of tissues for VTG analysis, the fish can be placed on a corkboard on the stage of a dissecting microscope.
   a. For zebrafish the head is cut right behind the pectoral fin and tail is cut right behind the dorsal fin.
   b. For Japanese medaka the abdomen is opened via a carefully made incision that extends along the ventral midline from the pectoral girdle to a point just cranial to the anus. Using the small forceps and small scissors, the liver is carefully removed.
6. Specimen for VTG analysis are placed in eppendorf tubes and immediately frozen in liquid nitrogen.
7. The carcass including the gonads is placed into a pre-labelled plastic tissue cassette, which is transferred into Davidson's or Bouin´s fixative. The volume of fixative should be at least 10 times the approximated volume of the tissues. The fixative container is gently agitated for five seconds to dislodge air bubbles from the cassette.
8. a. All tissues remain in Davidson's fixative overnight, followed by transfer to individual containers of 10 % neutral buffered formalin the next day. Containers with cassettes are gently agitated for 5 seconds to ensure adequate penetration of formalin into cassettes.
   b. Tissues remain in Bouins fixative for 24 h, followed by transfer to 70 % ethanol.

Tissue Processing

Objectives:

1. Dehydrate tissue for adequate penetration of paraffin.
2. Impregnate the tissue with paraffin to maintain tissue integrity and create a firm surface for microtomy.

Procedures:

1. Labelled tissue cassettes are removed from formalin/ethanol storage and the cassettes are placed in the processing basket(s). The processing basket is loaded in the tissue processor.
2. The processing schedule is selected.
3. After the tissue processor has completed the processing cycle, the basket(s) may be transferred to the embedded station.

Embedding

Objective:

Properly orient the specimen in solidified paraffin for microtomy.

Procedures:

1. The basket(s) of cassettes is/are removed from the processor and immersed in the paraffin-filled front chamber of the embedding station thermal console or the cassettes are moved to a separate paraffin heater.
2. The first cassette to be embedded is removed from the front chamber of the thermal console or the paraffin heater. The cassette lid is removed and discarded, and the cassette label is checked against the animal records to resolve potential discrepancies prior to embedding.
3. An appropriately sized embedding mould is selected.
4. The mould is held under the spout of the dispensing console and filled with molten paraffin.
5. The specimen is removed from the cassette and placed in the molten paraffin in the mould. This is repeated with 4-8 specimens for each paraffin mould. The position of individual fish is marked by putting fish no 1 in 180 degrees to fish 2-4/8.
6. Additional paraffin is added to cover the specimen.
7. The mould with the cassette base is placed on the cooling plate of the cryo console.
8. After the paraffin has solidified, the block (i.e., the hardened paraffin containing the tissues and the cassette base) is removed from the mould.

Microtomy

Objective:

Cut and mount histological sections for staining.

Procedures:

1. The initial phase of microtomy termed "facing" is conducted as follows:
    a. The paraffin block is placed in the chuck of the microtome.
    b. The chuck is advanced by rotating the microtome wheel and thick sections are cut from the paraffin surface of the block until the knife reaches the embedded tissues.
    c. The section thickness on the microtome is set between 3 – 5 microns. The chuck is advanced and multiple sections are cut from the block to remove any artefacts created on the cut surface of the tissue during rough trimming.
    d. The block can be removed from the chuck and placed facedown on ice to soak the tissue.
2. The next phase of microtomy is final sectioning and mounting of tissue sections on slides. These procedures are conducted as follows:
    a. If the block has been placed on ice, the block is removed from the ice and replaced in the chuck of the microtome.
    b. With the section thickness on the microtome set to 3 – 5 microns, the chuck is advanced by rotating the microtome wheel. Sections are cut from the block until a "ribbon" containing at least one acceptable section including the gonads has been produced. (As necessary during sectioning, the block may be removed from the chuck, placed on ice to soak the tissue, and replaced in the chuck.)
    c. The sections are floated flat on the surface of the water in the water bath. An attempt is made to obtain at least one section that contains no wrinkles and has no air bubbles trapped beneath it.
    d. A microscope slide is immersed beneath the best section, which is lifted out of the water using the slide. This process is referred to as "mounting" the section on the slide.
    e. Three sections are prepared for a set of fish. The second and third sections are taken at 50 micron intervals following the first section. If the fish are not embedded with their gonads in the same sectioning level, more sections are to be made to ensure that at least six sections including the gonads are obtained from each fish.
    f. With a slide-marking pen, the block number from which the slide was produced is recorded on the slide.
    g. The slide is placed in a staining rack.
    h. The block is removed from the chuck and placed facedown for storage.

