Non-OECD non-mammalian screens and tests (Conceptual Framework Levels 3-5)

C.2.14. Short-Term Juvenile Hormone Activity Screening Assay using *Daphnia magna* (SJHASA) (draft OECD TG)

Status: Assay being validated by the OECD.

483. Modality detected/endpoints: This short-term *in vivo* assay with *Daphnia magna* is expected to be responsive to juvenile hormone (JH) agonists which lead to the production of male offspring.

Background to the assay

484. This *in vivo* assay is in undergoing validation by the OECD, and may be approved as a test guideline (TG) in due course. The SJHASA exposes 17-day-old (i.e. adult) female *D. magna* to dilutions of the test chemical for 5-7 days. Their first brood after exposure is discarded, but all individuals of the second brood are sexed by observation of their longer first antenna. Juvenile hormone (JH) and other JH agonists cause the production of males due to exposure during a short critical period (52-53 hours after ovulation). An adverse outcome pathway for this process is under development – significant male production in a population could potentially lead to its decline. However, due to the very short-term nature of SJHASA, the endpoint of male production should not be considered as an adverse apical endpoint without further investigation in longer term tests.

485. OECD TG 211 (the *Daphnia magna* Reproduction Test) already has an option to measure male production as a response to JH agonists, but it is a much more resource-intensive test than the SJHASA and takes three times as long to perform.

When/why the assay may be used

486. Although the SJHASA could, in principle, be used at any stage in the hazard assessment process, the most likely use scenario will be when there are relatively few data available about the possible JH-disrupting properties of a chemical. The results from this assay are most likely to be available after deployment of a battery of *in vitro* screens, or as a supplement to existing data which suggest possible JH-related activity. Given the significant degree of endocrine system conservation across the arthropods, endocrine-linked effects in the SJHASA may also indicate the possibility of related activity in other arthropods such as copepods, decapods and insects.

487. It is possible that no endocrine-relevant data are available before the SJHASA is deployed (i.e. if the SJHASA has been used as a primary screen), but in that case a positive result in the screen should probably be followed up with relevant *in vitro* screening, if available, to investigate the suspected mode of action (MOA) in more detail. However, it should be noted that there are no standardised *in vitro* screens for JH agonists, although some are described in the scientific literature (for example, Cherbas, Koehler and Cherbas [1989]; Miyakawa and Iguchi [2017]).

488. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an 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

489. Existing information on endocrine-related effects from other arthropods should also be considered before deployment of the SJHASA, given the commonality of endocrine mechanisms in these taxa. Existing data available might also include one or more of a range of *in silico* or *in vitro* results which suggest that JH disruption may occur *in vivo* (but note the limitations of this approach, as indicated above). Such indicators of possible JH activity might include quantitative structure activity relationship (QSAR) predictions of JH activity, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen for JH agonist activity.

Scenarios: Positive and negative results combined with existing data

490. The scenarios (A to R) presented in Table C.2.14 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.

491. Positive results obtained with the SJHASA (Table C.2.14, Scenarios A-I) result in the conclusion that the test chemical is a possible JH disrupter *in vivo*, at least in crustaceans. However, as indicated above, although a positive response of the SJHASA indicates that the chemical is a possible JH agonist, a result of this type would generally need to be followed up with a more comprehensive screen. The most appropriate choice for this is the *Daphnia* Multigeneration Test (DMGT – draft OECD TG). However, if countries need further evidence concerning growth and sexual development, etc., a Harpacticoid Copepod Development and Reproduction Test – OECD GD 201 and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233 would be able to provide a precise no-observed-effect-concentration/x% effect concentration (NOEC/ECx)

for adverse effects. This may be particularly important because *Daphnia* are parthenogenic under certain circumstances, while *Amphiascus* and *Chironomus* reproduce sexually. In other words, in order to strengthen weight of evidence, a positive result in the SJHASA could be followed by the DMGT at Level 3, which if positive in turn might lead to conduct of OECD TG 233 (Level 5). Existing data suggesting endocrine-specific activity (e.g. positive *in vitro* data, or positive *in vivo* data from other species) will strengthen the case for additional testing still further.

492. The situation in which the SJHASA gives a negative result (Table C.2.14, Scenarios J-R) needs careful consideration of any existing data. If these data suggest that the chemical is endocrine active both *in vitro* and *in vivo* (Scenario J), then it is possible that the SJHASA is simply insufficiently sensitive.

493. If the SJHASA and existing *in vivo* data are all negative, but *in vitro* data reveal some JH activity (Scenario K), the probability is that the test chemical is not sufficiently potent to produce JH agonism *in vivo* in arthropods, or it may be rapidly metabolised. In such a situation, further testing is probably not necessary. However, if the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests have been insufficiently prolonged, in which case longer term testing with OECD TG 201 or OECD TG 233 might be justified.

494. On the other hand, if the SJHASA and the *in vitro* tests are negative (Scenario M), but there are positive existing *in vivo* data, the nature of those existing data should be considered. Unless the existing data are from another crustacean, the chemical is possibly not a JH agonist acting in crustaceans, but it may be more potent in species (e.g. insects) 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.

495. Finally, a negative SJHASA, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is probably not a JH agonist *in vitro* or *in vivo*, and further action is unnecessary.

496. In each of the above scenarios, it is possible that existing data will be equivocal, or there may be no existing data. This will weaken the conclusions which can be drawn about a negative SJHASA, and this is reflected in Table C.2.14. However, a lack of mechanistic data on JH activity should ideally be rectified before any further *in vivo* testing is finally conducted, although as indicated above, *in vitro* JH screens have not yet been internationally standardised. On the other hand, if the SJHASA is positive, further *in vivo* testing would generally be needed to quantify any adverse effects and/or to establish a NOEC or ECx for such effects, even if all existing data are equivocal, or if there are no existing data. Again, however, it may be useful 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. JH agonistic and antagonistic) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA could potentially reinforce effects on the SJHASA endpoint. 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.

497. The scenario in which the results of the SJHASA are themselves equivocal has not been dealt with in Table C.2.14, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. If possible reasons for false negatives are suspected, the SJHASA could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity). However, note that a repeat screen in the event of systemic toxicity would not be needed providing at least one tested concentration was not subject to such effects. It should also be borne in mind that changing environmental conditions such as shortening photoperiod, temperature and food shortages can also cause the production of male neonates in *D. magna*, so if these have accidentally occurred during the test, the results should be treated as suspect.

498. In summary, positive results in the SJHASA may indicate that a chemical is endocrine active *in vivo* via JH agonism. This suggests that more comprehensive *in vivo* testing would be needed if the intention is to derive a long-term NOEC/ECx and/or to confirm whether or not the chemical is an actual endocrine disrupter in arthropods due to the occurrence of adverse effects. Negative results in the SJHASA do not necessarily mean that the chemical is not a potential ED – a judgement about the endocrine disruption potential in other arthropods (especially sexually reproducing species) and the possible need for additional testing will have to be made based on a weight of evidence evaluation of existing *in vitro* and *in vivo* data.

References

Cherbas, L., M.M.D. Koehler and P. Cherbas (1989), "Effects of juvenile hormone on the ecdysone response of *Drosophila* Kc cells", *Developmental Genetics*, Vol. 10/3, pp. 177-188, https://doi.org/10.1002/dvg.1020100307.

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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.2.14. **Short-Term Juvenile Hormone Activity Screening Assay using *Daphnia magna* (SJHASA) (draft OECD TG): 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.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from available from juvenile hormone (JH-) based assays. JH assays concerning mechanisms of JH disruption may be available, but they are have not yet been internationally standardised. In practice, data from all assays may not be available and therefore this must be taken into account when deciding on the "next step".

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 a JH disrupter.

Scenarios	Result of SJHASA	Existing results		Possible conclusions	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	+	+	+	Strong evidence for *in vivo* juvenile hormone (JH) activity in crustaceans, plus possible JH effects in other arthropods.	Consider performing a *Daphnia* Multigeneration Test (DMGT – draft OECD TG).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist. The DMGT will show if sex ratio bias towards males carries over into the F2 generation and some regulatory authorities may consider that this provides sufficient information on adverse apical effects in crustaceans. However, as *Daphnia* are parthenogenetic, it would be desirable to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).
B	+	+	–	Strong evidence for *in vivo* JH activity in crustaceans.	Consider performing a DMGT (draft OECD TG).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist. The DMGT will show if sex ratio bias towards males carries over into the F2 generation and some regulatory authorities may consider that this provides sufficient information on adverse apical effects in crustaceans. However, as *Daphnia* are parthenogenetic, it would be desirable to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).
C	+	+	Eq/0	Strong evidence for *in vivo* JH activity in crustaceans.	Consider performing a DMGT (draft OECD TG).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist. The DMGT will show if sex ratio bias towards males carries over into the F2 generation and some regulatory authorities may consider that this provides sufficient information on adverse apical effects in crustaceans. However, as *Daphnia* are parthenogenetic, it would be desirable to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233). 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.
D	+	–	+	Moderate evidence for *in vivo* JH activity in crustaceans, plus possible JH effects in other arthropods.	Consider performing a DMGT (draft OECD TG).	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. The DMGT will show if sex ratio bias towards males carries over into the F2 generation and some regulatory authorities may consider that this provides sufficient information on adverse apical effects in crustaceans. However, as *Daphnia* are parthenogenetic, it would be desirable to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).

Scenarios	Result of SJHASA	Existing results		Possible conclusions	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)**			
E	+	–	–	Possible evidence for *in vivo* JH activity in crustaceans.	Consider performing a (DMGT (draft OECD TG).	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. The DMGT will show if sex ratio bias towards males carries over into the F2 generation and some regulatory authorities may consider that this provides sufficient information on adverse apical effects in crustaceans. However, as *Daphnia* are parthenogenetic, it would be desirable to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).
F	+	–	Eq/0	Possible evidence for *in vivo* JH activity in crustaceans.	Consider performing a DMGT (draft OECD TG). Given the absence or equivocal nature of existing *in vivo* data, it might also be sensible to conduct a JH-responsive insect assay (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. The DMGT will show if sex ratio bias towards males carries over into the F2 generation and some regulatory authorities may consider that this provides sufficient information on adverse apical effects in crustaceans. However, as *Daphnia* are parthenogenetic, it would be desirable to perform an additional apical test with sexually reproducing crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201) 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	+	Moderate evidence for *in vivo* JH activity in crustaceans, plus possible JH effects in other arthropods.	Consider performing a DMGT (draft OECD TG). Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for JH activity.	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH activity. The DMGT will show if sex ratio bias towards males carries over into the F2 generation and some regulatory authorities may consider that this provides sufficient information on adverse apical effects in crustaceans. However, as *Daphnia* are parthenogenetic, it would be desirable to perform an additional apical test with sexually reproducing crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201 and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233). 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.
H	+	Eq/0	–	Possible evidence for *in vivo* JH activity in crustaceans.	Consider performing a DMGT (draft OECD TG). Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for JH activity.	The DMGT will show if sex ratio bias towards males carries over into the F2 generation and some regulatory authorities may consider that this provides sufficient information on adverse apical effects in crustaceans. However, as *Daphnia* are parthenogenetic, it would be desirable to perform an additional apical test with sexually reproducing crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201 and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233). 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.

Scenarios	Result of SJHASA	Existing results		Possible conclusions	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)**			
I	+	Eq/0	Eq/0	Possible evidence for *in vivo* JH activity in crustaceans.	Consider performing a DMGT (draft OECD TG). Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for JH activity.	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH activity. The DMGT will show if sex ratio bias towards males carries over into the F2 generation and some regulatory authorities may consider that this provides sufficient information on adverse apical effects in crustaceans. However, as *Daphnia* are parthenogenetic, it would be desirable to perform an additional apical test with sexually reproducing crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201 and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233). 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.
J	–	+	+	The test chemical is probably a JH agonist without activity in crustaceans, although it is possible that *Daphnia magna* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist.
K	–	+	–	The test chemical is likely to have JH activity; however, without demonstrating sufficient activity to disrupt physiological processes *in vivo*.	If there is no activity in crustaceans or insects, further evidence is probably not needed.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist.
L	–	+	Eq/0	The test chemical is likely to have JH activity; however, without demonstrating sufficient activity to disrupt physiological processes *in vivo*.	Some regulatory authorities may conclude that no further evidence is required, but if insect data are absent, it might be desirable to conduct a Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist. 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.
M	–	–	+	The test chemical is probably without JH activity in crustaceans, although it is possible that *Daphnia magna* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. However, it is possible that the existing effects may not be due to JH activity.

