C.2.9. Fish Sexual Development Test (FSDT) (OECD TG 234)

Status: Assay validated by the OECD.

406. Modality detected/endpoints: estrogens (♀ and ♂ VTG ↑; phenotypic sex ratio ♀↑); anti-estrogens (♀ VTG ↓; phenotypic sex ratio ♂↑; sexually undifferentiated fish ↑); androgens (phenotypic sex ratio ♂↑; ♀ VTG ↓); anti-androgens (intersex fish ↑; ♀ VTG ↑; phenotypic sex ratio ♀↑); aromatase inhibitors (♀ VTG ↓; phenotypic sex ratio ♂↑); (optional endpoints – gonadal histopathology; genetic sex in medaka and stickleback). OECD TG 234 (FSDT) has been fully validated for Japanese medaka, zebrafish and stickleback. The test may also be responsive to certain thyroid-disrupting chemicals. It is known that thyroid hormone receptors TRα and TRβ are both present in fish early embryos and larvae (Power et al., 2001), and that maternally derived thyroxine (T4) is important for thyroid-dependent processes in fish early life stages (Nelson et al., 2014). One of these processes is swimbladder inflation, an endpoint which could be recorded in the FSDT test, and which is vital for the survival of fish fry. It has been shown, for example, that fathead minnow embryos exposed to a thyroid peroxidase (TPO) inhibitor (2-mercaptobenzothiazole) do not develop inflated swimbladders, probably because inhibition of TPO leads to decreased thyroid hormone synthesis (Villeneuve et al., 2013; Nelson et al., 2014). Also, Liu and Chan (2002) have shown that metamorphosis from embryo to larva in zebrafish is arrested by exposure to amiodarone (a TR antagonist) and by the goitrogen methimazole. Furthermore, Shi et al. (2008) demonstrated that the thyroid disrupter perfluorooctanesulfonic acid (PFOS) is able to delay hatching and cause developmental malformations in zebrafish embryos while upregulating two thyroid-related developmental genes, *hhex* and *pax8*. However, it is important to note that many non-ED chemicals will also cause these types of apical response, but by different mechanisms.

Background to the assay

407. This partial life cycle assay could potentially be used as a screen for the types of *in vivo* endocrine disruption activity in fish which are listed above (although it is considerably more expensive and time-consuming than the OECD TG 229/230 or EASZY screens), but should generally be used as a test which can also provide apical information of use in hazard identification/characterisation. It includes an endpoint (altered sex ratio), which is indicative of endocrine action, but more importantly indicates that adverse apical effects on sexual development are occurring. Major effects on phenotypic sex ratio would be expected to damage the ability of a fish population to reproduce itself, although small effects may be tolerated, but it is not possible to define the precise change in sex ratio beyond which adverse effects will occur unless specific information about a particular population is available. It should be noted that if the assay gives a positive result, this may be due to a positive indicator of hormonal activity (e.g. vitellogenin [VTG]), a positive for biased sex ratio, or a positive for both types of endpoint. Each of these three possible combinations of positive response should be considered separately (although the distinctions between indicators of hormonal activity and apical effects are not as clear in

OECD TG 234 [FSDT] as in other tests because it is acknowledged that sex ratio is both an apical endpoint [relevant for populations] as well as a biomarker endpoint [indicative of mode of action]), so they have been listed individually as points 1, 2 and 3 in the possible conclusions column of Table C.2.9.

408. If only three test concentrations are employed, a reliable NOEC or ECx for biased sex ratio may not be obtainable, so it may be desirable to use at least five test concentrations. However, if the test is used for hazard identification/characterisation, the stickleback should not be used because the validation data available so far show that in this species alterations of phenotypic sex ratio by test substances are uncommon. It should be noted that simultaneous measurement of both phenotypic and genotypic sex ratio (currently only possible in medaka and stickleback) will increase the statistical power of the test. However, power analysis of the validation results was used to prepare a test design providing sufficient power to detect changes in both sex ratio and VTG for the currently validated species. The ability of a substance with a suspected specific endocrine mechanism to change the sex ratio of fish should be considered during the choice of fish test species because some species are more susceptible to sex ratio changes caused by a specific endocrine mechanism than others. For example, zebrafish sex ratio is very sensitive to androgen agonists. Power analyses indicate that adequate power can be achieved with zebrafish as long as sufficient replication and fish per replicate are used (OECD, 2012). Given the high degree of endocrine system conservation across the vertebrates, adverse endocrine-linked effects in the FSDT may also indicate the possibility of related activity in other organisms such as amphibians, reptiles, birds or mammals.

