Report of Eight 21-day Fish Endocrine Screening Assays With Additional Test Substances for Phase-3 of the OECD Validation Program: Studies with Octylphenol in the Fathead Minnow (*Pimephales promelas*) and Zebrafish (*Danio rerio*) and with Sodium Pentachlorophenol and Androstenedione in the Fathead Minnow (*Pimephales promelas*)

This summary report was based upon 8 laboratory study reports.

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Background

Under the auspices of the Validation Management Group for Ecotoxicity Testing two protocols for a fish screening assay for endocrine active compounds have been through multi-laboratory testing. The data are reported as Phase-1A and Phase-1B for each of the two protocols, differing by the housing conditions of fish. The test substances in those studies tended to be potent, positive pharmaceutical compounds (e.g., 17β-estradiol and trenbolone in Phase-1A and Fadrozole, Prochloraz, and Flutamide in Phase-1B) and only a single weak positive, *t*-pentylphenol, in Phase-1B. In Phase-2, the Phase-1B protocol was tested with further negative substances: potassium permanganate and n-octanol in three species. Additionally, 2-methoxyethanol was voluntarily tested in as ingle laboratory with the fathead minnow. These results of these studies have already been reported in the OECD Phase 2 report of the validation of the 21-d fish endocrine screening assay and in the CEFIC study report with the three test substances in a single laboratory.

At the 4th meeting of the Validation Management Group for Ecotoxicity Testing in January 2006, the Phase-1B protocol was modified. It was recommended to reduce the mandatory endpoints to vitellogenin and secondary sexual characteristics. Additionally, the limited nature of the test substances in relation to the Guidance Document 34 criteria for interlaboratory substances with both positive and negative substances was noted.

CEFIC volunteered to conduct additional studies with another weak estrogen (octylphenol), a weak aromatizable androgen (androstenedione), and a difficult negative substance (sodium pentachlorophenol) in two laboratories each. This was designated Phase-3 and other laboratories were invited to participate. Denmark and Germany responded that they would support studies in zebrafish with octylphenol.

These additional Phase-3 studies have been conducted by a total of 5 laboratories. The present document is a detailed analysis of the Phase-3. The conclusions from these additional studies are in full concordance with the OECD Phase-2 report and the CEFIC study report of the validation of the 21-day fish endocrine screening assay. Further recommendations are proposed herein which may feed into further discussions on the Test Guideline by the appropriate group at the OECD, following the peer-review. However, these recommendations have not been examined and discussed by the VMG-eco or any other OECD group.

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SUMMARY

The eight studies in this report were conducted as part of OECD program to validate a standardized OECD protocol on adult fish for the detection of endocrine active substances (i.e. estrogen, aromatase inhibitors, androgen). Work in the previous Phases involved 3 fish species and a set of positive and negative compounds. In January of 2006, CEFIC volunteered to support additional work with three test substances in the fathead minnow (*Pimephales promelas*). Other laboratories were invited to participate, and both Denmark and Germany agreed to support studies with octylphenol in the zebrafish (*Danio rerio*). These studies were conducted in August 2006-May 2007 to support the scheduled peer review of the OECD 21 day fish endocrine screening assay. Given that the database with the third species, medaka (*Oryzias latipes*) was more extensive (e.g., octylphenol and pentachlorophenol have already been tested by the Ministry of Environment, Japan), the VMG-eco and the EDTA Task Force recognized that further studies were not necessary for the validation with medaka.

The objective of the Phase-3 studies was to test the relevance and specificity of two endocrine-related endpoints, vitellogenin (VTG) and secondary sexual characteristics (SSC), with the additional test substances. The objective was to challenge the protocol with a set of difficult test substances. The three test substances were a weak estrogen (octylphenol), a weak aromatizable androgen (androstenedione), and a difficult negative test substance (sodium pentachlorophenol).

- Octylphenol is considered a difficult weak estrogen as the substance has low affinity for the estrogen receptor (ER) ligand while becoming toxic to fish and leading to mortality within an approximate order of magnitude after achieving estrogenic doses capable of inducing VTG. Therefore, dose selection and the ability of laboratories to administer an estrogenic concentrations without mortality in aquatic test systems challenges the protocol.
- Androstenedione is a natural metabolite of testosterone present in most sewage systems receiving human wastes. Androstenedione is considered a difficult compound as it may present estrogenic properties if aromatized, it is a weak androgen receptor (AR) ligand, and it has limited water solubility. Therefore, it will present issues of VTG induction as an apparent estrogen, achieving detectable changes in female SSC characteristics and challenge laboratory dosing and analysis.
- Sodium pentachlorophenol is considered a difficult negative substance due to its toxicity, while the chorine atoms are known to inhibit ER binding in fish and mammals. It is negative in the mammalian uterotrophic bioassay. Under the conditions of the bioassay, the pKa of the phenolic group (4.74) will result in primarily the dissociated form which should not be active.

The target (nominal) concentrations were 3, 30 and 300 µg/l for *tert*-octylphenol (CASRN 140-66-9); 0.5, 5, and 50 µg/l for androst-4-ene-3,17-dione (CASRN 63-05-8), and 1, 10, and 100 µg/l for sodium pentachlorophenol (CASRN 131-52-2). The voluntary reference chemical was 17β-estradiol (CASRN) with a target concentration of 100 ng/l.

For validation, OECD Guidance Document 34 calls for studies to be conducted under code (OECD, 2005). However, aquatic toxicology studies themselves cannot be coded as the presence of the dilution systems for the test and reference substances clearly indicate which groups are being dosed, and also the approximate order of the test substance administration from low to high concentrations. Further, periodic chemical analyses are required to determine the actual or measured concentrations. Therefore, to ensure that these studies were in accordance with Guidance Document 34 in principle, the VTG analyses and the tubercle scoring exercises were conducted under code so that these values were achieved by blinded technical personnel. This is important to the success and acceptance of the 21-day fish endocrine screen validation program as none of the previous studies had been conducted where the observations were blind. These studies should then provide further support to the compliance of the OECD 21-day fish endocrine screening program with the criteria of Guidance Document 34.

The results of these studies support that interlaboratory studies have been done with additional positive and negative compounds which being of weak potency for the positives and a difficult negative test substance are representative compounds in a validation study for the protocol in regulatory applications. The results of these studies herein fully support the overall conclusion of the OECD validation program that VTG and SSC can be successfully employed to screen for certain endocrine modes of actions (i.e., estrogens, androgens, and aromatase inhibitors). At concentrations absent of overt signs of toxicity, there were no false positives or negatives observed with any of the three negative test substances directly related to the protocol itself.

Mortality, symptoms of intoxication, and other sublethal effects were occasionally evident and need to be incorporated into the overall interpretation. For example, in one laboratory, mortality at high sodium pentachlorophenol concentrations was coincident with absolute decreases in VTG and SCC, following the same concentration patterns as the intoxication symptoms and mortality. This is interpreted as related to general toxicity and not to any endocrine-related affect.

The VTG endpoint was consistently successful in both the zebrafish and the fathead minnow. Three of four laboratories observed statistically significant changes with octylphenol. The fourth laboratory suffered complete male mortality at the high dose of octylphenol where statistical significance occurred in the other laboratories. With androstenedione, both laboratories observed statistically significant increases in male VTG, indicating aromatization of certain androgens will result in both the induction of VTG in males and the emergence of tubercles in female fish. Sodium pentachlorophenol was judged a challenging negative substance with a size and hydrophobicity similar to an estrogen ligand. As expected for a robust endpoint, both laboratories failed to observe any absolute or statistically significant increases in VTG in either sex. Rather, absolute VTG decreases were observed at toxic levels of this compound in one laboratory. The latter finding is consistent with Phase-2 studies that indicated interpretative questions of about the role of systemic toxicity need to be taken into account. That is, systemic toxicity may impair the individuals' ability to produce VTG in such a group and should not be interpreted as an endocrine mediated disturbance. As noted, the latter is not interpreted as an endocrine mediated disturbance.

The SSC tubercle score endpoint was also successful in these studies in the fathead minnow. No SCC endpoints are available in the zebrafish. There was a decline in the absolute values of the fathead minnow male mean tubercle score at the high concentration of octylphenol and also with the reference compound, 17β-estradiol, consistent with previous observations with estrogens. The androstenedione did produce the statistically significant appearance of tubercles in females in both laboratories. There was an absolute decline in tubercle number and mean tubercle score at the high concentration of sodium pentachlorophenol in one laboratory synonymous with systemic toxicity and symptoms of intoxication. As noted, the latter is not interpreted as an endocrine mediated disturbance.

Several laboratories voluntarily employed the chosen concentration of the reference estrogen, 17β-estradiol. The findings reproduced the VTG and SCC changes observed previously in Phase-1B and Phase-2. Therefore, with this potent estrogen these studies support that the protocol is reproducible over time in different laboratories.

There were two other noteworthy observations.

- The first observation was for the VTG endpoint. In one laboratory, VTG baselines in males were below the ELISA detection limit. This presented issues of how to run statistical analyses as there was no real distribution of values to enter for the statistical analysis. Entering the detection limit or one half of the detection limit would present no coefficient of variation, sharply encouraging a finding of statistical significance with limited absolute differences with other groups. Given the natural variability in the VTG from group-to-group this could encourage false positives. To avoid this possibility, a random set of values was generated with a distribution equivalent to other groups and with a mean at one half of the detection limit. This did not impact the finding of statistical significance in this case. In another

laboratory, the VTG values exceeded the upper part of the calibration curve, but this presents no evident statistical issues.

- The second observation was for the tubercle endpoint. The female fish begin with a consistent baseline of zero. As with the VTG, this lack of variability increases the statistical sensitivity. Thus, there were no issues with detecting the relatively small increases in tubercle scores with the androstenedione. In this latter case, these circumstances then increase the basic sensitivity of the assay for androgens.

As difficulties had been encountered in previous studies with immature fish, the previous acceptance criteria for the age of fish on test were waived and older fish were accepted. These studies met the other previous test acceptance criteria for the fish to be placed on test, met the test criteria for mortality, O_2 saturation and temperature, and the measured values of the test substances are satisfactory to accept all three studies. The analytical recoveries of the test substances were acceptable, taking into account that the androstenedione was a difficult to test substance due to both its water solubility and potentially rapid biodegradation.

The following conclusions and recommendations are offered. These recommendations have not yet been discussed by an OECD group and should, therefore, be taken as proposals, and may or may not form the basis of further work/discussion on the future Test Guideline

- At non-toxic concentrations, the VTG and SSC endpoints consistently responded to the test substances as expected. The studies support the capability of these endpoints to correctly identify endocrine modes of action when other toxicities are not present. Collectively, with previous data, these findings support the validation of these endpoints.

- Given the evidence for impact of systemic toxicity and sublethal effects on several endpoints, several changes in the protocol and the interpretation of its results are recommended to resolve the issue of potential false positives in such circumstances:
    - Avoid concentrations with high mortality and symptoms of intoxication by refining concentration selection guidance beyond acute toxicity (LC50). The concentration selection should be informed with data from range finding studies, other species (as was done with medaka octylphenol results), and juvenile toxicity studies or early life stage studies.
    - Thus, in a straightforward solution, incorporate in the interpretative section of a future Test Guideline that positive findings for possible endocrine related effects should occur in the absence of confounders for systemic toxicity and symptoms of intoxication and that particularly vulnerable endpoints such as reproduction would be recognized.

- These study results with sodium pentachlorophenol, as with previous potassium permanganate studies, would support some caution in interpreting a decline in the tubercle number and score in the presence of severe mortality and symptoms of intoxication.

BACKGROUND, PROTOCOL, MATERIALS, AND METHODS

Background and Objectives.

These Phase-3 studies followed the Phase-2 of the validation of the 21-day fish screening assay with negative test substances in 3 test species: fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). The objective was to continue the testing of the relevance and reproducibility of two endocrine-related endpoints: vitellogenin (VTG) and secondary sexual characteristics (SSC). As the VMG-eco has not continued inter-laboratory validation work with three reproductive endpoints (spawning activity, fecundity, and gonadal histopathology), these endpoints were not included in this protocol although some laboratories did voluntarily include observations of spawning activity and egg production. The current studies employed difficult and challenging test substances: a weak estrogen (octylphenol), a weak aromatizable androgen (androstenedione), and a difficult negative test substance (sodium pentachlorophenol).

Overall Design & Protocol.

The protocol differed from that used in Phase-1B and Phase-2 in a) removing the reproductive endpoints, b) while continuing to employ five males and five females in two replicate tanks with zebrafish, the design was altered to four replicates with 2 males and 4 females per replicates (a total of 8 males and 16 females) with the fathead minnow. This latter basic design was followed by the fathead minnow laboratories in Phase-2. The protocol is provided in Annex I.

Reporting and Statistical Procedures.

The five laboratories were supplied with a standardized Excel spreadsheet for recording and transmittal of their results for analysis.

Effects VTG and SCC of the test substance concentrations were statistically analysed. The statistical procedures first evaluated the possibility that the two test vessels gave statistically different results using Student's T-Test. There were no occurrences of statistical significance in some 40 opportunities.

Then, the VTG and SCC results were evaluated for homogeneity of variance and normal distribution using Levene's test, which was considered more robust for the distribution normality than Bartlett's test. If the data fulfilled the qualifications, the data were analyzed using parametric statistical tests: ANOVA followed by Dunnett's procedures. If the data did not fulfill the qualifications, a Pairwise Mann-Whitney U-test was employed and, as this analysis is a one-to-one comparison of test groups with the control in a multi-test group protocol (pairwise), it was followed, if positive, by Bonferroni's adjustment to account for the possible error of the multiple groups. Differences were considered to be statistically significant at $p < 0.05$ in all tests. The statistical software used was SAS version 8.2 (SAS Institute, Cary, NC).

