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Guidance Document on a Juvenile Medaka anti-androgen screening assay (JMASA)

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Juvenile Medaka Anti-Androgen Screening Assay (JMASA)

Foreward

This document contains the Guidance Document (GD) on the Juvenile Medaka Anti-Androgen Screening Assay (JMASA). The JMASA is a 28-day in vivo screening assay which can identify the potential of chemicals to have endocrine disrupting properties with anti-androgenic (and androgenic) activity in fish using Japanese (minami) medaka (*Oryzias latipes*). The JMASA is not intended to determine toxicity values for risk assessment (i.e., no observed effect concentration [NOEC]).
This GD and its accompanying validation report are the result of project 2.57 of the Test Guidelines Programme (TGP), led by Japan, which was included in the TGP workplan in 2016. The JMASA protocol has been discussed in the Validation Management Group on Ecotoxicity testing (VMG-Eco) from 2016-2022, and subsequently reviewed in 2022. The document was subsequently approved by the WNT at its 35th meeting in April 2023, and the Chemicals and Biotechnology Committee agreed to its declassification on 20 June 2023.

This document is published under the responsibility of the Chemicals and Biotechnology Committee.

1 INTRODUCTION

1. This Guidance Document (GD) describes the Juvenile Medaka Anti-Androgen Screening Assay (JMASA), a 28-day *in vivo* screening assay which can identify the potential of chemicals to have endocrine disrupting properties with anti-androgenic (and androgenic) activity in fish using Japanese (minami) medaka (*Oryzias latipes*) but is not intended to determine toxicity values for risk assessment. The JMASA is placed at level 3 of the OECD conceptual framework for the testing of endocrine disrupting chemicals (EDCs) in GD 150 (1). The concept of the JMASA is based on studies of papillary processes (papillae) on the anal fin, whose induction and development is considered as main secondary sex characteristics (SSC) and is regulated by the androgen receptor signalling pathway (2, 3). In Japanese medaka, papillary processes (PP) normally develop on the latter half of the anal fin of males, between 42 and 49 days post-fertilisation (dpf) and grow under androgenic control, while anti-androgens or chemicals which interfere with androgen biosynthesis can prevent their appearance or limit their number. The current OECD Test Guidelines (TG), Nos. 229 and 230 can detect chemicals having anti-androgenic activity in addition to other endocrine disrupting activities but the statistical power or sensitivity of TG 229/230 to detect anti-androgen is low, as reported in the TG themselves (4,5) and in the GD150 (1). The GD No.148 provides the Androgenized Female Stickleback Screen (AFSS), a 21-day fish assay which can identify potential anti-androgenic activity of a test chemical based on the reduction of spiggin in the kidneys of female sticklebacks in which the level of the specific biomarker protein (i.e., spiggin) is moderately induced by exposure to 5α-dihydrotestosterone (DHT) (6), but the assay may include some limitations: (a) the recommended test species, the three spined stickleback (*Gasterosteus aculeatus*), has not been adopted in current TGs for EDCs (4, 5, 7, 8); (b) the assay may not be applicable to detection of the potential activities that interfere with steroid synthesis and metabolism, since androgenized females rather than intact males are used; and (c) the assay requires particular techniques and apparatus to maintain the same concentration of DHT in all test chambers over the test duration. Overcoming limitations of the AFSS, the Rapid Androgen Disruption Activity Reporter (RADAR) assay (TG 251) using eleutheroembryos of transgenic Japanese medaka was developed to detect androgen-axis active chemicals (9) but the required maintenance of the DHT concentration in all test wells still remains while it provides the advantage of possibly reducing the numbers of adult and juvenile fish in testing.

2. Japan started to develop and optimize the method of *in vivo* assay using juvenile Japanese medaka, which is applicable to the screening of anti-androgenic chemicals, in 2016, in the program on endocrine disruption “EXTEND2016” (currently updated as “EXTEND2022”) (10). Originally, possible inclusion of this assay into GD No. 148 was discussed, the separate document was developed due to the difference in test species, endpoints, and (co-)exposure scheme, and then this document is developed as GD rather than TG as with AFSS because of the limited regulatory needs mainly in EXTEND 2022 in Japan.

3. In the JMASA as described in this GD, juvenile medaka are exposed to the test chemical during a limited part of their lifecycle (28 days) and the SSC (i.e., papillary processes on anal fin) are assessed at the termination of the exposure. The JMASA can detect potential activities that interfere with steroid synthesis and metabolism in addition to androgen receptor antagonistic and agonistic action in medaka: anti-androgens can be identified by an inhibition of SSC in genetic males and androgenic potency of test chemicals can be distinguished by induction of papillary processes in genetic females. When hepatic

vitellogenin (VTG) levels (protein concentration or mRNA expression) are optionally measured, other potential activities of the test chemical (e.g., (anti-)oestrogenicity or aromatase inhibition) can also be assessed. Other measurements including survival and growth (e.g., total length and body weight) are not considered as endpoints specific to endocrine disruption but are needed to confirm the statistical robustness of the assay and to identify toxic effects (i.e., not related to endocrine disruption) of the test chemical. Special caution is needed for the fact that the disruption of PP can reflect endocrine disruption with mechanism of action other than interaction of the test chemicals as agonist/antagonist with the androgen receptor as presented in the validation report (11). For example, the significant inhibition of SSC in genetic males is found at a concentration with no significant growth inhibition, this is definitively considered as anti-androgenic activity.