When/why the assay may be used

409. Although OECD TG 234 (FSDT) could, in principle, be used at any stage in the hazard assessment process, the most likely use scenario will be when there are already some *in vitro* or *in vivo* screening data available about the possible endocrine disrupting properties of a chemical. It is unlikely that no other existing endocrine-relevant data will be available (i.e. if TG 234 has been used as a primary screen), but in that case a positive result in TG 234 should ideally be followed up with relevant *in vitro* screening to confirm the suspected mode of action (MOA) before any other *in vivo* testing is considered.

410. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an endocrine disrupter (ED), the study design has to be sufficiently robust to demonstrate the presence or absence of effects. In the dose selection, the investigator should also consider and ensure that data generated are adequate to fulfil the regulatory requirement across OECD countries as appropriate (e.g. hazard and risk assessment and labelling, ED assessment, etc.). The top dose or concentration should be sufficiently high to give clear systemic (i.e. non endocrine-specific) toxicity in order to ensure that a wide range of exposures (high to low) is tested. However, endocrine effects observed solely in the presence of clear systemic toxicity should be interpreted with caution and may be disregarded when sufficiently justified to be caused by secondary effects which are unlikely to be due to endocrine activity. The reason for this advice is a concern that some endocrine active substance (EAS) sensitive assays are being run at doses/concentrations of EASs that are too low to trigger direct impacts on the endocrine system. This guidance document is not the place to address this issue directly, but it should be considered when EAS-sensitive test guidelines (TGs) are revised in the future. In addition, the number and spacing of dose/concentration levels should also be adequate to

fulfil the objectives of the study (e.g. to demonstrate dose response relationships if this is required).

Existing data to be considered

411. Given the commonality of endocrine mechanisms in the vertebrates, relevant existing data available before deployment of OECD TG 234 (FSDT) might include *in vivo* results obtained with other vertebrates (e.g. a positive Uterotrophic Assay with rodents; positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies; or a positive result in the fish assays OECD TG 229 or 230), or one or more of a range of *in silico* or *in vitro* results which suggest that the modalities indicated above may occur *in vivo*. Such indicators of possible *in vivo* activity might include quantitative structure activity relationship (QSAR) predictions of endocrine activity, high throughput screening (HTS) data, "read-across" from *in vivo* results obtained with structurally related chemicals, or positive results from an *in vitro* screen for estrogen or androgen receptor-mediated activity, or for effects on steroidogenesis (especially aromatase inhibition). Further strong indication of *in vivo* estrogenic activity may also be available from an EASZY Assay with transgenic zebrafish embryos. Conduct of OECD TG 234 (FSDT) would be particularly relevant if the test chemical is suspected to act primarily on the sexual development phase of the fish life cycle (as opposed to the reproductive phase), because it provides apical information on phenotypic sex ratio which is fixed during the fry or juvenile stages of the species used in this test.

Scenarios: Positive and negative results combined with existing data

412. The scenarios (A to R) presented in Table C.2.9 represent all the possibilities of positive or negative results in combination with the presence or absence of existing data. The action taken will also depend on the regulatory environment, but the considerations given here are generally science based. Wherever possible, the recommended "next step which could be taken" avoids unnecessary animal testing. However, sometimes conducting an animal test will be indicated and then the relevance of species, strain and exposure route should always be considered. Further considerations specific to each scenario are given in the table.

413. Positive results obtained with one or more of OECD TG 234 (FSDT) indicators of hormonal activity but not with apical endpoints (Table C.2.9, Scenarios A-I, sub-section 2) result in the conclusion that the test chemical is a potential ED *in vivo*. If both an indicator of hormonal activity and sex ratio[1] give a correlated response (Table C.2.9, Scenarios A-I, sub-section 1), this provides evidence that the chemical is probably an actual ED (i.e. it causes adverse effects through an endocrine mechanism) if adverse population effects are expected as a consequence. If only sex ratio responds (Table C.2.9, Scenarios A-I, sub-section 3), it indicates that the chemical is probably an ED, but before drawing that conclusion, existing *in vitro* and *in vivo* data should be considered and a weight of evidence assessment carried out.