Fish Sources and Acclimation.

In all cases, the fish were acclimated for 2 weeks before placement on study. The sources for the fish were:

- Laboratory 1, zebrafish: Hørsholm Dyrehandel, Usserød Kongevej 43, 2970 Hørsholm, Denmark.
- Laboratory 2, zebrafish: West Aquarium, Bad Lauterberg, Germany.
- Laboratory 3, fathead minnow: Brixham Environmental Laboratory, Freshwater Quarry, Brixham, Devon, UK
- Laboratory 4, fathead minnow: New England Bioassay, 77 Batson Drive, Manchester, Connecticut 06042 USA.
- Laboratory 5, fathead minnow: Osage Catfisheries Inc., Osage Beach, MO 65065, USA

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As the previous validation studies encountered difficulties with the presence of immature fish, the previous criteria for fish age (20 weeks ± two weeks) was waived, and the use of older fish was encouraged. The ages of the fish on study were:

- Laboratory 1, zebrafish: 3 months.
- Laboratory 2, zebrafish: 8 months.
- Laboratory 3, fathead minnow: 11 months for octylphenol and 9 months for sodium pentachlorophenol.
- Laboratory 4, fathead minnow: 6.25 months for octylphenol and 9 months for androstenedione.
- Laboratory 5, fathead minnow: 5 months for both androstenedione and sodium pentachlorophenol.

The mean weights of the sexes at the beginning of the study were:

- Laboratory 1, zebrafish, octylphenol: Male weights were 0.347± 0.096 g, and females were 0.766 ± 0.206 g.
- Laboratory 2, zebrafish, octylphenol: Male weights were 0.357 ± 0.044 g, and females were 0.449 ± 0.0.84 g.
- Laboratory 3, fathead minnow, octylphenol: Male weights were 2.11 ± 0.294 g, and females were 1.34 ± 0.301 g.
- Laboratory 3, fathead minnow, sodium pentachlorophenol: Male weights 2.26 ± 0.54 g, and females were 1.42 ± 0.32 g.
- Laboratory 4, fathead minnow, octylphenol: Male weights were 1.9 ± 0.3 g, and females were 1.1 ± 0.2 g.
- Laboratory 4, fathead minnow, androstenedione: Male weights 2.32 ± 0.24 g, and females were 1.50 ± 0.21 g.
- Laboratory 5, fathead minnow, the two studies for androstenedione and sodium pentachlorophenol were run concurrently from one overall supply of fish: Male weights 2.03 ± 0.51 g, and females were 1.35 ± 0.30 g.

Test Materials.

For Laboratory 1, octylphenol was from Aldrich 29082-3 Lot 07604CD-146, and for Laboratory 2 Sigma Lot 05130 KD-266. For Laboratory 3, octylphenol was from Sigma Lot 07604CD-475. For Laboratory 4, octylphenol was from Sigma Lot 07604CD. For Laboratory 4, androstenedione was from Sigma, Lot 115K12921, and for laboratory 5, Sigma, Lot 115K1292. For Laboratory 3, sodium pentachlorophenol was from Aldrich Lot 07119HO-473, and for laboratory 5, Sigma-Aldrich, Lot 06324ED-196.

Some laboratories used 17β-estradiol as a positive reference compound. For laboratory 1, this was from Sigma Lot 044K1027; for Laboratory 4, this was from Sigma Lot 19217DE; and, for laboratory 5, this was from Sigma Lot 1203667.

Vitellogenin was for Laboratory 1 was performed at SDU, Denmark, and for Laboratory 2 by Henrik Holbech, Odense, Denmark. The ELISA kits were not identified. Vitellogenin was analysed in Laboratory 3 with fathead minnow vitellogenin ELISA Kit from Biosense Laboratories AS (Prod. No.: V01018401); in Laboratory 4 with fathead minnow vitellogenin ELISA Kit from the Cayman Chemical Company; Lot #:169395; and in Laboratory 5 with fathead minnow vitellogenin ELISA Kit from Biosense Laboratories AS.

Facility, Water, and Other Experimental Conditions.

For the zebrafish, the fish loading rate was <5 g/L. Water temperature was 25 ± 2 °C. Uneaten food and fecal material were removed from the bottom of the vessels every day by suction.

- Laboratory 1, water source was artificial reconstituted water, the test solution volume per tank was 10 L, the volume exchange was 5 tanks per day, and the fish were fed with brine shrimp (*Artemia* sp INVE Aquacultura Nutrition, Belgium.) *ad libitum* two times per day, with at least three hours between feedings.

- Laboratory 2, the water source was purified tap water, the test solution volume per tank was 12 L, the volume exchange was 5 tanks per day, and the fish were fed with brine shrimp (*Artemia* sp. TetraMin, Hauptfutter, Germany) *ad libitum* two times per day, with at least three hours between feedings.

For the fathead minnows, the fish loading rate was <5 g/L. There were 2 plastic spawning tiles sitting on a flat plastic base per replicate tank while on study. The tiles were observed daily, and, if eggs were found, then the tiles were removed, eggs counted, and the tiles replaced. Water temperature was 25 ± 2 °C. Uneaten food and fecal material were removed from the bottom of the vessels every day by suction.

- Laboratory 3, water source was dechlorinated tap water, , the test solution volume per tank was 45 L, the volume exchange was 5.12 tanks per day, and the fish were fed with a combination of pellets (BioOptimal pellet from BioMar) and brine shrimp (*Artemia* sp. Gamma Frozen Brineshrimp from Gamma Frozen Food) *ad libitum* two times per day, with at least three hours between feedings.
- Laboratory 4, the water source was Lake Huron water, the test solution volume per tank was 20 L, the volume exchange was 7.9 tanks per day, and the fish were fed with brine shrimp (*Artemia* sp.) *ad libitum* two times per day, with at least three hours between feedings.
- Laboratory 5, the water source was a mixture of tap water and deionized water, the test solution volume per tank was 20 L, the volume exchange was 5 tanks per day, and the fish were fed with brine shrimp (*Artemia* sp.) *ad libitum.*

Assay Validity Criteria.

The validity criteria for the test were met in all cases: a) mortality during acclimation did not exceed the limits, b) water temperature did not differ by more than ± 1°C and was always within the temperature range 25 ± 2°C specified for fathead minnow, and c) the oxygen concentration in the test media did not fall below 60% of air saturation during the study.

RESULTS

I. Dosing and Test Substance Analyses

Dosing procedures and analytical results for the studies are described below.

a. Octylphenol

The octylphenol stock solutions were prepared daily and introduced into the aquaria using semi-continuous diluters (flow through conditions). Nominal test concentrations were 3, 30 and 300 µg/l. The mixing vessels and the aquaria were connected by a tube. The water temperatures, pH-values and dissolved oxygen concentrations were measured daily in most laboratories. The mean concentrations of measured octylphenol for the four laboratories are shown in Table 1A.

Table 1A. Measured concentrations in the octylphenol studies and percent of the target nominal concentrations [a]

Laboratory	Solvent Control	Low Octylphenol (3 µg/l)	Intermediate Octylphenol (30 µg/l)	High Octylphenol (300 µg/l)	17β-Estradiol (100 ng/l)
1	ND	3.9 (128.8%)	17.5 (58.3%)	167.1 (55.7%)	72.2 (72.2%)
2	ND	2.4 (80.5%)	25.0 (83.4%)	269.0 (89.7%)	ND
3	Yes	2.35 (78.3%)	24.3 (81.1%)	205.6 (68.5%	ND
4	Yes	3.05 (101.8%)	29.7 (98.8%)	342.4 (114.1%)	67.6 (67.6%)

[a] Concentrations are the mean of the two vessels per group. ND: Not done

b. Androstenedione

The androstenedione stock solutions were prepared daily and introduced into the aquaria using semi-continuous diluters (flow through conditions). Nominal test concentrations were 0.5, 5, and 50 µg/l. The water temperatures, pH-values and dissolved oxygen concentrations were measured daily. The mean concentrations of measured androstenedione for the two laboratories are shown in Table 1B. Laboratory 4 measured the androstenedione in the initial diluter, and target concentrations were achieved. This laboratory also observed bacterial growth, particularly in the high concentration vessels. Laboratory 5 measured concentrations had a high standard deviation between the two replicate vessels at all three concentrations, but the mean concentrations were higher than Laboratory 4. The loss of androstenedione in Laboratory 4 and some Laboratory 5 vessels is attributed to possible loss on the surface of the system and possible biodegradation. This supports the original hypothesis that androstenedione was a difficult to test chemical.

Table 1B. Measured concentrations in the androstenedione studies and percent of the target nominal concentrations [a]

Laboratory	Solvent Control	Low Androstenedione (0.5 µg/l)	Intermediate Androstenedione (5 µg/l)	High Androstenedione (50 µg/l)	17β-Estradiol (100 ng/l)
4	Yes	0.21 (41.3%)	2.04 (40.8%)	10.9 (21.8%)	Not analyzed
5	Yes	0.423 (84.5%)	3.73 (74.7%)	40.5 (81%)	Not analyzed

[a] Concentrations are the mean of the two vessels per group. ND: Not done

c. Sodium Pentachlorophenol

The sodium pentachlorophenol stock solutions were prepared daily and introduced into the aquaria using semi-continuous diluters (flow through conditions). Nominal test concentrations were 1, 10, and 100 µg/l. The mixing vessels and the aquaria were connected by a tube. The water temperatures, pH-values and dissolved oxygen concentrations were measured daily. The mean concentrations of measured sodium pentachlorophenol for the two laboratories are shown in Table 1C. Measured concentrations were close to nominal in Laboratory 3 and at the intended nominal concentrations in Laboratory 5, This is consistent with the lack of degradation expected for the test substance.

Table 1C. Measured concentrations in the sodium pentachlorophenol (PCP) studies and percent of the target nominal concentrations [a]

Laboratory	Solvent Control	Low PCP (1 µg/l)	Intermediate PCP (10 µg/l)	High PCP (100 µg/l)	17β-Estradiol (100 ng/l)
3	ND	0.91 (91%)	7.2 (72.4%)	85.5 (85.5%)	Not analyzed
5	Yes	0.99 (99%)	11.43 (114.3%)	106.3 (106.3%)	Not analyzed

[a] Concentrations are the mean of the two vessels per group. ND: Not done

Dosing and Test Substance Analyses - Discussion and Conclusions

These measured values of administered test substances are satisfactory to accept all three studies. Together with the other test acceptance criteria, this indicates that the studies are themselves valid for use to assess the relevance and the reliability of the endpoints and the protocol.

II. Mortality, Intoxication, and Related Observations

During the test period all test fish were observed at least once daily for mortality and a variety of symptoms for intoxication. Dead fish were removed from the test vessel and discarded. Clearly distinguishable differences in appearance or intoxication behaviour between treated and control fish were documented daily.

a. Octylphenol

No mortality or symptoms of intoxication were observed in control replicates in any laboratory with octylphenol. Limited, non-dose related mortalities and no symptoms of intoxication or toxicity were observed in three laboratories. However, in laboratory 4, where the measured concentrations were greater than 300 µg/l mortality was extensive (all males and 5 of 10 females) and multiple symptoms of intoxication and toxicity were observed in fish prior to mortality.

b. Androstenedione

No mortality or symptoms of intoxication were observed in control replicates or solvent replicates in either laboratory with androstenedione. No mortality or symptoms of intoxication were observed in the test substance concentrations in laboratory 3. In laboratory 5, one male died in each of the low, intermediate, and high concentration groups, and two females died in the intermediate concentration group. There were no observations of intoxication or toxicity.

c. Sodium Pentachlorophenol

No mortality or symptoms of intoxication were observed in control replicates or solvent replicates in either laboratory with sodium pentachlorophenol. No mortality or symptoms of intoxication were observed in the test substance concentrations at the low and intermediate concentrations of sodium pentachlorophenol. However, in laboratory 3, 5 of 10 males and 5 of 10 females died at the high concentration of sodium pentachlorophenol. Symptoms of intoxication and toxicity were also observed. In laboratory 5, one male died in each of the low concentration, intermediate concentration, and positive reference groups, and one female died in the intermediate concentration group.

Mortality, Intoxication, and Related Observations - Discussion and Conclusions

Dose related mortality and symptoms of intoxication and toxicity were observed in two of the eight studies (laboratory 3 with sodium pentachlorophenol and laboratory 4 with octylphenol). Mortality and symptoms of intoxication were also related to the duration of exposure at a given concentration in these two studies. These observations are interpretated as evidence for systemic toxicity for the particular test substances at those doses.

III. Spawning Status and Fecundity

Although spawning status and fecundity were not integral to the protocol, both measured during the assay by several laboratories. For spawning status, the laboratory recorded the presence of eggs (yes/no answer) in

treated and control replicates on a daily basis. The data are presented as the total number of days that eggs were observed in each of the four replicates per group / the total number of days on test and then as the mean number of days that eggs were observed per group, e.g., 12 / 21. Laboratory 2 performed egg counts for their octylphenol studies.

a. Octylphenol

Laboratory 1 did not record spawning or fecundity observations. Laboratory 2 recorded total numbers of eggs produced per vessel by measuring the eggs daily, and the absolute number of eggs declined by about 50% in the high octylphenol concentration compared to the controls and the two lower doses. Laboratories 3 and 4 recorded the number of days that eggs were observed in the tiles for each vessel and expressed these as spawning activity (number of egg clutches observed divided by two as there were two vessels). Both laboratories observed dose related declines with octylphenol concentration. However, the spawning activity in laboratory 4 was only about one third to one fourth of the control and solvent control in laboratory 3, indicating lab-to-lab variation in this parameter. Further, the action of the reference 17β-estradiol is inconclusive with a value of 2 versus 3 in the control and 4 in the solvent control. Thus, the potent estrogenic compound may have only modestly affected spawning activity in laboratory 4, while spawning activity ceased at the high dose with octylphenol in that laboratory. The spawning status and fecundity data for octylphenol are presented in Table 2A.