DEFINITIONS

4. Definitions used in this Guidance Document are given in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. In Japanese medaka, previous studies have shown that the development of papillary processes on the anal fin is under the control of a signalling pathway initiated from the androgen receptor (2, 3). These papillary processes are externally visible and are readily quantifiable secondary sex characteristics of males. The method for the JMASA described in this GD has undergone inter-laboratory validation studies, which demonstrated that an inhibition of the formation of papillary processes, leading to the presence of fewer papillary processes on the anal fin, is a valid marker for screening anti-androgenic chemicals in male medaka (11). Because female medaka maintain the capacity to develop male secondary sex characteristics, the assay is applicable for screening of androgenic chemicals.

6. The test species for the JMASA is limited to Japanese (minami) medaka and closely related fish species, since the apical endpoint (i.e., papillary processes on the anal fin) is a species-specific SSC. In addition, this GD recommends the use of juvenile medaka at 5-6 week post fertilization (wpf) (i.e., approximately 10 days prior to the formation of the SSC) as test organisms. It should be noted that any specific characteristics (e.g., the age at which SSC appear) in the males to be deployed in the test should be determined beforehand as the historical data in the lab, because the development of SSC might be dependent on the strain of medaka and/or the culturing conditions (e.g., stocking density or feeding regime). At higher temperatures even those within a range considered acceptable in this assay (24 – 26°C) can lead to genotype-phenotype mismatches such as 4% or less XX males at 25°C (12). As described in TG240 (8), steps should be taken to minimize the proportion of XX males in the population. Since the incidence of XX males appears to have a genetic component and is therefore heritable, monitoring the culture stock and ensuring that XX males are not used to propagate the culture stock is an effective means to reduce the incidence of XX males in the population. Careful interpretation of growth data is also recommended in order to avoid false-positive results, since growth may be impacted/influenced by systemic or non-endocrine modes of action which can affect the development of SSC during the exposure period (13).

7. Vitellogenin (VTG) is a precursor of egg yolk proteins, and is normally produced by the liver of female oviparous vertebrates in response to circulating endogenous oestrogen. Although this GD does not include VTG as an essential endpoint for the JMASA, the measurement of VTG levels can identify chemicals which have an interaction with the oestrogen pathway (e.g., oestrogenic/anti-oestrogenic chemicals or aromatase inhibitors). Because of the importance of this endpoint to enhance the discussion on further assessment, it is recommended to checked the optionality with the competent authority depending on the use of the test results. VTG levels can be assessed based on the hepatic protein

concentration quantitatively measured by a specific Enzyme-Linked Immunosorbent Assay (ELISA) method or the expression of vtg mRNA in liver samples by the quantitative PCR method, and the test laboratory should consider from its own experience which method to use in the test. Measurement of VTG levels in both males and females should also be considered when it is suspected that a test chemical can affect the oestrogen pathway in fish, although it is noted that there is a possibility of production/reduction of VTG in females also being affected by general or non-endocrine toxicity of test chemical.

PRINCIPLE OF THE TEST

8. The assay is initiated with juvenile medaka in which sex cannot be identified externally (i.e., papillary processes on the anal fin are not yet visible in males). At the termination of the assay, genetic sex is identified by the presence of the sex determining gene (i.e., dmy gene), for all surviving fish. Overviews of the relevant assay conditions are provided in Annex 2. The assay is conducted using a range of test chemical exposure concentrations (at least three test concentrations are used because this assay is considered as screening test), as well as a water control and a solvent control (if needed). Four replicate vessels are used for each concentration including controls and each vessel contains 7 fish, which matches the number of replicates in TG229 (4) and that of fish for TG203 (14) and ensures at least one male in at least three out of four vessels tank by 99.8% even if sex ratio of males is minimum at 30%. The exposure is conducted for 28 days and sampling of fish is carried out at the end of this period. On sampling at day 28, all fish are killed humanely (14).

9. Measurements in the course of the exposure include indications of general toxicity (i.e., mortality, abnormal behaviour and growth (e.g., total length and body weight)), as well as the endpoints (SSC and VTG (optional)) to evaluate (potential) interaction with the endocrine system. All endpoints are analysed in the context of determination of the genetic sex of the individuals. The number of papillary processes on the anal fin of genetic males serves for the detection of androgen receptor antagonists and agonists (and chemicals active on other androgen axis such as aromatase inhibitors if VTG is measured). The fewer number of papillary processes formed in genetic males has been demonstrated following exposure to androgen antagonists compared to control fish (3, 15). Also, it has been documented that the detection of androgen agonists is possible based on the appearance of SSC in females, (15, 16, 17) but the specificity as a trigger of papillary formation in female still remains unknown. If the VTG levels in males and females are measured, potential (anti-)oestrogenic activity and/or aromatase inhibition can also be identified (17).

INFORMATION ON THE TEST CHEMICAL

10. The water solubility (e.g., by TG105) and the vapour pressure (e.g., by TG104) of the test chemical should be known. Other useful information includes the structural formula, purity of the test chemical, stability in water and light, pKa, Pow and biodegradability (e.g., by TG301 or 310). A reliable analytical method for the quantification of the test chemical in the test solutions, with known and reported accuracy and limit of quantification, should be available.

11. Although not necessary to conduct the test, results from acute toxicity tests or short-term (chronic) toxicity tests (e.g., by TG 203, 210, 212 or 215), or other screening assays for endocrine disrupting chemicals (e.g., by TG229, 230 or 234), preferably performed with the same species (Japanese medaka), may provide useful additional information. Results of *in vitro* assays, such as the androgen receptor transcriptional activation assay, might also be useful.

12. If this GD is applied to the testing of a mixture, its composition should as far as possible be characterised, e.g., by the chemical identity of its constituents, their quantitative occurrence and their test

chemical-specific properties. Before the use of the GD for regulatory testing of a mixture, it should be considered whether it will provide acceptable results for the intended regulatory purpose.

