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GUIDANCE DOCUMENT ON THE ANDROGENISED FEMALE STICKLEBACK SCREEN

Series on Testing and Assessment

No. 148

[In 2017, a small change and appropriate references were introduced to paragraph 17 for animal welfare reasons.]

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No. 148

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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

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

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

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FOREWORD

This Guidance Document describes a method to detect (anti-)androgenic chemicals using Androgenised females of the stickleback fish species (*Gasterosteus aculeatus*) exposed for 3 weeks to the chemical tested.

In 2008, the United Kingdom submitted a proposal to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) to develop a new Test Guideline for the detection of chemicals acting like (anti-)androgens through the use of androgenised females of the stickleback fish species (*Gasterosteus aculeatus*). The project was included in the work plan of the Test Guidelines Programme. The United Kingdom submitted a validation report on the test method in 2009, which has since been published in the Series on Testing and Assessment (No.128). A peer review of the validation was undertaken by the OECD and endorsed by the WNT in 2009 and has been published in the Series on Testing and Assessment (No.127).

The WNT stated in the peer-review report that until further clarification is made on the role of the Androgenised Female Stickleback Screening Assay in the OECD Conceptual Framework on Endocrine Disrupters Testing and Assessment and its sensitivity compared to other methods with similar objectives, this assay should be the subject of a Guidance document rather than a Test Guideline. The WNT also recommended that a negative substance should be tested in this assay to demonstrate the specificity of the method for (anti-) androgenic chemicals. The report on the negative substance testing, developed by the United Kingdom, will soon be available in the Series on Testing and Assessment as No.159.

This document was developed by the United Kingdom, reviewed and revised by a fish expert group, and approved at the 23rd Meeting of the Working Group of the National Coordinators of the Test Guidelines Programme in April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 22 June 2011.

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

INTRODUCTION

1. This Androgenised Female Stickleback Screen (AFSS) describes a 21-day *in vivo* assay for identifying endocrine active chemicals with (anti)androgenic activity in fish using female sticklebacks (*Gasterosteus aculeatus*). The concept of this assay is derived from work on the three-spined stickleback (3) (4) (5) (6) (7) (8), since the presence of the specific biomarker (i.e. spiggin) for androgens is present only in this species (4) (9). Although current OECD Test Guidelines 229 and to some extent 230 can detect androgen antagonism in addition to other endocrine disrupting actions, the activity detected is not always clearly specific to androgen antagonism (1)(2)(29)(30)(31)(32)(33). However, given the availability of other Test Guidelines (TGs), such as the OECD TG 441 on the Hershberger assay which can identify (anti)androgens in rats, the Working Group of the National Coordinators of the Test Guidelines Programme decided that the AFSS should be described in a Guidance Document rather than a Test Guideline, until a clear role is defined for the AFSS in the Conceptual Framework for Endocrine Disrupters Testing and Assessment (28). The AFSS protocol is in principle similar to the new OECD TG 230 (21-day Fish Assay: A short-term screening for oestrogenic and androgenic activity and aromatase inhibition), with two major differences: 1) only female fish are used, and 2) all groups except controls (water, solvent and test substance at the highest concentration used) receive 5µg/L dihydro-testosterone (DHT), in addition to the test substance compound. DHT is used in order to induce a fully controlled moderate level of the androgen regulated protein spiggin in the female stickleback kidney, to allow the detection of (anti)androgens. Following a chemical exposure period of 21 days, the AFSS detects androgen receptor agonists and antagonists. The validation work (3) has been reviewed by a panel of experts (28). The peer review report is supplemented with an addendum containing results of testing of a negative substance, ammonia.

2. This guidance document describes an *in vivo* screening assay where sexually mature female sticklebacks are exposed to suspected anti-androgenic chemicals during a limited part of their life-cycle (21 days). The AFSS can detect both androgens and anti-androgens; however its usefulness is greater in the detection of androgen antagonists. The protocol does not require an *in situ* pre-exposure period and measures one biomarker endpoint as indicator of (anti)androgenic activity, the level of spiggin in the female stickleback kidneys. Other measurements include survival and body weight; these are not considered endpoints in the AFSS but are needed to confirm statistical robustness of the assay (survival and biomarker level (spiggin/g body weight)).

INITIAL CONSIDERATIONS AND LIMITATIONS

3. The method described in this Guidance Document has undergone a thorough an inter-laboratory validation study that demonstrated the concept of spiggin as a marker of anti-androgenicity in female fish. The method requires a high level of expertise in flow-through fish tests, experience in rearing sticklebacks and differentiating males from females. Since the exposure involves more than one chemical (the test substance together with DHT) there is a theoretical potential of one chemical affecting the activity of the other. The presence of several control groups (water, solvent, DHT and test substance) warrants some security over this potential.

4. Spiggin is normally produced by the kidney of breeding male sticklebacks in response to circulating endogenous androgen, and is not normally found in female sticklebacks under natural conditions. In males,

it is briefly stored in the urinary bladder from where it is excreted by contractions and used as a cementing material for the construction of a nest. Spiggin is almost undetectable in the kidney of immature male and female sticklebacks because they lack sufficient circulating androgen; however, the kidney is capable of synthesising and excreting spiggin in response to exogenous androgen stimulation (4) (10).

5. The use of intact males for the detection of (anti)androgens has been proved to be difficult to reproduce in non-expert laboratories, due to the difficulties in fully controlling their reproductive status. In the AFSS this is not an issue as female fish are simultaneously treated with a model androgen (dihydro-testosterone, DHT) at 5µg/L and a range of concentrations of the putative (anti)androgen. Antiandrogenic activity is detected by the degree of reduction/inhibition of spiggin induction by the DHT treatment. The biological basis of the spiggin response following androgenic and antiandrogenic treatment is well established.

6. Spiggin protein levels can be measured by a specific Enzyme-Linked Immunosorbent Assay (ELISA) method using immunochemistry for the quantification of spiggin in kidneys collected from individual sticklebacks (4). [Annex 3](#) provides the recommended procedures for sample collection for spiggin analysis and [Annex 4](#) provides a validated protocol for spiggin analysis. The spiggin ELISA has demonstrated acceptable inter-laboratory reproducibility during a previous validation exercise (14).