Scenarios	Result of SJHASA	Existing results		Possible conclusions	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)**			
N	–	–	–	The test chemical is probably without JH activity in arthropods.	No further action is necessary.	–
O	–	–	Eq/0	The test chemical is probably without JH activity in arthropods.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. 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.
P	–	Eq/0	+	The test chemical is probably without JH activity in crustaceans, although it is possible that *Daphnia magna* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. Also, if clear *in vitro* mechanistic data are missing, it might be desirable to obtain some.	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH activity. However, it is possible that the existing effects may not be due to JH activity. 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 test chemical is probably without JH activity in arthropods.	No further action is necessary.	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 test chemical is probably without JH activity in crustaceans and possibly insects.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	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.

C.2.15. Androgenised Female Stickleback Screen (AFSS) (GD 148) (variant of OECD TG 230)

Status: Partially validated by the OECD.

499. Modality detected/endpoints: androgens (♀ spiggin ↑); anti-androgens (androgenised ♀ spiggin ↓).

Background to the assay

500. This assay is designed primarily as a screen for chemicals with *in vivo* anti-androgenic activity in fish, but it is also able to detect androgens. It has partially completed validation and has been published as an OECD guidance document (GD 148). The endpoints are indicators of hormonal activity and there are no apical measures of adverse effects **diagnostic of a specific estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modality**. This assay is a variant of the 21-Day Fish Assay (OECD TG 230) with a more limited range of endpoints, but it has more power to identify anti-androgens than OECD TG 229 or TG 230. An alternative *in vivo* assay with the scope for identifying anti-androgens is the Juvenile Medaka Anti-Androgen Screening Assay (JMASA).

When/why the assay may be used

501. Although the AFSS could, in principle, be used at any stage in the hazard assessment process, the most likely use scenario will be when there are relatively few data available about the possible endocrine disrupting properties of a chemical. The assay is most likely to be used either as part of a battery of *in vitro* and *in vivo* screens, or to follow up on existing data which suggest possible endocrine disruption activity at the androgen receptor. It would not be necessary for aquatic exposure to have been predicted (because a positive in the AFSS could potentially be extrapolated to terrestrial vertebrates), but such a prediction would provide additional justification for running the screen. It is also possible that no existing endocrine-relevant data are available (i.e. the AFSS has been used as a primary screen), but in that case a positive result in the screen should ideally be followed up with relevant *in vitro* screening in an attempt to confirm the suspected (anti)androgenic mode of action (MOA). Given the high degree of endocrine system conservation across the vertebrates, endocrine-linked effects in the AFSS may also indicate the possibility of related activity in other organisms such as amphibians, reptiles, birds or mammals.

502. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an endocrine disruptor (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

503. Given the commonality of endocrine mechanisms in the vertebrates, relevant existing data available before deployment of the AFSS might include *in vivo* results obtained with other vertebrates (e.g. a positive rodent Hershberger Bioassay – OECD TG 441, positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies), 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 data, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen for androgen receptor-mediated activity.

Scenarios: Positive and negative results combined with existing data

504. The scenarios (A to R) presented in Table C.2.15 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.

505. Positive results obtained with one of the endpoints (Table C.2.15, Scenarios A-I) result in the conclusion that the test chemical is a possible androgen or anti-androgen *in vivo*. If a regulatory authority required more evidence, positive results in the AFSS should be followed up with more comprehensive testing to show whether adverse apical effects occur at any part of the life cycle (and hence to provide evidence supporting a conclusion that the chemical is an actual ED). In other words, to increase confidence, a positive result in the AFSS would trigger fish life cycle testing at Level 5 (OECD TG 240 – MEOGRT or ZEOGRT), or possibly a Fish Sexual Development Test (FSDT) (OECD TG 234) at Level 4 if it is suspected that the most responsive part of the life cycle is sexual development. Existing data suggesting (anti)androgenic activity will strengthen the case for additional testing still further.

506. The situation in which the AFSS gives a negative result (Table C.2.15, Scenarios J-R) needs careful consideration of any existing data. If these data suggest that the chemical is (anti)androgenic both *in vitro* and *in vivo* (Scenario J), then the probability is that the AFSS is simply insufficiently sensitive. It might in these circumstances be appropriate to

conduct OECD TG 234 (FSDT), or alternatively, a fish life cycle test (OECD TG 240 – MEOGRT or ZEOGRT) to confirm that there is no endocrine activity in fish.

507. If the AFSS and existing *in vivo* data are all negative, but *in vitro* data reveal some (anti)androgenic activity (Scenario K), the probability is that the test chemical is not sufficiently potent to produce endocrine effects *in vivo* in fish or other organisms, or it may be rapidly metabolised or simply does not reach the receptor. In such a situation, further testing is probably not necessary. However, if the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests have been insufficiently prolonged, in which case longer term testing might be justified. Equally, if existing data suggest thyroid activity, consideration should be given to conducting the Amphibian Metamorphosis Assay (OECD TG 231).

508. On the other hand, if the AFSS and the *in vitro* tests are negative (Scenario M), but there are positive existing *in vivo* data, the chemical is probably not an ED with (anti)androgenic activity, but it may act via modes of action (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, or including life stages represented in TG 234 (FSDT) or in the MEOGRT or ZEOGRT.

509. Finally, a negative AFSS, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is not (anti)androgenic in fish, and no further testing for this modality will generally be necessary. It remains possible that it has thyroid activity, although if any existing tests for this modality are negative, it would suggest that this scenario is unlikely.

510. In each of the above scenarios, it is possible that existing data will be equivocal (Scenarios C, F-I, L and O-R), or there may be no existing data. This will weaken the conclusions which can be drawn about a negative AFSS, and this is reflected in Table C.2.15. However, a lack of mechanistic data on (anti)androgenic activity should ideally be rectified before any further *in vivo* testing is considered. On the other hand, if the AFSS is positive, further *in vivo* testing to obtain more evidence is generally desirable even if all existing data are equivocal, or if there are no existing data. Again, however, it will always be helpful 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. androgenic and anti-androgenic) could, depending on dose, lead to a minimisation or abolition of effects, while in others two different MOA (e.g. estrogenic and anti-androgenic) could potentially reinforce effects. 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.

511. The scenario in which the results of the AFSS are themselves equivocal has not been dealt with in Table C.2.15, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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, spiggin induction in females at a high concentration might be masked by any systemic toxicity (although it would not be sensible to run the assay at such high concentrations), while spiggin depression in androgenised females might just fail to reach a statistically

significant level because spiggin levels were relatively low to begin with. If these or other possible reasons for false negatives are suspected with good reason, the screen could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity, assuming systemic toxicity in the original test occurred at all concentrations), or a more appropriate version of it (e.g. ensure androgenised females have high spiggin levels at the start of the test) could be conducted. In particular, it might be appropriate to run the JMASA if anti-androgenic activity is suspected.

512. In summary, positive results in the AFSS indicate that a chemical is a possible (anti)androgen. If a regulatory authority required further evidence, more comprehensive *in vivo* testing would then be necessary to produce a long-term NOEC/ECx for adverse effects and/or to confirm whether or not the chemical is an actual (anti)androgen. Negative results in the AFSS do not necessarily mean that the chemical is not a possible (anti)androgen – a judgement about this will have to be made in the light of existing *in vitro* and *in vivo* data.

Reference

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.2.15. **Androgenised Female Stickleback Screen (AFSS) (OECD GD 148) (variant of OECD TG 230): 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.

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.

Note that this assay has been successfully validated, but it has not been published as an OECD test guideline.

Scenario	Result of AFSS	Existing results		Possible conclusions	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	+	+	+	Strong evidence for *in vivo* (anti)androgenic activity in fish and other organisms.	Consider performing fish life cycle test (ZEOGRT or MEOGRT – OECD TG 240).	An alternative approach would be to deploy TG 234 (Fish Sexual Development Test [FSDT]), especially if sexual development is expected to give a response at lower concentrations than reproduction.
B	+	+	–	Strong evidence for *in vivo* (anti)androgenic activity in fish.	Consider performing fish life cycle test (ZEOGRT or MEOGRT – OECD TG 240).	An alternative approach would be to deploy TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229 or TG 230), consider possible reasons for the disparity (e.g. differences in species sensitivity) before possibly conducting a life cycle test (OECD TG 240 – MEOGRT or ZEOGRT) or OECD TG 234 (FSDT).
C	+	+	Eq/0**	Strong evidence for *in vivo* (anti)androgenic activity in fish.	Consider performing fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) or OECD TG 234 (FSDT).	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of a life cycle test (MEOGRT 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.
D	+	–	+	Strong evidence for *in vivo* (anti)androgenic activity in fish and other organisms, but negative *in vitro* data suggest MOA may not be via interaction with the androgen receptor or interference with steroidogenesis, or that the test chemical may be metabolically activated *in vivo*.	Consider performing fish life cycle test (MEOGRT – OECD TG 240 or ZEOGRT).	An alternative approach would be to deploy TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction.
E	+	–	–	Strong evidence for *in vivo* (anti)androgenic activity in fish, but negative existing data raise doubts about the MOA, or suggest that the test chemical may be metabolically activated *in vivo*.	Consider performing fish life cycle test (MEOGRT – OECD TG 240 or ZEOGRT).	An alternative approach would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229 or TG 230), consider possible reasons for the disparity (e.g. differences in species sensitivity) before possibly conducting a life cycle test (FLCTT or OECD TG 240 – MEOGRT, or ZEOGRT) or TG 234 (FSDT).
F	+	–	Eq/0	Strong evidence for *in vivo* (anti)androgenic activity in fish, but negative or equivocal existing data raise doubts about the MOA, or suggest that the test chemical may be rapidly degraded in water or metabolically activated *in vivo*.	Consider performing a fish life cycle test (MEOGRT – OECD TG 240 or ZEOGRT) or TG 234 (FSDT).	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a life cycle test (MEOGRT or ZEOGRT) in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of a life cycle test. 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 AFSS	Existing results		Possible conclusions	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)**			
G	+	Eq/0	+	Strong evidence for *in vivo* (anti)androgenic activity in fish and other organisms, but mechanism unconfirmed.	Obtain more predictive mechanistic data and then consider performing fish life cycle test (MEOGRT – OECD TG 240 or ZEOGRT).	An alternative approach would be to deploy OECD TG 234 (FSDT). 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.
H	+	Eq/0	–	Strong evidence for *in vivo* (anti)androgenic activity in fish, but mechanism unconfirmed.	Obtain more predictive mechanistic data and then consider performing fish life cycle test (MEOGRT – OECD TG 240 or ZEOGRT).	An alternative approach would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229 or TG 230), consider possible reasons for the disparity (e.g. differences in species sensitivity) before possibly conducting a life cycle test (MEOGRT or ZEOGRT) or TG 234 (FSDT). 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	Strong evidence for *in vivo* (anti)androgenic activity in fish, but mechanism unconfirmed.	Obtain more predictive mechanistic data and then consider performing fish life cycle test (MEOGRT – OECD TG 240 or ZEOGRT) or TG 234 (FSDT).	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of a life cycle test (MEOGRT 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.
J	–	+	+	No evidence for (anti)androgenic activity *in vivo* in fish. However, the chemical is an (anti)androgen in other species and this mechanism has been confirmed *in vitro*.	Consider performing OECD TG 234 (FSDT).	It is possible that the failure to give a positive result in the AFSS was caused by the relatively short exposure time (three weeks). If this is suspected, it is worth considering whether to perform a fish life cycle test (MEOGRT or ZEOGRT) or OECD TG 234 (FSDT). Test design should be guided by the existing *in vivo* data.
K	–	+	–	There is no evidence that the chemical is an (anti)androgen *in vivo*, probably because it is very weakly acting or rapidly metabolised or degraded in water.	Probably no further action, but see comments in right-hand column.	It is possible that ndocrine disruptors which bioaccumulate slowly may only cause effects *in vivo* after exposure times longer than three weeks. If this is suspected, and depending on which part of the life cycle is suspected of being the most sensitive, consider performing OECD TG 234 (FSDT) or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). It is also possible that the chemical may be a thyroid-active chemical *in vivo* (consider performing the Amphibian Metamorphosis Assay [AMA] – OECD TG 231), an (anti)estrogen, or an aromatase inhibitor (consider performing OECD TG 229 or TG 230).