VALIDITY OF THE TEST

13. For a test to be valid the following conditions apply:

- the mean mortality in the water controls and, where relevant, in the solvent controls, should not exceed 10 per cent at the end of the exposure period;
- the concentrations of the test chemical in each test vessel is satisfactorily maintained within ±20% of the mean measured value in the treatment group;
- the dissolved oxygen concentration should be at least 60 % of the air saturation value (ASV) in each test vessel throughout the exposure period;
- the water temperature should not differ by more than ± 1 °C between test vessels at any one time during the exposure period and should be maintained within the temperature range specified (Annex 2);
- the number of males per tank should be at least one in at least three out of four replicates;

14. If a minor deviation from the validity 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 report.

15. While not a validity criterion, it is recommended that the mean of the number of papillary processes on the anal fin at the termination of the assay in water control (and solvent control, if used) exceed at least 60 (preferably 80) since relatively large values in controls contribute to ensuring adequate statistical power in the endpoint assessment. The mean number of papillary processes at the termination of the assay is normally in the range between 60 and 100 (11)), but can differ between Medaka strains.

DESCRIPTION OF THE METHOD

Selection of species

16. The test species is Japanese (minami) medaka, *O. latipes*. Although other species of medaka (e.g., *O. sakaizumii*) may be adapted to a similar test protocol (18), the specific methods and observational endpoints detailed in this guidance are applicable to *O. latipes* alone.

Equipment

17. Normal laboratory equipment and especially the following:

a) dissolved oxygen and pH meters;
b) equipment for determination of water hardness and alkalinity;
c) adequate apparatus for temperature control and preferably continuous monitoring;
d) equipment for the determination of concentration of test chemical in test solution;
e) suitably accurate balance (i.e. accurate to ± 0.5 mg).

Holding of animals/selection of test organisms

18. Test fish should be selected from a laboratory population, preferably from a single stock of the same age of 5-6 wpf (35 dpf to ensure 10 days prior to the expected development of papillary processes

for NIES-R strain in National Institute for Environmental Studies of Japan based on preliminary work on growth curve (Annex 3)); the fertilised eggs which are spawned on the same day are cultured and the larval fish hatched are bred in conditions appropriate for the test species (10). The fish to be used in the test should be acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test itself. It is noted that the loading rate and the stocking density during the breeding and the acclimation period should be sufficient (i.e., < 5 g per L) to ensure appropriate growth for the test fish (paragraph 19). For each population (or batch) of fish, the mortalities are recorded during the acclimation period and the population (batch) in which the cumulative mortality in the last seven days of acclimation is less than 5 % should be used in the test itself.

19. The test should be initiated with juvenile fish at the age of 5-6 wpf (i.e., 35-42 days post fertilisation). Prior to the determination of the age of test organisms at initiation, the historical data of the relationship between the growth (i.e., total length and body weight), age, and the formation of SSC (i.e., the number of papillary processes on anal fin) should be recorded for the strain and the breeding condition. The initiation of the test should be approximately 10 days before the expression of SSC (first appearance of the papillary processes on anal fin of male fish). The recommended body weight is 60-80 mg (total length of 17-18 mm) although these values depend on the strain and breeding condition. At the beginning of exposure, acclimated fish, selected as described in paragraph 18, are randomly distributed to the test chambers.

Water

20. Any water in which the test species shows suitable long-term survival and growth may be used 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 complexation of the test chemical), or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g., Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g., Ca, Mg, Na, K, Cl, SO_4), ammonia, total residual chlorine pesticides, total organic carbon and suspended solids should be made, for example, on a bi-annual basis where a dilution water is known to be relatively constant in quality. If the water is known to be of variable quality the measurements have to be conducted more often; the frequency is dependent on how variable the quality is. Some chemical characteristics of an acceptable dilution water are listed in Annex 4.

Test chambers

21. Any glass, stainless steel or other chemically inert vessels can be used. Because silicone is known to have a strong capacity to absorb lipophilic chemicals, it is recommended that the use of silicone seals in contact with water should be minimised or mono-block glass (or stainless steel) tanks used. The dimensions of the vessels should be large enough to allow normal growth, maintenance of dissolved oxygen concentration and compliance with the loading rate throughout the exposure period. The minimum capacity of test chambers applicable to the test is stated in Annex 2. The test chambers should be shielded from unwanted disturbance. The test chambers should be randomly positioned in the test area.

Exposure systems

22. For the purpose of this assay, the use of a flow-through exposure system is recommended (19, 20, 21, 22, 23, 24, 25, 26, 27, 28). For the exposure system, it is noted that use of silicone tubing, e.g., for test solution delivery, should be minimised, because of its strong capacity for absorbing lipophilic

chemicals. Glass, stainless steel, or other chemically inert material that has not been contaminated during previous tests can be used.

Test Solutions

23. Test solutions of the chosen concentrations are generally prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test chemical in dilution water using mechanical means (e.g. stirring and/or ultrasonication). Saturation columns (or solubility columns) can be used for achieving a suitable concentrated stock solution. The use of a solvent carrier is not recommended. However, in case a solvent is necessary, as a last resort, a solvent control should be run in parallel, at the same solvent concentration as the chemical treatments; i.e., the solvent level should preferably be equal across all concentrations as well as the solvent control. For difficult to test chemicals, the GD 23 on aquatic toxicity testing of difficult chemicals and mixtures should be consulted (29). If a solvent is used, the choice of solvent will be determined by the chemical properties of the test chemical. Although GD 23 recommends a maximum concentration of 100 µl/L, the solvent concentration should be as low as possible, to avoid any potential effect of the solvent on endpoints to be measured (30).

24. A flow-through exposure system (e.g., constructed of metering pump, proportional diluter and saturator system) will continually dispense and dilute a stock solution of the test chemical in order to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at appropriate intervals, at least twice a week, and should not vary by more than 10% throughout the test. In the case that a test chemical markedly accumulates in fish and its concentration in test solutions decreases due to fish growth, it is recommended that the renewal rate of the test solution in each chamber be adapted to maintain test concentrations as constant as possible.