7. Definitions used in this draft Guidance Document are given in [Annex 1](#).

PRINCIPLE OF THE TEST

8. Overviews of the relevant bioassay conditions are provided in [Annex 2](#). The assay is normally initiated with fish sampled from populations that are in spawning condition to facilitate selection of female fish. Spawning conditions can be readily induced in sticklebacks by temperature and photoperiod manipulations. Guidance on the age of fish and on the reproductive status is provided in the section *Selection of fish*. It should be noted that we recommend the use of sexually mature females, because sexual dimorphism in this species is present only when the fish are in active breeding; the reproductive status of the female (i.e., immature, early vitellogenic, later vitellogenic, spent) does not affect the female response to the treatment. At test termination, sex is confirmed by macroscopic examination of the gonads following ventral opening of the abdomen with scissors. The assay is conducted using a range of test substance exposure concentrations (at least three test concentrations are recommended), as well as a water control, a solvent control, a DHT control where DHT alone is administered at 5µg/L, and a test substance control where the test substance is administered alone at the highest concentration tested. Two vessels per treatment (replicates; each containing 5 female fish) are used. DHT is readily biodegradable in aqueous solutions; hence the test can only be conducted using flow-through conditions and a carrier solvent. In addition, since most of the androgen antagonists identified to date are highly hydrophobic molecules, the use of solvent carrier facilitates their administration. The exposure is conducted for 21-days and sampling takes place at the end of this period. Measurements include survival, whole body wet weight, whilst kidney spiggin levels is the only evaluated endpoint. The measurement of spiggin serves for the detection of chemicals with (anti)androgenic mode of action. The detection of androgen agonists is possible via the measurement of spiggin induction in female sticklebacks, and it has been well documented in the scientific peer-reviewed literature (4) (5) (6) (10) (11) (12) (13) (14) (15). Reduction of spiggin levels has also been demonstrated following exposure to androgen antagonists, both in intact male sticklebacks using a short-term reproductive assay (16) (17) (18) and in the androgenised (masculinised) female stickleback screen (3) (5) (6).

Test acceptance criteria

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

- the combined mortality in either the water or solvent controls does not exceed 10 per cent at the end of the exposure period;
- the dissolved oxygen concentration should be ≥ 60 per cent of the air saturation value (ASV) throughout the test;
- the water temperature should not differ by more than $\pm 1.5^{\circ}\text{C}$ between test chambers or between successive days at any time during the test, and should be within the temperature range of $15\text{-}19^{\circ}\text{C}$ (17 ± 2);
- evidence should be available to demonstrate that the measured concentrations of the test substance in solution have been satisfactorily maintained within $\pm 20\%$ of the mean measured concentrations;
- the sex of the fish sampled for spiggin determination is confirmed to be female.

DESCRIPTION OF THE METHOD

Apparatus

10. Standard 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) tanks made of chemically inert material (e.g. glass, stainless steel) and of a suitable capacity in relation to the recommended loading and stocking density (see [Annex 2](#)); It is desirable that test chambers be randomly positioned in the test area. The test chambers should be shielded from unwanted disturbance;
- (e) suitably accurate balance (i.e. accurate to $\pm 0.5\text{mg}$).

Water

11. 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. The pH of the water should be within the range 6.5 to 8.5, but during a given test it should be within a range of ± 0.5 pH units. It is recommended that dilution water hardness should be above 140 mg/l (as CaCO_3). In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of test substance), samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, and Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. Some chemical characteristics of acceptable dilution water are listed in [Annex 5](#).

Test solutions

12. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. Since DHT displays low solubility and stability in aqueous solutions the use of a solvent carrier is unavoidable. In addition, many suspected (anti)androgenic compounds are also highly hydrophobic, hence the use of solvent carrier benefits the practical aspects of their administration too. A solvent control group should be run in parallel, at the same solvent concentration as the chemical treatments. The choice of solvent will be determined by the chemical properties of the substance; for guidance consult the OECD Guidance Document on aquatic toxicity testing of difficult substances and mixtures (19). Methanol and ethanol can be problematic though, resulting in biofilms and turbidity of the water column. The validation data (3)

were produced using methanol at 1000µl/L, but other solvents such as ethanol, acetone or dimethyl sulfoxide (DMSO) have also been used effectively for the administration of DHT in aquaria. However, the OECD Guidance Document recommends that a maximum of 100µl/L of solvent should be observed in the aquaria whilst a recent review recommends that the solvent concentration in the aquaria should not exceed 20µl/L (20). These levels are achievable in the AFSS if stock solutions are made in 100% solvent and high dilution water flow rates are used. Nevertheless, statistical analysis of thousands of kidney samples for spiggin, from different exposures where solvents were employed at this high level, indicated that there were no differences in spiggin content between water control and solvent control female fish. The core endpoint employed by the test is based on a robust mechanistic response and does not appear to be affected by the high levels of solvent in the aquaria. It is recommended however, that the solvent concentration be minimised wherever technically feasible.

13. A flow-through test system should be used. Such a system continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump) in order to deliver a series of concentrations to the test chambers. In the test vessels that receive both DHT and the test substance, we recommend combining the two stock solutions to provide the desired concentrations. This is because the degree of reduction in spiggin levels by the test substance is directly related to the levels of DHT that induce spiggin production in the female kidney. By combining the stock solutions of DHT and the test substance slight differences in the flow rates between DHT solutions and test substance solutions that can adversely affect the response are avoided. In addition, the test becomes less labour intensive, as fewer flow rates need to be checked and calibrated on a daily basis. The flow rates of stock solutions and dilution water should be checked at intervals, preferably daily, during the test and should not vary by more than 15% throughout the test. Care should be taken to avoid the use of low-grade plastic tubing or other materials that may contain biologically active substances. When selecting the material for the flow-through system, possible adsorption of the test substance to this material should be considered.

14. Semi-static (static renewal) test conditions should be avoided unless there are compelling reasons associated with the test substance (e.g., stability, limited availability, high cost or hazard); if they were to be used however, then the preferred renewal procedure in the semi-static technique involves changing a proportion (at least two thirds) of the test water every 24 hours whilst retaining the test organisms in the test vessels. In this case, DHT at 5µg/L should be replaced by 17α-methyltestosterone (MT) at 0.5µg/L, since the latter steroid is stable in aqueous solutions over time and more potent than DHT (6). Aromatisation of MT to oestrogens has been reported in some fish species (21)(22) but vitellogenin induction in male sticklebacks was never observed after exposure to MT or DHT (personal communication-Ioanna Katsiadaki, Cefas Weymouth, UK).

Holding and selecting the fish

15. The only species that can be used is the three-spined stickleback (*Gasterosteus aculeatus*), as the androgen biomarker protein, spiggin, is not present in other teleost species.