Table 2A. Overall spawning status and fecundity parameters of zebrafish and fathead minnow during the 21-day screening assay exposure to octylphenol.

Laboratory		Control	Solvent control	Nominal Octylphenol (µg/l) 3	30	300	17β-Estradiol 100 ng/l
		Total Egg Numbers in Zebrafish					
2	**Vessel 1**	2048	NA	2602	2801	1295	NA
	Vessel 2	2976	NA	3143	3383	1326	NA
	Total	5024	NA	5745	6184	2621	NA
3		**Total spawning activity (daily observations of egg clutches per vessel/2)**					
		10.5	16	11.5	7	0	NA
4		**Total spawning activity (daily observations of egg clutches per vessel/2)**					
		3	4	2.5	1.5	0	2

b. Androstenedione

Androstenedione had no affect on the spawning activity of the fathead minnow in Laboratory 4. The spawning data for the control, the androstenedione concentrations, and 17β-estradiol were 3-6 fold higher that in the study with octylphenol suggesting study-to-study variability. Further, the 17β-estradiol reference concentration again failed to affect spawning activity in this laboratory. Laboratory 5 observed an absolute decrease in the solvent control; the low androstenedione concentration was similar to the dilution water control indicating no solvent affect, but rather variability of the measurement. At the intermediate and the high dose, there was a decrease in spawning activity, while the positive reference substance had no discernable affect. The spawning data for androstenedione are presented in Table 2B.

Table 2B. Overall spawning status and fecundity parameters of fathead minnows exposure to androstenedione.

Laboratory	Control	Solvent control	Nominal Androstenedione (µg/l) 0.5	5	50	17β-Estradiol 100 ng/l
4	**Total spawning activity (daily observations of egg clutches per vessel/2)**					
	9.5	NA	16	12	10.5	12.5
5	**Total spawning activity (daily observations of egg clutches per vessel/2)**					
	10.5	6	11.5	2	0	7

c. Sodium Pentachlorophenol

Sodium pentachlorophenol appeared to affect fathead minnow spawning activity in Laboratory 3 as spawning activity was decreased at the intermediate and high concentrations. Study-to-study variability was again suggested as Laboratory 3 spawning activity was only 20-40% of the control and solvent control in the octylphenol study. Laboratory 5 also observed decreased spawning activity in the test substance vessels, primarily versus the dilution water control and not the solvent control. The spawning data for sodium pentachlorophenol are presented in Table 2C.

Table 2C. Overall spawning status and fecundity parameters of fathead minnows exposed to sodium pentachlorophenol (PCP).

Laboratory	Control	Solvent control	Nominal PCP (µg/l) 1	10	100	17β-Estradiol 100 ng/l
3	**Total spawning activity (daily observations of egg clutches per vessel/2)**					
	4.5	3.5	2.5	0.5	1	NA
5	**Total spawning activity (daily observations of egg clutches per vessel/2)**					
	10.5	6	3.5	6	3.5	7

Spawning Activity and Fecundity – Discussion and Conclusions

As noted in Phase-1B, the presence of 5 males and 5 females in a single vessel did not result in optimal and consistent spawning activity in the fathead minnow. The results in Phase 3 studies were also highly variable within and among laboratories suggesting a lack of reproducibility. The relationship or lack of relationship with other observations was as follows:

- Octylphenol had a dose related effect on the total egg number of zebrafish and on the spawning activity of fathead minnows.
    - Although significant mortality occurred in Laboratory 4, Laboratories 2 and 3 did not observed mortality or significant signs of intoxication or toxicity.
    - 17β-estradiol had little or no effect on spawning activity in several cases, and, in Laboratory 4, the estradiol was barely different from the control and the low dose of the octylphenol.
    - Both octylphenol and 17β-estradiol significantly induced the production of VTG in males and increased the absolute values in females (see next section) so that a direct connection between VTG increases and decrements in spawning activity were not consistently observed.
    - Therefore, while octylphenol may have had an effect on fish reproduction at concentrations > 200 µg/l in these studies, but it cannot be related with confidence in these studies to an estrogenic mode of action.
- Androstenedione did not affect spawning activity in the fathead minnow in Laboratory 4 despite the induction of VTG in males and the appearance of tubercles in females. In Laboratory 5, the data suggest a decrease in spawning activity, and the measured concentrations were higher in this laboratory.
- Sodium pentachlorophenol appeared to affect spawning activity in Laboratory 3. This was coincident with mortality and other symptoms, absent increases of VTG in males or tubercles in females, and coincident with an absolute decrease in tubercles in males. However, no clear affect was seen in Laboratory 5 compared to the solvent control, and, in Laboratory 5, there was no general increase in mortality. Therefore, the decrease in spawning activity in Laboratory 3 is attributed to a systemic toxicity and mortality, and not an endocrine-related effect.

In conclusion, the spawning activity variability and inconsistency was similar to the results from the other fathead minnow laboratories in previous Phases of the validation program. Therefore, the previous decision of the VMG-eco to remove the spawning activity and fecundity measurements from the validation program appear to be justified. Further, spawning activity results need to be interpreted in an integrated way with the results from other compounds and other observations and some reproducibility needs to be established in order to relate this endpoint with any of the observations.

IV. Vitellogenin

VTG is a lipoprotein complex that is normally produced by the sexually active female in the liver, released into circulation, taken up by the developing oocyte, and processed to become part of the egg lipid and protein nutrients. VTG production is under estrogen control, where the mRNA transcription is under positive control by the ER-estradiol receptor-ligand complex. VTG production in the sexually active male is minimal. Therefore, the presence of elevated levels of VTG in the male is a sensitive indicator of estrogen exposure. Reduction in female VTG levels is considered an indicator of antiestrogens or aromatase exposure. VTG is measured by sampling serum at necropsy and analyzing using a species specific, monoclonal antibody ELISA kit. Due to some kit to kit and laboratory variations, a standardization curve is necessary for an analysis. There is typically a high variability of VTG values (high SD's), but true responses are sensitive and dramatic, and, thus, high enough to easily reach statistical significance.

a. Octylphenol

Octylphenol is a presumed estrogenic compound as several published studies have shown a statistically significant response in VTG to octylphenol exposure. Further, medaka respond with a statistically significant increase in VTG to octylphenol using protocols conducted in Japan similar to the current studies and in long-term fish life cycle studies.

The circulating levels of VTG in male fish increased in absolute value at the high octylphenol concentration and differed significantly from the control in three of four studies. In the fourth study, all males at the high concentration suffered mortality, and the VTG analyses for all males in other groups produced values below the detection limit in this study of 189 ng/ml.

In this laboratory, the uniform detection limit presented issues of how to run statistical analyses as there was no real distribution of values to enter for the statistical analysis. Entering the detection limit or one half of the detection limit would present no coefficient of variation, sharply encouraging a finding of statistical significance with limited absolute differences with other groups. Given the natural variability in the VTG from group-to-group this could encourage false positives. To avoid this possibility, a random set of values was generated with a distribution equivalent to other groups and with a mean at one half of the detection limit. This did not impact the finding of statistical significance in this case.

There was also an absolute increase in female circulating VTG at the high octylphenol concentration in three studies and a marginal decrease in a fourth. A statistically significance increase was observed in female VTG in three studies. VTG absolute values were not affected by the solvent in thee of the solvent controls, but there was an absolute increase in the females in laboratory 4 suggesting some background variation in the values. The VTG values were increased by exposure to 100 ng/l nominal values of the 17β-estradiol reference, and achieved statistical significance in the two male groups and one of the female groups. The data are presented in Table 3A.

b. Androstenedione

Published studies indicate that aromatizable androgens may lead to the formation of estrogens and the induction of VTG (Homung et al., 2004; Pawlowski et al., 2004). In previous validation studies with the non-aromatizable androgen, trenbolone, no VTG induction was observed. Based on its structure, androstenedione should be an aromatizable androgen, so that metabolic conversion to potentially estrogenic levels can be expected. Indeed, strong VTG induction was reproducibly observed in both laboratories in the males at the intermediate and high concentrations (5 and 50 µg/L). Increases in female VTG were observed in Laboratory 4 at both the intermediate and high concentrations and in Laboratory 5 at the high concentration. The data are presented in Table 3B.

Table 3A. Circulating VTG analyses for male and female fish exposed to octylphenol.

Laboratory	Group	Nominal Octylhenol (µg/L)	Circulating VTG (ng/ml) Male Mean Individual	Male *p*-value	Female Mean Individual	Female *p*-value
1	**Control**	0	2090		11,620,000	
	Solvent		753	0.4102	13,40,9376	0.9861
	Low	3	747	0.37530	10,303,000	0.9152
	Intermediate	30	1,510,464	0.4104	5,052,072	0.0811
	High	300	22,107,778	0.00011*	13,074,000	0.9995
	17β-estradiol	0.100	22,107,778	<0.00001*	8,267,000	0.5222
2	**Control**	0	416		3,546,250	
	Low	3	459	0.8804	5,335,714	0.2551
	Intermediate	30	582	0.7932	3,670,000	0.8985
	High	300	30,916,750	<0.0001*	12,147,778	0.1120 [a]
3	**Control**	0	479		3,414,625	
	Solvent	0	341	0.8603	3,154,300	0.9128
	Low	3	338	0.9176	2,875,350	0.9788
	Intermediate	30	2776	0.7817	2,900,563	0.9950
	High	300	1,652,886	<0.0001*	16,181,300	0.0001*
4	**Control**	0	180 [b]		1,950,162	
	Solvent	0	180 [b]	1.00	5,307,537	0.5678
	Low	3	180 [b]	1.00	3,341,474	0.9612
	Intermediate	30	180 [b]	1.00	3,501,709	0.9488
	High	300	[c]		21,002,657	<0.0001*
	17β-estradiol	0.100	9,258,481	<0.0001*	13,831,152	0.0003*

[a] The VTG values in this group where high variable, preventing the achievement of statistical significance after the application of Bonferroni's adjustment. [b] All values were at the detection limit in this laboratory.
[c] All male fish died at the high concentration before day 21 on test.
* Statistically significant change in replicates; NA – not applicable.

Table 3B. Circulating VTG analyses for male and female fathead minnows exposed to androstenedione.

Laboratory	Group	Nominal Androstenedione (µg/L)	Circulating VTG (ng/ml) Male Mean Individual	Male *p*-value	Female Mean Individual	Female *p*-value
4	**Control**	0	2517		3,219,186	
	Solvent	0.5	2459	1.00000 [a]	6,140451	0.9978
	Low	5	4806	1.00000 [a]	8,563841	0.9752
	Intermediate	50	15,523,068	0.00413*	26,359,886	0.1548 [b]
	High	0.100	55,591,289	0.00012*	46,063,909	0.0017*
5	**Control**	0	200		1,793,236	
	Solvent	0	1436	0.8621	2,081,554	0.9612
	Low	0.5	526	0.7434	1,644,264	0.9975
	Intermediate	5	667,797	0.00018*	2,902,962	0.0224*
	High	50	>3,150,000	<0.0001*	>3,150,000	0.0035*
	17β-estradiol	0.100	>3,150,000	<0.0001*	3,001,752	0.0074*

[a] The Bonferroni-adjustment results in a maximum p-value of 1
[b] The VTG values in this group where high variable, preventing the achievement of statistical significance.
* Statistically significant change in replicates; NA – not applicable.

c. Sodium pentachlorophenol

Sodium pentachlorophenol has been tested for ER binding in several systems, including medaka transcriptional assays, and was negative. This is expected as the phenolic hydroxyl group which is essential for ER affinity is stearically hindered by the adjacent chloro groups and the primary presence of the dissociated form of the compound as the pKa is 4.74. The mean circulating levels of VTG in the male and female were unchanged by sodium pentachlorophenol, although the female intermediate concentration in laboratory 4 was increased in absolute terms. This is attributed to normal variability. The positive reference, 17β-estradiol was included in the study from laboratory 3, and exposure did achieve statistical significance, indicating that the study was valid. The data are presented in Table 3C.

Table 3C. Circulating VTG analyses for fathead minnows exposed to sodium pentachlorophenol (PCP).

			Circulating VTG (ng/ml)			
			Male		Female	
Laboratory	**Group**	**Nominal PCP(μg/L)**	**Mean Individual**	***p*-value**	**Mean Individual**	***p*-value**
3	**Control**	0	545		2,464,350	
	Low	1	319	1.00000 [a]	3,193,833	0.7993
	Intermediate	10	1026	1.00000 [a]	4,067,800	0.1646
	High	100	705	1.00000 [a]	2,516,740	1.0000
	17β-estradiol	0.100	2,544,450	0.00073*	2,834,700	0.9749
5	**Control**	0	200		1,793,236	
	Solvent		1436	0.8621	2,081,554	0.9612
	Low	1	1906	0.9135	2,440,485	0.2613
	Intermediate	10	4096	0.2728	2,069,614	0.5036
	High	100	204	0.9935	2,023.854	0.9778
	17β-estradiol	0.100	>3,150,000	<0.0001*	3,001,752	0.0039*

[a] The Bonferroni-adjustment results in a maximum p-value of 1
* Statistically significant change, NA – not applicable.