414. As indicated above, an effect on sex ratio in OECD TG 234 (FSDT) shows that the test chemical causes an adverse apical effect, is a developmental toxicant and is probably also an ED (assuming that the concentration giving this response is not sufficiently high to cause systemic toxicity). If these results are combined with positive indicators of hormonal activity and/or positive *in vitro* screening assay data, some regulatory authorities may consider that this is sufficient to show the chemical is an ED, and/or that the information could be

used for hazard identification/characterisation (providing sufficient concentrations have been tested to give an acceptably precise no-observed-effect-concentration [NOEC] or x% effect concentration [ECx]). Other authorities might nevertheless require further data to demonstrate that adverse effects at lower concentrations do not occur during the reproductive phase of the life cycle, and in these circumstances, conduct of a fish life cycle test (Medaka Extended One-Generation Reproduction Test [MEOGRT] – OECD TG 240), or Zebrafish Extended One-Generation Reproduction Test [ZEOGRT]) would be appropriate. In principle, an extended version of OECD TG 229 (i.e. a Fish Reproduction Partial Life Cycle Assay) might also address this issue, but a suitable protocol for this has not been validated. Additional testing of this type might also be required if an indicator or indicators of hormonal activity in OECD TG 234 (FSDT), but not sex ratio, respond positively. Existing data suggesting endocrine activity would strengthen the case for any additional testing still further.

415. A situation in which OECD TG 234 (FSDT) gives a negative result needs careful consideration of any existing data. If these data suggest that the chemical is endocrine active both *in vitro* and *in vivo* (Table C.2.9, Scenario J), then the probability is that OECD TG 234 (FSDT) is simply insufficiently sensitive, perhaps because the main MOA acts during the reproductive phase of the life cycle. It might then be appropriate to conduct a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) to confirm that there is no adverse endocrine activity in fish.

416. If OECD TG 234 (FSDT) and existing *in vivo* data are all negative, but *in vitro* data reveal some endocrine activity (Scenario K), the probability is that the test chemical is not sufficiently potent to produce endocrine effects *in vivo* in fish, or it may be rapidly metabolised. In such a situation, further testing is probably not necessary. However, if there is good reason to believe that the reproductive part of the life cycle may be more responsive than sexual development, consider conducting OECD TG 229 or a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).

417. Furthermore, if OECD TG 234 (FSDT) and the *in vitro* tests are negative, but there are positive existing *in vivo* data (Scenario M), the chemical is probably not an ED acting on fish sexual development, but it may act via MOA not covered by the *in vitro* screens, or it may be more potent in species or life stages that have not been tested. In this situation, the existing *in vivo* data should be used to guide decisions about whether to conduct any further testing, either for modalities such as thyroid activity (e.g. OECD TG 231, or *Xenopus* Embryonic Thyroid Signalling Assay [XETA]), or including other life stages represented in OECD TG 229 or the MEOGRT/ZEOGRT.

418. Finally, a negative OECD TG 234 (FSDT), set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is probably not an ED acting on sexual development in fish, and no further testing for estrogenic, anti-estrogenic, androgenic or steroidogenic MOA should generally be considered unless there is reason to believe that reproduction may be more responsive than development. It remains possible that the chemical has thyroid activity, but this is unlikely if OECD TG 231 or the XETA are one of the negative *in vivo* assays.

419. In each of the above scenarios, it is possible that existing data will be equivocal, or there may be no existing data (Scenarios C, F-I, L and O-R). This will weaken the conclusions which can be drawn about a negative OECD TG 234 (FSDT). However, a lack of mechanistic data on endocrine activity should ideally be rectified before any further *in vivo* testing is finally rejected. On the other hand, if OECD TG 234 (FSDT) is positive, further *in vivo* testing may be needed, even if all existing data are equivocal, or if there are

no existing data. Again, however, it will always be desirable to obtain some mechanistic information before conducting further *in vivo* testing. There is also the possibility that equivocal mechanistic data may be the result of multiple modes of endocrine action. Under some circumstances, two opposite modes of simultaneous action (e.g. estrogenic and anti-estrogenic) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA (e.g. estrogenic and anti-androgenic) could potentially reinforce effects on certain apical endpoints. If multiple MOA are suspected, either from the existing results or based on QSAR/read-across/integrated approaches, this situation should be investigated further if needed for regulatory decision making.