16. Test fish should ideally be selected from a laboratory population of a single stock, preferably from the same spawning, which has been acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test (note, this acclimation period is not an *in situ* pre-exposure period). It is important to avoid using animals from the wild as they are often parasitised by plerocercoids of *Schistocephalus solidus*, which delays and/or inhibits sexual maturation in male fish (23) and results in the same phenotype as a gravid female; both effects may result in selecting parasitised males rather than females for the test.

17. Only female fish can provide meaningful information in this guidance document, as they lack high levels of endogenous androgens that could affect the response. In order to reduce the number of fish used

in the test, separation of sexes prior to the test is therefore essential. Sticklebacks display strong sexual dimorphism (the males develop blue irises and red throats, see [Annex 6](#)) only during their breeding season; hence the fish population used in the test should be adult fish (over 30 weeks of age assuming they have been cultured at $17\pm 2^{\circ}\text{C}$ throughout their life span) that are reproductively mature. If the fish supplier does not guarantee a female only population, selection is easily achievable in laboratory conditions by applying adequate photoperiod regime (18 hours light: 6 hours dark). Since social hierarchies are strong in this species, only few males in a single holding vessel will display nuptial coloration at one time. The easily recognised dominant males should be gradually removed on a daily basis, allowing the subordinate fish to become dominant. The time needed for this selection depends on the stocking density, the lower the density the more male fish will develop nuptial coloration at one time. As a guide, we recommend the use of 0.5 g/l over a two-week period under high photoperiod to ensure an all female population. Female fish can be further identified by their gravid appearance. Alternatively, fish can be sexed using DNA techniques (24) (25), which can also be applied to a skin swab (34) (35), waving the need for fin or spine clipping and associated welfare issues. A sex ratio of 1:1 should be assumed in a mixed population, although often we observe a slightly biased sex ratio towards females. At least 5 female fish per test vessel are needed to provide sufficient statistical power in the AFSS. Fish should be fed *ad libitum* throughout the holding period and during the exposure phase. Note- fish should not be fed within 12 hours of necropsy.

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

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

19. Fish should not receive treatment for disease in the two-week period preceding the test, or during the exposure period.

TEST DESIGN

20. At least three concentrations of the chemical (along with DHT at $5\mu\text{g/l}$), a water control, a solvent control, a DHT control and the highest concentration of the chemical tested with no DHT (test substance control) are used per experiment (all in duplicate test vessels). The data may be analysed in order to determine statistically significant differences between treatment and control responses. Calculation of these statistical parameters will be useful in order to establish whether any further longer term testing for adverse effects (namely, survival, development, growth and reproduction) is required for the chemical.

21. At initiation of the experiment on day-0, five females from the stock population are sampled for the measurement of kidney spiggin. At termination of the assay after 21 days of exposure, all five female fish in each vessel are euthanized and their kidneys are excised for the measurement of spiggin.

Selection of test concentrations

22. For the purposes of this test, the highest test concentration should be set by the maximum tolerated concentration (MTC) determined from a range finder or from other toxicity data, or 10 mg/l whichever is lowest (26). The MTC is defined as the highest test concentration of the chemical, which results in less than 10% mortality, no abnormal external characteristics and behaviour. Using this approach assumes that there are existing empirical acute toxicity data or other toxicity data from which the MTC can be estimated. Estimating the MTC can be inexact and typically requires some professional judgment.

23. Three test concentrations, spaced by a constant factor not exceeding 10, (in addition to the water, solvent, DHT and test substance controls) are required. A range of spacing factors between 3.2 and 10 is recommended. A typical test should include the following treatments (all in duplicate vessels):

- Water control
- Solvent control (solvent at the same level as in the treatment vessels)
- Test substance control (test substance at high concentration + solvent)
- DHT control (DHT at 5 µg/l + solvent)
- High test substance concentration +DHT at 5 µg/l + solvent
- Medium test substance concentration +DHT at 5 µg/l + solvent
- Low test substance concentration +DHT at 5 µg/l + solvent

PROCEDURE

Selection and weighing of test fish

24. It is only moderately important to minimise variation in weight of the fish at the beginning of the assay. This is because spiggin units per kidney are divided by the body weight to normalise the response. Suitable size range is 1-2g. For the whole batch of fish used in the test, the range in individual wet weights at the start of the test should be kept to within $\pm 30\%$ of the arithmetic mean wet weight. It is recommended to weigh a subsample of the fish stock before the test in order to estimate the mean weight.

Conditions of Exposure

Duration

25. The test duration is 21 days with no pre-exposure period needed.

Feeding

26. The fish should be fed *ad libitum* with appropriate food ([Annex 2](#)) at a sufficient rate to maintain body weight. Care should be taken to avoid microbial growth and water turbidity. The daily ration may be divided into two equal portions for multiple feeds per day, separated by at least six hours between each feed. The ration is based on the initial total fish weight for each test vessel. Food should be withheld from the fish for 12 hours prior to the day of sampling.

27. Fish foods should be evaluated for the presence of contaminants including heavy metals, organochlorine pesticides, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs).

28. Uneaten food and faecal material should be removed from the test vessels each day by carefully cleaning the bottom of each tank using suction.

Light and temperature

29. The photoperiod during the test should be 12 hours dark: 12 hours light (light intensity 540 to 1000 lux) and the water temperature should be 15-19°C ([see Annex 2](#)).

Frequency of Analytical Determinations and Measurements

30. Prior to initiation of the exposure period, proper function of the chemical delivery system should be ensured. All analytical methods needed should be established, including sufficient knowledge on the substance stability in the test system. During the test, the concentrations of the test substance and DHT are determined at regular intervals, as follows: the flow rates of diluent and toxicant stock solution should be checked preferably daily, but as a minimum twice per week, and should not vary by more than 20% throughout the test. It is recommended that the actual test substance concentrations be measured in all vessels at the start of the test and at weekly intervals thereafter.

31. It is recommended that results are based on measured concentrations.

32. Samples should be filtered (e.g. using a 0.45 µm pore size) or centrifuged prior to analysis if there is evidence of precipitate or a reasonable expectation (i.e. when it is known that the solubility of the test system has been exceeded). If required, then centrifugation is the recommended procedure. However, if the test material does not adsorb to filters, filtration may also be acceptable.