Scenario	Result of AFSS	Existing results		Possible conclusions	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)**			
L	–	+	Eq/0	The chemical may not be an (anti)androgen *in vivo*, but the confidence in this conclusion is relatively low as there is only one unequivocal *in vivo* test result (a negative).	Consider performing a fish assay (OECD TG 229 or TG 230) with a different species, or consider a longer term test (TG 234 [FSDT] or life cycle [MEOGRT – OECD TG 240, or ZEOGRT]).	It is also possible that the chemical may be a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or *Xenopus* Embryonic Thyroid Signalling Assay [XETA]), an (anti)estrogen, or an aromatase inhibitor (consider performing OECD TG 229, TG 230 or EASZY). 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.
M	–	–	+	The chemical is probably not an (anti)androgen in fish. However, it may act through MOA not covered by the available *in vitro* assays, or it may be more potent in a species other than that tested, or over a longer exposure period.	Use the existing *in vivo* data to help choose a possible longer term test with an appropriate species.	It is also possible that the chemical may be a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA), an (anti)estrogen, or an aromatase inhibitor (consider performing OECD TG 229, TG 230 or EASZY), although lack of *in vitro* binding affinity with the estrogen or androgen receptors suggests the two former possibilities are unlikely. Use the existing *in vivo* data to guide any further testing.
N	–	–	–	The chemical is probably not an (anti)androgen in fish or other organisms.	No further action with respect to (anti)androgenic MOA.	It is still possible that the chemical may be a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA), an (anti)estrogen, or an aromatase inhibitor (consider performing OECD TG 229, TG 230 or EASZY), although lack of *in vitro* binding affinity with the estrogen or androgen receptors suggests the two former possibilities are unlikely.
O	–	–	Eq/0	The chemical is probably not an (anti)androgen in fish or other organisms.	Probably no further action. However, see comments in right-hand column.	If the paucity of *in vivo* data are a concern, performance of a screening test (OECD TG 229 or TG 230) with a different species, or a longer term test (i.e. TG 234 [FSDT] or life cycle [MEOGRT – OECD TG 240, or ZEOGRT]) could be considered. It is also possible that the chemical may be a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA), an (anti)estrogen, or an aromatase inhibitor (consider performing OECD TG 229, TG 230 or EASZY), although lack of *in vitro* binding affinity with the estrogen or androgen receptors suggests the two former possibilities are unlikely. 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 AFSS	Existing results		Possible conclusions	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 (anti)androgen in fish, but confidence in this conclusion is low given the lack of more predictive *in vitro* data and the availability of positive existing *in vivo* data.	Obtain more predictive mechanistic data, then consider possible further testing.	If the mechanistic data confirm that the chemical has potential (anti)androgenic action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (TG 234 [FSDT] or life cycle [MEOGRT – OECD TG 240, or ZEOGRT]). Use the existing *in vivo* data as a guide to test design. If the mechanistic data reveal (anti)estrogenic/aromatase inhibition activity, perform a fish assay (OECD TG 229 or TG 230). If any existing data suggest thyroid activity, consider an AMA (OECD TG 231) or XETA. 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 (anti)androgen in fish or other organisms, but the lack of more predictive mechanistic data are a concern.	Obtain more predictive mechanistic data, then consider possible further testing.	If the mechanistic data confirm that the chemical has potential (anti)androgenic action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (TG 234 [FSDT] or life cycle [MEOGRT – OECD TG 240, or ZEOGRT]). Use the existing *in vivo* data as a guide to test design. If the mechanistic data reveal (anti)estrogenic/aromatase inhibition activity, perform a fish assay (OECD TG 229 or TG 230). If any existing data suggest thyroid activity, consider an AMA (OECD TG 231) or XETA. 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 is probably not an (anti)androgen in fish, but confidence in this conclusion is low given the lack of more predictive *in vitro* and existing *in vivo* data.	Obtain more predictive mechanistic data, then consider possible further testing.	If the mechanistic data confirm that the chemical has potential (anti)androgenic action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (TG 234 [FSDT] or life cycle [MEOGRT – OECD TG 240, or ZEOGRT]). Use the existing *in vivo* data as a guide to test design. If the mechanistic data reveal (anti)estrogenic/aromatase inhibition activity, perform a fish assay (OECD TG 229 or TG 230). If any existing data suggest thyroid activity, consider an AMA (OECD TG 231) or XETA. 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.

C.2.16. EASZY Assay: Detection of Substances Acting through Estrogen Receptors using Transge nic cyp19a1b GFP Zebrafish Embryos (draft OECD TG)

Status: Assay being validated by the OECD.

513. Modality detected/endpoints: This *in vivo* zebrafish assay is sensitive to estrogen receptor (ER) agonists; pro-estrogens that can be metabolised to become ER agonists; androgens that can be aromatised to ER agonists; and some non-aromatisable androgens. It relies on transgenic zebrafish embryos which fluoresce when exposed to an ER agonist.

Background to the assay

514. This assay is currently being validated by the OECD, and one round of validation with up to five participating laboratories for each test chemical was initiated in 2014. The assay is based on a transgenic zebrafish line expressing green fluorescent protein (GFP) under the control of the promoter of the ER-regulated cyp19a1b gene coding for brain aromatase. The newly fertilised embryos are exposed for 96 hours to dilutions of the test chemical, after which they are scanned using a fluorescence imaging microscope, and the intensity of fluorescence recorded. From the concentration-response curve, ECx concentrations are derived and relative estrogenic potency can be calculated.

When/why the assay may be used

515. Although data from EASZY could, in principle, be available at any stage in the hazard assessment process, the most likely scenario will be when there are relatively few data available about the possible endocrine disrupting properties of a chemical. The assay is most likely to be used either as part of a battery of *in vitro* and *in vivo* screens, or to follow up on existing data which suggest possible endocrine disruption activity. Given the high degree of endocrine system conservation across the vertebrates, adverse endocrine-linked effects in the EASZY Assay may also indicate the possibility of related activity in other organisms such as amphibians, reptiles, birds or mammals. It is also possible that no existing endocrine-relevant data are available (i.e. EASZY has been used as a primary screen), but in that case a positive result in the screen should ideally be followed up with relevant *in vitro* screening in an attempt to confirm the precise mode of action (MOA). Furthermore, a positive EASZY result would also need to be followed up with an additional *in vivo* fish test such as the Fish Short-Term Reproduction Assay (FSTRA – OECD TG 229) or Fish Sexual Development Test (FSDT – OECD TG 234), which will give some indication of any adverse apical effects. Possible conclusions to be derived from the results of EASZY, and guidance about potential additional studies to strengthen weight of evidence, are summarised in Table C.2.16.

516. Caution should be used when negative results are obtained with certain types of chemicals because absorption into the embryo via the chorion may have been impeded. Development of the OECD Fish Embryo Acute Toxicity (FET) test (OECD TG 236) with zebrafish showed that this applies in particular to chemicals with a molecular weight ≥3kDa

and a very bulky molecular structure. Absorption of these chemicals will take place at a higher rate after hatching, but delayed hatch may therefore also protect the embryo from estrogenic effects. Although it is known that fish embryos have some metabolic capacity (e.g. Weigt et al. [2011]), and that EASZY is able to detect pro-estrogens such as methoxychlor that require metabolic activation (Brion et al., 2012), metabolism may be less efficient than in juveniles and adults, so use of the test with endocrine disrupting chemicals that require metabolic activation may give some false negatives. Nonetheless, a recent study comparing the metabolism of two estrogenic substances (BPS and BP2) in zebrafish embryos and adults reported that metabolic profiles were qualitatively the same between embryos and adults, with no major differences, although the biotransformation of both molecules was more extensive in adults (Le Fol et al., 2017).

517. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an endocrine disruptor (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

518. Existing information on endocrine-related effects from other vertebrates (up to and including mammals, e.g. from OECD TG 407) should always be considered, given the commonality of endocrine mechanisms in these taxa. Existing data available before deployment of EASZY might include *in vivo* results obtained with other vertebrates (e.g. a Uterotrophic Bioassay with rodents, positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies), 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 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).

Scenarios: Positive and negative results combined with existing data

519. The scenarios (A to R) presented in Table C.2.16 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.

520. Positive results obtained with one or more of the endpoints (Table C.2.16, Scenarios A-I) result in the conclusion that the test chemical is a potential ED *in vivo*. When a positive response is observed in the EASZY Assay, confirmatory experiments can be conducted by co-exposing embryos to the test chemical and the estrogen receptor (ER) antagonist ICI 182 780. If a significant down-regulation of the GFP is then observed, the involvement of functional ERs is indicated. Positive responses would ideally need to be followed up with more comprehensive testing to show whether adverse apical effects related to endocrine impacts occur at any part of the life cycle (and hence to discover whether the chemical is an ED acting through certain estrogen/androgen/thyroid/steroidogenesis [E,A,T,S] pathways). In other words, a positive result in the EASZY Assay may trigger TG 234 (FSDT) at Level 4 or fish life cycle testing (e.g. MEOGRT – TG 240) at Level 5. Existing data suggesting endocrine activity will strengthen the case for additional testing.

521. The situation in which the EASZY Assay gives a negative result (Table C.2.16, Scenarios J-R) needs careful consideration of any existing data. If the weight of evidence of these data suggests that the chemical is endocrine active both *in vitro* and *in vivo* in other species (Scenario J), then the probability is that the EASZY may simply be insufficiently responsive in that case, or fish in general may be unresponsive. In some of these circumstances, it might be appropriate to conduct a FSDT (OECD TG 234), or alternatively, a fish life cycle test (e.g. MEOGRT, TG 240) to confirm that there is no endocrine activity in fish.

522. If the EASZY Assay 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 may or may not be necessary. If the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests are not of sufficient duration, in which case longer term testing might be justified. If the *in vitro* data reveal anti-androgenic or thyroid activity, consideration should be given to conducting the Androgenised Female Stickleback Screen (AFSS – OECD GD 148) or Juvenile Medaka Anti-Androgen Screening Assay (JMASA), or the Amphibian Metamorphosis Assay (AMA – OECD TG 231), respectively.

523. On the other hand, if the EASZY Assay and the *in vitro* tests are negative, but there are positive existing *in vivo* data (Scenario M), the chemical is probably not a potential ED with the modalities listed above, but it may act via estrogen- or androgen-related 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 relevant existing *in vitro* and *in vivo* data should be used to guide decisions about whether to conduct any further testing, either for modalities such as anti-androgenicity or including life stages represented in OECD TG 234 (FSDT) or in TG 240 (MEOGRT).

524. Finally, a negative EASZY, set against a background of negative *in vitro* and *in vivo* data (Scenario N) that includes relevant *in vivo* data for fish, suggests that the test chemical is not a potential ED in fish or other vertebrates, and no further testing for estrogenic, anti-estrogenic, androgenic or steroidogenic MOA will generally be necessary. It remains possible that it has anti-androgenic or thyroid activity, although negative *in vitro* tests for these modalities would suggest that this scenario is unlikely.

525. 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 EASZY test, and this is reflected in Table C.2.16. However, a lack of mechanistic data on endocrine activity should usually be rectified before any further *in vivo* testing is finally decided on. Indeed, as a general principle, it is desirable to obtain mechanistic data before any *in vivo* testing. On the other hand, if EASZY is positive, further *in vivo* testing is generally indicated, particularly when existing data are equivocal, or if there are no existing data. 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 androgenic) could potentially reinforce effects on EASZY. 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.

526. The scenario in which the results of the EASZY Assay are themselves equivocal has not been dealt with in Table C.2.16, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. If false negatives (e.g. systemic toxicity) are suspected with good reason, the screen could be repeated if none of the test concentrations have given reliable data (e.g. conduct it at lower concentrations which avoid systemic toxicity). However, note that a repeat test in the event of systemic toxicity would not be needed providing at least one tested concentration was not subject to such effects.

527. In summary, positive results in the EASZY Assay indicate that a chemical is a possible endocrine disrupter. More predictive *in vivo* testing would then be necessary to produce a long-term no-observed-effect-concentration/x% effect concentration (NOEC/ECx) and/or to confirm whether or not the chemical is an actual endocrine disrupter with adverse effects *in vivo*. Negative results in EASZY do not necessarily mean that the chemical is not a potential ED – a judgement about its endocrine disruption potential and the possible need for additional testing will have to be made based on a weight of evidence evaluation of existing *in vitro* and *in vivo* data.

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Table C.2.16. **EASZY Assay: Detection of substances acting through estrogen receptors using transgenic cyp19a1b GFP zebrafish embryos (draft OECD TG): 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. Note that there are no apical endpoints in this assay considered to be diagnostic of an estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modality.

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 little evidence at present that equivalent *in vitro* assays with systems derived from fish offer advantages over their mammalian counterparts, although some differences in response have been observed.

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.