420. The scenario in which the results of OECD TG 234 (FSDT) are themselves equivocal has not been dealt with in Table C.2.9, for reasons of brevity. In this context, an equivocal result might be a non-monotonic concentration-response (e.g. no effect at a high concentration but effects at a lower concentration), or a result which borders on statistical significance. Without knowing the exact circumstances, reliable advice cannot be given, but the opinions of an experienced ecotoxicologist should be sought. Clearly, however, such equivocal results do not necessarily rule out the existence of *in vivo* endocrine activity. For example, an effect on sex ratio might just fail to reach a statistically significant level due to a random imbalance in the control sex ratio. If these or other possible reasons for false negatives are suspected with good reason, the test could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity), or a more appropriate version of it (e.g. more fish per replicate) could be conducted.

421. In summary, an adverse apical response (i.e. biased sex ratio) in OECD TG 234 (FSDT) indicates that a chemical is a probable ED. A combination of biased sex ratio and a positive endocrine-responsive mechanistic endpoint (e.g. VTG) is even stronger evidence that the chemical is an actual ED. If sufficient test concentrations have been tested, this will allow a precise NOEC or ECx to be calculated. In such cases, some regulatory authorities may consider that no more data are required, while others may wish to investigate whether the reproductive stage of the life cycle is even more sensitive than the developmental part. On the other hand, negative results in OECD TG 234 (FSDT) do not necessarily mean that the chemical is not an ED – a judgement about this will have to be made in the light of existing *in vitro* and *in vivo* data.

Note

1. Note that sex ratio can be considered as an indicator or biomarker of endocrine activity in its own right, as well as an apical measurement of adverse effects, although some types of non-EDCs may hypothetically be able to affect this endpoint in some species. None of these non-EDCs have yet been found.

References

Liu, Y.-W. and W.K. Chan (2002), "Thyroid hormones are important for embryonic to larval transitory phase in zebrafish", *Differentiation*, Vol. 70/1, pp. 36-45, https://doi.org/10.1046/j.1432-0436.2002.700104.x.

Nelson, K. et al. (2014), "Evaluation of hypothesized adverse outcome pathway linking thyroid peroxidase to fish early life stage toxicity", presentation, Society of Environmental Toxicology and Chemistry, Vancouver, British Columbia, Canada, 9-13 November 2014.

OECD (2012), "Validation report (Phase 2) for the Fish Sexual Development Test for the detection of endocrine active substances (updated 2012)", OECD Series on Testing and Assessment, No. 142, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2011)23/REV1&doclanguage=en.

Power, D.M. et al. (2001), "Thyroid hormones in growth and development of fish", *Comparative Biochemistry and Physiology Oart C: Toxicology & Pharmacology*, Vol. 130/4, pp. 447-459, https://doi.org/10.1016/S1532-0456(01)00271-X.

Shi, X. et al. (2008), "Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS", *Toxicology and Applied Pharmacology*, Vol. 230/1, pp. 23-32, https://doi.org/10.1016/j.taap.2008.01.043.

Villeneuve, S. et al. (2013), "Investigating alternatives to the fish early-life stage test: A strategy for discovering and annotating adverse outcome pathways for early fish development", *Environmental Toxicology and Chemistry*, Vol. 33/1, pp. 158-169, https://doi.org/10.1002/etc.2403.

WHO/IPCS (2002), "Global assessment of the state-of-the-science of endocrine disrupters", Damstra, T. et al. (eds.) WHO/PCS/EDC/02.2, World Health Organization, Geneva, www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en.

Table C.2.9. **Fish Sexual Development Test (FSDT) (OECD TG 234): Guidance for scenarios of combinations of results with existing data**

This table represents possible conclusions to be drawn from assay data, and a next step which could be taken if further evidence is required about possible endocrine disrupting properties and/or effects. The guidance offered is not meant to be prescriptive, but provides science-based considerations. It encourages the use of all available data and expert judgement in a weight of evidence approach. Regional and national interpretation of results and "next steps" may vary.

The conclusions are grouped into a series of scenarios (A-R), each scenario representing a different combination of assay results, existing *in vitro* data and existing *in vivo* data. The symbol "+" indicates that the data in question represent a positive result, «-" indicates a negative result, and "Eq/0" indicates that the data are either equivocal or are not available.

The assay under discussion could either be positive for both apical and indicators of endocrine activity endpoints, or positive just for apical endpoints, or positive just for indicators of endocrine activity. However, note that sex ratio could in most cases be considered as both an indicator of endocrine activity and an apical endpoint, and as yet, no chemicals have been found which are able to alter sex ratios by way of mechanisms other than endocrine disruption. For each scenario, each of these three possibilities is addressed separately in the possible conclusions column.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from estrogen receptor (ER-), androgen receptor (AR-) and steroidogenesis-based assays (Level 2). Thyroid hormone receptor (TR) and other assays concerning mechanisms of thyroid disruption may be available, but they are not in common use. In practice, data from all assays may not be available and therefore this must be taken into account when deciding on the "next step". Quantitative structure activity relationship (QSAR) predictions of estrogen and androgen binding/activation may be made for some substances. There is no evidence at present that equivalent *in vitro* assays with systems derived from fish offer advantages over their mammalian counterparts.