33. During the test, dissolved oxygen, temperature, and pH should be measured in all test vessels at least once per week. Total hardness and alkalinity should be measured in the controls and one vessel at the highest concentration at least once per week. Temperature should preferably be monitored continuously in at least one test vessel.

Observations

34. A number of general measures (survival, behaviour and appearance) and a single core biomarker endpoint (spiggin) are assessed over the course of the AFSS. Evaluation of these measurements or endpoint and their utility are described below:

Survival

35. Fish should be examined daily during the test period and any mortality should be recorded and the dead fish removed, as soon as possible. Dead fish should not be replaced in either the control or treatment vessels. The sex of the fish that die during the test should be confirmed by macroscopic evaluation of the gonads.

Behaviour and appearance

36. Any abnormal behaviour (relative to controls) should be noted daily; this might include signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. Additionally, external abnormalities (such as haemorrhage, discoloration) should be noted daily. Such signs of toxicity should be considered carefully during data interpretation since they may indicate concentrations at which measurements of the biomarker are not reliable.

Humane killing of fish

37. At day 0 and day 21 (conclusion of the exposure), the fish should be euthanised with appropriate amounts of Tricaine (Tricaine methane sulfonate, Metacain, MS-222 (CAS.886-86-2), 100-500 mg/L buffered with 300 mg/L NaHCO₃ (sodium bicarbonate, CAS.144-55-8) to reduce mucous membrane irritation; the fish are then individually weighed as wet weights (blotted dry) and the kidney is excised for spiggin level determination ([Annex 3](#)).

Sampling of fish for spiggin evaluation

38. The kidney from each fish is excised ([Annex 3](#)) and placed in individually labelled 2ml tubes with screw caps (do not use clip top type tubes as the spiggin measurement protocol involves heating up in the presence of a strong denaturing buffer, so high pressure is built up which can force open the caps of such tubes). The measurement of spiggin protein in the kidney should be based upon a validated homologous ELISA method, using homologous spiggin standard and homologous antibodies. All necessary reagents are commercially available.

39. Quality control of spiggin analysis will be accomplished through the use of standards, blanks and at least duplicate analyses. Each ELISA plate used for spiggin assays should include the following quality control samples: at least 8 calibration standards covering the range of expected spiggin concentrations, and at least one non-specific binding assay blank (analysed in duplicate). At least two aliquots (well-duplicates) of each sample dilution will be analysed. Well-duplicates that differ by more than 20% should be re-analysed.

40. The correlation coefficient (R^2) for calibration curves should be greater than 0.99. However, a high correlation is not sufficient to guarantee adequate prediction of concentration in all ranges. In addition to having a sufficiently high correlation for the spiggin calibration curve, the concentration of each standard, as calculated from the calibration curve, should all fall between 80 and 120 % of its nominal concentration. If the nominal concentrations trend away from the calibration regression line (e.g. at lower concentrations), it may be necessary to split the calibration curve into low and high ranges or to use a nonlinear model to adequately fit the absorbance data. If the curve is split, both line segments should have $R^2 > 0.99$.

41. The limit of detection (LOD) is defined as the concentration of the lowest analytical standard, and limit of quantification (LOQ) is defined as the concentration of the lowest analytical standard multiplied by the lowest dilution factor.

DATA AND REPORTING***Evaluation of Biomarker Responses by Analysis of Variance (ANOVA)***

42. To identify potential endocrine activity of a chemical, responses are compared between treatments and control groups using analysis of variance (ANOVA). An appropriate statistical test should be performed between the dilution water and solvent controls for spiggin. Guidance on how to handle dilution water and solvent control data in the subsequent statistical analysis can be found in OECD, 2006c (27). The biological response of any male fish present in the vessels should be removed from analysis. The data are logarithmically transformed prior to performing the ANOVA. Dunnett's test (parametric) on multiple pairwise comparisons or a Mann-Whitney with Bonferroni adjustment (non-parametric) may be used for non-monotonous dose-response. Other statistical tests may be used (e.g. Jonckheere-Terpstra test or Williams test) if the dose-response is approximately monotone. In all cases, the level of significance should be reported. A statistical flowchart is provided in [Annex 7](#) to help in the decision on the most appropriate statistical test to be used.

Reporting of test results

43. Study data should include:

Testing facility:

- Responsible personnel and their study responsibilities;

- Each laboratory should have demonstrated proficiency using DHT as a model androgen and Flutamide as a model antiandrogen;

Test Substance:

- Characterization of test substance;
- Physical nature and relevant physicochemical properties;
- Method and frequency of preparation of test concentrations;
- Information on stability and biodegradability;

Solvent:

- Characterization of solvent (nature, concentration used);
- Justification of choice of solvent;

Test animals:

- Species and strain;
- Supplier and specific supplier facility;
- Age of the fish at the start of the test and reproductive/spawning status;
- Details of animal acclimation procedure;
- Whole body wet weight of the fish at the start of the exposure (from a sub-sample of the fish stock). Suitable range is 1-2g (± 0.3);
- Confirmation of sex upon necropsy as female

Test Conditions:

- Test procedure used (test-type, loading rate, stocking density, etc.);
- Method of preparation of stock solutions and flow-rate;
- The nominal test concentrations, weekly measured concentrations of the test solutions and analytical method used, means of the measured values and standard deviations in the test vessels and evidence that the measurements refer to the concentrations of the test substance in true solution;
- Dilution water characteristics (including pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels, total organic carbon, suspended solids and any other measurements made);
- Photoperiod (duration and intensity);
- Water quality within test vessels: 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 relevant contaminants if available (e.g. heavy metals, PCBs, PAHs and organochlorine pesticides);

Results:

- Evidence that the controls met the acceptance criteria of the test;
- Data on mortalities occurring in any of the test concentrations and control;
- Statistical analytical techniques used, treatment of data and justification of techniques used;

- Data on biological observations of gross morphology and behaviour and spiggin levels;
- Results of the data analyses preferably in tabular and graphical form;
- Incidence of any unusual reactions by the fish and any visible effects produced by the test substance.

GUIDANCE FOR THE INTERPRETATION AND ACCEPTANCE OF THE TEST RESULTS

44. This section contains a few considerations to be taken into account in the interpretation of test results for the main endpoint measured. The results should be interpreted with caution where the test substance appears to cause overt toxicity or to impact on the general condition of the test animal.