Scenarios	Result of EASZY Assay	Existing results		Possible conclusions	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	+	+	+	Strong evidence for *in vivo* endocrine activity in fish and other organisms.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental no-observed-effect-concentration/x% effect concentration (NOEC/ECx).	An alternative approach would be to deploy OECD TG 234 (Fish Sexual Development test [FSDT]), especially if sexual development is expected to give a response at lower concentrations than reproduction.
B	+	+	–	Moderate evidence for *in vivo* endocrine activity in fish, despite lack of *in vivo* effects in existing tests.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	An alternative approach would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.
C	+	+	Eq/0**	Moderate evidence for *in vivo* endocrine activity in fish despite equivocal or absent *in vivo* data in other species.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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 sufficient transformation to endocrine-active products 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.
D	+	–	+	Strong evidence for *in vivo* endocrine activity in fish and other species, but confidence about MOA is reduced by negative mechanistic data.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo* (such activation is possible in the EASZY Assay), or it may operate via mechanisms not covered by the *in vitro* screens. An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT).
E	+	–	–	Some evidence for *in vivo* endocrine activity in fish, but confidence is reduced by negative *in vitro* data and negative *in vivo* activity in other species.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo* (such activation is possible in the EASZY Assay), or it may operate via mechanisms not covered by the *in vitro* screens. An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.

Scenarios	Result of EASZY Assay	Existing results		Possible conclusions	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	Some evidence for *in vivo* endocrine activity in fish, but confidence is reduced by negative *in vitro* data and equivocal or absent *in vivo* activity in other species.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo* (such activation is possible in the EASZY Assay), or it may operate via mechanisms not covered by the *in vitro* screens, or may not be metabolically activated *in vitro*. If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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	+	Strong evidence for *in vivo* endocrine activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240).	An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT). 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.
H	+	Eq/0	–	Some evidence for *in vivo* endocrine activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240).	An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before possibly conducting a life cycle test. 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	Moderate evidence for *in vivo* endocrine activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240).	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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.

Scenarios	Result of EASZY Assay	Existing results		Possible conclusions	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	–	+	+	Based on the existing data, the chemical has endocrine activity *in vivo*. The lack of response in the EASZY Assay suggests that fish are not responsive, unless the existing data are from fish.	Consider performing OECD TG 234 (FSDT).	It is possible that the failure to give a positive result in the EASZY Assay was caused by the short exposure time (96 hours). If this is suspected (e.g. if the chorion has impeded uptake and/or the chemical only bioaccumulates slowly), or if the existing *in vivo* data are from a fish, OECD TG 234 (FSDT) or potentially a life cycle test (e.g. TG 240 – MEOGRT) would be able to study the effects of longer exposure and confirm whether there is a hazard to fish. Choice of test should be guided by the existing *in vivo* data.
K	–	+	–	There is no evidence that the chemical is a potential endocrine disruptor (ED) *in vivo*, probably because it is very weakly acting or rapidly metabolised.	Probably no further action, but see comments in right-hand column.	It is known that uptake of some chemicals can be impeded by the chorion, and it is possible that EDs which bioaccumulate slowly may only cause effects *in vivo* after exposure times longer than 96 hours. If this is suspected, and depending on which part of the life cycle is suspected of being the most sensitive, consider performing OECD TG 229/230 (FSTRA or 21-Day Fish Assay) or TG 234 (FSDT). It is also possible that the chemical may be an anti-androgen *in vivo* (consider performing the Androgenised Female Stickleback Screen [AFSS] or Juvenile Medaka Anti-Androgen Screening Assay [JMASA]), or a thyroid-active chemical *in vivo* (consider performing the Amphibian Metamorphosis Assay [AMA] – OECD TG 231).
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 (a negative).	Probably no further action, but see comments in right-hand column.	If the equivocal existing data are from a fish assay, consider performing a fish assay (e.g. OECD TG 229 or TG 230) with a different species, or a longer term test (e.g. TG 234 [FSDT] or life cycle test [MEOGRT TG 240]) if the chemical is a slow bioaccumulator. It is also possible that the chemical may be an anti-androgen *in vivo* (consider performing an AFSS), or a thyroid-active chemical *in vivo* (consider performing an AMA– OECD TG 231). 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.
M	–	–	+	The chemical is apparently not a potential ED in fish but it does have activity in another species.	Use the existing *in vivo* data to help decide whether a longer term test with an appropriate fish species is needed.	It is also possible that the chemical may be an anti-androgen *in vivo* (consider performing an AFSS), or a thyroid-active chemical *in vivo* (consider performing an AMA – OECD TG 231), although lack of *in vitro* binding affinity with the androgen receptor suggests the former is unlikely. Use the existing *in vivo* data to guide any further testing.
N	–	–	–	The chemical is probably not a potential ED *in vivo*.	No further action with respect to estrogenic, anti-estrogenic, androgenic or steroidogenic MOA.	It is still possible that the chemical may be an anti-androgen *in vivo* (consider performing an AFSS), or a thyroid-active chemical *in vivo* (consider performing an AMA – OECD TG 231), although lack of *in vitro* binding affinity with the androgen receptor suggests the former is unlikely.

Scenarios	Result of EASZY Assay	Existing results		Possible conclusions	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)**			
O	–	–	Eq/0	The chemical is probably not a potential ED in fish.	Probably no further action. However, see comments in right-hand column.	If the paucity of *in vivo* data is a concern, performance of a screening test (OECD TG 229 or TG 230) with a different species, or a longer term test (i.e. FSDT – TG 234 or life cycle test [MEOGRT]) could be considered. It is also possible that the chemical may be an anti-androgen *in vivo* (consider performing an AFSS), or a thyroid-active chemical *in vivo* (consider performing an AMA – OECD TG 231), although lack of *in vitro* binding affinity with the androgen receptor suggests the former is unlikely. 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.
P	–	Eq/0	+	The chemical is probably not a potential ED in fish, but confidence in this conclusion is low given the lack of mechanistic *in vitro* data and the availability of positive existing *in vivo* data.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential endocrine action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (TG 234 – FSDT or life cycle test [MEOGRT]). Use the existing *in vivo* data as a guide to test choice. If the mechanistic data reveal anti-androgenic or thyroid activity, consider performing an AFSS (OECD GD 148)/JMASA, or AMA (OECD TG 231), respectively. 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 a potential ED in fish, but the lack of mechanistic *in vitro* data are a concern, even though the existing *in vivo* data are negative.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential endocrine action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (TG 234 – FSDT or life cycle test [MEOGRT]). Use the existing *in vivo* data as a guide to test choice. If the mechanistic data reveal anti-androgenic or thyroid activity, consider performing an AFSS (OECD GD 148)/JMASA, or AMA (OECD TG 231), respectively. 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.

Scenarios	Result of EASZY Assay	Existing results		Possible conclusions	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)**			
R	-	Eq/0	Eq/0	The chemical is probably not a potential ED in fish, but confidence in this conclusion is low given the lack of mechanistic *in vitro* and existing *in vivo* data.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential endocrine action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (TG 234 [FSDT] or life cycle test [MEOGRT]). Use the existing *in vivo* data as a guide to test choice. If the mechanistic data reveal anti-androgenic or thyroid activity, consider performing an AFSS (OECD GD 148)/JMASA, or AMA (OECD TG 231), respectively. 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.

C.2.17. JMASA: Juvenile Medaka Anti-Androgen Screening Assay (draft OECD GD)

Status: Assay being validated by the OECD.

528. Modality detected/endpoints: This draft *in vivo* medaka assay is sensitive to androgen antagonists and to chemicals interfering with androgen biosynthesis. In principle, it can also be used to identify estrogen agonists and antagonists, as well as aromatase inhibitors. However, this guidance will restrict itself to the detection of anti-androgenicity alone, as data on responses to the other modalities have not yet been published.

Background to the assay

529. This assay is currently starting validation by the OECD for possible approval as a guidance document (GD). No validation data have yet been produced, but some developmental data are available. It is planned to have a GD ready by 2019 at the earliest. The assay is based on juvenile medaka (*Oryzias latipes*), the males of which develop secondary sexual characteristics known as papillary processes (PP) on the anal fin between 42 and 49 days post-fertilisation (dpf). The PP grow under androgenic control, and anti-androgens or chemicals which interfere with androgen biosynthesis can prevent their appearance or limit their number. Juvenile medaka (both sexes at 42 dpf) are exposed to test chemical for 28 days, to 70 dpf, after which their genotypic sex is determined using the *dmy* gene. This enables the males alone to be evaluated for the presence, reduction or absence of PP. It is optionally possible to measure vitellogenin, so the assay can in principle also be used to detect estrogen agonists and antagonists, and aromatase inhibitors. However, this option will only be considered further in future editions of this document when supporting data have been evaluated.

When/why the assay may be used

530. Although data from the JMASA could, in principle, be available at any stage in the hazard assessment process, the most likely scenario will be when there are relatively few data available about the possible endocrine disrupting properties of a chemical. The assay is most likely to be used either as part of a battery of *in vitro* and *in vivo* screens, or to follow up on existing data which suggest possible endocrine disruption activity. Given the high degree of endocrine system conservation across the vertebrates, adverse endocrine-linked effects in the JMASA assay may also indicate the possibility of related activity in other organisms such as amphibians, reptiles, birds or mammals. It is also possible that no existing endocrine-relevant data are available (i.e. the JMASA has been used as a primary screen), but in that case a positive result in the screen should ideally be followed up with relevant *in vitro* screening in an attempt to confirm the precise mode of action (MOA). Furthermore, a positive JMASA result would also need to be followed up with an additional *in vivo* fish test such as the Fish Short-Term Reproduction Assay (FSTRA – OECD TG 229) or Fish Sexual Development Test (FSDT – OECD TG 234), which will give some

indication of any adverse apical effects. Possible conclusions to be derived from the results of the JMASA, and guidance about potential additional studies to strengthen weight of evidence, are summarised in Table C.2.17.

531. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an endocrine disruptor (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 GD 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

532. Existing information on endocrine-related effects from other vertebrates (up to and including mammals, e.g. positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies) should always be considered, given the commonality of endocrine mechanisms in these taxa. Existing data available before deployment of the JMASA might include *in vivo* results obtained with other vertebrates (e.g. a Hershberger Bioassay in Rats – OECD TG 441), or one or more of a range of *in silico* or *in vitro* results which suggest that anti-androgenicity 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 data, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen for androgen receptor-mediated activity, or for effects on androgen biosynthesis.

533. It should be noted that a sensitive *in vivo* assay for anti-androgenicity is already available: the Androgenised Female Stickleback Screen (AFSS – OECD GD 148). This is slightly shorter than the JMASA (21 days), but relies on the pre-treatment of female sticklebacks (*Gasterosteus aculeatus*) with an androgen before measuring anti-androgenic effects of the test chemical (reduction in induced spiggin glue protein).

Scenarios: Positive and negative results combined with existing data

534. The scenarios (A to R) presented in Table C.2.17 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.

535. Positive results obtained with the secondary sexual characteristics endpoint (Table C.2.17, Scenarios A-I) result in the conclusion that the test chemical is a possible anti-androgen *in vivo*. This would ideally need to be followed up with more comprehensive testing to show whether adverse apical effects related to endocrine impacts occur at any part of the life cycle (and hence to discover whether the chemical is an ED acting through certain estrogen/androgen/thyroid/steroidogenesis [E,A,T,S] pathways). In other words, a positive result in the JMASA may trigger OECD TG 234 (FSDT) at Level 4 or fish life cycle testing (e.g. MEOGRT – TG 240) at Level 5. Existing data suggesting anti-androgenic activity will strengthen the case for additional testing.

536. The situation in which the JMASA gives a negative result (Table C.2.17, Scenarios J-R) needs careful consideration of any existing data. If the weight of evidence of these data suggests that the chemical is endocrine active both *in vitro* and *in vivo* in other species (Scenario J), then the probability is that the JMASA may simply be insufficiently responsive in that case, or fish in general may be unresponsive. In some of these circumstances, it might be appropriate to conduct an FSDT (OECD TG 234), or alternatively, a fish life cycle test (e.g. MEOGRT, TG 240) to confirm that there is no endocrine activity in fish.

537. If the JMASA 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 may or may not be necessary. If the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests are not of sufficient duration, in which case longer term testing might be justified.

538. On the other hand, if the JMASA and the *in vitro* tests are negative, but there are positive existing *in vivo* data (Scenario M), the chemical is probably not a potential ED with anti-androgenic activity, but it may act via estrogen- or androgen-related 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 relevant existing *in vitro* and *in vivo* data should be used to guide decisions about whether to conduct any further testing, including life stages represented in OECD TG 234 (FSDT) or in TG 240 (MEOGRT).

539. Finally, a negative JMASA, set against a background of negative *in vitro* and *in vivo* data (Scenario N) that includes relevant *in vivo* data for fish, suggests that the test chemical is not a potential ED in fish or other vertebrates, and no further testing for anti-androgenic or anti-steroidogenic MOA will generally be necessary.