Discussion and Conclusions – Vitellogenin Analyses

The VTG results were consistent with the expected modes of action of the test substances and were reproducible across laboratories. The results were achieved using blinded analysts. In conclusion, these studies support the validation of the VTG endpoint in the fish endocrine screen:

1. The VTG results were reproducible with difficult and challenging compounds.
2. The VTG results with 17β-estradiol were consistent with different laboratories from other validation program Phases over time.
3. The VTG results were the least variable and most consistent with the males. Some variability was noted in females, and on at least two occasions the p-values were of groups not expected to reach significant were < 0.10.
4. The potential of aromatizable androgens to lead to VTG induction was confirmed.
5. The VTG analyses were performed with coded samples.

V. Secondary Sexual Characteristics

The primary observation for secondary sexual characteristics (SCC) in the fathead minnow is based on the presence and size of tubercles in the normal sexually active male and their diminution in inactive males and their absence in the normal female. The tubercles are counted per individual, and the size of the tubercles on the individual are scored to generate a semi-quantitative value to represent the reproductive status of that male. The rating scores for the tubercle size are: 1=present, 2=enlarged, 3=pronounced. Representative photographs have been introduced in the OECD protocol. The individual values are converted into an overall mean tubercle number and score per replicate and mean tubercle number and score per concentration. A secondary

observation could be used: the presence in the fathead minnow of vertical colouration stripes in the sexually active male and their absence or diminution in the non-spawning male and their absence in the normal female.

a. Octylphenol

Octylphenol exposure resulted in decreases in the absolute values of both the tubercle score at the high concentration in laboratory 3, but did not achieve statistical significance. All males died at the high dose in laboratory 4. The reference 17β-estradiol, however, resulted in statistically significant decreases in the males in both laboratories. This reproduces the effect of the reference compound on males observed in most previous studies. The data are presented in Table 4A.

Table 4A. Mean tubercle scores for fathead minnows exposed to octylphenol.

Laboratory	Group	Nominal Octylphenol (µg/L)	Mean Tubercle Scores			
			Male		Female	
			Mean Individual	*p*-value	Mean Individual	*p*-value
3	**Control**	0	17.10		0.0	
	Solvent	0	18.20	0.9936	0.0	NA
	Low	3	16.50	0.9994	0.0	NA
	Intermediate	30	9.00	0.0842	0.0	NA
	High	300	1.13	0.0004*	0.0	NA
4	**Control**	0	15.7		0.0	
	Solvent	0	13.7	0.8612	0.0	NA
	Low	3	14.2	0.9953	0.0	NA
	Intermediate	30	16.6	0.8120	0.0	NA
	High	300	[a]	- -	0.0	NA
	17β-estradiol	0.100	6.9	0.0088*	0.0	NA

[a] All male fish died at the high concentration before day 21 on test. * Statistically significant decrease; NA - Not applicable

b. Androstenedione

Androstenedione exposure resulted in the appearance of tubercles in female fish in the fathead minnow studies, and the increases were statistically significant. There was a modest absolute increase in the tubercle score in males in laboratory 4. The data are presented in Table 4B.

Table 4B. Mean tubercle scores for fathead minnows exposed to androstenedione.

Laboratory	Group	Nominal Androstenedione (µg/L)	Mean Tubercle Scores			
			Male		Female	
			Mean Individual	*p*-value	Mean Individual	*p*-value
4	**Control**	0	13.5		0.0	
	Solvent	0	14.5	0.9745	0.0	1.00000 [a]
	Low	0.5	10.0	0.3299	0.0	1.00000 [a]
	Intermediate	5	16.3	0.5245	1.9	0.13993 [b]
	High	50	16.9	0.3547	2.3	0.02366*
5	**Control**	0	20.8		0.0	
	Solvent Control	0	16.8	0.5011	0.0	1.00000 [a]
	Low	0.5	17.8	0.9895	0.2	1.00000 [a]
	Intermediate	5	15.4	0.5690	0.63	1.00000 [a]
	High	50	16.6	0.8439	3.5	0.00862*
	17β-estradiol	0.100	11.7	0.0713	0.0	1.00000 [a]

[a] The Bonferroni-adjustment results in a maximum *p*-value of 1

[b] The tubercle score in this group was significant by the pairwise Mann-Whitney, but was not significant after the application of Bonferroni's adjustment. * Statistically significant increase

c. Sodium pentachlorophenol

In laboratory 3, there was a decrease in the male absolute mean tubercle scores with increasing sodium pentachlorophenol concentration and with the reference estrogen 17β-estradiol, but the absolute decline did not attain statistical significance. In laboratory 5, there was also a decrease in the male absolute mean tubercle scores with increasing sodium pentachlorophenol concentration and with the reference estrogen 17β-estradiol. While the absolute decline again did not attain statistical significance, the decline with the 17β-estradiol was approaching statistical significance. In addition, there was a modest absolute difference between the dilution water control and the dimethylforamide solvent control. The data are presented in Table 4C.

Table 4C. Mean tubercle scores for fathead minnows exposed to sodium pentachlorophenol (PCP).

			Mean Tubercle Scores			
			Male		Female	
Laboratory	**Group**	**Nominal PCP (μg/L)**	**Mean Individual**	***p*-value**	**Mean Individual**	***p*-value**
3	**Control**	0	18.9		0.0	
	Low	1	17.5	0.9909	0.0	NA
	Intermediate	10	14.5	0.6574	0.0	NA
	High	100	11.2	0.3516	0.0	NA
	17β-estradiol	0.100	11.4	0.2093	0.0	NA
5	**Control**	0	20.8		0.0	
	Solvent Control		16.8	0.5011	0.0	NA
	Low	1	19.3	0.9995	0.0	NA
	Intermediate	10	19.2	0.9998	0.0	NA
	High	100	13.0	0.1633	0.0	NA
	17β-estradiol	0.100	11.7	0.0713	0.0	NA

[a] No surviving males in the replicate or concentration.
* Statistically significant decrease; NA - Not applicable

Secondary Sexual Characteristics – Discussion and Conclusions

The SSC results were consistent overall with the expected modes of action of the test substances and were reproducible across laboratories. The results were achieved using blinded analysts.

In conclusion, these studies support the validation of the SCC endpoint in the fish endocrine screen:

1. The SSC results were reproducible with difficult and challenging compounds.
2. The SCC results with 17β-estradiol were consistent with different laboratories from other validation program Phases over time in decreasing the tubercle scores in males.
3. The SCC results with octylphenol were consistent with this estrogenic affect of decreasing tubercle scores in males.
4. The SCC results with androstenedione were consistent with the expected androgenic affect of this compound leading to the appearance of tubercles in females.
5. The SCC results with sodium pentachlorophenol are cautionary that the tubercle scores in males can also be decreased by systemic toxicity, which is consistent with observations made with several other toxic, negative compounds in Phase-2. Therefore, interpretation of an estrogenic mode of action should rely primarily on the VTG endpoint and only secondarily on the SSC endpoint. Further, a decrease in the tubercle score should not be interpreted as antiandrogenic given evidence of the limited impact of flutamide and the decreases observed with other toxic negatives.
6. The SSC analyses were performed with coded samples.

OVERALL DISCUSSION AND CONCLUSIONS

The eight studies in this report were conducted as part of OECD program to validate a standardized OECD protocol on adult fish for the detection of endocrine active substances (i.e. estrogen, aromatase inhibitors, androgen). In January of 2006, CEFIC volunteered to support additional work with three test substances in the fathead minnow (*Pimephales promelas*). Other laboratories were invited to participate, and both Denmark and Germany agreed to support studies with octylphenol in the zebrafish (*Danio rerio*). These studies were conducted in August 2006-May 2007 to support the scheduled peer review of the OECD 21 day fish endocrine screening assay.

The objective of the Phase-3 studies was to test the relevance and specificity of two endocrine-related endpoints, vitellogenin (VTG) and secondary sexual characteristics (SSC), with the additional test substances. The objective was to challenge the protocol with a set of difficult test substances. The three test substances were a weak estrogen (octylphenol), a weak aromatizable androgen (androstenedione), and a difficult negative test substance (sodium pentachlorophenol).

- Octylphenol is considered a difficult weak estrogen as the substance has low affinity for the estrogen estrogen receptor (ER) ligand while becoming toxic and leading to mortality within an approximate order of magnitude after achieving estrogenic doses capable of inducing VTG. Therefore, dose selection and the ability of laboratories to administer an estrogenic concentrations without mortality in aquatic test systems challenges the protocol.
- Androstenedione is a natural metabolite of testosterone present in most sewage systems receiving human wastes. Androstenedione is considered a difficult compound as it may present estrogenic properties if aromatized, it is a weak androgen receptor (AR) ligand, and it has limited water solubility. Therefore, it will present issues of VTG induction as an apparent estrogen, achieving detectable changes in female SSC characteristics and challenge laboratory dosing and analysis.
- Sodium pentachlorophenol is considered a difficult negative substance due to its toxicity while the chorine atoms are known to inhibit ER binding in fish and mammals. It is negative in the mammalian uterotrophic bioassay. Under the conditions of the bioassay, the pKa of the phenolic group (4.74) will result in primarily the dissociated form which should not be active.

The target (nominal) concentrations were 3, 30 and 300 µg/l for *tert*-octylphenol (CASRN 140-66-9); 0.5, 5, and 50 µg/l for androst-4-ene-3,17-dione (CASRN 63-05-8), and 1, 10, and 100 µg/l for sodium pentachlorophenol (CASRN 131-52-2). The voluntary reference chemical was 17β-estradiol (CASRN) with a target concentration of 100 ng/l.

For validation, Guidance Document 34 calls for studies to be conducted under code. However, aquatic toxicology studies themselves cannot be coded as the presence of the dilution systems for the test and reference substances clearly indicate which groups are being dosed, and also the approximate order of the test substance administration from low to high concentrations. Further, periodic chemical analyses are required to determine the actual or measured concentrations. Therefore, to ensure that these studies were in accordance with Guidance Document 34 in principle, the VTG analyses and the tubercle scoring exercises were conducted under code so that these values were achieved by blinded technical personnel. This is important to the success and acceptance of the 21-day fish endocrine screen validation program as none of the previous studies had been conducted where the observations were blind. These studies should then provide further support to the compliance of the OECD 21-day fish endocrine screening program with the criteria of Guidance Document 34.

The results of these studies support that interlaboratory studies have been done with additional positive and negative compounds which being of weak potency for the positives and a difficult negative test substance are representative compounds in a validation study for the protocol in regulatory applications. The results of these studies herein support the overall conclusion of the OECD validation program that VTG and SSC can be successfully employed to screen for certain endocrine modes of actions (i.e., estrogens, androgens, and

aromatase inhibitors). At concentrations absent of overt signs of toxicity, there were no false positives or negatives observed with any of the three negative test substances directly related to the protocol itself.

Mortality, symptoms of intoxication, and other sublethal effects were occasionally evident and need to be incorporated into the overall interpretation. Some symptom observations, mortality at high sodium pentachlorophenol concentrations approached statistical significance versus the controls with absolute decreases in VTG and SCC, following the same concentration patterns as the intoxication symptoms and mortality.

The VTG endpoint was consistently successful in both the zebrafish and the fathead minnow. Three of four laboratories observed statistically significant changes with octylphenol. The fourth laboratory suffered complete male mortality at the high dose of octylphenol where statistical significance occurred in the other laboratories. With androstenedione, both laboratories observed statistically significant increases in VTG, indicating aromatization of certain androgens will result he emergence of tubercles in female fish. With sodium pentachlorophenol, both laboratories did not observe any absolute or statistically significant increases in VTG. Rather, absolute VTG decreases were observed at toxic levels of this compound. The latter finding is consistent with Phase-2 studies that indicated interpretative questions about the role of systemic toxicity need to be taken into account. That is, systemic toxicity may impair the individuals' ability to produce VTG in such a group and should not be interpreted as an endocrine mediated disturbance.

The SSC tubercle score endpoint was also successful in the fathead minnow. No SCC endpoints are available in the zebrafish. There was a decline in the absolute values of the fathead minnow male mean tubercle score at the high concentration of octylphenol consistent with observations with estrogens. The androstenedione did produce the statistically significant appearance of tubercles in females in both laboratories. There was an absolute decline in tubercle number and mean tubercle score at the high concentration of sodium pentachlorophenol synonymous with systemic toxicity and symptoms of intoxication. The latter is not interpreted as an endocrine mediated disturbance.

Several laboratories voluntarily employed the chosen concentration of the reference estrogen, 17β-estradiol. The findings reproduced the VTG and SCC changes observed previously in Phase-1B and Phase-2. Therefore, with this potent estrogen these studies support that the protocol is reproducible over time in different laboratories.

There were two other noteworthy observations.

- The first observation was for the VTG endpoint. In one laboratory, VTG baselines in males were below the ELISA detection limit. This presented issues of how to run statistical analyses as there was no real distribution of values to enter for the statistical analysis. Entering the detection limit or one half of the detection limit would present no coefficient of variation, sharply encouraging a finding of statistical significance with limited absolute differences with other groups. Given the natural variability in the VTG from group-to-group this could encourage false positives. To avoid this possibility, a random set of values was generated with a distribution equivalent to other groups and with a mean at one half of the detection limit. This did not impact the finding of statistical significance in this case. In another laboratory, the VTG values exceeded the upper part of the calibration curve, but this presents no evident statistical issues.
- The second observation was for the tubercle endpoint. The female fish begin with a consistent baseline of zero. As with the VTG, this lack of variability increases the statistical sensitivity. Thus, there were no issues with detecting the relatively small increases in tubercle scores with the androstenedione. In this latter case, these circumstances then increase the basic sensitivity of the assay for androgens.