45. In setting the range of test concentrations, care should be taken not to exceed the Maximum Tolerated Concentration (MTC) to allow a meaningful interpretation of the data. It is important to have at least one treatment where there are no signs of toxic effects. Signs of disease and signs of toxic effects should be thoroughly assessed and reported. For example, it is possible (although not documented) that production of spiggin in females can also be affected by general toxicity and non-endocrine toxic modes of action, e.g. nephrotoxicity. However, interpretation of effects may be strengthened by other treatment levels that are not confounded by systemic toxicity.

45. There are a few aspects to consider for the acceptance of test results. As a guide, the spiggin levels in control groups (water, solvent) and the DHT control (DHT alone at 5µg/l) should be distinct and separated by approximately two orders of magnitude. Examples of the range of values encountered in control and treatment groups are available in the literature and the validation report (3) (4) (5) (6).

46. If a laboratory has not performed the assay before, or substantial changes (e.g. change of fish supplier) have been made, it is advisable that a technical proficiency study is conducted. In practice, each laboratory is encouraged to build its own historical data for control (water and solvent, DHT and test substance) females; these can be compared to available data from the validation studies (3) (4) (5) (6) to ensure laboratory proficiency.

47. In general, spiggin response is positive (the substance has antiandrogenic activity) if there is a statistically significant decrease in female spiggin levels ($p < 0.05$), in the treated groups (at least at the highest dose tested) compared to the DHT control group whilst the mean response of spiggin levels in the control groups (water and solvent) and in the test substance control group is below 100 spiggin units/g body weight and in the absence of signs of general toxicity. Spiggin response is also positive (the substance has androgenic activity) if there is a significant increase ($p < 0.05$) in female spiggin levels in the test substance control group (test substance at the highest dose tested) compared to the water and solvent control group. A positive result is further supported by the demonstration of a biologically plausible relationship between the dose and the response curve. As mentioned earlier, the spiggin decrease may not entirely be of endocrine origin; however a positive result should generally be interpreted as evidence of endocrine activity *in vivo*, and should normally initiate actions for further clarification.

48. Spiggin response is negative (the substance has no antiandrogenic activity at least in the highest concentration tested) when there is no statistically significant decrease in female spiggin levels ($p > 0.05$) in the treated groups compared to DHT control group. It should be noted that the ability of the AFSS to detect antiandrogens is relevant only to chemicals that interact with the androgen receptor. Compounds that display antiandrogenic activity via other mechanisms (i.e. disruption of steroidogenesis) will not be identified as such. Spiggin response is also negative (the substance has no androgenic activity) if the mean spiggin response in the test substance control group is comparable to the water and solvent control groups (all below 100 spiggin units/g body weight).

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35. Breacker, C., Barber, I., Norton, W.H., McDearmid, J.R., and Tilley, C.A. 2016. A Low-Cost Method of Skin Swabbing for the Collection of DNA Samples from Small Laboratory Fish. *Zebrafish*, DOI: 10.1089/zeb.2016.1348.

ANNEX 1

ABBREVIATIONS & DEFINITIONS

ASV: Air Saturation Value

ELISA: Enzyme-Linked Immunosorbent Assay

Loading rate: the wet weight of fish per volume of water

LOD: limit of detection

LOQ: limit of quantification

DHT: dihydrotestosterone

DMSO: Dimethyl sulfoxide

MT: 17 α -Methyltestosterone

MTC: Maximum Tolerated Concentration

PAH: Polycyclic aromatic hydrocarbons

PCB: polychlorinated biphenyls

R²: correlation coefficient

Stocking density: number of fish per volume of water

ANNEX 2**EXPERIMENTAL CONDITIONS FOR THE AFSS**

1. Species	Three-spined stickleback (<i>Gasterosteus aculeatus</i>)
2. Test type	Flow-through
3. Water temperature	17 ± 2°C
4. Illumination quality	Fluorescent bulbs (wide spectrum)
5. Light intensity	540-1000 lux
6. Photoperiod (dawn / dusk optional)	12 h light, 12 h dark
7. Loading rate	<5 g per L
8. Test chamber size	10 L (minimum)
9. Test solution volume	8 L (minimum)
10. Volume exchanges of test solutions	Minimum of 6 daily
11. Age of test organisms	See paragraph 17 (>30 weeks)
12. Approximate wet weight of adult fish (g)	Females: 1.5 ± 30%
13. No. of fish per test vessel	5 (all females)
14. No. of treatments	= 3 (plus appropriate controls)
15. No. vessels per treatment	2 minimum
16. No. of fish per test concentration	10 females
17. Feeding regime	Frozen bloodworm or other acceptable food once or twice daily (<i>ad libitum</i>)
18. Aeration	None unless DO concentration falls below 60% ASL
19. Dilution water	Clean surface, well or reconstituted water or dechlorinated tap water

- | | |
|--------------------------------------|---|
| 20. Acclimation period | 7 days |
| 21. Chemical exposure duration | 21-d |
| 22. Biological measures and endpoint | - Survival (measure)
- Wet weight (measure)
- Spiggin (endpoint) |
| 23. Test acceptability | Dissolved oxygen $\geq 60\%$ of saturation;
mean temperature of $17 \pm 2^\circ\text{C}$; 90%
survival of fish in the controls; measured
test concentrations within 20% of mean
measured concentrations. |

STICKLEBACK SOURCE AND HUSBANDRY INFORMATION

The stickleback is an emerging model in the field of chemical testing and as such there aren't many commercial suppliers of disease-free animals. However, the species has long been used as a model fish for other disciplines such as behaviour and evolution and therefore a number of universities and research institutes have established colonies that could be used as a disease free genetic stock to produce the animals required in the AFSS.

The male stickleback becomes very territorial during their breeding season and as such natural reproduction in this species does not take place in groups of many fish but requires the use of a single tank per male. This procedure requires a lot of space and effort so the routine production of stickleback stocks utilises an *in vitro* fertilization (IVF) technique. The stickleback IVF is a straightforward easy to do and high yield procedure. As a guide, the production of 1,000 stickleback fertilised eggs can be achieved with the use of sperm from a single male and eggs from 10 females (average egg clutch of 100 eggs) in less than an hour. The limiting factor is the readiness of females to release their eggs when applying mild abdominal pressure; however, photoperiodic manipulation readily provides female sticklebacks in a breeding condition. The stickleback sperm is fertile even outside the breeding period so as long as the fish is more than 30 weeks of age, its sperm can be used to fertilise eggs.

The following websites provide either contact details or detailed husbandry and IVF protocols for laboratory rearing of three-spined sticklebacks (the list is by no means exhaustive but only indicative).

