INTERCALIBRATION EXERCISE USING A STICKLEBACK ENDOCRINE DISRUPTER SCREENING ASSAY

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Introduction

The Organisation for Economic Co-operation and Development (OECD) is currently in the process of validating a fish screening guideline for endocrine disrupters. This guideline currently uses three core fish species – Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*), and employs three core endpoints – vitellogenin (VTG) induction, gross morphology (secondary sexual characteristics and gonadosomatic index) and gonad histology.

None of the core species are indigenous to the UK or other European waters and none of them have an unambiguous, diagnostic endpoint for the detection of androgens and their antagonists.

Research under the UK Endocrine Disruption in the Marine Environment (EDMAR) programme produced an androgen-specific biomarker using the three-spined stickleback (*Gasterosteus aculeatus* – Figure 1). This biomarker is spiggin, a proteinaceous glue normally produced by males for constructing nests during the breeding season, but which is also produced by females exposed to exogenous androgens. This work was advanced further by the development of a specific marker for oestrogenic exposure in this species – VTG induction.

Figure 1: Stickleback Gasterosteus aculeatus. *Male displaying breeding colours.*

The work under EDMAR led to the realisation that the UK has a possible additional/alternative test species which could be included in the OECD fish screening guideline and this was brought to the attention of the OECD Validation Management Group-Ecotoxicology (VMG-Eco), which is responsible for overseeing the validation of the fish screen.

The VMG-Eco agreed that the UK should conduct a small interlaboratory calibration to evaluate the feasibility of the OECD fish screening assay, adapted for stickleback. This would serve to generate information on the reproducibility of the stickleback-based method, thus bringing available information into line with the core species and justifying further validation with larger-scale intercalibrations.

Three laboratories participated in the intercalibration:

Centre for Environment, Fisheries and Aquaculture Science, UK (CEFAS – Lead Laboratory);

Centre for Ecology and Hydrology (CEH), UK;

University of Bergen, Norway.

Experimental design

- The experimental design (Figure 2) mimicked, as far as possible, the first OECD intercalibration that was conducted with the core species (named Phase 1a).

17 ß-OESTRADIOL	17 ß -TRENBOLONE
Dilution water control	Dilution water control
Solvent (methanol 0.0001%) control	Solvent (methanol 0.001%) control
10 ng/l	50 ng/l
32 ng/l	500 ng/l
100 ng/l	5000 ng/l

FOR BOTH CHEMICALS

Measurements/observations made on each individual animal:
wet weight, vitellogenin, spiggin
gonad histology, GSI

	DAY 0	DAY 14	DAY 21
Control	8F + 8M	10F + 10M	10F + 10M
Concentration 1		10F + 10M	10F + 10M
Concentration 2		10F + 10M	10F + 10M
Concentration 3		10F + 10M	10F + 10M

Total number of fish sampled per sampling event:

Day 0	Day 14	Day 21
16 fish in control only	80 fish in treatments and control	80 fish in treatments and control

Total number of fish used for both chemicals: (16+80+80)* 2 chemicals = **352** fish

Figure 2: Experimental design for the intercalibrations

- Three-week continuous exposure via water
- Two "replicates" per treatment – separate male and female test vessels
- Endpoints measured (both males and females): VTG induction in plasma (ELISA), spiggin induction in kidney (ELISA), Gonadosomatic Index (GSI), gonad histology.
- Lead lab distributed ELISA reagents and standards to other participants -polyclonal antiserum against spiggin, polyclonal antiserum against stickleback vitellogenin, spiggin standard and purified stickleback vitellogenin standard (lyophilised powder).
- Criteria for the histological assessment of gonadal development of male and female sticklebacks were developed by the lead laboratory.
- Histology was conducted by CEFAS and CEH only.

Statistical analyses

- Since raw data demonstrated non-equal variances and non-normality, VTG and spiggin values were firstly log transformed.
- General Linear Model (GLM) procedure (analogous to an ANOVA, but for unequal sample sizes per treatment) followed by a two-tailed Dunnet's Test, in order to assess the ability of each laboratory to detect increases or decreases in the endpoints measured. All treatments (including the water only control) were compared with the solvent control.

Results

E2 exposure

- The concentration of VTG in males exposed to E2 is shown in Figure 3. Limit of detection for the VTG ELISA is 0.01 µg/ml.

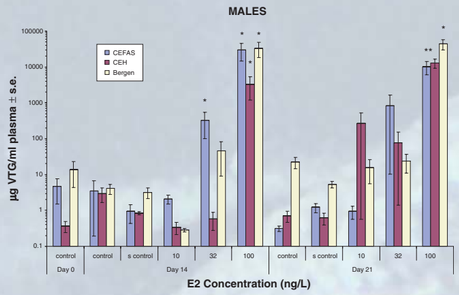

*Figure 3: Vitellogenin (VTG) induction in male stickleback exposed to E2 for 14 and 21 days. Values plotted as means ± standard error on a log scale. *denotes significantly different (p<0.0001) from the relevant solvent control.*

- Baseline levels of VTG (i.e. concentrations typically found in the initial control and in the experimental controls) were low, and generally in the region of <10µg/ml.
- All three laboratories detected a significant increase in VTG in males after 14 and 21 days at a concentration of 100 ng/L.
- Lowest Observed Effect Concentration (LOEC) = 100 ng/L.
- No Observed Effect Concentration (NOEC) of 32 ng/L.
- One laboratory (CEFAS) detected a significant increase at 32 ng/L after 14 days; this was not the case after 21 days exposure, although a trend of increasing VTG with concentration appeared to exist.
- No significant concentration-related VTG response in females (data not shown).
- No response in the spiggin endpoint in either males or females (data not shown).