Staining, Cover slipping, and Slide Labelling

Objectives:

- Stain the sections for histopathological examination
- Permanently seal mounted and stained tissues.

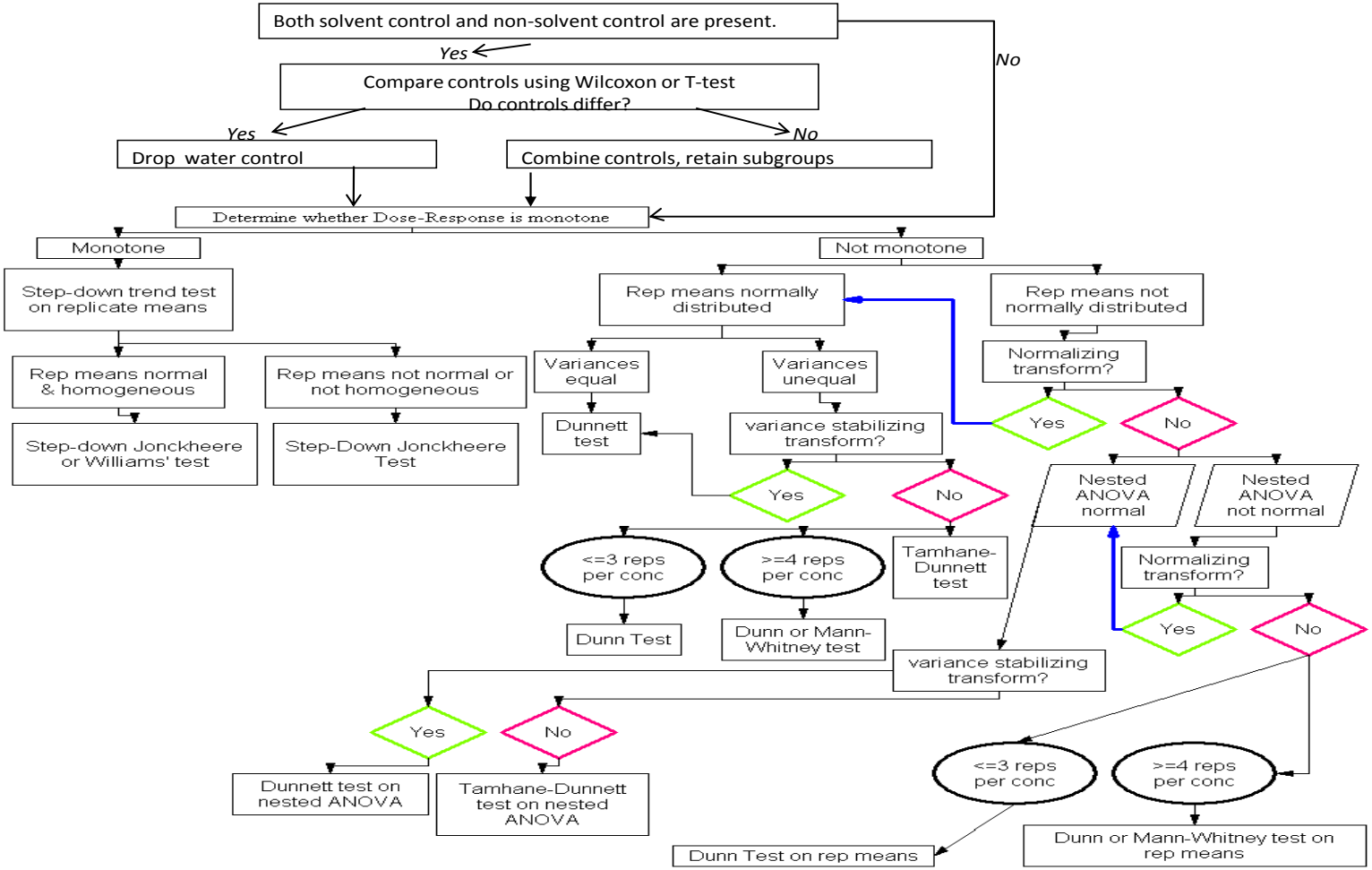
- Permanently identify stained sections in a manner that allows complete traceability.