The Cefas Weymouth, UK (<http://www.cefas.co.uk/our-science/animal-health-and-food-safety/environment-and-animal-health/stickleback-as-a-model-fish.aspx>)

The University of Oregon, US
(http://stickleback.uoregon.edu/index.php/Stickleback_Research)

The University of British Columbia, Canada (<http://www.zoology.ubc.ca/~schluter/lab.html>)

Stony Brook University, US (<http://life.bio.sunysb.edu/ee/belllab/protocols.html>)

Stanford University, US (<http://kingsley.stanford.edu/>)

ANNEX 3

RECOMMENDED PROCEDURE FOR KIDNEY EXCISION FOR SPIGGIN ANALYSIS

Removal of the test fish from the test chamber

- (1) Test fish should be removed from the test chamber using a small spoon-net.
- (2) In principle, the test fish should be removed in the following order to avoid risk of contamination: control, solvent control, test substance control, highest test concentration, middle test concentration, lowest test concentration and DHT control. In addition, if any obvious males are present in the test vessels they should be removed after the presumed females are removed.
- (3) The sex of each test fish is identified on the basis of external secondary sex characteristics (e.g., nuptial coloration).
- (4) Place the test fish in a container for transport and carry it to the workstation for excision of the kidney. Check the labels of the test chamber and the transport container for accuracy and to confirm that the number of fish that have been removed from the test chamber and that the number of fish remaining in the test chamber are consistent with expectation.
- (5) The sex to be confirmed by macroscopic observation of gonadal gross morphology.

Excision of the kidney

- (1) Transfer the test fish from the container for transport to the anaesthetic solution using a net.
- (2) After the test fish is anaesthetised, transfer the test fish on the filter paper (or a paper towel) using tweezers (commodity type). When grasping the test fish, apply the tweezers to the sides of the head to prevent breaking the tail.
- (3) Wipe down the fish on the filter paper (or the paper towel).
- (4) Determine the wet weight of the fish to the nearest mg (two decimal points)
- (5) Separate the head of the fish from the body of the fish by a single cut made right behind the pectoral fin using a scalpel or dissection scissors (figure 1).
- (6) Place the fish abdomen side up. Then make a small transverse incision partway between the ventral neck region and the mid-abdominal region using dissection scissors (figure 2).
- (7) Insert the dissection scissors into the small incision, and incise the abdomen from a point caudal to the branchial mantle to the cranial side of the anus along the midline of the abdomen. Be careful not to insert the dissection scissors too deeply so as to avoid damaging the internal organs (figure 3).
- (8) Place the test fish abdomen side up on the paper towel.
- (9) Extend the walls of the abdominal cavity with precision tweezers to exteriorise the internal organs and confirm the sex by visual inspection of the gonads; the testes have a characteristic black appearance and are much smaller than the ovaries. Record sex (figure

- 4).
- (10) Grasp the oesophagus using another pair of precision tweezers and excise the gastrointestinal tract, including the liver, the gallbladder and the gonads (figure 5). Excise the caudal gastrointestinal tract from the anus and remove from the abdominal cavity (figure 6).
 - (11) Grasp the gonads (one lobe at a time) and separate taking care not to pull the kidney along with it (figure 7).
 - (12) Excise the gonadal tract from the anus and remove from the abdominal cavity (figure 8).
 - (13) Expose the kidney that is lying along the spine (figure 9). With the help of fine precision tweezers disconnect the kidney from the spine (figure 10). Grasp the caudal end of the kidney using the precision tweezers and remove the kidney from the abdominal cavity (figure 11).
 - (14) In rare occasions the kidney is firmly attached to the ovary; in this case seek for the kidney (or parts of it) along the dorsal line of the ovary. Disconnect, grasp and remove as above.
 - (15) Trim any remnants of the head kidney that may still be attached. Ensure the whole kidney is collected even if it's in many different pieces.
 - (16) Place the kidney in a pre-labelled 2ml screw cap vial and freeze at -70°C immediately.



Figure 1: Separate the head of the fish from the body of the fish by a single cut made right behind the pectoral fin using a scalpel or dissection scissors.

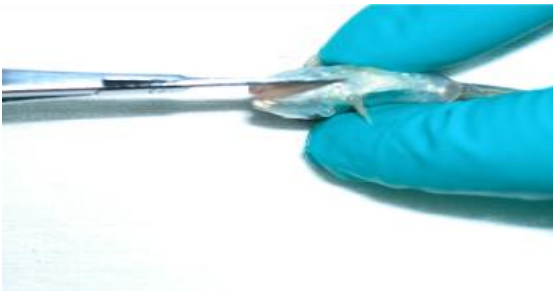


Figure 2: Place the fish abdomen side up. Then make a small transverse incision partway between the ventral neck region and the mid-abdominal region using dissection scissors.

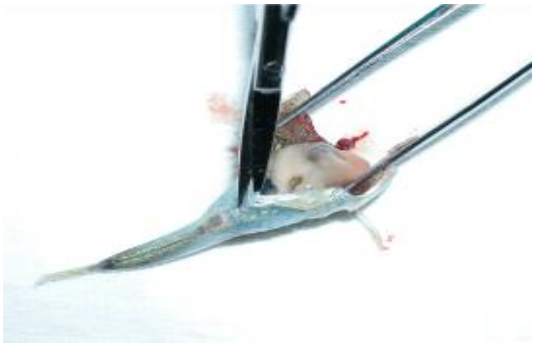


Figure 3: Insert the dissection scissors into the small incision, and incise the abdomen from a point caudal to the branchial mantle to the cranial side of the anus along the midline of the abdomen. Be careful not to insert the dissection scissors too deeply so as to avoid damaging the internal organs.



Figure 4: Extend the walls of the abdominal cavity with precision tweezers to exteriorise the internal organs. Confirm the sex by visual inspection of the gonads; the testes have a characteristic black appearance and are much smaller than the ovaries. Record sex.

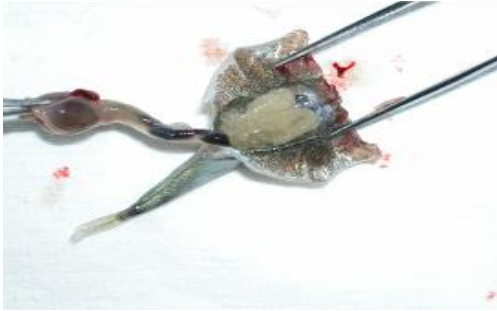


Figure 5: Grasp the oesophagus using another pair of precision tweezers and excise the gastrointestinal tract, including the liver and the gallbladder.