Conditions of exposure

25. A complete summary of test parameters and conditions can be found in Annex 2.

Duration of exposure

26. This test requires the exposure duration to be 28 days (i.e., 4 weeks). This exposure period will allow a satisfactory development of SSC in control fish in order to ensure reliable statistical results. At the termination of the exposure, all fish exposed to the test chemical and control(s) are sampled to assess the endpoints.

Number of test fish and loading

27. The number of test fish at the termination of the test should be sufficient to meet statistical requirements. Since sex ratio (% males or females) is normally ranged within 30-70 % in Japanese medaka (8), the use of 7 fish per tank for all treatments and controls is recommended in order to ensure at least one male per tank at the end of the exposure. Although this GD allows the use of less than 7 fish per tank at the initiation of the exposure, it should be considered that the results of the assay may be invalid, e.g., if a couple of tanks for the control group include no male fish at the end of the exposure. The test fish should be randomly distributed to the test chambers among the treatment and the control groups.

28. The loading rate (biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60% of the ASV value can be maintained without aeration throughout the test. For flow-through exposure condition, a loading rate not exceeding 5 g/L of solution at any time is recommended.

Light and temperature

29. The photoperiod and water temperature should be maintained appropriately for the test species throughout the test (Annex 2).

Feeding

30. Fish should be fed *ad libitum* with an appropriate food at a sufficient rate to support normal growth (refer to Table 1 of Annex 5 of TG240). Feeding should be approximately equal across replicates unless adjusted to account for mortality. As a general guide, the daily ration may be divided into two or three equal portions for multiple feeds per day, separated by at least three hours between each feed. A single, larger ration is acceptable, particularly over weekends. Feeding should be withheld 24 hours before ending the test. During the test, uneaten food and faecal material should be removed from the test chambers at appropriate intervals (e.g., by siphoning twice a week).

31. Fish can be fed live brine shrimp (*Artemia* spp.) *nauplii*, supplemented with a commercially available flake food if necessary. Food should be regularly analysed for contaminants such as organochlorine pesticides, polycyclic aromatic hydrocarbons (PAHs) and, polychlorinated biphenyls (PCBs). Food with an elevated level of endocrine active chemicals (e.g., phytoestrogens) that could compromise the response of the test should be avoided.

Test concentrations

32. Normally three test concentrations, spaced by a constant factor not exceeding 10, and a water control (and solvent control, if necessary) are required. A range of spacing factors between 3.2 and 10 is recommended. Each test concentration should include four replicate test chambers.

33. If available, prior knowledge of the toxicity of the test chemical, preferably conducted with the same species and life stage, can help in selecting appropriate test concentrations, e.g., from acute or chronic toxicity tests (14, 31, 32, 33), a range finding test, or other screening assays for endocrine disrupting chemicals (4, 5, 9). For this test, the highest test concentration should be set by the maximum tolerated concentration (MTC), 10 mg/L, or the maximum solubility in water, whichever is lowest. MTC is defined as the highest test concentration of the chemical which results in less than 10% acute mortality and is usually close to 1/10 or 1/3 of the LC_{50} of test chemical. Estimating MTC may require some professional judgment based on toxicity data and the chemical properties of the test chemical.

Controls

34. A water control and, if needed, a solvent control containing the solvent carrier which is evident to have no significant influence on the test organisms should be run in parallel with the test concentration series. Four replicate chambers should be used in controls.

35. Where solvent is used to prepare test solutions, the final concentration of the solvent in test solutions should not be greater than 0.1 ml/L and every effort to minimize the solvent concentration used should be made. Although it should be the same concentration in all test chambers (except the water control), should the diluter system being used make it technically difficult to achieve the same solvent concentration in all treatments, the solvent concentration in the solvent control should be equal to the highest solvent concentration in the treatment groups.

Frequency of Analytical Determinations and Measurements

36. Analytical measurement of test concentrations is compulsory. Prior to initiation of the exposure period, proper functioning of the chemical delivery system should be ensured (e.g., by measuring test concentrations). Analytical methods required should be established, including an appropriate limit of quantification (LOQ) and sufficient knowledge acquired on the test chemical stability in the test system. During the test, the concentrations of the test chemical should be determined at least once a week for all replicates in each treatment group, rotating among replicate tanks of the same treatment group, to characterise exposure conditions.

37. It is recommended that results be based on mean measured concentrations. However, if measured concentrations of the test chemical in solution have been satisfactorily maintained within ± 20% of the nominal concentrations throughout the test, then the results can either be based on nominal or mean measured values but the study author should check the preference of the regulatory authority.

38. During the test, the flow rates of dilution water and stock solution should be checked at intervals suitable for the system used (e.g., three times a week). For all test chambers, dissolved oxygen, temperature, and pH of test solutions should be measured at least once a week. Additionally, temperature of test solution should preferably be monitored continuously in at least one replicate tank for all treatments. Total hardness and alkalinity should be measured in the controls and one replicate at the highest concentration during the test.

Observations and measurements of fish

39. During the test, observations on survival of fish should be made daily. Any mortality should be recorded in each test chamber and the dead fish should be removed from the chambers as soon as possible but should not be replaced. Sex determination of dead fish is not needed.

40. Abnormalities in behaviour (e.g., hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding) and external appearances (e.g., haemorrhage, discoloration) on fish should also be noted. These abnormal responses may include signs of general toxicity of the test chemical and support the interpretation of data. Additionally, reproductive behaviour such as spawning status in females and territorial behaviour in males should preferably be noted, since such data may provide useful qualitative information to the evaluation of effect of test chemical on endocrine system of test fish.