Existing results: ** "Effects (*in vivo* effects of concern)" assumes effects have been observed in other *in vivo* screens/tests which give rise to concern that the test chemical may be an endocrine disrupter.

Scenario	Result of TG 234 (FSDT)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
A	+	+	+	1) Strong evidence for adverse effects in fish and other organisms by an endocrine mechanism. 2) Strong evidence for endocrine effects, but uncertainty about whether they are adverse in fish. 3) Strong evidence for adverse effects in fish and other organisms. There is a possibility that the apical endpoint sex ratio is more sensitive to the test chemical than the mechanistic endpoint vitellogenin (VTG), or mechanism may hypothetically not be via direct interaction with estrogen receptor (ER), androgen receptor (AR) or by aromatase inhibition, even though it is noted that currently there is no evidence for sex ratio change in fish caused by other mechanisms than those mentioned here at otherwise non-toxic concentrations of chemicals.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) should be considered.	If OECD TG 234 (Fish Sexual Development Test [FSDT]) was only performed with three test concentrations, this may not be sufficiently precise to establish a reliable no-observed-effect-concentration/x% effect concentration [NOEC/ECx]. Also, note that some endocrine disruptors (EDs) may be more toxic to reproduction than to sexual development, in which case TG 234 (FSDT) would be less responsive than a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).
B	+	+	–	1) Strong evidence for adverse effects in fish by an endocrine mechanism. 2) Strong evidence for endocrine effects in fish, but uncertainty about whether they are adverse. 3) Strong evidence for adverse effects in fish and other organisms. There is a possibility that the apical endpoint sex ratio is more sensitive to the test chemical than the mechanistic endpoint VTG, or mechanism may hypothetically not be via direct interaction with ER, AR or by aromatase inhibition, even though it is noted that currently there is no evidence for sex ratio change in fish caused by other mechanisms than those mentioned here at otherwise non-toxic concentrations of chemicals.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) should be considered.	If OECD TG 234 (FSDT) was only performed with three test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case TG 234 (FSDT) would be less responsive than a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).
C	+	+	Eq/0**	1) Strong evidence for adverse effects in fish by an endocrine mechanism. 2) Strong evidence for endocrine effects in fish, but uncertainty about whether they are adverse. 3) Strong evidence for adverse effects in fish and other organisms. There is a possibility that the apical endpoint sex ratio is more sensitive to the test chemical than the mechanistic endpoint VTG, or mechanism may hypothetically not be via direct interaction with ER, AR or by aromatase inhibition, even though it is noted that currently there is no evidence for sex ratio change in fish caused by other mechanisms than those mentioned here at otherwise non-toxic concentrations of chemicals.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) should be considered. This would be particularly helpful given the equivocal *in vivo* effects, or lack of *in vivo* tests, in other taxa.	If OECD TG 234 (FSDT) was only performed with three test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case TG 234 (FSDT) would be less responsive than a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple modes of action (MOA). If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenario	Result of TG 234 (FSDT)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
D	+	–	+	1) Strong evidence for adverse effects in fish and other organisms, possibly by an unknown endocrine mechanism. 2) Medium-strong evidence for endocrine effects in fish, but they do not appear to be adverse. 3) Strong evidence for adverse effects in fish and other organisms. There is a possibility that the apical endpoint sex ratio is more sensitive to the test chemical than the mechanistic endpoint VTG, or mechanism may hypothetically not be via direct interaction with ER, AR or by aromatase inhibition, even though it is noted that currently there is no evidence for sex ratio change in fish caused by other mechanisms than those mentioned here at otherwise non-toxic concentrations of chemicals.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) should be considered.	If OECD TG 234 (FSDT) was only performed with three test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case TG 234 (FSDT) would be less responsive than a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). If *in vitro* data are negative or equivocal, it might be unsafe to conclude that an effect on sex ratio was definitely caused by endocrine disruption, although this is the most probable explanation, especially if endocrine disruption has been shown in other species.
E	+	–	–	1) Strong evidence for adverse effects in fish, possibly by an unknown endocrine mechanism. 2) Medium-strong evidence for endocrine effects in fish, but they do not appear to be adverse. 3) Strong evidence for adverse effects in fish and other organisms. There is a possibility that the apical endpoint sex ratio is more sensitive to the test chemical than the mechanistic endpoint VTG, or mechanism may hypothetically not be via direct interaction with ER, AR or by aromatase inhibition, even though it is noted that currently there is no evidence for sex ratio change in fish caused by other mechanisms than those mentioned here at otherwise non-toxic concentrations of chemicals.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) should be considered.	If OECD TG 234 (FSDT) was only performed with three test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case TG 234 (FSDT) would be less responsive than a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). If *in vitro* data are negative or equivocal, it might be unsafe to conclude that an effect on sex ratio was definitely caused by endocrine disruption, although this is the most probable explanation, especially if endocrine disruption has been shown in other species.