540. 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 JMASA, and this is reflected in Table C.2.17. However, a lack of mechanistic data on endocrine activity should usually be rectified before any further *in vivo* testing is finally decided on. Indeed, as a general principle, it is desirable to obtain mechanistic data before any *in vivo* testing. On the other hand, if the JMASA is positive, further *in vivo* testing is generally indicated, particularly when existing data are equivocal, or if there are no existing data. 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. androgenic and anti-androgenic) could, depending on dose, lead to a minimisation or

abolition of adverse effects, while in others two different MOA (e.g. anti-steroidogenic and androgenic) could potentially reinforce effects on the JMASA. 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.

541. The scenario in which the results of the JMASA are themselves equivocal has not been dealt with in Table C.2.17, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. If false negatives (e.g. systemic toxicity) are suspected with good reason, the screen could be repeated if none of the test concentrations have given reliable data (e.g. conduct it at lower concentrations which avoid systemic toxicity). However, note that a repeat test in the event of systemic toxicity would not be needed providing at least one tested concentration was not subject to such effects.

542. In summary, positive results in the JMASA indicate that a chemical is a possible endocrine disrupter. More predictive *in vivo* testing would then be necessary to produce a long-term no-observed-effect-concentration/x% effect concentration (NOEC/ECx) and/or to confirm whether or not the chemical is an actual endocrine disrupter with adverse effects *in vivo*. Negative results in the JMASA do not necessarily mean that the chemical is not a potential ED – a judgement about its endocrine disruption potential and the possible need for additional testing will have to be made based on a weight of evidence evaluation of existing *in vitro* and *in vivo* data.

Reference

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.2.17. **JMASA: Juvenile Medaka Anti-Androgen Screening Assay (draft OECD GD): 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. Note that there are no apical endpoints in this assay considered to be diagnostic of an estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modality.

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.

Scenarios	Result of JMASA	Existing results		Possible conclusions	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	+	+	+	Strong evidence for *in vivo* anti-androgenic activity in fish and other organisms.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental no-observed-effect-concentration/x% effect concentration (NOEC/ECx).	An alternative approach would be to deploy OECD TG 234 (Fish Sexual Development Test [FSDT]), especially if sexual development is expected to give a response at lower concentrations than reproduction.
B	+	+	–	Strong evidence for *in vivo* anti-androgenic activity in fish, despite lack of *in vivo* effects in existing tests.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	An alternative approach would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.
C	+	+	Eq/0**	Strong evidence for *in vivo* anti-androgenic activity in fish despite equivocal or absent *in vivo* data in other species.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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.
D	+	–	+	Strong evidence for *in vivo* anti-androgenic activity in fish and other species, but confidence about MOA is reduced by negative mechanistic data.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo*, or it may operate via mechanisms not covered by the *in vitro* screens. An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction.
E	+	–	–	Moderate-strong evidence for *in vivo* anti-androgenic activity in fish, but confidence is reduced by negative *in vitro* data and negative *in vivo* activity in other species.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo*, or it may operate via mechanisms not covered by the *in vitro* screens. An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.

Scenarios	Result of JMASA	Existing results		Possible conclusions	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	Moderate-strong evidence for *in vivo* anti-androgenic activity in fish, but confidence is reduced by negative *in vitro* data and equivocal or absent *in vivo* activity in other species.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo*, or it may operate via mechanisms not covered by the *in vitro* screens. If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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	+	Strong evidence for *in vivo* anti-androgenic activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240).	An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. 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.
H	+	Eq/0	–	Strong-moderate evidence for *in vivo* anti-androgenic activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240).	An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before possibly conducting a life cycle test. 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	Moderate evidence for *in vivo* endocrine activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240).	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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.

Scenarios	Result of JMASA	Existing results		Possible conclusions	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	–	+	+	Based on the existing data, the chemical has anti-androgenic activity *in vivo*. The lack of response in the JMASA suggests that fish are not responsive, unless the existing data are from fish.	Consider performing OECD TG 234 (FSDT).	It is possible that the failure to give a positive result in the JMASA was caused by the relatively short exposure time (28 days). If this is suspected (e.g. the chemical only bioaccumulates slowly), or if the existing *in vivo* data are from a fish, OECD TG 234 (FSDT) or potentially a life cycle test (e.g. TG 240 – MEOGRT) would be able to study the effects of longer exposure and confirm whether there is a hazard to fish. Choice of test should be guided by the existing *in vivo* data.
K	–	+	–	There is no evidence that the chemical is a possible anti-androgenic endocrine disruptor (ED) *in vivo*, probably because it is very weakly acting or rapidly metabolised.	Probably no further action, but see comments in right-hand column.	It is possible that EDs which bioaccumulate slowly may only cause effects *in vivo* after exposure times longer than 28 days. If this is suspected, and depending on which part of the life cycle is suspected of being the most sensitive, consider performing OECD TG 234 (FSDT).
L	–	+	Eq/0	The chemical may not be an anti-androgenic ED *in vivo*, but the confidence in this conclusion is relatively low as there is only one unequivocal *in vivo* test result (a negative).	Probably no further action, but see comments in right-hand column.	If the equivocal existing data are from a fish assay, consider performing a fish assay (e.g. OECD TG 229 or TG 230) with a different species, or a longer term test (e.g. TG 234 – FSDT or life cycle test [MEOGRT]) if the chemical is a slow bioaccumulator. 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.
M	–	–	+	The chemical is apparently not a possible anti-androgenic ED in fish but it does have activity in another species.	Use the existing *in vivo* data to help decide whether a longer term test with an appropriate fish species is indicated.	
N	–	–	–	The chemical is probably not a possible anti-androgenic ED *in vivo*.	No further action with respect to anti-androgenic or anti-steroidogenic MOA.	
O	–	–	Eq/0	The chemical is probably not a possible anti-androgenic ED in fish.	Probably no further action. However, see comments in right-hand column.	If the paucity of *in vivo* data is a concern, performance of a screening test (OECD TG 229 or TG 230) with a different species, or a longer term test (i.e. TG 234 – FSDT or life cycle test [MEOGRT]) could be considered. 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.
P	–	Eq/0	+	The chemical is probably not a possible anti-androgenic ED in fish, but confidence in this conclusion is low given the lack of mechanistic *in vitro* data and the availability of positive existing *in vivo* data.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential anti-androgenic action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (TG 234 – FSDT or life cycle test [MEOGRT]). Use the existing *in vivo* data as a guide to test choice. 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.

Scenarios	Result of JMASA	Existing results		Possible conclusions	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)**			
Q	–	Eq/0	–	The chemical is probably not a possible anti-androgenic ED in fish, but the lack of mechanistic *in vitro* data are a concern, even though the existing *in vivo* data are negative.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential endocrine action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (TG 234 – FSDT or life cycle test [MEOGRT]). Use the existing *in vivo* data as a guide to test choice. 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 is probably not a possible anti-androgenic ED in fish, but confidence in this conclusion is low given the lack of mechanistic *in vitro* and existing *in vivo* data.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential anti-androgenic action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (TG 234 – FSDT or life cycle test [MEOGRT]). Use the existing *in vivo* data as a guide to test choice. 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.

C.2.18. *Xenopus* Embryonic Thyroid Signalling Assay (XETA) (draft OECD TG)

Status: Assay being validated by the OECD.

543. Modality detected/endpoints: This *in vivo* assay with transgenic THbZIP *Xenopus laevis* tadpoles is responsive to pro-thyroid chemicals and anti-thyroid chemicals, but not to estrogens or (probably) other steroid hormone (ant)agonists.

Background to the assay

544. This *in vivo* assay is currently being validated by the OECD, and may be approved as an OECD test guideline (TG) in due course. It is designed as a screen for thyroid activity in amphibians, not to provide information on endocrine activity for use in assessing the environmental risks of an individual chemical based on a predicted environmental concentration/predicted no-effect concentration (PEC/PNEC) approach. It is important to note that there are several types of thyroid disruption, not all of which involve direct interactions with the thyroid receptor. Although it is to be expected that the assay will be responsive to all chemicals that interact with thyroid hormone (TH) receptors, or that lead to either an increase or a decrease in TH levels (thyroxine – T4; or the active form triiodothyronine – T3), it cannot be used to provide an unequivocal identification of the precise mode of action (MOA) of a chemical.

545. The XETA uses transgenic *Xenopus laevis* tadpoles into which a THbZIP promoter has been inserted that is regulated by TH and other TH agonists (Morvan-Dubois, Demeneix and Sachs, 2008). The promoter is linked to the gene coding for green fluorescent protein, and thus the degree of receptor response can be measured by fluorescence in the transparent tadpoles. Transgenic tadpoles are produced by mating wild-type females with THbZIP males, and these tadpoles are then exposed to dilutions of the test chemical from six-day post-fertilisation (N&F stage 45) for three days (to stage 47), after which their degree of fluorescence is recorded. The XETA has to be run both with and without a T3 internal spike because *Xenopus* tadpoles produce a very low level of TH at these developmental stages. Without a T3 internal spike, the XETA will respond only to pro-thyroid chemicals with an increase in fluorescence. If run with a T3 spike, it will respond to anti-thyroid chemicals and to pro-thyroid chemicals. The latter will be detected with a higher sensitivity in unspiked mode. Anti-thyroid chemicals will produce either an increase or a decrease in fluorescence, depending on the MOA. The tadpoles are metabolically competent, so the XETA is expected to be responsive to active metabolites.

When/why the assay may be used

546. Although the XETA could, in principle, be used at any stage in the hazard assessment process, the most likely use scenario will be when there are relatively few data available about the possible thyroid disrupting properties of a chemical. The results from this assay are most likely to be available after deployment of a battery of *in vitro* and *in vivo* screens,

or as a supplement to existing data which suggest potential endocrine disruptor (ED) activity. Given the high degree of endocrine system conservation across the vertebrates, endocrine-linked effects in the XETA may also indicate the possibility of related activity in other organisms such as fish, reptiles, birds or mammals. A number of mammalian (rat) assays are sensitive to thyroid disruption, particularly thyroid antagonists, including the pubertal assay (male or female), the enhanced repeat dose assay (OECD TG 407), and the intact male screening assay. Note that these assays utilise different routes of exposure than the XETA and therefore, depending on the properties of the chemical, have differing potentials for the test substance to be metabolised. It should also be noted that, at present, the only validated screening assay for thyroid-active chemicals is the Amphibian Metamorphosis Assay (AMA – OECD TG 231). The AMA uses more tadpoles than the XETA and is more time-consuming (21 days compared with 3), so the XETA may be more appropriate for rapid screening of large numbers of chemicals. On the other hand, the AMA measures several apical endpoints including speed of development and metamorphosis, whereas conclusions about adverse responses cannot be drawn from the XETA.

547. It is possible that no endocrine-relevant data are available before the XETA is deployed (i.e. if the XETA has been used as a primary screen), but in that case a positive result in the screen should probably be followed up with relevant *in vitro* screening, if available, to investigate the suspected MOA in more detail. It should be noted that *in vitro* screens exist for thyroid agonists and antagonists (e.g. GH_3 rat pituitary somatotroph cell proliferation; solid state thyroid receptor binding assays; transfected reporter gene assays in yeast or mammalian cell lines), but also for thyroid disruption occurring at other points in the endocrine system (e.g. porcine thyroperoxidase assay, TBG/TTR binding assays, FRTL-5 rat cell lines sensitive to iodide uptake inhibitors (see Table A.1). However, most of these screens are still at the research stage and none have yet been validated and standardised at the international level.

548. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an 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

549. Existing information on endocrine-related effects from other vertebrates (up to and including mammals, e.g. positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies) should also be considered before deployment of the

XETA, given the commonality of endocrine mechanisms in these taxa. Existing data available might also include one or more of a range of *in silico* or *in vitro* results which suggest that thyroid disruption may occur *in vivo* (but note the limitations of this approach, as indicated above). Such indicators of possible thyroid activity might include quantitative structure activity relationship (QSAR) predictions of thyroid activity, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen for thyroid agonist/antagonist activity.

Scenarios: Positive and negative results combined with existing data

550. The scenarios (A to R) presented in Table C.2.18 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.

551. Positive results obtained with the XETA (Table C.2.18, Scenarios A-I) result in the conclusion that the test chemical is a possible thyroid disrupter *in vivo*, at least in amphibians. However, as indicated above, although a positive response of the XETA indicates that the chemical is a possible thyroid disrupter, a result of this type would generally need to be followed up with a more comprehensive screen. The most appropriate choice for this is the Amphibian Metamorphosis Assay (AMA – OECD TG 231). However, if countries need further evidence concerning growth and sexual development, a Larval Amphibian Growth and Development Assay (LAGDA – OECD TG 241) would be able to provide a precise no-observed-effect-concentration/x% effect concentration (NOEC/ECx) for adverse effects. In other words, in order to strengthen weight of evidence, a positive result in the XETA could be followed by an AMA at Level 3, which if positive in turn might lead to conduct of a LAGDA (Level 4). Existing data suggesting endocrine-specific activity (e.g. positive *in vitro* data, or positive *in vivo* data from other species) will strengthen the case for additional testing.