41. At the termination of exposure, for all surviving fish, morphometric measurements are performed (e.g., total length and body weight). It is important to evaluate growth during the exposure period between the treatments and the controls, based on the results of the morphometry at the termination of exposure, since reduced growth, caused by a general toxic effect of the test chemical, may lead to a significant delay in development of papillary processes on the anal fin (15). In the case where an influence on growth is evident in the fish exposed to test chemical, the results of SSC should be interpreted in consideration of a possible toxic effect of the test chemical.

Sampling and endpoint assessment

Sampling of fish

42. At day 28 of the exposure (i.e., at the termination of the exposure), the fish should be euthanised with appropriate amounts of anaesthetic solution (e.g., Tricaine methane sulfonate, MS-222 (CAS.886-86-2), 100-500 mg/L) buffered with 300 mg/L $NaHCO_3$ (sodium bicarbonate, CAS.144-55-8) to reduce mucous membrane irritation. If fish are showing signs of considerable suffering (very severe and death can be

reliably predicted) and considered moribund, animals may be anaesthetised and euthanised and treated as mortality for data analysis. When a fish is euthanised due to morbidity, this should be noted and reported.

Tissue sampling

43. If VTG assessment is required, the fish are dissected and the liver is collected from each individual. The liver samples should immediately be stored at ≤-70 °C until the VTG protein (or *vtg* mRNA) measurements. For verification of genetic sex, a small portion of tissue, e.g., caudal fin, is sampled from each fish, and the samples adequately preserved e.g., in deep-freezing at ≤–30 °C or in ethanol, until *dmy* gene analysis. The caudal region of the fish, including the anal fin, is preserved in an appropriate fixative (e.g., 10% neutral buffered formalin) or photographed so that papillary processes on the anal fin can be counted at a later date. If desired, other tissues (i.e., gonad) may be sampled and preserved at this time. Special caution should be taken to minimize the cross-contamination of the tissue samples.

Secondary sex characteristics

44. In Japanese medaka, only sexually mature males have papillary processes, which are formed on the joint plates of certain anal fin rays as an SSC under normal circumstances, providing a potential biomarker for androgen receptor agonists and antagonists. The method of quantification of SSC, i.e., counting the number of joint plates with papillary processes, is given in Annex 5. For genetic male fish, the mean numbers of joint plates with papillary processes in each test chamber are used to identify the anti-androgenic potency of a test chemical. Also, the mean values in genetic females can be used to assess any androgenic activity of a test chemical. However, the results of SSC should be interpreted with caution if an influence of the test chemical on growth can be identified from the morphometric data (15).

Vitellogenin

45. VTG analysis, based on VTG protein concentrations or *vtg* mRNA expression, may optionally be conducted, to assess the potential for activities other than (anti-)androgenic effects of the test chemical (e.g., (anti-)oestrogenicity or aromatase inhibition). Liver VTG concentration should be quantified by ELISA method with a homologous antibody. It is also recommended that the ELISA used should be capable of detecting VTG levels as low as a few ng/mg tissue (e.g., 1 ng/mg liver), which is the background level in the control males (34). Example procedures for VTG ELISA are given in Annex 6 of OECD TG230 (5). Alternatively, the methods for *vtg* mRNA quantification which have been established by the U.S. EPA, i.e., *vtg* I gene mRNA extraction from a liver sample and quantification of the number of copies of the *vtg* I gene (per ng of total mRNA) by quantitative RT-PCR, can be used (35). For the VTG assessment, the selection of techniques used in the test may depend on the experience of the laboratory.

Determination of genotypic sex

46. The genotypic sex of test fish is determined by an identified and sequenced gene (i.e., the *dmy* gene) which is located on the Y chromosome (36). The presence of the *dmy* gene indicates an XY individual, regardless of phenotype, while the absence of the *dmy* gene indicates an XX individual, regardless of phenotype (37, 38). For *dmy* gene analysis, isolation of deoxyribose nucleic acid from biological samples (e.g., caudal fin) can be performed with commercially available reagents. The presence or absence of the *dmy* gene can be determined by the polymerase chain reaction (PCR) method. Several suggested examples of protocols and possible primers can be referred to for the determination of genetic sex (9, 36, 38, 39). Instead of the PCR method, real-time PCR assay methods, which the laboratory of the U.S. EPA have established, can be used (35). It is recommended to re-analyse for the *dmy* gene using

different primers if an individual in which phenotypic sex may be inconsistent with genetic sex is found (i.e., a possible spontaneous sex-reversed XX male) (37).

2 DATA AND REPORTING

Statistical analysis

47. Since genotypic sex is determined for all test fish, the data (on replicate mean basis) should be analysed for each genotypic sex separately (i.e., XY males and XX females). Failure to do this will greatly reduce the statistical power of any analysis. Statistical analyses of the data should preferably follow procedures described in the GD; Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (40). The decision tree for statistical analysis is provided in Annex 6.

Solvent controls

48. If a solvent is used, the potential effects of the solvent on test fish should be evaluated. For the endpoint measurements, a statistical comparison between the solvent and the water control group is performed. Effects on survival or growth occurring in the solvent control, when compared to the water control, should be reported and discussed in the context of the reliability of the test data. If the two control groups statistically differ, the data from the treatment groups exposed to the test chemical should be compared to the solvent control unless it is evident that comparison to the water control should be preferred. If there is no statistically significant difference between the two control groups, it is recommended that the treatments exposed to the test chemical are compared with the pooled data of the two controls, unless it is known that comparison to either the water or solvent control group only should be preferred. The study author should check the preference of the regulatory agencies before the handling of these control data.

Test report

49. The test report should include the following:

Test chemical:

Mono-constituent substance

- chemical identification (e.g., IUPAC, CAS name, CAS number, SMILES, InChI code);
- physical appearance, water solubility, and additional relevant physicochemical properties; structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

Multi-constituent substance, UVCBs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test species:

- scientific name, strain if available, source and method of harvesting of the fertilised eggs and subsequent handling.