Procedures:

1. Staining
    a. Slides are air-dried overnight before staining.
    b. The sections are stained by Hematoxylin-Eosin.
2. Cover slipping
    a. Cover slips can be applied manually or automatically.
    b. A slide is dipped in xylene or TissueClear, and the excess xylene/TissueClear is gently knocked off the slide.
    c. Approximately 0.1 ml of mounting medium is applied near the end of the slide opposite to the frosted end or on the cover slip.
    d. The cover slip is tilted at a shallow angle as it is applied to the slide.
3. Labelling
    a. Each slide label should contain the following information.
        i. Laboratory name
        ii. Species
        iii. Specimen No. / Slide No.
        iv. Chemical / Treatment group
        v. Date

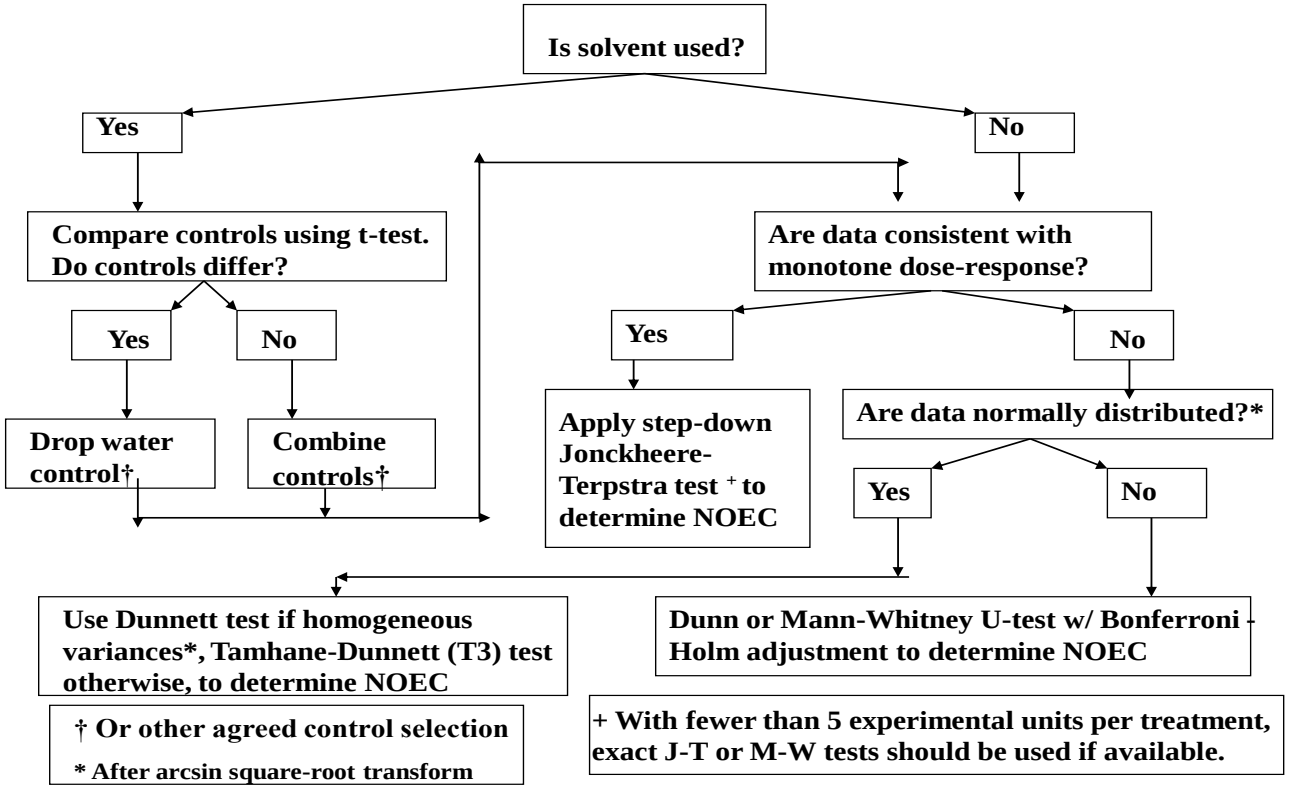
ANNEX 8

Statistical Flow Chart for vitellogenin analysis


Both solvent control and non-solvent control are present.
Yes
No
Compare controls using Wilcoxon or T-test
Do controls differ?
Yes
No
Drop water control
Combine controls, retain subgroups
Determine whether Dose-Response is monotone
Monotone
Not monotone
Step-down trend test on replicate means
Rep means normal & homogeneous
Rep means not normal or not homogeneous
Step-down Jonckheere or Williams' test
Step-Down Jonckheere Test
Rep means normally distributed
Rep means not normally distributed
Variances equal
Variances unequal
Dunnett test
variance stabilizing transform?
Yes
No
<=3 reps per conc
>=4 reps per conc
Tamhane-Dunnett test
Dunn Test
Dunn or Mann-Whitney test
Normalizing transform?
Yes
No
Nested ANOVA normal
Nested ANOVA not normal
Normalizing transform?
Yes
No
variance stabilizing transform?
Yes
No
Dunnett test on nested ANOVA
Tamhane-Dunnett test on nested ANOVA
<=3 reps per conc
>=4 reps per conc
Dunn Test on rep means
Dunn or Mann-Whitney test on rep means

Statistical Flow Chart for sex ratio analysis

Statistics Flow-Diagram for Sex Ratio Response-NOEC



ANNEX 9

GUIDANCE FOR TISSUE SAMPLING FOR GENETIC SEX DETERMINATION AND FOR GENETIC SEX DETERMINATION BY PCR-METHOD

Tissue sampling, preparation and storage before determination of genetic sex by PCR-method in medaka (Prepared by the Laboratory for Aquatic Organisms of Bayer CropScience AG)

1. With fine scissors the anal or the dorsal fin will be cut off in each individual fish and placed into a tube filled with 100 µL of extraction-buffer 1 (details on buffer preparation see below). The scissors will be cleaned after each single fish in a beaker filled up with distilled H_2O and dried with a paper tissue.