As difficulties had been encountered in previous studies with immature fish, the previous acceptance criteria for the age of fish on test were waived and older fish were accepted. These studies met the other previous test acceptance criteria for the fish to be placed on test, met the test criteria for mortality, O_2 saturation and

temperature, and the measured values of the test substances are satisfactory to accept all three studies. The analytical recoveries of the test substances were acceptable, taking into account that the androstenedione was a difficult to test substance due to both its water solubility and potentially rapid biodegradation. Therefore, the studies are themselves useful to assess the relevance and the reliability of the endpoints in Phase-2 of the validation program.

The following conclusions and recommendations are offered. These recommendations have not yet been discussed by an OECD group and should, therefore, be taken as proposals, and may or may not form the basis of further work/discussion on the future Test Guideline

- At non-toxic concentrations, the VTG and SSC endpoints consistently responded to the test substances as expected. The studies support the capability of these endpoints to correctly identify endocrine modes of action when other toxicities are not present. Collectively, with previous data, these findings support the validation of these endpoints.

- Given the evidence for impact of systemic toxicity and sublethal effects on several endpoints, several changes in the protocol and the interpretation of its results are recommended to resolve the issue of potential false positives in such circumstances:
    - Avoid concentrations with high mortality and symptoms of intoxication by refining concentration selection guidance beyond acute toxicity (LC50). The concentration selection should be informed with data from range finding studies, other species (as was done with medaka octylphenol results), and juvenile toxicity studies or early life stage studies.
    - Thus, in a straightforward solution, incorporate in the interpretative section of a future Test Guideline that positive findings for possible endocrine related effects should occur in the absence of confounders for systemic toxicity and symptoms of intoxication and that particularly vulnerable endpoints such as reproduction would be recognized.

- These study results with sodium pentachlorophenol, as with previous potassium permanganate studies, would support some caution in interpreting a decline in the tubercle number and score in the presence of severe mortality and symptoms of intoxication.

REFERENCES

OECD. (2005). Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. OECD EHS Series on Testing and Assessment No. 34. OECD, Paris, 2005 (96 pp). [ENV/JM/MONO(2005)14].

Hornung MW, KW Jensen, JJ Korte, MD Kahl, EJ Durhan, JS Denny, TR Henry, GT Ankley. (2004). Mechanistic basis for estrogenic effects in fathead minnow (*Pimephales promelas*) following exposure to the androgen 17α-methyltestosterone: conversion of 17α-methyltestosterone to 17α-methylestradiol. *Aquatic Toxicology* 66:15–23.

Pawlowski S, A. Sauer, J.A. Shears, C.R. Tyler, T. Braunbeck. (2004). Androgenic and estrogenic effects of the synthetic androgen 17α-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*) determined using the gonadal recrudescence assay. *Aquatic Toxicology* 68:277–291.

ANNEX I
PROTOCOL FOR PHASE-3 STUDIES FOR THE VALIDAITON OF THE OECED 21-DAY FISH ENDOCRINE SCREENING ASSAY

PROTOCOL FOR PHASE-3 OF THE VALIDATION
For the OECD 21-day Fish Screening Assay for Endocrine Active Substances

INTRODUCTION

1. This protocol describes an *in vivo* screening assay for identifying endocrine active chemicals in sexually dimorphic fish. The fish screening assay exposes fish to chemicals for up to 21 days. The *in vivo* fish screening assay is intended to detect chemicals that show androgenic or estrogenic activity in fish exposed during a limited part of their life-cycle in which they are reproductively active. The core endpoints in the assay respond to estrogens, antiestrogens, androgens, and aromatase inhibitors. However, the responses should not be considered definitive and should be used as part of a weight of evidence assessment in combination with other information (such as structure-activity relationships, hormone receptor binding, *in vitro* assays specific for endocrine modes of action, and mammalian responses specific for endocrine modes of action). The concept for this protocol is derived from work on the fathead minnow *(Pimephales promelas)* (1) (2) (3) (37), the Japanese medaka (*Oryzias latipes*) (4) (5) (6) and the zebrafish (*Danio rerio*) (7) (8) (9) (10). There is limited evidence that the protocol could also be used for the three-spined stickleback (*Gasterosteus aculatus*) (11). The following protocol refers only to the fathead minnow.

2. This protocol does not require *in situ* pre-exposure period of the fish. There are two core endpoints as indicators of endocrine disrupter activity, namely: i) gross morphology (e.g., secondary sexual characteristics (SCC)) and ii) vitellogenin levels (VTG).

PRINCIPLE OF THE ASSAY

3. Adult fish of both sexes are the test organisms, and adult features of actively reproducing individuals that respond directly to estrogens and androgens are the core endpoints, VTG and SCC, respectively. To address these core endpoints under relevant conditions, the assay uses adult individuals in spawning condition; senescent animals should not be used. Guidance on the age of fish is provided in the section on selection of test organisms.

4. The assay is conducted using a water control and three chemical exposure concentrations for each test substance. A solvent carrier control is not obligatory, but, in case a solvent would be used to dissolve a test substance, a solvent control should be considered, using the same solvent concentration as in the chemical treatments. A positive reference control may be included (1 concentration). Two vessels (replicates) per treatment will be used (each vessel containing 5 males and 5 females). The exposure is conducted for 21-days. Daily observations of the spawning status for each test vessel qualitatively confirm that the fish are in spawning condition. These observations will be recorded as a Yes/No type of answer, and any eggs will be removed daily from the test chamber. Overviews of the relevant bioassay conditions are provided in Appendix 1 to this protocol.

5. On sampling at day 21 (test termination), 20 fish (10 males and 10 females) per treatment level are killed humanely and blood samples are collected for VTG determination. SCC are evaluated in fathead minnow.

6. SCC are under endocrine control in sexually dimorphic species, such as the fathead minnow. Experience to date with fathead minnows suggests that some endocrine active chemicals may initially induce changes in the following external characteristics (decreases in males or appearance in females: body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized SSC (size of dorsal nape pad, number of nuptial tubercles) (3) (12) (13) (14) (15). For example, estrogen receptor agonists can decrease nuptial tubercle numbers and size of the dorsal nape pad in adult males, and androgens can cause the appearance of nuptial tubercles in females.

7. VTG is a phospholipoglycoprotein precursor to egg yolk protein. VTG is normally produced by sexually-active females of all oviparous species in the liver. VTG production is controlled by interaction of estrogens with the estrogen receptor. VTG is released into the circulation and taken up by the developing follicles to produce the egg yolk. Significantly, males maintain the capacity to produce VTG in response to stimulation with estrogen receptor agonists. As such, induction of VTG in males and immature females has been successfully exploited as a biomarker specific for estrogenic compounds in a variety of OECD fish species (1) (2) (3) (28) (29) (30) (31) (32) (33). Reduction of VTG levels in females can be used as an indicator of decreased circulating endogenous oestrogen and this response is used as a biomarker for potential anti-oestrogenic activity and aromatase inhibitors.

INFORMATION ON THE TEST SUBSTANCE

8. Characterisation of the test substances is the responsibility of the original chemical supplier. It is not the responsibility of the lead or participating laboratories. The test substances for Phase-3 are octylphenol (CASRN 140-66-9), 4-androstenedione (CASRN 63-05-8), and sodium pentachlorophenol (CASRN 131-52-2). Each substance will be recorded by name, supplier, batch number, structural formula, purity, appearance, storage conditions, and expiry date. An objective of Phase-3 is to assess the assay's performance with an unknown test substance. Therefore, the staff performing core biological measurements, i.e. the VTG and SCC measurements, will be blinded to the nature of the test substances and their concentrations, effectively testing under code as recommended by OECD Guidance Document 34 (48). This recognizes that full coding cannot be achieved for the chemical dilution and administration to the fish tanks and the chemical analyses that are required.

TEST ACCEPTANCE CRITERIA

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

- the mortality in the control(s) does not exceed 10 per cent at the end of the exposure period; as well, signs of disease are visible in less than 10 per cent of control animals during the course of the test.
- the dissolved oxygen concentration has been at least 60 per cent of the air saturation value (ASV) throughout the exposure period.
- the water temperature did not differ by more than ± 1 °C between test vessels at any one time during the exposure period and was maintained within a range of 2°C within the temperature ranges specified for the test species (Appendix 1).

DESCRIPTION OF THE METHOD

Apparatus

10. Normal laboratory equipment and especially the following:

(a) 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 and of a suitable capacity in relation to the recommended loading and stocking density (see Appendix 1);
(e) spawning substrate for fathead minnow, Appendix 5 gives the necessary details.
(f) suitably accurate balance (i.e. accurate to ± 0.5%).

Water

11. Any water in which the test species shows suitable long-term survival, growth and reproduction 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. In order to ensure that the dilution water will not unduly influence the test result (for example by complexion of test substance), 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), 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. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every six months). Some chemical characteristics of acceptable dilution water are listed in Appendix 2.

Test solutions

12. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns (solubility columns) can be used for achieving a suitable concentrated stock solution. The use of solvents and dispersants should be avoided unless clearly necessary and justified (e.g. in order to produce a suitably concentrated stock solution. The quantities of any solvent should be minimized, and the choice of solvent should consider other negative impacts such as rapid biodegradation which would lead to fouling of the tanks.

13. A flow-through test system will be used. Such a system continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) in order to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water are essential to maintain the test concentrations. Flow rates should be checked at intervals of at least twice a week and preferably daily during the test. Flow rates should not vary by more than 10% 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 that could dissolve or leach into the water.

Selection of test organisms

14. The species selected for this phase of the validation program is the zebrafish (*Danio rerio*) and the fathead minnow *(Pimephales promelas)*. The exposure phase will be started with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals, in spawning condition (namely, with clear SSC visible in males) and evidence of actively spawning individuals (fertilized egg clutches). Test fish shall be selected from a laboratory population, preferably from a single stock. For general guidance only (and not to be considered in isolation from observing the actual reproductive status of a given batch of fish), fathead minnows should be a minimum of 20 weeks of age, assuming they have been cultured at 25 ± 2°C throughout their lifespan. Older fish are acceptable due to difficulties in previous studies with the presence of immature fish.

15. After receipt by the laboratory, fish 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 (note, this acclimation period is not an *in situ* pre-exposure period). Fish should be fed *ad libitum* throughout the holding and acclimation period and during the exposure phase. The same feed should be used during acclimation and during exposure.

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

- 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.

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

TEST DESIGN

18. Three concentrations of each test substance are used per experiment. In order to assess reproducibility across the laboratories, the doses of the test substances will be specified in Phase-3.

19. On day-21 of the experiment, 10 males and 10 females from each treatment level (5 males and 5 females for each of the two replicates) and from the control are sampled for the measurement of the core endpoints (see Appendix 3 for diagram of test design).

PROCEDURE

Selection and weighing of test fish

20. It is important to minimise variation in weight of the fish at the beginning of the assay. Suitable size ranges for the different species recommended for use in this test are given in Appendix 1. For the whole batch of fish used in the test, the range in individual weights at the start of the test should be kept, if possible, within ± 20% of the arithmetic mean weight. It is recommended to weigh a subsample of fish before the test in order to estimate the mean weight.

Conditions of exposure

Duration

21. The test duration is 21 days.

Loading rates and stocking densities

22. The loading rate and stocking density of the vessels are important (for definitions, see Appendix 4) and vary by the test species used (see Appendix 1). If the stocking density is too high, then overcrowding stress will occur leading to reduced growth rates and possibly to disease. If it is too low, territorial behavior may be induced which could also affect growth. In any case, the loading rate should be low enough in order that a dissolved oxygen concentration of at least 60% ASV can be maintained without aeration.

Feeding

23. The fish should be fed *ad libitum* with an appropriate food (Appendix 1) at a sufficient rate to maintain body condition (2). Care should be taken to avoid microbial growth and water turbidity, see also paragraph 25. 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. Food should be withheld from the fish on the day of sampling for 12 hours prior to necropsy in order to aid in histology processing of small fish.

24. Fish food, other than the one recommended in Appendix 1, should be evaluated for the presence of contaminants such as organochlorine pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and any estrogenic activity.

25. Uneaten food and fecal material should be removed from the test vessels at least twice weekly, e.g. by carefully cleaning the bottom of each tank using suction.

Light and temperature

26. The photoperiod and water temperature should be appropriate for the test species (see Appendix 1).

Test concentrations and controls

27. Each participating laboratory will perform studies on two of the three test substances in Phase 3. With three participating laboratories, each test substance will then be run twice for comparison of inter-laboratory assay replication. To facilitate this comparison, the doses are specified for each test substance. This minimizes the number of laboratories and animals necessary to assess the assay's reproducibility. The doses are spaced at one order of magnitude intervals. Based on available data, this should result in a No Observed Effect Concentration (NOEC) and a dose having a detectable response for the positive substances. The nominal doses will be as follows:

- octylphenol: 3, 30, 300 µg/L
- 4-androstenedione: 0.5, 5, 50 µg/L
- sodium pentachlorophenol: 1, 10, 100 µg/L

28. Each participating laboratory should perform a positive control with the octylphenol and the sodium pentachlorophenol test substances. The positive reference substance is 17β-estradiol (CASRN 58-50-2) is voluntary. If positive estradiol control is run, only one dose is necessary at the nominal concentration of 100 nanograms/L.

Frequency of analytical determinations and measurements

29. 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 substances stability in the test system. During the test, the concentrations of the test substance are determined at regular intervals in all vessels, as a minimum, at the start of the test and at weekly intervals thereafter (7, 14, and 21 days).