Test conditions:

- test procedure used (e.g., flow-through or semi-static, loading);
- photoperiod(s);
- test design (e.g., number of test chambers and replicates, material of test chamber, size and water volume of chamber, number of test fish per replicate);
- method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration should be given, when used);
- method of dosing the test chemical (e.g., pumps, diluting systems);
- 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 (e.g., the recovery efficiency, the limit of quantification), evidence that the measurements refer to the concentrations of the test chemical in true solution;
- water quality within test chambers (e.g., pH, hardness, temperature, dissolved oxygen concentration);
- information on feeding (e.g., type of food(s), source, amount given and frequency, analyses for contaminants such as PCBs, PAHs and organochlorine pesticides, if relevant).

Results:

- evidence that controls met the overall validity criteria;
- data on mortalities occurring in any of the test concentrations and controls;
- approach for the statistical analysis and treatment of data (i.e., statistical methods used);
- data on biological observations including SSC and VTG protein/*vtg* mRNA (if measured);
- Lowest observed effect concentration (LOEC) assessed (at p = 0.05) and no observed effect concentration (NOEC) for each response assessed (optional);
- any deviation from the Guidance and deviations from the validity criteria, and considerations of potential consequences on the outcome of the test.

50. For the results of endpoint measurements, mean values and their standard deviations (on both replicate and concentration basis, if possible) should be presented.

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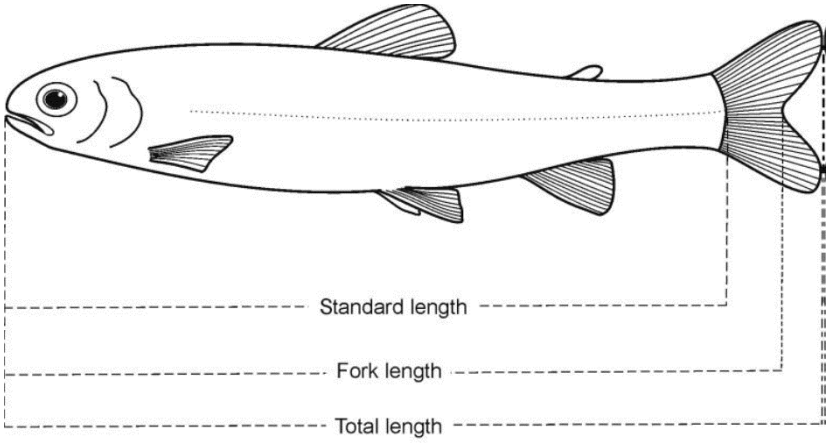
ANNEX 1 - Definitions

- **ASV**: air saturation value, expressed as a percentage.
- **Body weight**: fish wet weight in the condition of blotted dry.
- **CAS**: Chemical Abstracts Service.
- **DHT**: 5α-dihydrotestosterone.
- **dmy**: sex determining gene of medaka; a Y-specific DM domain gene.
- **dpf**: days post fertilisation.
- **EDC**: endocrine disrupting chemical.
- **ELISA**: Enzyme-Linked Immunosorbent Assay.
- **Flow-through test**: a test with continued flow of test solutions through the test system during the duration of exposure.
- **GD**: guidance document.
- **InChI**: International Chemical Identifier.
- **IUPAC**: International Union of Pure and Applied Chemistry.
- **JMASA**: Juvenile Medaka Anti-Androgen Screening Assay.
- **LC_{50}**: Median lethal concentration; the concentration of a test chemical that kills 50% of exposed test organisms within a given time period.
- **LOQ**: limit of quantification.
- **Loading rate**: the wet weight of fish per volume of water.
- **LOEC**: Lowest observed effect concentration; the lowest tested concentration of a test chemical at which the chemical is observed to have a statistically significant effect (at $p < 0.05$) when compared with the control.
- **MEOGRT**: Medaka Extended One Generation Reproduction Test.
- **mRNA**: messenger ribonucleic acid.
- **MTC**: maximum tolerated concentration; the highest test concentration of the chemical which results in less than 10% acute mortality.
- **NOEC**: No observed effect concentration; the test concentration immediately below the LOEC.
- **PAH**: polycyclic aromatic hydrocarbon.
- **PCB**: polychlorinated biphenyl.
- **PCR**: polymerase chain reaction.
- **RT-PCR**: Reverse Transcriptase Polymerase Chain-Reaction.
- **SMILES**: simplified molecular-input line-entry system.
- **SSC**: secondary sex characteristics.
- **Total length**: refer to figure below.



- **TG**: OECD guideline for the testing of chemicals.
- **U.S. EPA**: United States Environmental Protection Agency.
- **UVCB**: Substances of unknown or variable composition, complex reaction products or biological materials.
- **VTG**: Vitellogenin; a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active females of all oviparous species.
- **wpf**: weeks post fertilisation.