2. Now the fin-tissues will be homogenized by a micro tube teflon pistil for the lysis of cells. For each tube a new pistil will be used to prevent any contaminations. The pistils will be placed overnight in 0.5 M NaOH, rinse for 5 minutes in distilled H_2O and stored in ethanol or sterile after autoclave until use.
3. It is also possible to store the fin tissue without any extraction-buffer 1 on dry-ice and then at -80°C refrigerator to prevent any degeneration of the DNA. But the extraction runs better, if you extract the DNA at the same time (handling see above; samples should be thawed on ice after storaging at -80°C before the buffer will be filled in the tubes).
4. After homogenizing all tubes will be placed in a water bath and boiled for 15 minutes at 100°C.
5. Then 100 µL of the extraction buffer 2 (details on buffer preparation see below) will be pipetted into each tube. The samples will be stored at room temperature for 15 minutes and in the meantime they will be sometimes gently shaken by hand.
6. Afterwards all tubes will be placed in the water bath again and boiled for another 15 minutes at 100°C.
7. Until further analysis the tubes will be frozen at -20°C.

Buffer preparation:

1. PCR-buffer 1:
   a. 500 mg N-Lauroylsarcosine (e.g. Merck KGaA, Darmstadt, GE)
   b. 2 mL 5M NaCl
   c. ad 100 mL dest. H_2O
   → autoclave

2. PCR-buffer 2:
   a. 20 g Chelex (e.g. Biorad, Munich, GE)
   b. To swell in 100 mL dest. H_2O
   → autoclave

Determination of genetic sex (by PCR-method) in medaka (Prepared by the Laboratory for Aquatic Organisms of Bayer CropScience AG and Universität Würzburg Biozentrum)

The prepared and frozen tubes (described in the above section) will be thawed on ice. After that, they will be centrifuged using an Eppendorf centrifuge (30sec at max. speed, at room temperature). For the PCR, the clear supernatant separated from the precipitate will be used. It has absolutely to be avoided that any traces of Chelex (localized in the precipitate) are transferred to the PCR reaction, because this will interfere with the "Taq"-polymerase activity. The supernatant will be used directly or can be stored frozen (at -20 °C) and rethawed again in several cycles without negative impact on the DNA for later analyses.