30. Results (e.g. NOEC for VTG and SCC) should always be based on measured concentrations and included in the report.

31. Samples may need to be filtered (e.g. using a 0.45 µm pore size) or centrifuged. If needed, then centrifugation is the recommended procedure. If the test material is known not adsorb to filters, filtration may also be acceptable.

32. During the test, total hardness and alkalinity should be measured in the controls and one vessel at the highest concentration at least once per week. Dissolved oxygen and pH should be measured at least twice a week and preferably daily in all vessels. Temperature should preferably be monitored continuously in at least one test vessel.

Observations during exposure

33. A number of general observations are assessed during the course of the assay. Collection of these endpoints and their utility are described below:

Survival

34. Fish should be examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) noted. 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.

Behaviour

35. Any abnormal behavior (relative to controls) should be noted; this might include signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. Such behavioral observations may provide useful qualitative information to inform potential future fish testing requirements (for example, territorial aggressiveness in normal males or masculinized females has been observed in fathead minnows under androgenic exposure).

Spawning status

36. Daily qualitative observations of spawning in each test vessel will be recorded as Yes for the presence of eggs, and No in the absence of eggs in the control tanks. These data will be recorded daily in the case of two replicate tanks with values of 2 for two Yes's, 1 for one Yes and one No, and 0 for two No's. Eggs will be removed daily from the test chambers. A spawning substrate must be placed in the test chamber for the fathead minnow and zebrafish to enable fish to spawn in normal conditions. Appendix 5 gives further details of recommended spawning substrates for fathead minnow.

Appearance and observation of secondary sex characteristics

37. Both male and female fish should be observed daily. Decreases in the size of the dorsal nape pads and nuptial tubercles in males as well as any changes in the number of nuptial tubercles and the visual appearance and number of nuptial tubercles in females should be recorded (see Paragraph 40 for observations at necropsy).

Necropsy

Humane killing of fish

38. At day 21, i.e. at conclusion of the exposure, the fish should be anaesthetized with appropriate anesthetics (e.g. MS-222 (100 - 500 mg per L buffered with $NaHCO_3$) or FA-100 and ice-cold water).

Body weights

39. **A**t the end of the test, all surviving fish will be blotted dry individually weighed to the nearest 0.1 g. If dry weights are used, this will be measured after 24 hours at 60°C.

Secondary Sexual Characteristics (SSC)

40. The number and size of nuptial tubercles in both sexes of the fathead minnow can be quantified directly or in preserved specimens (3). Standard operating procedures for the evaluation of SSC in fathead minnow are available in Appendix 6.

Vitellogenin (VTG)

41. Blood will be collected from the caudal artery/vein with a heparinised microhematocrit capillary tube or by cardiac puncture using a heparinised syringe. Depending upon the size of the fish, collectable blood volumes generally range from 20 to 80 µl per individual for fathead minnows (1) (16). Plasma is separated from the blood via centrifugation (3 min; 15,000 g; room temperature), and stored with protease inhibitors at -80^{o}C, until analyzed for VTG. The measurement of VTG should be based upon a homologous ELISA method. Information should be available upon the intra-assay and inter-assay variability and on the lower detection limit of the method. It is recommended to use a method capable for detecting VTG levels as low as a few ng/ml plasma. For further details on vitellogenin sampling and determination methods refer to Appendix 7.

42. A complete transparency of the ELISA method is needed to understand potential sources of variability. Therefore, polyclonal fathead minnow VTG antibody and purified VTG protein from the fathead minnow are utilized (28) (29) for the determination of VTG for this species. The most suitable methods for determining VTG in this species are provided in Appendix 7.

DATA AND REPORTING

Treatment of results

43. Excel Spreadsheets for the collection of test results will be made available to participating laboratories before the start of Phase 3. All laboratories will use the same template for data collection. It is the responsibility of each laboratory to check the data for accuracy before submitting the completed Excel Spreadsheets to the Lead laboratory.

Evaluation of Biomarker Responses by Analysis of Variance (ANOVA) or by Regression Analysis

44. To identify potential endocrine activity by a chemical, responses are compared between treatments versus controls groups using analysis of variance (ANOVA). All biological response data should be analyzed and reported separately by sex (either females or males). If the required assumptions for parametric methods are not met 1) normal distribution (e.g. Shapiro-Wilk's test) and 2) homogeneous variance (Bartlett's test or Levene’s test),

then consideration should be given to transforming the data to homogenize variances prior to performing the ANOVA, or to carrying out a weighted ANOVA. Dunnett's test (parametric) on multiple pair-wise comparisons or a Mann-Whitney with Bonferroni's adjustment (non-parametric) are examples of procedures that may be used. Additional information can be obtained from the OECD Draft Guidance Document on Statistical Analysis of Ecotoxicity Data, available on the OECD public website (47).

45. To identify potential endocrine activity following chemical exposure, responses across the different treatment concentrations could also be examined based on regression analysis (based on three exposure concentrations per chemical). All biological response data should be analyzed and reported separately by sex (either females or males). Direct observation of concentration-related trends in biological responses can also usefully inform the outcome of such a study for the intrinsic endocrine activity of a test chemical.

Interpretation of results

46. The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method. For the core endpoints, the following will be noted as positive outcomes:

- the statistically significant appearance of male secondary sexual characteristics in females,
- the statistically significant decrease in male secondary sexual characteristics,
- the statistically significant increases in male VTG levels, and
- the statistically significant increase or decreases in female VTG levels.

Apparent differences observed in qualitative reproduction and spawning of the treatment groups from the controls will be noted and reported. Changes in body weight induced by the test substance from the controls will be noted and reported, and, with behavioral and other changes, the potential effects of other toxicities on the test results will be considered.

47. The consistency of the significant changes with other information (e.g., receptor binding, *in vitro* data, other fish data, and other mammalian data), if available, will be considered in the interpretation.

Test report

48. The test report must include the following information:

Test substance:
- physical nature and, where relevant, physicochemical properties;
- chemical identification data, including purity and analytical method for quantification of the test substance where appropriate.

Test species:
- scientific name of the species,
- strain,
- supplier source, and
- procedures and observations during receipt and acclimation.

Test conditions:
- test design and procedures (number of replicates, number of fish for each sex per replicate, loading rate, stocking density, photoperiod, temperature, etc.);
- dilution water characteristics (including pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels, total organic carbon, suspended solids and any other measurements made);

- 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 necessary (e.g. PCBs, PAHs and organochlorine pesticides);
- the nominal test concentrations;
- method of preparation of stock solutions and flow-rate (justification and identification of any solubilising agent or solvent, and its concentration);
- the sampling times, the measured substance values, and the means and standard deviations; and
- the analytical method for the test substance and evidence that the measurements refer to the concentrations of the test substance in true solution;

Results:

- evidence that controls met the validity criterion for survival;
- data on mortalities occurring in any of the test concentrations;
- data for the qualitative reproduction and spawning of the controls and test groups, preferably in tabular from;
- data for any behavioral observations during the exposure, including incidence, if any;
- data for any secondary sexual characteristic observations during the exposure, including incidence, if any;
- data for body weight;
- data on biological observations of the including secondary sex characteristics from necropsy specimens, preferably in both tabular and graphical form;
- data for vitellogenin analyses, preferably in both tabular and graphical form;
- statistical analytical techniques used, including treatment of data and justification of techniques used; and vitellogenin; results of the statistical analysis preferably in tabular and graphical form;
- no observed effect concentration for each response assessed (NOEC);
- lowest observed effect concentration (at $p = 0.05$) for each response assessed (LOEC) with any concentration-response data and curves available
-

LITERATURE

1. Panter GH, Thompson RS, JP Sumpter. 1998a. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. Aquat Toxicol 42:243-253.
2. Panter GH, Campbell PM, Hutchinson TH, Kime DE, Länge R, Lye C, McCahon P, Sumpter JP, Tattersfield LJ, Tyler CR. 1998b. Effects of endocrine disruptors in the fathead minnow (*Pimephales promelas*). *Abstracts*, 19th Annual Meeting, Society of Environmental Toxicology and Chemistry, Charlotte, NC, USA.
3. Ankley GT, Jensen, KM, Kahl, MD, Korte, JJ, Makynen, EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). Environ Tox Chem 20:1276-1290.
4. Gray MA and Metcalfe CD. 1999. Toxicity of 4-*tert*-octylphenol to early life stages of Japanese medaka (*Oryzias latipes*) Aquat Toxicol 46:149-154.
5. Groonen S, Denslow N, Manning S, Barnes S, Barnes D and Brouwer M. 1999. Serum vitellogenin levels and reproductive impairment of male Japanese medaka (*Oryzias latipes*) exposed to 4-tert-octylphenol. Environ Hlth Perspect 10:385-390.
6. Shioda T and Wakabayashi M. 2000. Effect of certain chemicals on the reproduction of medaka (*Oryzias latipes*) Chemosphere 40:239-243.
7. Bresch H, Beck H, Ehlermann D, Schlaszus H, Urbanek M. 1990. A long-term test comprising reproduction and growth of zebrafish (*Danio rerio*) with 4-chloroaniline. Arch Environ Contam Toxicol 19:419-427
8. Andersen L, Bengtsson BE, Björk M, Gessbo Å, Holbech H, Hylland K, Norrgren L, Pedersen KL, Lundgren A, Petersen GI, Steinholz Å, Örn S. 2000. Zebrafish for testing endocrine disrupting chemicals. TemaNord 2000:555. ISBN:92-893-0480-4.
9. Andersen L, Holbech H, Gessbo Å, Norrgren L, Petersen GI. 2003. Effects of exposure to 17α-ethinylestradiol during early development on the sexual differentiation and induction of vitellogenin in zebrafish (*Danio rerio*). Comp Biochem Physiol C Toxicol Pharmacol 134:365-374.
10. Van den Belt K, Verheyen R and Witters H. 2001. Reproductive effects of ethynylestrdadiol and 4t-octylphenol on the Zebrafish (*Danio rerio*). Arch Environ Contam Toxicol 41:458-467.
11. Katsiadaki I, Scott AP, Mayer I. 2002. The potential of the three-spined stickleback (*Gasterosteus aculeatus L.*) as a combined biomarker for oestrogens and androgens in European waters. Mar Environ Res 54(3-5):725-728.
12. Smith RJF. 1974. Effects of 17α-methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). Can J Zool 52:1031-1038.
13. Panter GH, Hutchinson TH, LängeR, Whale G, Sumpter JP, Tyler CR. (in preparation). Detection of (anti-) androgens in an adult fathead minnow (*Pimephales promelas*) non-spawning assay. Draft CEFIC-EMSG research report reference AQ006. CEFIC, Brussels, Belgium.
14. Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, Giesy JP. 1999b. Effects of waterborne exposure of 17α-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquat Toxicol 47:129-145.
15. Holbech H, Andersen L, Petersen GI, Korsgaard B, Pedersen KL, Bjerregaard P. 2001. Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (*Danio rerio*). Comp. Biochem. Physiol. C. 130: 119-131.
16. Jensen KM, Korte JJ, Kahl MD, Pasha MS, Ankley GT. 2001. Aspects of basic reproductive biology and

endocrinology in the fathead minnow (*Pimephales promelas*). Comp Biochem Physiol 28:127-141.

17. Van den Belt K, Wester PW, Van der Ven LT, Verheyen R, Witters H. 2002. Effects of ethynilestradiol on the reproductive physiology in zebrafish (*Danio rerio*): time dependency and reversibility. Env Toxicol Chem 21:767-775.
18. Panter GH, Hutchinson TH, Länge R, Whale G, Sumpter JP, Tyler CR. 2001. Quantifying gonadal development in sexually maturing fathead minnows (*Pimephales promelas*). CEFIC-EMSG research report reference AQ004. CEFIC, Brussels, Belgium.
19. Makynen EA, Kahl MD, Jensen KM, Tietge JE, Wells KL, Van Der Kraak G, Ankley GT. 2000. Effects of the mammalian anti-androgen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). Aquat Toxicol 48:461-475.
20. Roberts RJ. 1978. Fish Pathology. Baillere Tindall, London. ISBN 0 7020 0674.
21. West G. 1990. Methods of assessing ovarian development in fish: a review. Aust J Mar Freshwater Res 41:199-222.
22. Wester PW and Vos JG. 1994. Toxicological pathology in laboratory fish: an evaluation with two species & various environmental contaminants. Ecotoxicology 3:21-44.
23. Miles-Richardson SR, Pierens SL, Nichols KM, Kramer VJ, Snyder EM, Snyder SA, Render JA, Fitzgerald SD, Giesy JP. 1999a. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Environ Res 80:122-137.
24. Harries JE, Runnalls T, Hill E, Harris C, Maddix S, Sumpter JP, Tyler CR. 2000. Development and validation of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environ Sci Technol* (in press).
25. Kramer VJ, Miles-Richardson S, Pierens SL, Giesy JP. 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure in fathead minnows (*Pimephales promelas*) exposed to waterborne 17â-estradiol. Aquat Toxicol 40:335-360.
26. Panter GH, Hutchinson TH, Länge R, Whale G, Sumpter JP, van Aerle R, Tyler CR. 2002. Quantifying histological development in the gonads of sexually maturing fathead minnow: phase 2. CEFIC-EMSG research report reference AQ005. CEFIC, Brussels, Belgium.
27. Thorpe KL, Hutchinson TH, Hetheridge MJ, Scholze M, Sumpter JP, Tyler CR. 2001. Assessing the biological potency of binary mixtures of environmental estrogens using vitellogenin induction in juvenile rainbow trout (Oncorhynchus mykiss). Environ Sci Technol 35:2476-81
28. Parks LG, Cheek AO, Denslow ND, Heppell SA, McLachlan JA, LeBlanc GA, Sullivan CV. 1999. Fathead minnow (*Pimephales promelas*) vitellogenin: Purification, characterization, and quantitative immunoassay for the detection of estrogenic compounds. Comp Biochem Physiol 132C:113-125.
29. Korte JJ, Kahl MD, Jensen KM, Pasha MS, Parks LG, LeBlanc GA, Ankley GT. 2000. Fathead minnow vitellogenin: Complementary DNA sequence and messenger RNA and protein expression after 17β-estradiol treatment. Environ Toxicol Chem 19:972-981.
30. Tyler CR, Vandereerden B, Jobling S, Panter G, Sumpter JP. 1996. Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. J Comp Physiol 166B:418-426.
31. Rose J, Holbech H, Lindholst C, Noerum U, Povlsen A, Korsgaard B, Bjerregaard P. 2002. Vitellogenin induction by 17β-estradiol and 17α-ethinylestradiol in male zebrafish (*Danio rerio*). Comp. Biochem. Physiol. C. 131: 531-539.
32. Nishi K, Chikae M, Hatano Y, Mizukami H, Yamashita M, Sakakibara R, Tamiya E. 2002. Development

and application of a monoclonal antibody-based sandwich ELISA for quantification of Japanese medaka (Oryzias latipes) vitellogenin. Comp. Biochem. Phys. C 132:161-169.