Scenario	Result of TG 234 (FSDT)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
F	+	–	Eq/0	1) Strong evidence for adverse effects in fish, possibly by an unknown endocrine mechanism. 2) Medium-strong evidence for endocrine effects in fish, but they do not appear to be adverse. 3) Strong evidence for adverse effects in fish and other organisms. There is a possibility that the apical endpoint sex ratio is more sensitive to the test chemical than the mechanistic endpoint VTG, or mechanism may hypothetically not be via direct interaction with ER, AR or by aromatase inhibition, even though it is noted that currently there is no evidence for sex ratio change in fish caused by other mechanisms than those mentioned here at otherwise non-toxic concentrations of chemicals.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) should be considered. This would be particularly helpful given the equivocal *in vivo* effects, or lack of *in vivo* tests, in other taxa.	If OECD TG 234 (FSDT) was only performed with three test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case TG 234 (FSDT) would be less responsive than a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). If *in vitro* data are negative or equivocal, it might be unsafe to conclude that an effect on sex ratio was definitely caused by endocrine disruption, although this seems the most probable explanation, especially if endocrine disruption has been shown in other species. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
G	+	Eq/0	+	1) Strong evidence for adverse effects in more than one organism, possibly by an unknown endocrine mechanism. 2) Medium-strong evidence for endocrine effects, but they do not appear to be adverse in fish. 3) Strong evidence for adverse effects in fish and other organisms. There is a possibility that the apical endpoint sex ratio is more sensitive to the test chemical than the mechanistic endpoint VTG, or mechanism may hypothetically not be via direct interaction with ER, AR or by aromatase inhibition, even though it is noted that currently there is no evidence for sex ratio change in fish caused by other mechanisms than those mentioned here at otherwise non-toxic concentrations of chemicals.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) should be considered. Given uncertainty about the mechanism of action, any further *in vivo* testing should be preceded by *in vitro* mechanistic studies.	If OECD TG 234 (FSDT) was only performed with three test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case TG 234 (FSDT) would be less responsive than a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). If *in vitro* data are negative or equivocal, it might be unsafe to conclude that an effect on sex ratio was definitely caused by endocrine disruption, although this seems the most probable explanation, especially if endocrine disruption has been shown in other species. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenario	Result of TG 234 (FSDT)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
H	+	Eq/0	–	1) Strong evidence for adverse effects in fish, possibly by an unknown endocrine mechanism. 2) Medium-strong evidence for endocrine effects in fish, but they do not appear to be adverse. 3) Strong evidence for adverse effects in fish and other organisms. There is a possibility that the apical endpoint sex ratio is more sensitive to the test chemical than the mechanistic endpoint VTG, or mechanism may hypothetically not be via direct interaction with ER, AR or by aromatase inhibition, even though it is noted that currently there is no evidence for sex ratio change in fish caused by other mechanisms than those mentioned here at otherwise non-toxic concentrations of chemicals.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) should be considered. Given uncertainty about the mechanism of action, any further *in vivo* testing should be preceded by *in vitro* mechanistic studies.	If OECD TG 234 (FSDT) was only performed with three test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case TG 234 (FSDT) would be less responsive than a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). If *in vitro* data are negative or equivocal, it might be unsafe to conclude that an effect on sex ratio was definitely caused by endocrine disruption, although this seems the most probable explanation, especially if endocrine disruption has been shown in other species. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
I	+	Eq/0	Eq/0	1) Strong evidence for adverse effects in fish, possibly by an unknown endocrine mechanism. 2) Moderate-strong evidence for endocrine effects in fish, but they do not appear to be adverse. 3) Strong evidence for adverse effects in fish and other organisms. There is a possibility that the apical endpoint sex ratio is more sensitive to the test chemical than the mechanistic endpoint VTG, or mechanism may hypothetically not be via direct interaction with ER, AR or by aromatase inhibition, even though it is noted that currently there is no evidence for sex ratio change in fish caused by other mechanisms than those mentioned here at otherwise non-toxic concentrations of chemicals.	Some regulatory authorities may consider that further evidence is not required, especially if adverse effects have been demonstrated. However, if more evidence is needed about adverse effects in fish, performance of a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) should be considered. Given uncertainty about the mechanism of action, any further *in vivo* testing should be preceded by *in vitro* mechanistic studies.	If OECD TG 234 (FSDT) was only performed with three test concentrations, this may not be sufficiently precise to establish a reliable NOEC/ECx. Also, note that some EDs may be more toxic to reproduction than to sexual development, in which case TG 234 (FSDT) would be less responsive than a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). If *in vitro* data are negative or equivocal, it might be unsafe to conclude that an effect on sex ratio was definitely caused by endocrine disruption, although this seems the most probable explanation, especially if endocrine disruption has been shown in other species. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity, lack of metabolic activation or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information