Figure 6: Excise the caudal gastrointestinal tract from the anus and remove from the abdominal cavity.



Figure 7: Grasp the gonads (one lobe at a time) and separate taking care not to pull the kidney along with it.



Figure 8: Excise the gonadal tract from the anus and remove from the abdominal cavity.



Figure 9: Expose the kidney that is lying along the spine. With the help of fine precision tweezers disconnect the kidney from the spine.



Figure 10: Grasp the caudal end of the kidney using the precision tweezers and remove the kidney from the abdominal cavity.



Figure 11: Excise the kidney and ensure there are no remnants attached to the spine.



Figure 12: The complete procedure. The whole kidney is visibly excised whilst the spine is clear of kidney tissue.

ANNEX 4**GUIDANCE FOR QUANTIFICATION OF KIDNEY SPIGGIN PROTEIN
(MODIFIED FROM KATSIADAKI ET AL, 2002a)***Consumables/Equipment*

High Protein Binding (Polystyrene)-Flat-bottom 96 well microplates

Low Protein Binding (Polypropylene)-Round-bottom 96 well microplates

Plate sealers

Multi-pipettes (12 and 8 channel) and multipipette troughs

Plate Washer: Program to wash 3 times using 300ul for each well, Consult operating manual.

Plate reader at 405 nm

*Chemicals*Denaturing Buffer: 100 mM Tris-HCl (pH:8.5), 10mM EDTA, 8M Urea, 2% SDS, 200mM β -mercaptoethanol

Coating Buffer: 0.05 M sodium bicarbonate-carbonate; pH 9.6; 1 Tablet in 100 ml DW

Washing Buffer: 0.1 M sodium phosphate [72mM di-basic, 28mM monobasic] 140 mM NaCl, 27mM potassium chloride, 0.05% Tween 20 (v/v). Dissolve first all constituents in 1 L DW and then add the remaining water. Tween is difficult to handle as it's very viscous to be volumetrically measured using convectional pipettes. Use a plastic graduated Pasteur pipette where the end is cut with scissors to allow suction.

Note: Approximately 3L wash buffer required per 10 plates

Chemical	1L	2L	3L	4L	5L
NaH ₂ PO ₄ (MW: 120)	3.36	6.71	10	13.44	16.80
Na ₂ HPO ₄ (MW:142)	10.2	20.4	30.6	40.8	51
NaCL	8.2	16.4	24.6	32.8	41
KCl	2g	4g	6g	8g	10g
Tween 20	0.5ml	1ml	1.5ml	2ml	2.5ml

Assay Buffer: washing buffer + 0.1% BSA (w/v) = **0.5g BSA per 500ml** washing buffer + 0.15 mM sodium azide
(Dissolve **0.065g** of sodium azide in **1ml** wash buffer and Add **70 μ l** to **500 ml** assay buffer)

Spiggin Standard (SS): Spiggin standard (5,000 units/ml) is available either commercially or at the Cefas Weymouth laboratory (<http://www.cefas.co.uk/our-science/animal-health-and-food-safety/environment-and-animal-health/stickleback-as-a-model-fish.aspx>).

For the standard curve:

Create S1 (HIGH) by adding **50 μ l** of SS into **450 μ l** assay buffer.**Create S2 (LOW)** by adding **10 μ l** of SS into **490 μ l** assay buffer.For coating: Add **100 μ l** of SS into **100mls** of coating buffer (see above). Use **100 μ l** per well to coat (10ml per plate).

Antisera: **1° antibody:** Polyclonal anti-spiggin (raised in a rabbit) is available commercially and at Cefas Weymouth at 1/100 dilution. Dilute further as follows: 100µl of the antiserum to 20mls assay buffer (working dilution of 1/20,000). You would need to add 65 µl per well (135µl). This will give a final dilution of antiserum of approximately 1/60,000.

2° antibody: Alkaline phosphatase conjugated Anti-rabbit IgG whole molecule. Dilute 1/15,000 (1µl per 15ml of assay buffer). You would need to add 150µl to all wells. Be aware that the fresher this preparation the faster the reaction the following morning with the substrate. Ideally prepare 6 hours before addition to the plates, particularly when a large number of plates are involved.

Substrate (tracer) pNPP Kit (1 tablet of 0.2 M Tris and 1 tablet of pNPP per 20ml DW).

Kidney preparation for the spiggin ELISA

- 1) Place each sample in a 2ml freestanding tube (Fisher: FB56123) with a gasket sealed screw cap (Fisher: FB56133) and add 200 µl of denaturing buffer. Do not use clip top tubes. If kidney size is visually judged to be too big to be solubilised into 200µl (i.e. this volume is not enough to fully cover the tissue), use 400µl instead and record. Only in rare cases (usually big males with substantial kidney hypertrophy) the addition of 400µl is required.
- 2) Place the tubes in a rack and heat at 80°C for 45 minutes. Vortex samples after 20min to facilitate tissue digestion. Again, in exceptional cases you may need to extend heating to more than 45 minutes. Do this only for the kidneys that have not been fully coaxed after this period. Assay samples immediately after digestion or store at -80°C until ready to assay. If samples are to be assayed next morning, they can be left overnight in the fridge (4°C).

ELISA Procedure

DAY 1: STEP 1 – Coating wells with spiggin.

- 1) Use High Protein Binding (Polystyrene) flat-bottom 96 well microplates
- 2) Dilute SS 1:1000 with coating buffer (100µl in 100ml)
- 3) Add 100µl of coating solution to each well (multi-pipette)
- 4) Seal plates, wrap in wet paper and store overnight at 4°C