ANNEX 2 - Test conditions for Juvenile Medaka anti-Androgen Screening Assay

1.	Test species (recommended)	Japanese medaka (*Oryzias latipes*)
2.	Test type	Flow-through test
3.	Water temperature	25 ± 1 °C (the recommended mean temperature throughout the test in each tank is 25 ± 1 °C)
4.	Illumination	Fluorescent bulbs (wide spectrum)
5.	Illumination level	10-20 µE/m²/s, 540-1000 lux, or 50-100 ft-c
6.	Photoperiod	12-16 hours light, 12-8 hours dark
7.	Loading rate	<5 g/L
8.	Test chamber size	Minimum of 1.8 L
9.	Volume exchanges of test solutions	Minimum of 5 daily
10.	Age of test organisms at initiation	35-42 dpf (5-6 wpf)
11.	Number of organisms per replicate	7 fish/replicate tank (recommended)
12.	Number of treatments	3 test chemical treatments plus appropriate control(s)
13.	Number of replicates per treatment	4 replicates per treatment for test chemical and control (minimum)
14.	Number of organisms per test	Minimum 112 fish (minimum 140 fish, if solvent control is used)
15.	Feeding regime	Live brine shrimp (*Artemia* spp.) *nauplii*, supplemented with a commercially available flake food if necessary, two or three times daily, *ad libitum*
16.	Aeration	None unless dissolved oxygen falls below 60 % ASV
17.	Dilution water	Clean surface, well or reconstituted water or dechlorinated tap water.
18.	Chemical Exposure duration	28 days (pre-exposure period is not required)

19. Biological endpoints	Survival; abnormal response (e.g., in behavior and appearances); growth (total length and body weight); secondary sex characteristics (number of papillary processes); vitellogenin (VTG protein or *vtg* mRNA, as an optional endpoint)
20. Test validity criteria	Mean mortality of ≤10% in the controls; the concentrations of the test chemical in each test vessel are satisfactorily maintained within ±20% of the mean measured value in the treatment group; dissolved oxygen of ≥60% ASV; water temperature of 25±1 °C throughout the test and ±1 °C between test vessels at any one time; the number of males per tank should be at least one in at least three out of four replicates

ANNEX 3 - Typical growth curve of Japanese medaka

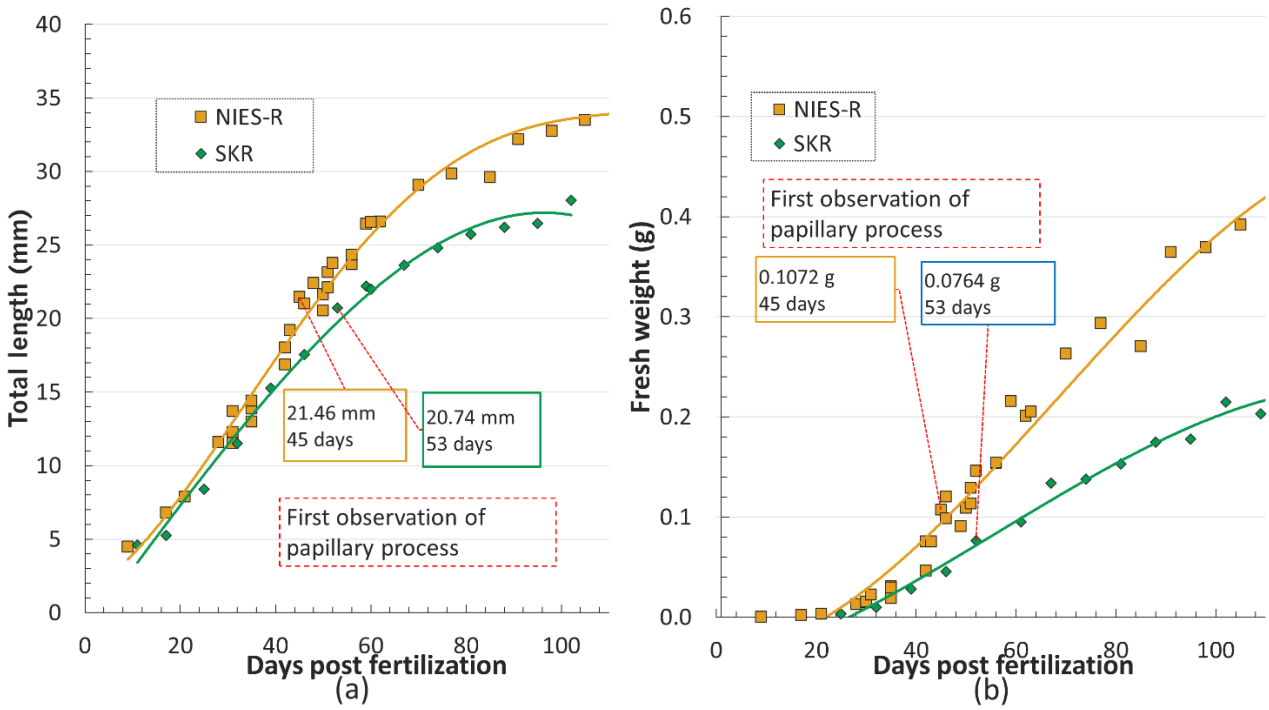


Figure 1. Growth curve of two Japanese medaka strains (NIES-R and SKR) with the first observation of secondary sex characteristics (papillary processes) based on (a) total length and (b) fresh weight.

ANNEX 4 - Some chemical characteristics of an acceptable dilution water

Substance	Limit concentration
Particulate matter	5 mg/L
Total organic carbon	2 mg/L
Un-ionised ammonia	1 µg/L
Residual chlorine	10 µg/L
Total organophosphorous pesticides	50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls	50 ng/L
Total organic chlorine	25 ng/L
Aluminium	1 µg/L
Arsenic	1 µg/L
Chromium	1 µg/L
Cobalt	1 µg/L
Copper	1 µg/L
Iron	1 µg/L
Lead	1 µg/L
Nickel	1 µg/L
Zinc	1 µg/L
Cadmium	100 ng/L
Mercury	100 ng/L
Silver	100 ng/L

ANNEX 5 - Counting of joint plates with papillary processes on anal fin

Background

In Japanese medaka, papillary processes normally appear in adult males but not in females. In males, papillary processes can be found on fin rays from 2nd to 8th or 10th from the posterior end of the anal fin, depending on the strain and age, although papillae are rarely produced on the 1st fin ray (Figure 1a: male anal fin, 1b: female anal fin).