1. Preparation of the "Reaction Mix" (25 µL per sample) :

Volume		Final Concentration	
Template DNA 0.5µl-2µl			
10xPCR-buffer with MgCl2		2.5µl	1x
Nucleotides (each of dATP, dCTP, dGTP, dTTP)		4µl (5mM)	200µM
Forward Primer (10µM)	(see below 3-5)	0.5µl	200nM
Reverse Primer (10µM)	(see below 3-5)	0.5µl	200nM
DMSO 1.25µl 5%			
Water (PCR grade)		up to 25µl	
Taq E- Polymerase		0.3µl	1.5U

10xPCR-buffer with $MgCl_2$: 670mM Tris/HCl (pH8.8 at 25°C), 160mM $(NH_4)_2SO_4$, 25mM MgCl2, 0.1%Tween 20

For each PCR (see below 3-5) the special primer as a new combination of "Reaction-Mix" and the adequate needed amount of template DNA for each sample (see above) is needed. The respective volumes will be transferred into new tubes using pipettes. After that all tubes will be closed, stirred (ca. 10 sec) and centrifuged (10 sec, at room temperature). Now the respective PCR-programs can be started. Additionally a positive control (exemplary DNA sample with known activity and clear results) and a negative control (1 µL dest. H_2O) will be used in each PCR-program.

2. Preparation of the agarose gel (1 %) – During running PCR-programs:

- Solve 3 g agarose in 300mL 1 x TAE-buffer (1% agarose gel)
- This solution should be boiled using an microwave (ca. 2-3 min)
- Transfer the hot solution into a special casting box, which lies on ice
- After ca. 20 min the agarose gel is ready to use
- Storage the agarose gel in 1x TAE-buffer until the end of the PCR-programs

3. Actin-PCR-program:

This PCR-reaction is aimed to demonstrate that the DNA in the sample is not harmed.

- Special primer:
  - "M act 1(upper/forward)" ➔ TTC AAC AGC CCT GCC ATG TA
  - "M act 2(lower/reverse)" ➔ GCA GCT CAT AGC TCT TCT CCA GGG AG
- Program:
  - 5 min 95 °C
  - Cycle (35-times):
    - Denaturation ➔ 45 sec at 95 °C
    - Annealing ➔ 45 sec at 56 °C
    - Elongation ➔ 1 min at 68 °C
  - 15 min 68 °C

4. X- and Y-Gene-PCR-program:

The samples with intact DNA will be used in this PCR-program to detect the X- and Y-Genes. Male DNA should show one double-band and female DNA should show one single band (after staining and gel-electrophoresis). For this program-run one positive control for males (XY-sample) and one for females (XX-sample) should be included.

- Special primer:
  - "PG 17.5" (upper/forward) ➔ CCG GGT GCC CAA GTG CTC CCG CTG
  - "PG 17.6" (lower/reverse) ➔ GAT CGT CCC TCC ACA GAG AAG AGA
- Program:
  - 5 min 95 °C
  - Cycle (40-times):
    - Denaturation ➔ 45 sec at 95 °C
    - Annealing ➔ 45 sec at 55 °C
    - Elongation ➔ 1 min 30 sec at 68 °C
  - 15 min 68 °C

5. Y-Gene-PCR-program as "control" for X- and Y-Gene-PCR-program:

This PCR-program verifies the results of the "X- and Y-Gene-PCR-program". The "male-samples" should show one band and the "female-samples" shouldn't show any band (after staining and gel-electrophoresis).