33. Yokota H, Morita H, Nakano N, Kang IJ, Tadokoro H, Oshima Y, Honjo T, Kobayashi K. 2001. Development of an ELISA for determination of the Hepatic vitellogenin in Medaka (*Oryzias latipes*). Jpn J Environ Toxicol 4:87–98.
34. Panter GH, Tyler CR, Maddix S, Campbell PM, Hutchinson TH, Länge R, Lye C, Sumpter JP. 1999. Application of an ELISA to quantify vitellogenin concentrations in fathead minnows (*Pimephales promelas*) exposed to endocrine disrupting chemicals. CEFIC-EMSG research report reference AQ001. CEFIC, Brussels, Belgium.
35. Fenske M, van Aerle, RB, Brack, SC, Tyler, CR, Segner, H. 2001. Development and validation of a homologous zebrafish (*Danio rerio* Hamilton- Buchanan) vitellogenin enzyme-linked immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals. Comp. Biochem. Phys. C 129:217-232.
36. Tyler CR, Aerle RV, Hutchinson TH, Maddix S, Trip H. 1999. An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. Environ Toxicol Chem 18:337-347.
37. Ankley GT, Jensen KM, Makynen EA, Kahl MD, JJ Korte, MW Hornung, TR Henry, JS Denny, RL Leino, VS Wilson, MC Cardon, PC Hartig, Gray LE. 2003. Effects of the androgenic growth promoter 17-B-trenbolone on fecundity and reproductive endocrinology of the fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 22:1350-1360.
38. Kiparissis Y, Metcalfe TL, Balch GC, Metcalfe CD. 2003. Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*). Aquat Toxicol 63:391-403.
39. Bayley M, Junge M, Baatrup E. 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat Toxicol 56:227-39.
40. Panter GH, Hutchinson TH, Lange R, Lye CM, Sumpter JP, Zerulla M, Tyler CR. 2002. Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. Environ Toxicol Chem 21:319-26.
41. Gimeno S, Komen H, Gerritsen AGM, Bowmer T. 1998. Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during sexual differentiation. Aquat Toxicol 43:77-92.
42. Seki M, Yokota H, Matsubara H, Maeda M, Tadokoro H, Kobayashi K. 2003. Fish full life–cycle testing for the weak estrogen 4-tert-pentylphenol on medaka (*Oryzias latipes*). Env Toxicol Chem 22:1487–1496.
43. Comparative Evaluation of Vitellogenin Methods, draft final report. USEPA, 2002.
44. Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, Leino RL. 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). Toxicol Sci. 67:121-30.
45. Panter GH, Thompson RS, and Sumpter JP. 2000. Intermittent Exposure of Fish to Estradiol. Environ Sci Technol 34:2756–2760.
46. Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Yamaguchi T, Maeda M, Imada N, Tadokoro H, Honjo T. 2002. Effect of 17β-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). Chemosphere 47:71-80.
47. OECD Draft Guidance Document on Statistical Analysis of Ecotoxicity data (URL: http://www.oecd.org/document/12/0,2340,en_2649_34377_1898188_1_1_1_1,00.html)
48. OECD. 2005. Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. OECD SERIES ON TESTING AND ASSESSMENT Number 34. ENV/JM/MONO(2005)14. OECD, Paris.

Phase-3 OECD 21-day Fish Screening Assay Validation Report
Additional Test Substance Studies

APPENDIX 1: EXPERIMENTAL CONDITIONS FOR THE NON-SPAWNING FISH ENDOCRINE SCREENING PROTOCOL

1. Recommended species	Fathead minnow (*Pimephales promelas*)	Medaka (*Oryzias latipes*)	Zebrafish (*Danio rerio*)
2. Test type	Flow-through	Flow-through	Flow-through
3. Water temperature	25 ± 2°C	24± 2°C	26 ± 2°C
4. Illumination quality	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum)
5. Light intensity	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
6. Photoperiod (dawn / dusk transitions are optional, however not considered necessary)	16 h light, 8 h dark	16 h light, 8 h dark	16 h light, 8 h dark
7. Loading rate	<5 g per L	<5 g per L	<5 g per L
8. Test chamber size	20 L (minimum)	2 L (minimum)	4 L (minimum)
9. Test solution volume	16 L (minimum)	1.5 L (minimum)	4 L (minimum)
10. Volume exchanges of test solutions	Minimum of 5 daily	Minimum of 5 daily	Minimum of 5 daily
11. Age of test organisms	Reproductively mature fish (not senescent)	Reproductively mature fish (not senescent)	Reproductively mature fish (not senescent)
12. Wet weight of adult fish (g)	Females: 1.5 ± 20% Males: 2.5 ± 20%	Females: 0.35 ± 20% Males: 0.35 ± 20%	Females: 0.65 ± 20% Males: 0.5 ± 20%
13. No. of fish per test vessel	10	10	10
14. No. of treatments	= 3 (plus appropriate controls)	= 3 (plus appropriate controls)	= 3 (plus appropriate controls)
15. No. vessels per treatment	2 minimum	2 minimum	2 minimum
16. No. of fish per test concentration	10 adult females and 10 males (5 females and 5 males in each replicate vessel)	10 adult females and 10 males (5 females and 5 males in each replicate vessel)	10 adult females and 10 males (5 females and 5 males in each replicate vessel)

APPENDIX 1 (continued)

17. Feeding regime	Frozen adult brine shrimp twice daily (ad libitum)	Brine shrimp nauplii twice daily (ad libitum)	Frozen adult brine shrimp twice daily (ad libitum)
18. Aeration	None unless DO concentration falls below 4.9 mg per L	None unless DO concentration falls below 4.9 mg per L	None unless DO concentration falls below 4.9 mg per L
19. Dilution water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water
20. Dilution factor	max 10	max 10	max 10
21. Pre- exposure period	none	None	None
22. Chemical exposure duration	21-d	21-d	21-d
23. Biological endpoints	Non-specific: - survival - behavior Endocrine biomarkers: - gross morphology (incl. SSC) - VTG	Non-specific: - survival - behavior Endocrine biomarkers: - gross morphology (incl. SCC), - VTG	Non-specific: - survival - behavior Endocrine biomarkers: - gross morphology, - VTG
24. Test acceptability	Dissolved oxygen ≥60% of saturation; mean temperature of 25 ± 2°C; 90% survival of fish in the controls	Dissolved oxygen ≥60% of saturation; mean temperature of 24 ± 2°C; 90% survival of fish in the controls	Dissolved oxygen ≥60% of saturation; mean temperature of 26 ± 2°C; 90% survival of fish in the controls

APPENDIX 2: SOME CHEMICAL CHARACTERISTICS OF ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATIONS
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 □g/l
Residual chlorine	< 10 □g/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

APPENDIX 3: SCHEMATIC OF EXPERIMENTAL DESIGN FOR THE NON-SPAWNING FISH ASSAY

FOR EACH OF THE TEST SUBSTANCES

Measurements/observations made on each individual animal:
Wet weight, secondary sexual characteristics, spawning status, vitellogenin

21d

Control	(5F+5M) in **Control Replicate 1** and (5F+5M) in **Control Replicate 2**
Positive Control (if needed)	(5F+5M) in **Positive Control Replicate 1** and (5F+5M) in **Positive Control Replicate 2**
Concentration 1	(5F+5M) in **Concentration 1 Replicate 1** and (5F+5M) in **Concentration 1 Replicate 2**
Concentration 2	(5F+5M) in **Concentration 2 Replicate 1** and (5F+5M) in **Concentration 2 Replicate 2**
Concentration 3	(5F+5M) in **Concentration 3 Replicate 1** and (5F+5M) in **Concentration 3 Replicate 2**

Total number of fish sampled per test:
Day 21, assuming 100% survival, 80 fish in control and treatment concentrations,
and 20 additional fish if the positive control is run

APPENDIX 4: ABBREVIATIONS & DEFINITIONS

CV – coefficient of variation

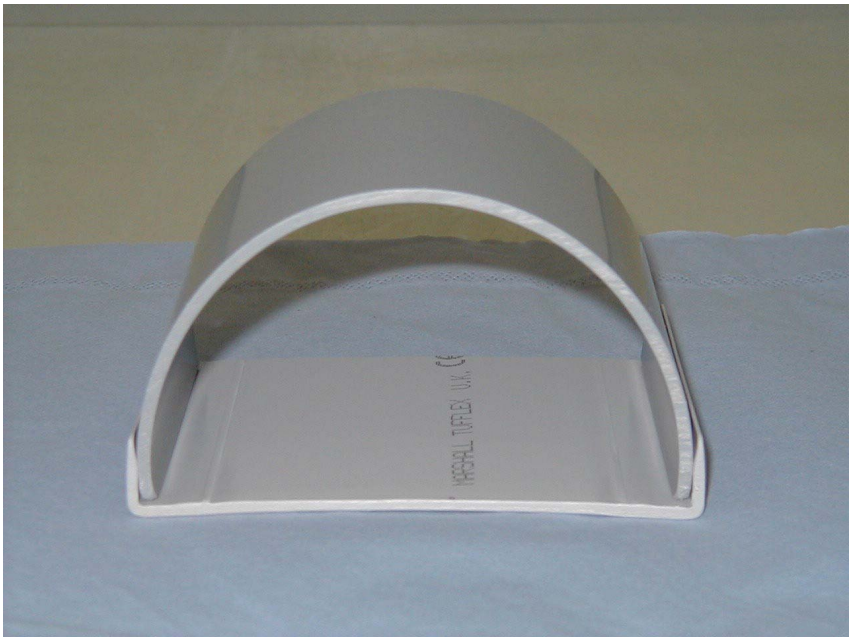
Loading rate - the wet weight of fish per volume of water.

Stocking density - is the number of fish per volume of water.

VTG - vitellogenin is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active females of all oviparous species.

APPENDIX 5: GUIDANCE ON THE USE OF A SPAWNING SUBSTRATE FOR FATHEAD MINNOW

As the adults are in spawning condition, provision for their spawning activity such as adequate space and spawning substrates are necessary. A spawning tile is essential and tray is optional. For example, in the figure, the plastic spawning tile is sitting on a flat plastic base (i.e. 80 mm length of grey semi-circular guttering sitting on a lipped tray of 130 mm length). As the fathead male is territorial, one tile should be provided per male in each tank. The laboratories should observe daily the spawning status in each test vessel as a 'Yes/No.' A "Yes" is a clutch of eggs in at least one of the spawning tiles. A "No" is the absence of eggs in all tiles. If eggs are present, they should be removed each day.



The base is designed to contain any eggs that do not adhere to the tile surface and would therefore fall to the bottom of the tank (or those eggs laid directly onto the flat plastic base). Please note, all spawning substrates should be leached for a minimum of 12 hours, in dilution water, before use.

APPENDIX 6: SECONDARY SEXUAL CHARACTERISTICS (NUPITAL TUBERCLES) IN THE FATHEAD MINNOW

Introduction and Background

1. Potentially important characteristics of physical appearance in adult fathead minnows in endocrine disrupter testing include body color (i.e., light/dark), coloration patterns (i.e., presence or absence of vertical bands), body shape (i.e., shape of head and pectoral region, distension of abdomen), and specialized secondary sex characteristics (i.e., number and size of nuptial tubercles, size of dorsal pad and ovipositor).

2. Nuptial tubercles are located on the head (dorsal pad) of reproductively-active male fathead minnows, and are usually arranged in a bilaterally-symmetric pattern (Jensen et al. 2001). Control females and juvenile males and females exhibit no tubercle development (Jensen et al. 2001). There can be up to eight individual tubercles around the eyes and between the nares of the males. The greatest numbers and largest tubercles are located in two parallel lines immediately below the nares and above the mouth. In many fish there are groups of tubercles below the lower jaw; those closest to the mouth generally occur as a single pair, while the more ventral set can be comprised of up to four tubercles. The actual numbers of tubercles is seldom more than 30 (range, 18-28; Jensen et al. 2001). The predominant tubercles (in terms of numbers) are present as a single, relatively round structure, with the height approximately equivalent to the radius. Most reproductively-active males also have, at least some, tubercles which are enlarged and pronounced such that they are indistinguishable as individual structures.