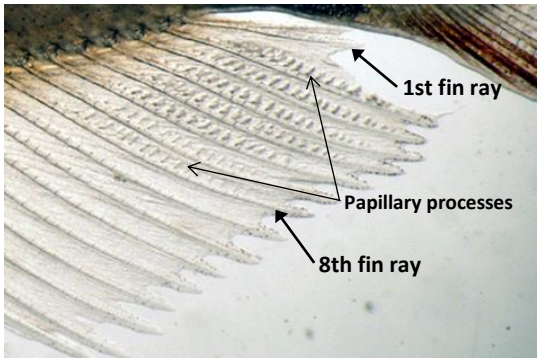


Figure 1a. Anal fin of control male medaka.

Figure 1b. Anal fin of control female medaka.

Treatment of samples

After necropsy (i.e., sampling of the caudal region including the anal fin), the anal fin should be imaged to allow for convenient counting of joint plates with papillary processes for each sample, or the samples should be stored in appropriate fixative (e.g., 10% neutral buffered formalin) until use. When imaging or fixing anal fin samples, it is important to keep the anal fin in a flat and spread-out condition, to allow for easier location of papillary processes.

Counting of joint plates with papillary processes

For each image or sample of anal fin, the number of joint plates with papillae (Figure 2) should be counted in each fin ray (e.g., from the posterior margin) and recorded and the total number of joint plates determined in each fish.

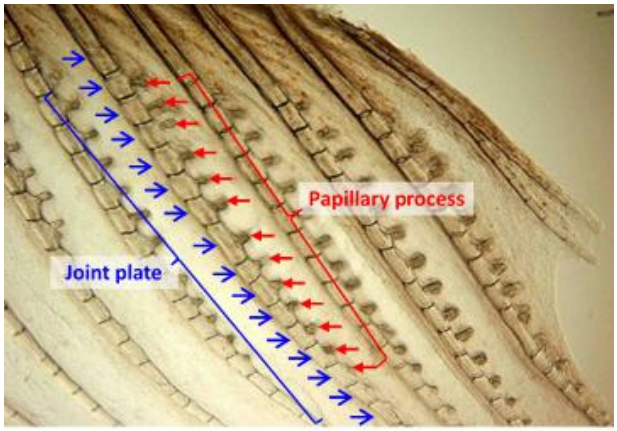


Figure 2. Joint plates with papillary process.

ANNEX 6 - Statistical analysis

If the data (normally, replicate means) are assumed to be a monotone trend (in an increase or a decrease), it is recommended to analyse the data by a trend-based step-down methods (e.g., step-down Williams test, Jonckheere-Terpstra test). To assess monotonicity, a visual check from a scatter plot can be used, although data should preferably be evaluated by using linear and quadratic contrasts. If the data are non-monotonic, a multiple comparison test, such as the Dunnett test (parametric data) or Mann-Whitney test according to Holm (non-parametric data), should be performed.

As a preliminary, the data should be assessed for normality (e.g., using the Shapiro-Wilk test) and homogeneity of the variance (e.g., using Levene's test) among the treatment groups where parametric methods (e.g., Williams test, Dunnett test) are employed for analysis. Where the assumption of normality or variance homogeneity is not met, data transformation, to achieve these requirements, can be sought or non-parametric methods employed.

It is important that the strongest, valid tests are employed for statistical analysis of the endpoints. For example, the power properties of the step-down Jonckheere-Terpstra test are very similar to those of the step-down Williams test, when the data are normally distributed with homogeneous variances, and are superior to Williams when those conditions are violated. On the other hand, for datasets with few replicates, the power properties of the Mann-Whitney and Dunn tests are worse, sometimes much worse, than those of Dunnett's test.

An example of statistical flow chart for biological data (e.g., SSC, VTG) from JMASA provides below.

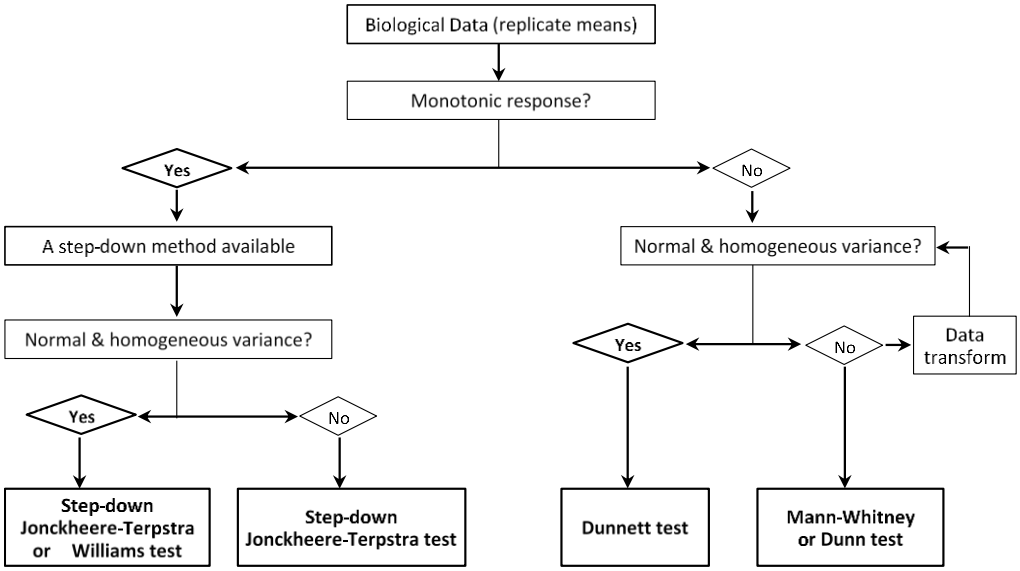


Figure 1. An example of statistical flow chart