552. The situation in which the XETA gives a negative result (Table C.2.18, Scenarios J-R) needs careful consideration of any existing data. If these data suggest that the chemical is endocrine active both *in vitro* and *in vivo* (Scenario J), then it is possible that the XETA is simply insufficiently sensitive.

553. If the XETA 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 thyroid effects *in vivo* in amphibians or other organisms, or it may be rapidly metabolised. In such a situation, further testing is probably not necessary. However, if the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests have been insufficiently prolonged, in which case longer term testing with the AMA or LAGDA might be justified.

554. On the other hand, if the XETA and the *in vitro* tests are negative (Scenario M), but there are positive existing *in vivo* data, the nature of those existing data should be considered. Unless the existing data are from another amphibian, the chemical is possibly not an ED acting in amphibians, 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.

555. Finally, a negative XETA, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is probably not a thyroid-active ED, and further action is unnecessary.

556. In each of the above scenarios, it is possible that existing data will be equivocal, or there may be no existing data. This will weaken the conclusions which can be drawn about a negative XETA, and this is reflected in Table C.2.18. However, a lack of mechanistic data on thyroid activity should ideally be rectified before any further *in vivo* testing is finally conducted, although as indicated above, many thyroid modalities are not detectable in *in vitro* screens. On the other hand, if the XETA is positive, further *in vivo* testing would generally be needed to quantify any adverse effects and/or to establish a NOEC or ECx for such effects, even if all existing data are equivocal, or if there are no existing data. Again, however, it may be useful 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. thyroidogenic and anti-thyroidogenic) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA could potentially reinforce effects the XETA endpoint. 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.

557. The scenario in which the results of the XETA are themselves equivocal has not been dealt with in Table C.2.18, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. If possible reasons for false negatives are suspected, the XETA could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity). However, note that a repeat screen in the event of systemic toxicity would not be needed providing at least one tested concentration was not subject to such effects.

558. In summary, positive results in the XETA may indicate that a chemical is a possible endocrine disrupter via one or more of several types of thyroid activity. This suggests that more comprehensive *in vivo* testing would be needed if the intention is to derive a long-term NOEC/ECx and/or to confirm whether or not the chemical is an actual endocrine disrupter due to the occurrence of adverse effects. Negative results in XETA do not necessarily mean that the chemical is not a potential ED – a judgement about the endocrine disruption potential and the possible need for additional testing will have to be made based on a weight of evidence evaluation of existing *in vitro* and *in vivo* data.

References

Morvan-Dubois, G., B.A. Demeneix and L.M. Sachs (2008), "*Xenopus laevis* as a model for studying thyroid hormone signalling: From development to metamorphosis", *Molecular and Cellular Endocrinology*, Vol. 293/1-2, pp. 71-79, https://doi.org/10.1016/j.mce.2008.06.012.

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.2.18. ***Xenopus*** **Embryonic Thyroid Signalling Assay (XETA) (draft OECD TG): 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.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from thyroid hormone receptor (TR) and other assays concerning mechanisms of thyroid disruption although these are not yet 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 TR binding/activation may be made for some substances.

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 thyroid disrupter.

Scenarios	Result of XETA	Existing results		Possible conclusions	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	+	+	+	Strong evidence for *in vivo* thyroid activity in amphibians, plus thyroid effects in other species.	Consider performing an Amphibian Metamorphosis Assay (AMA – OECD TG 231).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist. Note that although the AMA will provide some data on apical effects, more definitive information on adverse outcomes would be provided by the Larval Amphibian Growth and Development Assay (LAGDA – OECD TG 241).
B	+	+	–	Strong evidence for *in vivo* thyroid activity in amphibians.	Consider performing an AMA (OECD TG 231).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist. Note that although the AMA will provide some data on apical effects, more definitive information on adverse outcomes would be provided by the LAGDA (OECD TG 241).
C	+	+	Eq/0	Strong evidence for *in vivo* thyroid activity in amphibians.	Consider performing an AMA (OECD TG 231).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist. Note that although the AMA will provide some data on apical effects, more definitive information on adverse outcomes would be provided by the LAGDA (OECD TG 241). 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.
D	+	–	+	Strong evidence for *in vivo* thyroid activity in amphibians, plus thyroid effects in other species.	Consider performing an AMA (OECD TG 231).	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens. Note that although the AMA will provide some data on apical effects, more definitive information on adverse outcomes would be provided by the LAGDA (OECD TG 241).
E	+	–	–	Strong evidence for *in vivo* thyroid activity in amphibians.	Consider performing an AMA (OECD TG 231).	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens. Note that although the AMA will provide some data on apical effects, more definitive information on adverse outcomes would be provided by the LAGDA (OECD TG 241).
F	+	–	Eq/0	Strong evidence for *in vivo* thyroid activity in amphibians.	Consider performing an AMA (OECD TG 231). Given the absence or equivocal nature of existing *in vivo* data, it might also be sensible to conduct a thyroid-responsive mammalian assay (e.g. rat pubertal).	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens. Note that although the AMA will provide some data on apical effects, more definitive information on adverse outcomes would be provided by the LAGDA (OECD TG 241). 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.

Scenarios	Result of JMASA	Existing results		Possible conclusions	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)**			
G	+	Eq/0	+	Strong evidence for *in vivo* thyroid activity in amphibians, plus thyroid effects in other species.	Consider performing an AMA (OECD TG 231). Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for thyroid (ant)agonistic activity.	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no thyroid activity. Note that although the AMA will provide some data on apical effects, more definitive information on adverse outcomes would be provided by the LAGDA (OECD TG 241). 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.
H	+	Eq/0	–	Strong evidence for *in vivo* thyroid activity in amphibians.	Consider performing an AMA (OECD TG 231). Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for thyroid (ant)agonistic activity.	Note that although the AMA will provide some data on apical effects, more definitive information on adverse outcomes would be provided by the LAGDA (OECD TG 241). 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	Strong evidence for *in vivo* thyroid activity in amphibians.	Consider performing an AMA (OECD TG 231). Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for thyroid (ant)agonistic activity. Given the absence or equivocal nature of existing *in vivo* data, it might also be sensible to conduct a thyroid-responsive mammalian assay (e.g. rat pubertal).	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no thyroid activity. Note that although the AMA will provide some data on apical effects, more definitive information on adverse outcomes would be provided by the LAGDA (OECD TG 241). 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.
J	–	+	+	The test chemical is probably a thyroid (ant)agonist without activity in amphibians, although it is possible that *Xenopus laevis* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.
K	–	+	–	The test chemical is probably a thyroid (ant)agonist without activity in amphibians or other taxa, although it is possible that *Xenopus laevis* responds atypically in this case.	If there is no activity in amphibians or mammals, further evidence is probably not needed.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.

Scenarios	Result of JMASA	Existing results		Possible conclusions	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)**			
L	–	+	Eq/0	The test chemical is probably a thyroid (ant)agonist without activity in amphibians, although it is possible that *Xenopus laevis* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required, but if mammalian data are absent, it might be desirable to conduct a thyroid-responsive rodent screen (e.g. rat pubertal).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist. 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.
M	–	–	+	The test chemical is probably without thyroid activity in amphibians, although it is possible that *Xenopus laevis* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required.	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens.
N	–	–	–	The test chemical is probably without thyroid activity in amphibians or other taxa.	No further action is necessary.	–
O	–	–	Eq/0	The test chemical is probably without thyroid activity in amphibians.	Some regulatory authorities may conclude that no further evidence is required, but if mammalian data are absent, it might be desirable to conduct a thyroid-responsive rodent screen (e.g. rat pubertal).	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens. 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.
P	–	Eq/0	+	The test chemical is probably without thyroid activity in amphibians, although it is possible that *Xenopus laevis* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. Also, if clear *in vitro* mechanistic data are missing, it might be desirable to obtain some.	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no thyroid activity. 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 test chemical is probably without thyroid activity in amphibians or other taxa.	No further action is necessary.	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 test chemical is probably without thyroid activity in amphibians.	Some regulatory authorities may conclude that no further evidence is required, but if mammalian data are absent, it might be desirable to conduct a thyroid-responsive rodent screen (e.g. rat pubertal).	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.

C.2.19. Harpacticoid Copepod Development and Reproduction Test with *Amphiascus* (OECD GD 201)

Status: Partially validated by the OECD.

559. Modality detected/endpoints: This long-term *in vivo* assay with the marine copepod crustacean *Amphiascus tenuiramis* could not be fully validated as an OECD test guideline (TG) due to the complexity of the methodology which requires considerable operator training. Although the assay has not been conducted with hormonally based insect growth regulators, it is expected (by extension from experience with other copepods) to be responsive to juvenile hormone (JH) (ant)agonists and ecdysteroid (Ec) (ant)agonists which can interfere with such processes as metamorphosis, moulting, growth and reproduction. *A. tenuiramis* is known to contain the ecdysone receptor (Gaertner et al., 2012) and the moulting hormone 20-hydroxyecdysone (Block, Bejarano and Chandler, 2003). The assay exposes the test organisms over at least one generation. It is important to note, however, that none of the endpoints in this apical test are specifically responsive to JH- or Ec-active chemicals, and the assay will give positive results with many other substances. The lack of internationally validated mechanistic assays for endocrine activity in crustaceans may prevent firm conclusions about whether test chemicals are endocrine disruptors (EDs) in this taxon, although *in vitro* assays for JH and Ec activity are available in the literature. However, the data from the test may nevertheless be of value for classification and hazard identification/characterisation.

Background to the assay

560. This is a one-generation growth and reproduction assay using the marine benthic copepod crustacean *Amphiascus tenuiramis*. It is not specifically sensitive to JH or Ec (ant)agonists, but is expected to be apically responsive to some of them. However, many non-endocrine toxicants will also produce a response. The assay is technically demanding and operators require significant training before it can be conducted repeatably. Newly hatched larvae (F0) <24-hours-old are exposed individually until adulthood, at which time they are paired and allowed to breed. The test can be terminated after two clutches of offspring (F1) have been produced, and the whole test from F0 to F1 larvae takes 36 days. The test can optionally be extended to study survival and reproduction of the F1 animals. Endpoints include survival of the F0 generation, developmental rate, time to production of the first and second clutches, reproductive success, clutch size, fertility, and number of viable hatched F1 offspring (nauplii).

When/why the assay may be used

561. Although OECD GD 201 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* and *in vivo* data available about the possible JH or Ec activity and/or effects of a chemical. Given the significant degree of endocrine system conservation across the arthropods, effects in OECD GD 201 may also indicate the possibility of related activity in other crustaceans (e.g. cladocera and decapods) and insects.

562. It is not recommended that OECD GD 201 be deployed as a primary test for JH or Ec activity and effects, but it should be noted that there are no standardised *in vitro* screens for JH or Ec (ant)agonists, although some are described in the scientific literature (e.g. Cherbas, Koehler and Cherbas [1989]; Dinan et al. [2001]; Smagghe et al. [2003]; Swevers et al. [2003]).

563. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an 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 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

564. Existing information on endocrine-related effects from other arthropods should also be considered before deployment of OECD GD 201, given the commonality of endocrine mechanisms in these taxa. Existing data available might also include one or more of a range of *in silico* or *in vitro* results which suggest that JH or Ec disruption may occur *in vivo* (but note the limitations of this approach, as indicated above). Such indicators of possible JH or Ec activity might include quantitative structure activity relationship (QSAR) predictions of JH/Ec activity, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen for JH/Ec (ant)agonist activity. In addition, *in vivo* data should ideally be available from one or more of several assays, possibly including the Short-Term Juvenile Hormone Activity Screening Assay (SJHASA), the Sediment-Water Chironomid Toxicity Test Using Spiked Sediment or Water (OECD TG 218/219), the Sediment Water Chironomid Life Cycle Toxicity Test (OECD TG 233), the *Daphnia magna* Reproduction Test with male neonate option (OECD TG 211) or the *Daphnia* Multigeneration Test (DMGT).

Scenarios: Positive and negative results combined with existing data

565. The scenarios (A to R) presented in Table C.2.19 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.

566. Positive results obtained with OECD GD 201 (Table C.2.19, Scenarios A-I) result in the conclusion that the test chemical has adverse apical effects, at least in crustaceans, but these are not necessarily caused by JH or Ec activity. If countries need further evidence concerning growth and sexual development, etc. in this phylum, a Chironomid Life Cycle Toxicity Test (OECD TG 233) would be able to provide information on adverse effects in insects. In other words, in order to strengthen weight of evidence, a positive result in OECD GD 201 could be followed by the OECD TG 233 (Level 5). Existing data suggesting endocrine-specific activity (e.g. positive *in vitro* data, or positive *in vivo* data from other species) will strengthen the case for additional testing still further.