Acknowledgment

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Trenbolone exposure

- The concentration of spiggin in females exposed to trenbolone is shown in Figure 4. Limit of detection is 0.8 units/ml of kidney homogenate (when expressed in spiggin units/g of body weight, values up to 80 spiggin units are regarded as negative, due to multiplications needed to normalise the samples with the standard dilutions).

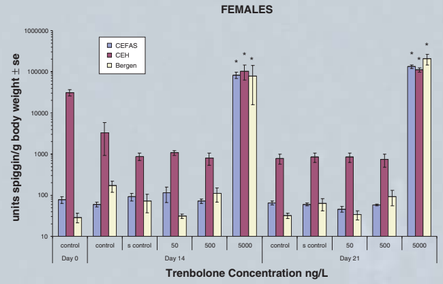

*Figure 4: Spiggin induction in female stickleback exposed to the synthetic androgen Trenbolone, for 14 and 21 days. Values plotted as means ± standard error on a log scale. * denotes significantly different (p<0.0001) from the relevant solvent control.*

- Baseline levels of spiggin in CEFAS and Bergen females were below 100 Units/g body weight.
- All three laboratories detected a significant increase in spiggin in females after 14 and 21 days at the highest concentration 5000 ng/L
- LOEC = 5000 ng/L
- NOEC = 500 ng/L.
- Induction of spiggin in female stickleback is the main diagnostic endpoint for androgenic activity, which is unambiguous and unique to the stickleback. Since this endpoint is absent in the OECD core species, the main endpoint for androgenic activity is VTG suppression in females, an effect which is not androgen-specific.
- VTG levels in females presented in Figure 5. Females showed varying responses to the androgen and, taken together, the results did not support the identification of a NOEC or LOEC.
- There was no consistent concentration-related effect of trenbolone on male spiggin or VTG levels

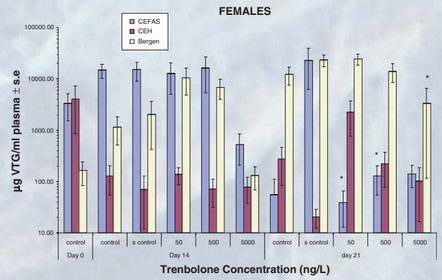

*Figure 5: Vitellogenin (VTG) concentrations in female stickleback exposed to the synthetic androgen Trenbolone, for 14 and 21 days. Values plotted as means ± standard error on a log scale. * denotes significantly different (p<0.05) from the relevant solvent control.*

- GSIs showed no consistent pattern in either exposure (Figure 6), indicating that this endpoint did not respond to E2 or trenbolone. This is in agreement with Phase 1a findings.
- Histological assessment of the gonads indicated that the majority of fish from both CEFAS and CEH had mature-stage gonads and there was no significant effect of either test compound on development.

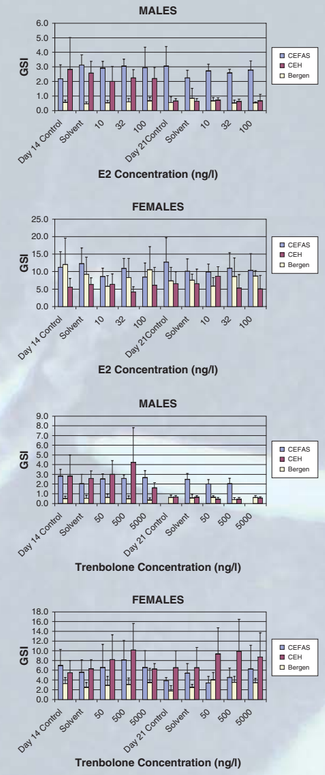

Figure 6: GSI of males and females in the E2 and trenbolone exposures at Days 14 and 21.

- A single case of intersex was observed in one male from the CEFAS E2 exposure (Figure 7), but this was an isolated incidence and is unlikely to be the result of exposure to the oestrogen.

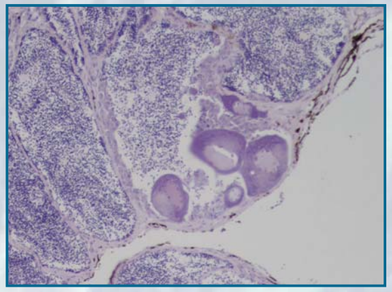

Figure 7: Histological section of a male stickleback testis showing testis-ova (intersex), from the CEFAS E2 (32 ng/L) exposure.

Conclusions

- This intercalibration exercise successfully demonstrated that the stickleback is a suitable test species for inclusion in the OECD fish screening guideline, possessing valid, reproducible, diagnostic endpoints for the detection of potent oestrogens (VTG induction in males) and androgens (spiggin induction in females).
- VTG suppression in females cannot be considered to be a diagnostic or reliable endpoint for the detection of androgens in a stickleback screening assay.
- GSI as a core endpoint therefore has limited value within a screening assay for endocrine disrupters.
- The results obtained here are comparable with those obtained with the core species. Although medaka and fathead minnow demonstrated slightly greater sensitivity, this may be due to the significantly warmer temperatures used (25°C vs 15°C). A slower metabolic rate in stickleback may lead to a slower response to the oestrogen/androgen.
- A further intercalibration is required to mimic the second OECD intercalibration that recently took place with the core species, using a weak oestrogen (4-tert-pentylphenol), an anti-androgen (flutamide) and an aromatase inhibitor (prochloraz).
- This will fully align the stickleback validation with the core species
- The UK is currently gauging international interest in participating in this larger-scale exercise and funds are being sought.

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