- Special primer:
  - "DMTYa (upper/forward)" ➔ GGC CGG GTC CCC GGG TG
  - "DMTYd (lower/reverse)" ➔ TTT GGG TGA ACT CAC ATG G
- Program:
  - 5 min 95 °C
  - Cycle (40-times):
    - Denaturation ➔ 45 sec at 95 °C
    - Annealing ➔ 45 sec at 56 °C
    - Elongation ➔ 1 min at 68 °C
  - 15 min 68 °C

6. Staining of the PCR-samples:

- Staining solution:
  - 50 % Glycerin
  - 100 mM EDTA
  - 1 % SDS
  - 0.25 % Bromphenolblue

- 0.25 % Xylenxyanol

- Pipette 1 µL of the staining solution into each single tube

7. Start of the Gel-Electrophoresis:

- The prepared 1 % agarose gel will be transferred into a gel-electrophoresis-chamber filled with 1 x TAE-Puffer
- 10 - 15 µL of each stained PCR-sample will be pipetted into an agarose gel slot
- Also 5 - 15 µL of the 1kb-"Ladder"(Invitrogen) will be pipetted into a separate slot
- Start the electrophoresis by 200 V
- Stop after 30-45 min

8. Determination of the bands:

- Clean the agarose gel in distilled H_2O
- Now transfer the agarose gel into Ethidiumbromid for 15 - 30 min
- After that, a picture of the agarose gel should be taken in an UV-light-box
- Finally the samples are analyzed in comparison to the positive control-band (or bands) and the ladder

ANNEX 10

GUIDANCE ON TISSUE SAMPLING FOR GENETIC SEX DETERMINATION BY PCR METHOD IN THE THREE-SPINED STICKLEBACK

Tissue sampling and DNA extraction

DNA can be extracted using a variety of commercially available reagents and both manual and automated extraction systems. The protocol used at the Cefas Weymouth laboratory is outlined below, and the alternative approaches have been added where appropriate.

1. With fine scissors, a small piece of tissue (10-20 mg) from the dorsolateral area (after removing the heaed and tail for VTG analysis), is removed from each individual fish. The tissue is added into a tube and either placed directly in liquid nitrogen (for storage at -80°C) or filled with 70% ethanol (for transport and subsequest storage at 4°C). The scissors are cleaned after each single fish in 70% ethanol then in distilled water and dried with tissue paper.

2. The ethanol (if present) is removed by aspiration and the tissue is digested overnight with proteinase K in 400 µl of ATL buffer (Qiagen). An aliquot (200 µl) of the digest is transferred to a 96-well S-block (Qiagen) and the DNA extracted in a 96-well format using the Qiagen Universal BioRobot and the QIamp Investigator BioRobot kit. The DNA is eluted in a 50 µl of DNase and RNase free water. If using hard tissues to extract DNA (such as a spine or a pectoral fin) it may be necessary to homogenise the sample in the lysis buffer using a FastPrep® tissue lyser or equivalent tissue disruption system.

Alternatively,

(a) the tissue is digested overnight with proteinase K in 400 µl of G2 lysis buffer (Qiagen) and DNA is extracted from 200 µl of the digest using either the EZ-1 DNA easy tissue kit and the EZ-1 biorobot or the DNA easy mini tissue kit. The DNA is eluted in a 50 µl volume.

(b) The tissues are processed using the DNAzol reagent. Briefly, tissue samples are lysed in 1ml of DNAzol for 10 minutes in a 1.5 ml micro centrifuge tube and then centrifuged at 13,000 rpm for 5 minutes to remove any particulate matter. The lysed sample is then transfered to a new 1.5 ml micro centrifuge tube containing 500 µl of 100% molecular grade ethanol and then centrifuged at 13,000 rpm for 10 minutes to precipitate the DNA. The ethanol is removed and replaced with 400 µl of 70% molecular grade ethanol, centrifuged at 13,000 rpm for 5 minutes and the DNA pellet is dissolved in 50 µl molecular DNase and RNase free water. Again, when using the hard tissues (pectoral fin) it may be necessary to homogenise the sample in the lysis buffer using a FastPrep® tissue lyser or equivalent tissue disruption system prior to extracting the DNA.

3. The DNA is stored at -20°C until required.

Important note: gloves must be worn during the procedures.