567. The situation in which OECD GD 201 gives a negative result (Table C.2.19, Scenarios J-R) needs careful consideration of any existing data. If these data suggest that the chemical is endocrine active both *in vitro* and *in vivo* (Scenario J), then it is possible that OECD GD 201 is simply insufficiently sensitive.

568. If OECD GD 201 and existing *in vivo* data are all negative, but *in vitro* data reveal some JH or Ec activity (Scenario K), the probability is that the test chemical is not sufficiently potent to produce JH/Ec (ant)agonism *in vivo* in arthropods, or it may be rapidly metabolised. In such a situation, further testing is probably not necessary.

569. On the other hand, if OECD GD 201 and the *in vitro* tests are negative (Scenario M), but there are positive existing *in vivo* data, the nature of those existing data should be considered. Unless the existing data are from another crustaceans, the chemical is possibly not a JH or Ec (ant)agonist acting in crustaceans, but it may be more potent in species (e.g. insects) 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 (e.g. with OECD TG 233).

570. Finally, a negative OECD GD 201, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is probably not a JH or Ec (ant)agonist *in vitro* or *in vivo*, and further action is unnecessary.

571. In each of the above scenarios, it is possible that existing data will be equivocal, or there may be no existing data. This will weaken the conclusions which can be drawn about a negative OECD GD 201, and this is reflected in Table C.2.19. However, a lack of mechanistic data on JH or Ec activity should ideally be rectified before any further *in vivo* testing is finally conducted, although as indicated above, *in vitro* JH/Ec screens have not yet been internationally standardised. On the other hand, if OECD GD 201 is positive, further *in vivo* testing would generally be needed to quantify any adverse effects in insects, even if all existing data are equivocal, or if there are no existing data. Again, however, it may be useful 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. JH or Ec agonistic and antagonistic) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA could potentially reinforce effects on the OECD GD 201 endpoint. 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.

572. The scenario in which the results of OECD GD 201 are themselves equivocal has not been dealt with in Table C.2.19, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. If possible reasons for false negatives are suspected, OECD GD 201 could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity).

573. In summary, positive results in OECD GD 201 indicate that a chemical has adverse effects in crustaceans which may or may not be via JH or Ec (ant)agonism. This may need to be followed up with further apical testing with insects. Negative results in OECD GD 201 do not necessarily mean that the chemical is not a potential ED – a judgement about the endocrine disruption potential in other arthropods will have to be made based on a weight of evidence evaluation of existing *in vitro* and *in vivo* data.

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Table C.2.19. **Harpacticoid Copepod Development and Reproduction Test with *Amphiascus* (OECD GD 201): 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.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from available from juvenile hormone (JH-) or ecdysteroid (Ec-) based assays. JH or Ec assays concerning mechanisms of disruption may be available, but they are have not yet been internationally standardised. In practice, data from all assays may not be available and therefore this must be taken into account when deciding on the "next step".

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 a JH or Ec disrupter.

Scenarios	Result of OECD GD 201	Existing results		Possible conclusions	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	+	+	+	Strong evidence for adverse *in vivo* effects in crustaceans, possibly but not necessarily caused by juvenile hormone (JH) or ecdysteroid (Ec) (ant)agonists, plus possible JH or Ec effects in other arthropods.	It would be desirable (if not already conducted) to perform an apical test with insects (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH or Ec (ant)agonist.
B	+	+	–	Moderate evidence for adverse *in vivo* effects in crustaceans, possibly but not necessarily caused by JH or Ec (ant)agonists.	It would be desirable (if not already conducted) to perform an apical test with insects (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH or Ec (ant)agonist.
C	+	+	Eq/0	Moderate evidence for adverse *in vivo* effects in crustaceans, possibly but not necessarily caused by JH or Ec (ant)agonists.	It would be desirable (if not already conducted) to perform an apical test with insects (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH or Ec (ant)agonist. 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.
D	+	–	+	Strong evidence for adverse *in vivo* effects in crustaceans, possibly but not necessarily caused by JH or Ec (ant)agonists, plus possible JH or Ec effects in other arthropods.	It would be desirable (if not already conducted) to perform an apical test with insects (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233).	The lack of *in vitro* JH or Ec activity is not evidence against any JH/Ec activity, due to the limited nature of current *in vitro* JH and Ec screens.
E	+	–	–	Some evidence for adverse *in vivo* effects in crustaceans, possibly but not necessarily caused by JH or Ec (ant)agonists.	It would be desirable (if not already conducted) to perform an apical test with insects (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233).	The lack of *in vitro* JH or Ec activity is not evidence against any JH/Ec activity, due to the limited nature of current *in vitro* JH and Ec screens.
F	+	–	Eq/0	Some evidence for adverse *in vivo* effects in crustaceans, possibly but not necessarily caused by JH or Ec (ant)agonists.	Given the absence or equivocal nature of existing *in vivo* data, it might also be sensible to conduct a JH-responsive crustacean assay (e.g. the *Daphnia* Multigeneration Test [DMGT] and/or a JH/E-responsive insect assay [e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233]).	The lack of *in vitro* JH/Ec activity is not evidence against any JH/Ec activity, due to the limited nature of current *in vitro* JH/Ec screens. 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.

Scenarios	Result of OECD GD 201	Existing results		Possible conclusions	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)**			
G	+	Eq/0	+	Strong evidence for adverse *in vivo* effects in crustaceans, possibly but not necessarily caused by JH or Ec (ant)agonists, plus possible JH or Ec effects in other arthropods.	Given the absence or equivocal nature of existing *in vitro* data, it would be desirable to obtain further *in vitro* data on JH/Ec activity if possible. It might also be sensible to conduct a JH/Ec-responsive insect assay if not already performed (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233).	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH/Ec activity. 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.
H	+	Eq/0	–	Some evidence for adverse *in vivo* effects in crustaceans, possibly but not necessarily caused by JH or Ec (ant)agonists.	Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for JH/Ec activity. It might also be sensible to conduct a JH/Ec-responsive insect assay if not already performed (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233).	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH/Ec activity. 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	Strong evidence for adverse *in vivo* effects in crustaceans, possibly but not necessarily caused by JH or Ec (ant)agonists.	Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for JH/Ec activity. It might also be sensible to conduct a JH/Ec-responsive insect assay (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233).	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH/Ec activity. 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.
J	–	+	+	The test chemical is probably a JH or Ec (ant)agonist without adverse effects in crustaceans, although it is possible that *Amphiascus* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. However, it might also be sensible to conduct a JH/Ec-responsive insect assay if not already performed (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH/Ec (ant)agonist.
K	–	+	–	The test chemical is probably a JH or Ec (ant)agonist without adverse effects in crustaceans or other taxa, although it is possible that *Amphiascus* responds atypically in this case.	If there is no activity in crustaceans or insects, further evidence is probably not needed.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH/Ec (ant)agonist.

Scenarios	Result of OECD GD 201	Existing results		Possible conclusions	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)**			
L	–	+	Eq/0	The test chemical is probably a JH or Ec (ant)agonist without adverse effects in crustaceans, although it is possible that *Amphiascus* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required, but if insect data are absent, it might be desirable to conduct a Chironomid Life Cycle Toxicity Test (OECD TG 233).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH/Ec agonist. 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.
M	–	–	+	The test chemical is probably without JH or Ec activity in crustaceans, although it is possible that *Chironomus* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	The lack of *in vitro* JH/Ec activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens.
N	–	–	–	The test chemical is probably without JH or Ec activity in arthropods.	No further action is necessary.	–
O	–	–	Eq/0	The test chemical is probably without JH or Ec activity in arthropods.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	The lack of *in vitro* JH/Ec activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. 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.
P	–	Eq/0	+	The test chemical is probably without JH or Ec activity in crustaceans, although it is possible that *Amphiascus* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. Also, if clear *in vitro* mechanistic data are missing, it might be desirable to obtain some.	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH/Ec activity. 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.

Scenarios	Result of OECD GD 201	Existing results		Possible conclusions	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)**			
Q	–	Eq/0	–	The test chemical is probably without JH or Ec activity in arthropods.	No further action is necessary.	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 test chemical is probably without JH or Ec activity in crustaceans and possibly insects.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	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.

C.2.20. *Daphnia* Multigeneration Test for Assessment of Endocrine-Active Chemicals (DMGT) (proposed OECD TG)

Status: Assay proposed for validation by the OECD.

574. Modality detected/endpoints: This long-term *in vivo* assay with *Daphnia magna* is responsive to juvenile hormone (JH) agonists which lead to the production of male offspring. It exposes the test organisms over two generations. The lack of internationally validated mechanistic assays for endocrine activity in crustaceans may prevent firm conclusions about whether test chemicals are endocrine disruptors (EDs) in this taxon, although *in vitro* assays for JH and ecdysteroid (Ec) activity are available in the literature. However, the data from the test may nevertheless be of value for classification and hazard identification/characterisation.

Background to the assay

575. This *in vivo* assay has been proposed but not yet approved as an OECD project. The validation by the OECD has not yet begun and the proposal is not expected to be approved as a test guideline (TG) until 2019 at the earliest. As such, the guidance in this section should be regarded as provisional, and it should be noted that the protocol outlined below may change. The DMGT at present consists of three linked exposure experiments. It begins with <24-hour-old neonates, exposes them continuously to dilutions of the test chemical, allows them to grow to adulthood, then produce at least three successive broods (termed the "F1 test", run for 21 days). The second test (termed the "F2: F1 exposed test") takes neonates from the third or subsequent brood in each concentration of the F1 test and exposes them to the same range of test concentrations for a further 21 days. The third test (termed the "F2: F1 unexposed test") solely takes control neonates from the third or subsequent brood in the F1 test and again exposes them for 21 days to the same range of concentrations as in the other tests. At the end of each test, all individual neonates are sexed by observation of their longer first antenna. JH and other JH agonists cause the production of males due to exposure during a short critical period (52-53 hours after ovulation). An adverse outcome pathway for this process has been described[1] – significant male production in a population would be expected to lead to its decline. The production in this test of a sex ratio significantly skewed towards males can therefore probably be considered as an adverse apical endpoint.

576. Limited data produced during the development of this test suggest that JH agonists may give have a more pronounced effect on sex ratio in the "F2: F1 exposed" test than the "F2: F1 unexposed" test. However, more data are required for this to be substantiated.

When/why the assay may be used

577. Although the DMGT 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* and *in vivo* data available about the possible JH-disrupting properties of a chemical. Given the significant degree of endocrine system conservation across the arthropods,

endocrine-linked effects in the DMGT may also indicate the possibility of related activity in other arthropods such as copepods, decapods and insects.

578. It is possible that no endocrine-relevant data are available before the DMGT is deployed (i.e. if the DMGT has been used as a primary test, or has been deployed to test a chemical for non-endocrine related chronic toxicity), but in that case a positive result in the test should probably be followed up with relevant *in vitro* screening, if available, to investigate the suspected mode of action (MOA) in more detail. However, it should be noted that there are no standardised *in vitro* screens for JH agonists, although some are described in the scientific literature (e.g. Cherbas, Koehler and Cherbas [1989]).

579. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an 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 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

580. Existing information on endocrine-related effects from other arthropods should also be considered before deployment of the DMGT, given the commonality of endocrine mechanisms in these taxa. Existing data available might also include one or more of a range of *in silico* or *in vitro* results which suggest that JH disruption may occur *in vivo* (but note the limitations of this approach, as indicated above). Such indicators of possible JH activity might include quantitative structure activity relationship (QSAR) predictions of JH activity, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen for JH agonist activity. In addition, *in vivo* data should ideally be available from one of two assays, the Short-Term Juvenile Hormone Activity Screening Assay (SJHASA) or the *Daphnia magna* Reproduction Test with male neonate option (OECD TG 211).

Scenarios: Positive and negative results combined with existing data

581. The scenarios (A to R) presented in Table C.2.20 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.

582. Positive results obtained with the DMGT (Table C.2.20, Scenarios A-I) result in the conclusion that the test chemical is a possible JH disrupter *in vivo* with adverse apical effects, at least in crustaceans. However, although a positive response of the DMGT indicates that the chemical is a possible JH agonist with adverse effects in crustaceans, it should be noted that *Daphnia*'s parthenogenetic reproductive strategy is not shared by many other arthropods. Therefore, if countries need further evidence concerning growth and sexual development, etc. in this phylum, a Harpacticoid Copepod Development and Reproduction Test (OECD GD 201) and/or the Sediment-Water Chironomid Life Cycle Toxicity Test (OECD TG 233) would be able to provide information on adverse effects in sexually reproducing species. In other words, in order to strengthen weight of evidence, a positive result in the DMGT could be followed by OECD GD 201 (Level 4) and/or OECD TG 233 (Level 5). Existing data suggesting endocrine-specific activity (e.g. positive *in vitro* data, or positive *in vivo* data from other species) will strengthen the case for additional testing still further.