Scenario	Result of TG 234 (FSDT)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
J	–	+	+	The chemical is an ED *in vivo* in other species but does not appear to act on sexual development in fish. If any other fish tests are also negative, fish may not be responsive at all to the test chemical.	Some regulatory authorities may consider that further evidence is not required. However, if it is suspected that the reproductive part of the life cycle may be responsive, consider conducting OECD TG 229 or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	As OECD TG 229 only uses three test concentrations and exposes fish for just three weeks, an extended version which runs more concentrations for longer would provide more comprehensive data about interference with reproduction than OECD TG 229 unmodified. However, an agreed protocol for such an extended test is not available, so an option would be to run a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).
K	–	+	–	Despite the *in vitro* mechanistic data for potential endocrine activity, there is no evidence for endocrine disruption *in vivo*. This may be because the chemical is quickly transformed/degraded to an inactive metabolite, or because it only interacts very weakly with the endocrine system. However, it is also possible that the chemical only acts on the reproductive part of the fish life cycle which is not exposed in OECD TG 234 (FSDT).	Some regulatory authorities may consider that sufficient data are available to show that the chemical is not an ED *in vivo*. However, if it is suspected that the reproductive part of the life cycle may be responsive, consider conducting either OECD TG 229 or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	As OECD TG 229 only uses three test concentrations and exposes fish for just three weeks, an extended version which runs more concentrations for longer would provide more comprehensive data about interference with reproduction than OECD TG 229 unmodified. However, an agreed protocol for such an extended test is not available, so an option would be to run a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).
L	–	+	Eq/0	The chemical may not be an ED *in vivo*, but the confidence in this conclusion is relatively low as there is only one unequivocal *in vivo* test result (the negative OECD TG 234). However, it is also possible that the chemical only acts on the reproductive part of the fish life cycle which is not exposed in TG 234 (FSDT).	Some regulatory authorities may consider that sufficient data are available to show that the chemical is not an ED *in vivo*. However, such a conclusion is not well supported. If it is suspected that the reproductive part of the life cycle may be responsive, consider conducting either OECD TG 229 or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	As OECD TG 229 only uses three test concentrations and exposes fish for just three weeks, an extended version which runs more concentrations for longer would provide more comprehensive data about interference with reproduction than OECD TG 229 unmodified. However, an agreed protocol for such an extended test is not available, so an option would be to run a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenario	Result of TG 234 (FSDT)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
M	–	–	+	The chemical is probably not an ED acting on sexual development in fish, but it does have endocrine activity in other species. However, it may act through MOA not covered by the available *in vitro* assays, or it may be more potent in a fish species other than that tested. It is also possible that the chemical only acts on the reproductive part of the fish life cycle which is not exposed in OECD TG 234 (FSDT), although such action is presumably not via one of the mechanisms mentioned above.	Some regulatory authorities may consider that sufficient evidence is available. However, if it is suspected that the reproductive part of the life cycle may be responsive, consider conducting either OECD TG 229 or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT), possibly using a different species to that employed in OECD TG 234 (FSDT). If thyroid activity is suspected, it may be helpful to conduct an Amphibian Metamorphosis Assay (TG 231) or *Xenopus* Embryonic Thyroid Signalling Assay.	As OECD TG 229 only uses three test concentrations and exposes fish for just three weeks, an extended version which runs more concentrations for longer would provide more comprehensive data about interference with reproduction than OECD TG 229 unmodified. However, an agreed protocol for such an extended test is not available, so an option would be to run a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).