DAY 1: STEP 2 – Sample dilutions and incubation with 1° antiserum

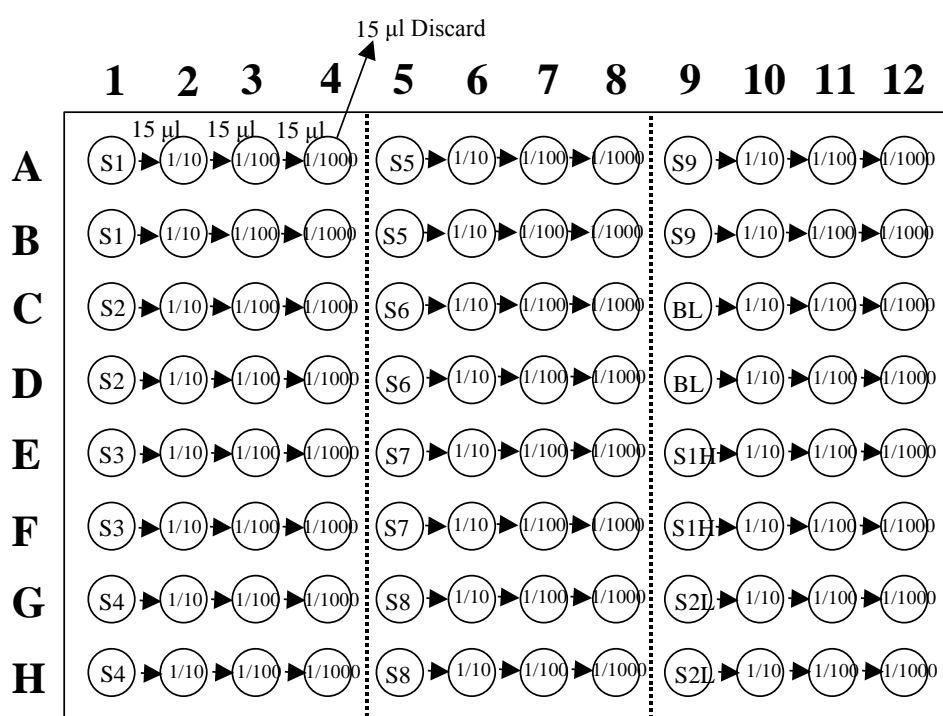
- 1) Use Low Protein Binding (Polypropylene) round-bottom 96 well microplates
- 2) Add 135 µl of assay buffer to each well
- 3) Dilute SS: S1 HIGH 1:10=500 Units/ml; S2 LOW 1:50=100 Units/ml
- 4) Add 15 µl of sample to wells in column 1, 5 and 9 (in duplicate) (S1-S9)
- 5) Add 15 µl of denaturing buffer in column 9 (in duplicate) (BL)
- 6) Add 15 µl of HIGH Spiggin standard (in duplicate) (S1H)
- 7) Add 15 µl of LOW Spiggin standard (in duplicate) (S2L)
- 8) Mix using multi-pipette (10x) and transfer 15 µl to next column*
- 9) Repeat 3 times and change pipette tips between different columns
- 10) Discard last 15 µl. All wells should have 135µl.



- 11) Dilute 1° antiserum 1:20,000 in assay buffer as described (note the antiserum is usually provided already in 1/100 dilution so dilute a further 1/200 only).
- 12) Add 65 µl of 1° antiserum to each well containing sample dilutions and standard dilutions. Note this is providing a final dilution of the antiserum in the plate of 1/60,000.
- 13) Place on plate shaker for about 4 min. Take care to avoid spillage by increasing speed gradually.
- 14) Seal plates, wrap in wet paper and incubate overnight at 4°C.

* Mixing can be facilitated by placing the plate on the plate shaker while transferring samples between columns.

Figure 1: Spiggin ELISA plate layout



Example Spiggin Standards wells in detail

E9 = 500 units	E10 = 50 units	E11 = 5 units	E12 = 0.5 units
F9 = 500 units	F10 = 50 units	F11 = 5 units	F12 = 0.5 units
G9 = 100 units	G10 = 10 units	G11 = 1 units	G12 = 0.1 units
H9 = 100 units	H10 = 10 units	H11 = 1 units	H12 = 0.1 units

DAY 2: Transfer incubations to high binding plates.

- 1) Rinse the wells of the coated plates 3X with washing buffer

- 2) Starting from last column (column 12) transfer 150µl of the sample incubations (in the low binding plates) to the corresponding wells on the coated plates (the high binding). Discharge pipettes tips after transferring column 9 and before starting transferring column 8. Repeat for columns 8 and 4.
- 3) Seal plates, wrap in wet paper, place in a plastic bag and incubate for 4-6h at room temperature.
- 4) After 4-6 hours, rinse wells 3X with washing buffer.
- 5) Add 150µl of 2° antibody (anti-rabbit alkaline phosphatase) diluted 1:15,000 in assay buffer to all wells (15ml buffer / plate).
- 6) Seal plates, wrap in wet paper, place in a plastic bag and incubate overnight at 4°C
- 7) Prepare 0.2 M Tris buffer from pNPP Kit (by dissolving 1 tablet in 20ml DW), mix and store at 4°C. We found that preparation of the substrate buffer the day before reduces the amount of cloudiness in the final buffer and gives better results.

DAY 3

- 1) Remove pNPP tablets from freezer and warm to room temperature (1 per 20ml)
- 2) Remove plates and wash 3X with distilled water (Note, do not use wash buffer for this wash).
- 3) Dissolve pNPP tablet in 0.2 M Tris buffer (prepared the evening before) but be aware that colour development is affected by light. Cover flask with foil or dark paper while dissolving.
- 4) Add 150µl of pNPP to each well as quickly as possible
- 5) Allow colour to develop (~40min) until standards read 2.0-2.4 optical density (by checking the first plate every 5 min)
- 6) Take the reading and save the file

Calculation of spiggin units

The reading taken directly from the file represents spiggin units/ml of sample digest. Quite often more than one value is within the standard curve so an average value should be calculated. The closer the absorbance reading to the middle of the standard curve is, the more accurate the calculated value.

Calculate “spiggin units /kidney” by multiplying the spiggin units/ml (value from plate reader) by 0.2 (representing the 200µl of buffer or 0.4 if 400µl were added) followed by 100 (this represents the dilutions made to the original standard made using fully hypertrophied kidneys from males).

Divide “spiggin units/kidney” by the body weight of the fish to obtain the preferred expression of Spiggin Units /g of body weight. Usually up to 100 units/g of body weight means there is practically no glue in the kidney. Values over 500 units are indicative of spiggin presence in the kidney. Samples that fall in between these values should be re-assayed to confirm if there was a slight contamination from near-by high values or a real low reading.

ANNEX 5**CHEMICAL CHARACTERISTICS OF ACCEPTABLE DILUTION WATER**

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

ANNEX 6

PHOTOGRAPHS OF MALE (TOP) AND FEMALE (BOTTOM) STICKLEBACK



Sticklebacks display strong sexual dimorphism (the males develop blue irises and red throats only during their breeding season).

ANNEX 7

DECISION FLOWCHART FOR THE STATISTICAL ANALYSIS

