Appendix A6 – Fish Short-term Reproduction (Fish Assay)

Fish Short-term Reproduction (Fish Assay)	
Purpose	The fish short-term reproduction assay is a screening assay intended to identify changes in morphology, histopathology, spawning, and specific biochemical endpoints which may reflect interference with the normal function of the hypothalamic-pituitary-gonadal (HPG) axis. It is not intended to quantify or confirm endocrine disruption, or to provide a quantitative assessment of risk, but rather to provide suggestive evidence that endocrine-regulated processes may be sufficiently perturbed to warrant more definitive testing.
Design	The fish short-term reproduction assay entails exposing reproductively mature fathead minnows (*Pimephales promelas*) to a minimum of three concentrations of a test chemical and appropriate control(s) for 21 days. Successful spawning is established during a pre-exposure period of at least 14 days. Each of the four replicate tanks in each treatment level contains four females and two males.
Endpoints	Survival Behavior Body length Body weight Fecundity* • # of spawns • # of eggs/female reproductive day Fertilization Success* • # fertile eggs/female reproductive day • % fertile eggs Gonadal Histopathology* Gonadosomatic Index (GSI)* Appearance and Secondary Sex Characteristics* • Overall body coloration, vertical banding • Fatpad (weight, score, index) • Tubercles (count, score) • Ovipositor size Biochemical measures* • Vitellogenin • Estradiol • Testosterone * key endpoints

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Interpretation	The fish short-term reproduction assay as presented is intended to serve in a screening capacity to provide an indication of potential endocrine activity, not to confirm any specific mechanism, mode of action, or adverse effect. Therefore, a significant effect in one or more of the key endpoints of this assay (fecundity, fertilization success, histopathology, GSI, biochemical measures, and secondary sex characteristics) should be considered indicative of possible endocrine system disturbance. The suite of endpoints included is necessary to provide a fully comprehensive assessment of the disrupting potential to the HPG-axis in a representative fish. It is important to note however that if a given exposure level results in substantial mortality or other overt signs of toxicity, responses in other endpoints may be due to general toxicity, not necessarily mediated primarily via interaction with the endocrine system. The lower treatment level(s) should be examined for effects outside of the range of general toxicity. If all test concentrations exhibit mortality, then the assay would need repeating before inference on possible endocrine activity can be made. It is recognized that some endpoints may be responsive to non-endocrine stresses in addition to endocrine-mediated pathways, particularly fecundity. Although reductions in fecundity indicate adverse organismal and, potentially, population level effects (*i.e.*, reproductive toxicity), these cannot be definitively distinguished from direct endocrine-mediated effects by this assay when changes in other core endpoints are not present. Nevertheless, reductions in fecundity are considered a positive effect in this assay because they may be endocrine-mediated and should be considered in concert with results of the other assays in the Tier 1 battery. Results that would be considered equivocal for this single assay should be considered indications of potential endocrine activity and evaluated in light of the weight-of-evidence from the other assays in the Tier I battery of assays for the EDSP.
Main peer review comments	• Agreed that the assay is biologically and toxicologically relevant to the stated purpose. • Agreed that the overall design and endpoints selected are generally highly appropriate for screening for HPG perturbing chemicals, particularly (anti-) estrogenic and (anti-) androgenic compounds. • Recommend that fish are as similar as possible in egg production at the beginning of exposure. • Recommend that fish are sexually mature and of similar and optimal age for reproduction and to avoid mistaking immature males for females. • Recommend clarifying guidance for equal distribution of spawning groups among treatments to avoid bias.

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	• Suggest clarifying use of behavior observations. [EPA accepts the recommendations given and will revise the protocol guidance accordingly.]
Strengths (within the context of the proposed battery)	• Incorporates a standard, easily acquired laboratory model species, *Pimephales promelas*, and utilizes common aquatic toxicology methods; • Straightforward, cost effective, reasonably short-term assay; • Detects (anti-)estrogen and (anti-)androgen perturbations in addition to disruptors of the entire HPG axis using reproductively active male and female fish; • Employs an intact HPG axis and hence is relevant to other taxa when conserved elements of the HPG axis are considered; • Reproducible results demonstrated in multiple laboratories; • Informs the appropriate concentration range to be used in Tier 2 testing, which avoids the need for an additional range-finding study and reduces the number of animals needed.
Limitations (within the context of the proposed battery)	• Inherent technical difficulties testing substances that are poorly soluble in water in aquatic systems, and methods for delivering such substances to the test system. (Generally addressed on OECD Guidance Document 23); • Some measurements (*e.g.*, plasma steroids) will require specialized technical expertise