3. Some types of endocrine-disrupting chemicals (EDCs) can cause the abnormal occurrence of certain secondary sex characteristics in the opposite sex; for example, androgen receptor agonists, such as 1 7 -methyltestosterone or 1 7 -trenbolone, can cause female fathead minnows to develop nuptial tubercles (Smith 1974; Ankley *et al.* 2001; 2003), while estrogen receptor agonists may decrease number or size of nuptial tubercles in males (Miles-Richardson et al. 1999; Harries et al. 2000).

4. This protocol describes characterization of nuptial tubercles in fathead minnows based on procedures used at the U.S. Environmental Protection Agency lab in Duluth, MN. Specific products and/or equipment can be substituted with comparable materials available in participating labs.

Protocol

Anesthetic

5. MS-222 is used as an anesthetic for fish sampling/assessment (Kahl et al. 2001). Sodium bicarbonate is used as an buffering agent for the sedative.

Reagents

6. MS-222 - Fenquel^{TM} (Tricaine Methanesulfonate) and sodium bicarbonate - $NaHCO_3$.

Anesthetic Preparation Procedure:

7. Collect 1 L of control test water at nominal test temperature (e.g., 25^{o}C) in a beaker

- allocate 100 mg of MS-222 to weigh pan
- allocate 200 mg of sodium bicarbonate to weigh pan

8. Add weighed chemicals to control water and stir (ca., 1 minute)

9. Transfer dissolved chemical solution to stainless steel bowl for easy fish handling

10. Solution will accommodate 20 to 30 organisms (added individually); fresh solution will need to be prepared for additional animals

Sampling Methods

Procedure

11. Using a 12.5cmX10cm (125mm) fine mesh nylon net, carefully net organism from culture or test chamber.

- If handling toxicant-exposed fish, start with control fish and work up with increasing EDC concentrations.

12. Place organisms in MS-222 solution.

- Activity level may be momentarily high with rapid swimming or darting. Activity will decrease but gill ventilation rate may become elevated or rapid.

13. Within about 1 minute fish will start to show loss of equilibrium.

- Spiral or erratic swimming.
- Loss of movement, listlessness.
- Gentle probing with the net will cause little physical response. Organisms are still actively ventilating.

14. Remove fish from anaesthetic with a net. Wipe excess moisture from net and fish into an absorbent towel. Gently place fish on petri dish - lack of movement occurs.

- Fish should not be actively moving, muscle tissue should still be rigid without loss of character. Continued emersion into MS-222 may be required. If potency of MS-222 is not adequate, additional chemical (< 10 mg) may be added to strengthen effectiveness.

15. Viewing is best accomplished using an illuminated magnifying glass or 3X illuminated dissection scope. View fish dorsally and anterior forward (head toward viewer).

a. Place fish in small petri dish (e.g., 100 mm in diameter), anterior forward, ventral down. Focus viewfinder to allow identification of tubercles. Gently and slowly roll fish from side to side to identify tubercle areas. Count and score tubercles.

b. Repeat the observation on the ventral head surface by placing the fish dorsal anterior forward in the petri dish.

c. Observations should be completed within 2 min for each fish.

d. Return fish to control water to revive, if desired.

16. If fish are handled in a gentle manner within a reasonable amount of time during tubercle assessment recovery will occur within a few minutes without lasting adverse affects (Kahl et al. 2001). To avoid mortality during and after this procedure be alert to the following details.

- Keep fish moist during procedure.
- Limit the amount of time used to score tubercles.
- When placing fish into clean water gently move the fish back and forth, aiding water movement across the gill membranes.

17. If tubercles are assessed at test conclusion, animal may be subjected to additional sampling at this time (e.g., removal of blood for vitellogenin measurements; dissection of gonads).

Tubercle Counting and Rating

18. Six specific areas have been identified for assessment of tubercle presence and development in adult fathead minnows. A template was developed to map the location and quantity of tubercles present (attachment 1). The number of tubercles is recorded and their size can be quantitatively ranked as: 1-present, 2-enlarged and 3-pronounced for each organism (Fig. 1).

19. Rating criteria for tubercle size. Ratings are to be applied only when tubercles are present.

Rating 1-present, is identified as any tubercle having a single point whose height is nearly equivalent to its radius (diameter).

Rating 2- enlarged, is identified by tissue resembling an asterisk in appearance, usually having a large radial base with grooves or furrows emerging from the center. Tubercle height is often more jagged but can be somewhat rounded at times.

Rating 3- pronounced, is usually quite large and rounded with less definition in structure.

20. At times these tubercles will run together forming a single mass along an individual or combination of areas (B, C and D, described below). Coloration and design are similar to rating 2 but at times are fairly indiscriminate. Using this rating system generally will result in overall tubercle scores of <50 in a normal control male possessing a tubercle count of 18 to 20 (Jensen et al. 2001).

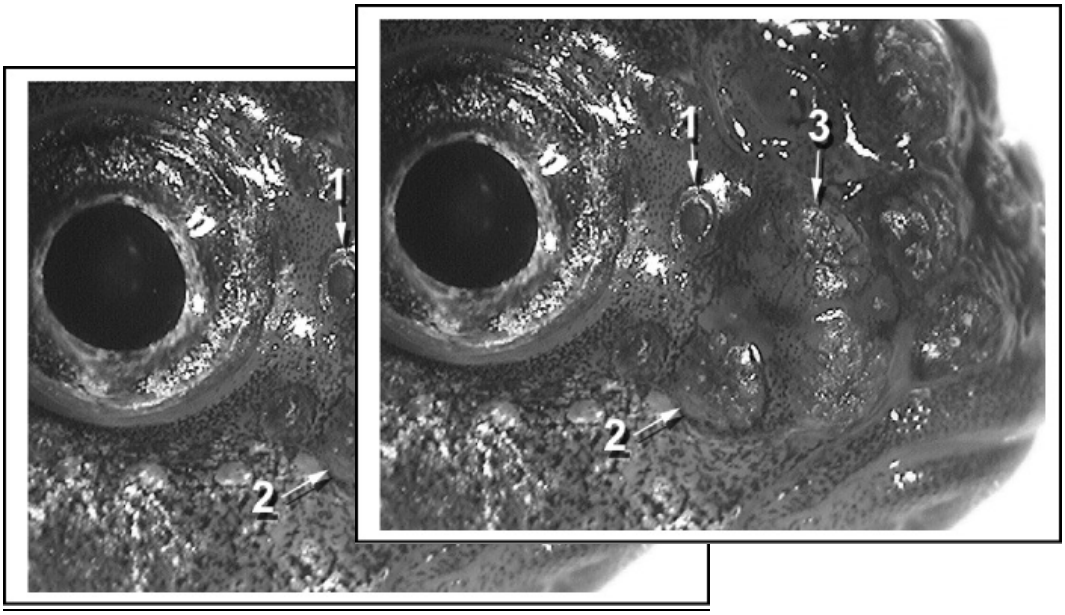


Figure 1

21. The actual number of tubercles in some fish may be greater than the template boxes (Attachment 1) for a particular rating area. If this happens, additional rating numbers may be marked within, to the right or to the left of the box. The template therefore does not have to display symmetry. An additional technique for mapping tubercles which are paired or joined vertically along the horizontal plane of the mouth could be done by double-marking two tubercle rating points in a single box.

Mapping regions:

A - Tubercles located around eye. Mapped dorsal to ventral around anterior rim of eye. Commonly multiple in mature control males, not present in control females, generally paired (one near each eye) or single in females exposed to androgens.

B - Tubercles located between nares, (sensory canal pores). Normally in pairs for control males at more elevated levels (2- enlarged or 3- pronounced) of development. Not present in control females with some occurrence and development in females exposed to androgens.

C - Tubercles located immediately anterior to nares, parallel to mouth. Generally enlarged or pronounced in mature control males. Present or enlarged in less developed males or androgen-treated females.

D - Tubercles located parallel along mouth line. Generally rated developed in control males. Absent in control females but present in androgen-exposed females.

E - Tubercles located on lower jaw, close to mouth, usually small and commonly in pairs. Varying in control or treated males, and treated females.

F - Tubercles located ventral to E. Commonly small and paired. Present in control males and androgen-exposed females.

TUBERCLE TEMPLATE

I. The following format is suggested for uniform entry of the tubercle numerical rating and score.

After counting the number of clearly visible tubercles, their size and location are to be recorded. The size will be scored based upon rating criteria defined in paragraph 19. For each individual fish, each tubercle will be rated and then an overall score for the individual will be calculated. The suggested template follows.

Date Observations Made: _____

Dosage group _____

Individual Fish Sex _____

Individual Fish ID _____

Total Tubercle Number: _____

Tubercle Number	Rating 1	Rating 2	Rating 3	Score
1				_____
2				_____
3				_____
4				_____
5				_____

Continue until all tubercles for the individual have been scored
Note: the numbered rows should correspond to the total tubercle number entered above.

TOTAL INDIVIDUAL SCORE
(Sum of individual tubercle scores) _____

APPENDIX 7: VITELLOGENIN (VTG) MEASUREMENT IN FATHEAD MINNOW

The Fish Drafting Group agreed, based on several comparative evaluation studies, that homologous ELISA methods (i.e. species-specific antibodies and VTG standards) should be used for the validation exercise. The VTG standards will be prepared centrally and delivered to all participating labs. VTG purification is conducted according to the following procedure:

VTG Purification Methods

Purification of VTG from plasma or homogenate will be accomplished by anion exchange chromatography using POROS 20HQ resin and the BIOCAD Sprint TM purification system as described by Denslow et al (1999). Plasma or homogenate will be diluted 1:10 with loading buffer (20 mM bis-tris-propane, 75 mM NaCl, pH 9.0) and then injected onto the anion exchange resin. Non-binding proteins will be eluted from the column by several volumes of running buffer (20 mM bis-tris-propane, 150 mM NaCl, pH 9.0). VTG will be selectively released from the column using a linear gradient of NaCl (150 - 800 mM) and collected in several 1.0 ml fractions. VTG is the last protein to elute from the column. The identity of the VTG peak during anion exchange purification will be verified by comparison to uninduced males and the fact that the VTG becomes the dominant protein in the blood (e.g., >50%) when induced.

After pooling the VTG fractions, the pH will be adjusted to 7 using 500 mM bis-tris- propane, pH 6. The protease inhibitor, Aprotinin, and bactericide, sodium azide, will be added to the purified VTG at 10KIU/ml and 0.02%, respectively. The VTG solution will be filtered (0.45 ml) to remove any possible flocculants or proteins that may have come out of solution. The cryoprotectant, glycerol, will be added to the VTG at 50% (v/v). A VTG aliquot without glycerol will be collected for amino acid quantification and sent to Battelle for Mass Spectroscopy Analysis.

Quantitative analysis of the purified VTGs will be accomplished by several different methods.

- Prior to the addition of glycerol, the absorbance of the purified VTG will be measured at 280nm. Proteins absorb light at this wavelength and this method has been used by several investigators to determine VTG content. We will use a molar extinction coefficient for fathead minnow, medaka, and zebrafish VTGs to calculate the concentration in mg/ml.
- The amino acid composition and concentration of purified VTG (without glycerol) will be determined using amino acid analysis. This procedure will be done in triplicate for each species.
- The total protein of the VTG in glycerol will be measured using the Bradford assay (Coomassie Plus TM, Pierce) using bovine serum albumin as a standard.

The purity of the purified VTGs will be determined by polyacrylamide gel electrophoresis (PAGE using both native and denaturing gels), Western, and ELISA analysis. In most fish species, VTG is a dimer, and yields 2 bands with high molecular weights (120,000 - 180,000 Daltons) after SDS-PAGE. Western analysis of the purified VTG should recognize the two high molecular weight VTG bands; however, it may also recognize smaller fragments depending on primary antibody specificity and degree of VTG degradation. Finally, the VTG will be used to generate a standard curve by direct ELISA to verify its integrity.

The protein VTG is sensitive to freeze/thaw events that can fracture the protein and affect ELISA results (Kroll & Denslow, unpublished results). To control for this variation, the purified VTG should be frozen only once after purification. After thawing an aliquot, it is stable for 1 year+ at -20ºC and remains in liquid form because it contains 50% glycerol. VTG stability at -20ºC has been verified by ELISA using positive controls over a 1-year period. Single use aliquots is also a method to avoid freeze/thaw effects.

Important note: According to preliminary studies undertaken by a supplier of commercial ELISA kits, glycerol concentrations > 10% may impair the analytical reactions!

Sample collection can be accomplished by either blood collection from caudal vein / artery or blood collection by cardiac puncture.

Two methods are considered appropriate for VTG analysis in fathead minnow:

- The ELISA method established at US EPA MED as described by Korte et al. 2000 (reference 29.), which employs a polyclonal fathead minnow VTG antibody.
- A commercially available ELISA kit, based on a monoclonal antibody for fathead minnow VTG.

Both methods have been compared in an evaluation study conducted by US EPA MED, which stated the need for some amendments of the commercially available kit. These amendments are supposed to be completed by April 2004. Further details are available *via* the fathead minnow lead lab AstraZeneca BEL / UK or the OECD Secretariat.

Acceptable kits using homologous antibodies for these methods are:

Biosense: Prod. no. V01018401
(see: http://www.biosense.com/docs/FHM-kit.pdf)

GE Healthcare [formerly Amersham]: Prod. no. RPN5942 see:
http://www4.amershambiosciences.com/aptrix/upp01077.nsf/Content/Products?OpenDocument&parentid=25900130&moduleid=165643