Polymerase chain reaction (PCR) analysis

Amplifications were performed using 2.5 µl of the DNA extract in a 50 µl reaction volume using the Idh locus primers (as described by Peichel et al., 2004. Current Biology 1:1416-1424):

- Forward primer 5’ GGG ACG AGC AAG ATT TAT TGG 3’
- Reverse primer 5’ TAT AGT TAG CCA GGA GAT GG 3’

There are numerous suppliers of suitable PCR reagents. The method outlined below is that currently used at the Cefas Weymouth laboratory.

1. Preparation of the “Reaction Mix” (50 µl per sample):

- A mastermix is prepared as follows. This can be prepared in advance and stored frozen at -20 °C until required. Make sufficient mastermix for a negative control (molecular biology grade water only).

	Volume (stock conc.)/ sample	Final Concentration
5xGoTaq® Reaction Buffer	10µl	1x
MgCl2	5 µl (25 mM)	2.5 mM
Nucleotides (dATP, dCTP, dGTP, dTTP)	0.5 µl (25 mM each)	250 µM each
Forward Primer	0.5µl (0.1 nmol/µl)	2.0 µM
Reverse Primer	0.5µl (0.1 mol/µl)	2.0µM
Molecular biology grade water	30.75 µl	
GoTaq polymerase	0.25 µl	1.25U

- Dispense 47.5 µl to a labeled 0.5ml thin walled PCR tube.
- Add 2.5 µl of the purified DNA to the appropriately labeled tube. Repeat for all samples and the negative control.
- Over lay with 2 drops of mineral oil. Alternatively, use a thermal cycler with a heated lid.
- Close the lids.
- Samples were denatured in a Peltier PTC-225 thermal cycler at 94 ± 2°C for 5 minutes followed by 39 cycles of 94 ± 2°C for 1 minute, 55 ± 2°C for 1 minute, 72 ± 2°C for 1 minute, and a final extension of 72 ± 2°C for 10 minutes.

2. Preparation of the agarose gel (2 %):

Traditionally the PCR products are resolved on a 20% agarose gel containing ethidium bromide. Capillary based electrophoresis systems can also be used.

- Weigh 2 g agarose in 100 ml 1 x TAE-buffer
- Heat in a microwave (ca. 2-3 min) to dissolve the agarose.

- Add 2 drops of ethidium bromide final concentration 0.5µg/ml
- Transfer the hot solution into the gel casting equipment.
- Allow the gel to harden

3. Gel-Electrophoresis:

- Transferred the agarose gel to the electrophoresis equipment and submerge in 1 x TAE-buffer
- Load 20 µl of each sample to a separate well, adding a molecular weight marker (100bp DNA ladder, Promega) to a spare well.
- Electrophoresis is performed at 120 V for 30-45 minutes.

4. Visualisation of the amplification products

If the ethidium bromide was incorporated in to the agarose gel as described above, the DNA products are visualised under a UV source. Alternatively the agarose gel is stained by covering the gel in a dilute solution of ethidium bromide (0.5 µg/ml in water) for 30 minutes prior to visualisation.

ANNEX 11

GUIDANCE FOR ARTIFICIAL FERTILISATION PROCEDURE FOR THE THREE-SPINED STICKLEBACK

The purpose of this section is to describe the procedures to obtain fertilised eggs from the three-spined stickleback in view of using them in the FSDT.

Procedures

Obtaining sperm from the males

1. A well-coloured male of the desired population is euthanized.
2. The testes are dissected from each side of the fish. *The testes are generally heavily pigmented, rod shaped structures that are readily apparent at the lateral midline of the body*. Use either of the following methods:
   a. Using a pair of fine scissors, begin at the cloaca and make a 1-1.5cm incision with a single snip angled at about 45 degrees.
   b. Use a scalpel to make a small incision in the side of the fish slightly posterior to the pelvis and just ventral of the lateral plates.
3. The testes are removed using fine forceps and placed into a petri dish.
4. Each testis is covered with 100 µl freshly made **Hank's final solution***.
5. The testes are finely diced by using a razor blade or scalpel. This will release sperm and give the Hank's solution a milky appearance.
6. The fluid containing sperm is added into a tube, while trying not to include any pieces of testes tissue when pipetting.
7. 800 µl of Hank's final solution are added into the tube and mixed well.
8. If required, the male can be preserved by fixing in 100% ethanol or other desired fixative. This is particularly important if the study is assigning parental origin of offsprings.