N	–	–	–	The chemical is probably not an ED acting on sexual development in fish, or *in vivo* in other species. It is possible that the chemical is able to interfere with the reproductive part of the fish life cycle but the probability of this is low given the apparent absence of estrogenic, androgenic or steroidogenic properties *in vitro* or *in vivo*.	Some regulatory authorities may consider that sufficient data are available to show that the chemical is not an ED *in vivo*. However, if it is suspected that the reproductive part of the life cycle may be responsive, consider conducting either OECD TG 229 or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	As OECD TG 229 only uses three test concentrations and exposes fish for just three weeks, an extended version which runs more concentrations for longer would provide more comprehensive data about interference with reproduction than OECD TG 229 unmodified. However, an agreed protocol for such an extended test is not available, so an option would be to run a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).
O	–	–	Eq/0	The chemical is probably not an ED acting on sexual development in fish. It is possible that the chemical is able to interfere with the reproductive part of the fish life cycle, but the probability of this is low given the apparent absence of estrogenic, androgenic or steroidogenic properties.	Some regulatory authorities may consider that sufficient data are available to show that the chemical is not an ED *in vivo*. However, if it is suspected that the reproductive part of the life cycle may be responsive, consider conducting either OECD TG 229 or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	As OECD TG 229 only uses three test concentrations and exposes fish for just three weeks, an extended version which runs more concentrations for longer would provide more comprehensive data about interference with reproduction than OECD TG 229 unmodified. However, an agreed protocol for such an extended test is not available, so an option would be to run a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenario	Result of TG 234 (FSDT)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
P	–	Eq/0	+	The chemical is probably not an ED acting on sexual development in fish, but confidence in this conclusion is low given the lack of comprehensive *in vitro* data and the availability of positive existing *in vivo* data. However, it is possible that the chemical only acts on the reproductive part of the fish life cycle which is not exposed in OECD TG 234 (FSDT).	Some regulatory authorities may consider that sufficient evidence is available. However, if it is suspected that the reproductive part of the life cycle may be responsive, consider conducting either OECD TG 229 or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). However, it would be desirable to obtain comprehensive mechanistic data before possibly proceeding to further *in vivo* testing.	As OECD TG 229 only uses three test concentrations and exposes fish for just three weeks, an extended version which runs more concentrations for longer would provide more comprehensive data about interference with reproduction than OECD TG 229 unmodified. However, an agreed protocol for such an extended test is not available, so an option would be to run a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
Q	–	Eq/0	–	The chemical is probably not an ED acting on sexual development in fish, or *in vivo* on other species, but the lack of more predictive mechanistic data are a concern, even though the existing *in vivo* data are negative. It is nevertheless possible that the chemical is able to interfere with the reproductive part of the fish life cycle.	Some regulatory authorities may consider that sufficient data are available to show that the chemical is not an ED *in vivo*. However, if it is suspected that the reproductive part of the life cycle may be responsive, consider conducting either OECD TG 229 or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). It would be desirable to obtain comprehensive mechanistic data before any further *in vivo* testing.	As OECD TG 229 only uses three test concentrations and exposes fish for just three weeks, an extended version which runs more concentrations for longer would provide more comprehensive data about interference with reproduction than OECD TG 229 unmodified. However, an agreed protocol for such an extended test is not available, so an option would be to run a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
R	–	Eq/0	Eq/0	The chemical may not be an ED acting on sexual development in fish, but confidence in this conclusion is low given the lack of comprehensive *in vitro* and existing *in vivo* data. It is nevertheless possible that the chemical is able to interfere with the reproductive part of the fish life cycle.	Some regulatory authorities may consider that sufficient data are available to show that the chemical is not an ED *in vivo*. However, if it is suspected that the reproductive part of the life cycle may be responsive, consider conducting either OECD TG 229 or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). However, it would be desirable to obtain comprehensive mechanistic data before any further *in vivo* testing.	As OECD TG 229 only uses three test concentrations and exposes fish for just three weeks, an extended version which runs more concentrations for longer would provide more comprehensive data about interference with reproduction than OECD TG 229 unmodified. However, an agreed protocol for such an extended test is not available, so an option would be to run a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.