* Hank's Buffered Salt Solution (HBSS):

HBSS is needed to preserve the sperm whilst preparing for fertilisation. Important note*: Although most of the stock solutions required can be made in advance**, stock 5** and subsequently the **final solution**, should be made up **fresh** on the day of use.*

Stock 1

NaCl	8.00 g
KCl	0.40 g
Distilled water (DW)	100 ml

Stock 2

Na_2HPO_4 (anhydrous)	0.358 g
KH_2PO_4	0.60 g
DW	100 ml

Stock 3

$CaCl_2$	0.72 g

DW 50 ml

Stock 4
$MgSO_4,7H_2O$ 1.23 g
DW 50 ml

Stock 5 (FRESHLY PREPARED)
$NaHCO_3$ 0.35 g
DW 10 ml

Note: If you already have some of the above salts but with different water content (i.e. $2H_2O$ instead of anhydrous) you can still use it but first adjust weight based on molecular weight).

For Hank's final solution combine in the following order:

1. stock 1 1.0 ml
2. stock 2 0.1 ml
3. stock 3 0.1 ml
4. DW 8.6 ml
5. stock 4 0.1 ml
6. stock 5 0.1 ml

Mix well before use.

Fertilisation

1. Large, gravid females are identified from the desired population; females are ready for squeezing only when you can see eggs protruding from the cloaca. Ready females have the characteristic 'head up' posture.
2. Gently run a finger or thumb down the side of the fish towards the tail to encourage the expulsion of an egg sack into a fresh petri dish. Repeat on the other side and return the fish to its tank.
3. The eggs can be spread out (forming a monolayer) using a fine paintbrush. It is important to try and expose as many eggs as possible to the sperm so maximising the surface area of the eggs is helpful. Important note: *Keep the eggs humid by laying damp tissue around them (it is important the eggs do not touch water directly as this can prematurely harden the chorion preventing fertilisation).* There is a large variation in the number of eggs each female can produce but as an average, about 150 eggs should be easily obtained from a single gravid female.
4. 25µl of sperm in Hank's mixture is spread evenly over the whole surface of the eggs using the paintbrush. The eggs will quickly harden and change colour (within a minute) once fertilisation has begun. If the estimated number of eggs is more than 150, repeat the procedure. Similarly if the eggs don't harden within a minute add a bit more sperm. Important note: *Adding more sperm does not necessarily improve fertilisation rate.*
5. The eggs and the sperm solution should be left to 'interact' for at least 15 minutes and the fertilised eggs should be placed into the exposure aquaria within 1.5 hours post fertilisation.
6. The procedure is repeated using another female until the desired number of eggs is collected.
7. Spare few eggs from the last batch and fix them in 10% acetic acid.

Counting and distributing eggs in test aquaria

1. Eggs should be evenly distributed between each treatment level to avoid genetic bias. Each batch of fertilised eggs should be separated into equal size groups (as many as the treatment levels) by the use of a blunt instrument (i.e. wide-blade entomology forceps or use of an inoculation loop). If you aim for 4 replicates per treatment, with 20 eggs each then you need to distribute 80 eggs per exposure aquatia. Important note: It is advisable to add an extra 20% (i.e. 96 eggs per treatment level) until you are confident that you obtain 100% fertilisation rates.
2. Stickleback eggs are very prone to fungal infections outside the father-guarded nest. In this respect, treatment of all eggs with methylene blue during the first 5 days of the test is critically important. A stock solution of methylene blue is prepared at 1 mg/ml and added to the exposure aquaria to give a maximum final concentration of 2.125 mg/L. Important note: *Sticklebacks should not be exposed to methylene blue once hatched soothe system should be free of methylene blue by day 6.*
3. The eggs are inspected daily and any dead or unfertilised eggs are recorded as such. Important note: *The eggs should never be outside water until they hatch even for very brief periods.*