583. The situation in which the DMGT gives a negative result (Table C.2.20, Scenarios J-R) needs careful consideration of any existing data. If these data suggest that the chemical is endocrine active both *in vitro* and *in vivo* (Scenario J), then it is possible that the DMGT is simply insufficiently sensitive.

584. If the DMGT and existing *in vivo* data are all negative, but *in vitro* data reveal some JH activity (Scenario K), the probability is that the test chemical is not sufficiently potent to produce JH agonism *in vivo* in arthropods, or it may be rapidly metabolised. In such a situation, further testing is probably not necessary. However, if the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests have been insufficiently prolonged, in which case longer term testing with OECD GD 201 or OECD TG 233 might be justified.

585. On the other hand, if the DMGT and the *in vitro* tests are negative (Scenario M), but there are positive existing *in vivo* data, the nature of those existing data should be considered. Unless the existing data are from another crustacean, the chemical is possibly not a JH agonist acting in crustaceans, but it may be more potent in species (e.g. insects) 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 (e.g. with OECD TG 233).

586. Finally, a negative DMGT, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is probably not a JH agonist *in vitro* or *in vivo*, and further action is unnecessary.

587. In each of the above scenarios, it is possible that existing data will be equivocal, or there may be no existing data. This will weaken the conclusions which can be drawn about a negative DMGT, and this is reflected in Table C.2.20. However, a lack of mechanistic data on JH activity should ideally be rectified before any further *in vivo* testing is finally conducted, although as indicated above, *in vitro* JH screens have not yet been internationally standardised. On the other hand, if the DMGT is positive, further *in vivo* testing would generally be needed to quantify any adverse effects in crustaceans and/or insects, even if all existing data are equivocal, or if there are no existing data. Again, however, it may be useful 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. JH agonistic and antagonistic) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA could potentially reinforce effects on the DMGT endpoint. 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.

588. The scenario in which the results of the DMGT are themselves equivocal has not been dealt with in Table C.2.20, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. If possible reasons for false negatives are suspected, the DMGT could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity). It should also be borne in mind that changing environmental conditions such as shortening photoperiod, temperature, and food shortages can also cause the production of male neonates in *D. magna*, so if these have accidentally occurred during the test, the results should be treated as suspect.

589. In summary, positive results in the DMGT indicate that a chemical is a probable endocrine disrupter with adverse effects in crustaceans via JH agonism. This may need to be followed up with further apical testing with sexually reproducing arthropods. Negative results in the DMGT do not necessarily mean that the chemical is not a potential ED – a judgement about the endocrine disruption potential in other arthropods will have to be made based on a weight of evidence evaluation of existing *in vitro* and *in vivo* data.

Note

1. See: https://aopwiki.org/wiki/index.php/Aop:201.

References

Cherbas, L., M.M.D. Koehler and P. Cherbas (1989), "Effects of juvenile hormone on the ecdysone response of *Drosophila* Kc cells", *Developmental Genetics*, Vol. 10/3, pp. 177-188, https://doi.org/10.1002/dvg.1020100307.

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.2.20. ***Daphnia*** **Multigeneration Test for Assessment of Endocrine-Active Chemicals (DMGT) (proposed OECD TG – SPSF not yet agreed): 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.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from available from juvenile hormone (JH-) based assays. JH assays concerning mechanisms of JH disruption may be available, but they are have not yet been internationally standardised. In practice, data from all assays may not be available and therefore this must be taken into account when deciding on the "next step".

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 a JH disrupter.

Scenarios	Result of DMGT	Existing results		Possible conclusions	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	+	+	+	Evidence for adverse *in vivo* effects in crustaceans caused by juvenile hormone (JH) and JH mimics, plus possible JH effects in other arthropods.	As *Daphnia* are parthenogenetic, it would be desirable (if not already conducted) to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist.
B	+	+	–	Evidence for adverse *in vivo* effects in crustaceans caused by JH and JH mimics.	As *Daphnia* are parthenogenetic, it would be desirable (if not already conducted) to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist.
C	+	+	Eq/0	Evidence for adverse *in vivo* effects in crustaceans caused by JH and JH mimics.	As *Daphnia* are parthenogenetic, it would be desirable (if not already conducted) to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist. 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.
D	+	–	+	Evidence for adverse *in vivo* effects in crustaceans caused by JH and JH mimics, plus possible JH effects in other arthropods.	As *Daphnia* are parthenogenetic, it would be desirable (if not already conducted) to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens.
E	+	–	–	Evidence for adverse *in vivo* effects in crustaceans caused by JH and JH mimics.	As *Daphnia* are parthenogenetic, it would be desirable (if not already conducted) to perform an additional apical test with sexually reproducing crustaceans and/or insects (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233).	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens.

Scenarios	Result of DMGT	Existing results		Possible conclusions	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	Evidence for adverse *in vivo* effects in crustaceans caused by JH and JH mimics.	Given the absence or equivocal nature of existing *in vivo* data, and the fact that *Daphnia* are parthenogenetic, it might also be sensible to conduct a JH-responsive insect assay (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) or crustacean assay (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201).	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. 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	+	Evidence for adverse *in vivo* effects in crustaceans caused by JH and JH mimics, plus possible JH effects in other arthropods.	Given the absence or equivocal nature of existing *in vitro* data, it would be desirable to obtain further *in vitro* data on JH activity if possible. Also, as *Daphnia* are parthenogenetic, it might also be desirable to conduct a JH-responsive insect assay (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) or crustacean assay (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201).	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH activity. 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.
H	+	Eq/0	–	Evidence for adverse *in vivo* effects in crustaceans caused by JH and JH mimics.	Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for JH activity. Also, as *Daphnia* are parthenogenetic, it might also be desirable to conduct a JH-responsive insect assay (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) or crustacean assay (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201).	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH activity. 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	Evidence for adverse *in vivo* effects in crustaceans caused by JH and JH mimics.	Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for JH activity. Also, as *Daphnia* are parthenogenetic, it might also be desirable to conduct a JH-responsive insect assay (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) or crustacean assay (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201).	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH activity. 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.
J	–	+	+	The test chemical is probably a JH agonist without adverse effects in crustaceans, although it is possible that *Daphnia magna* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist.

Scenarios	Result of DMGT	Existing results		Possible conclusions	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)**			
K	–	+	–	The test chemical is probably a JH agonist without adverse effects in crustaceans or other taxa, although it is possible that *Daphnia magna* responds atypically in this case.	If there is no activity in crustaceans or insects, further evidence is probably not needed.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist.
L	–	+	Eq/0	The test chemical is probably a JH agonist without adverse effects in crustaceans, although it is possible that *Daphnia magna* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required, but if insect data are absent, it might be desirable to conduct a Sediment-Water Chironomid Life Cycle Toxicity Test (OECD TG 233).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH agonist. 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.
M	–	–	+	The test chemical is probably without JH activity in crustaceans, although it is possible that *Daphnia magna* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens.
N	–	–	–	The test chemical is probably without JH activity in arthropods.	No further action is necessary.	–
O	–	–	Eq/0	The test chemical is probably without JH activity in arthropods.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	The lack of *in vitro* JH activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. 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.
P	–	Eq/0	+	The test chemical is probably without JH activity in crustaceans, although it is possible that *Daphnia magna* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. Also, if clear *in vitro* mechanistic data are missing, it might be desirable to obtain some.	If a new *in vitro* mechanistic assay is conducted, note that a negative does not mean that the test material has no JH activity. 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.

Scenarios	Result of DMGT	Existing results		Possible conclusions	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)**			
Q	–	Eq/0	–	The test chemical is probably without JH activity in arthropods.	No further action is necessary.	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 test chemical is probably without JH activity in crustaceans and possibly insects.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from insects (e.g. the Sediment-Water Chironomid Life Cycle Toxicity Test – OECD TG 233) if these are not already available.	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.

C.2.21. Fish Life Cycle Toxicity Test (FLCTT) (US EPA OPPTS 850.1500)

Status: Assay validated at national level.

590. Modality detected/endpoints: The basic FLCTT as described by Benoit (1981), US EPA (1996) and others does not contain endpoints which solely respond to endocrine disrupters. However, many of the endpoints in this apical test are nevertheless affected by estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) endocrine disruptors (EDs). Of particular interest in the context of estrogens, androgens and steroidogenesis disrupters are time to sexual maturity, sex ratio of adults, fecundity and fertility, but other endpoints may also be responsive to some EDs (e.g. growth may respond to some thyroid disrupters). It should be noted that no cases are known in which altered sex ratio was caused by a substance other than an ED.

Background to the assay

591. This assay is designed primarily as an apical test for chemicals with suspected reproductive or long-term toxicity. It has not been adopted for publication as an OECD test gideline (TG), but has been widely used for several decades by regulatory agencies for assessing possible chronic effects in fish. The endpoints are all apical measures of development, growth or reproduction. Exposure of the test organisms (fathead minnow *Pimephales promelas*, in the case of Benoit [1981], but other species can be successfully used with minor changes in the protocol, including sheepshead minnow *Cyprinodon variegatus*, zebrafish *Danio rerio* and medaka *Oryzias latipes*) usually continues from the freshly fertilised eggs of the F0 generation to the fry or young fish of the F1 generation (four to eight weeks post-hatch in the case of fathead minnow [Benoit, 1981]).

592. It should be noted that it would be relatively straightforward to include ED-specific endpoints in this test. However, this is no longer necessary as a fish life cycle test that includes such endpoints, the Medaka Extended One-Generation Reproduction Test (MEOGRT), has recently been validated and published by the OECD (TG 240). A similar test using zebrafish – the Zebrafish Extended One-Generation Reproduction Test (ZEOGRT) – is currently in validation. If there is a need to examine the apical effects of a suspected ED, it would therefore be preferable to use the MEOGRT or ZEOGRT rather than the FLCTT.

When/why the assay may be used

593. As stated above, the FLCTT has essentially been superseded by the MEOGRT or ZEOGRT for the purposes of evaluating endocrine active substances (EAS), because it does not have any EAS-specific endpoints. Therefore, if new life cycle data are required in an assessment, the MEOGRT or ZEOGRT would be the assays of choice. However, if FLCTT data on adverse effects are already available when conducting a hazard assessment of an E,A,T,S chemical, they should certainly be considered in a weight of evidence

evaluation as "*in vivo* effects of concern" alongside any other relevant *in vitro* and *in vivo* data.

594. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an 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 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 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

595. Given the commonality of endocrine mechanisms in the vertebrates, relevant existing data available before deployment of the FLCTT for endocrine disruption hazard assessment might include *in vivo* results obtained with other vertebrates (e.g. a positive Uterotrophic Bioassay with rodents, positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies), as well as information on possible modes of action (MOA) from quantitative structure activity relationships (QSARs) and/or *in vitro* screens. These will probably be accompanied by *in vivo* fish assay data from OECD TG 229, TG 230 or EASZY, and may also include data from the Fish Sexual Development Test (OECD TG 234). It would not be advisable or ethically desirable to conduct an unmodified FLCTT without mechanistic or *in vivo* screening data because it would then not be possible to link any apical effects with endocrine disruption. Furthermore, data from OECD TG 229 and/or TG 234 (FSDT) could be of use in focusing attention in the FLCTT on particularly vulnerable parts of the life cycle. Given the high ethical and financial cost of the FLCTT, it is important to make full use of existing endocrine-related data, both before the test is begun and during data evaluation.

Scenarios: Positive and negative results combined with existing data

596. The scenarios (A to R) presented in Table C.2.21 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.

597. Positive results obtained with one of the FLCTT endpoints result in the conclusion that the test chemical is able to cause adverse effects *in vivo* (Table C.2.21, Scenarios A-I), but not necessarily that it is an ED. Note that if doubt exists about the test performance (e.g. highly unusual results in controls), a comparison with historical control data with respect to overall test performance might be helpful. However, the nature of these effects and any existing data will require careful consideration. If *in vitro* and/or *in vivo* data already exist which reveal possible endocrine disrupting properties (Scenarios A, B and D), a positive endpoint in the FLCTT could lead to a conclusion that the test chemical is an actual ED. Such a conclusion will be strengthened considerably if the endocrine modality previously identified is plausibly linked to the responding endpoint. For example, if the chemical has estrogenic properties and reduced fecundity of the F0 adults has been observed in the FLCTT, this gives added confidence in this conclusion. On the other hand, it may be harder to argue a plausible link between estrogenic properties on the one hand, and an endpoint such as growth or survival on the other, although it is known that some estrogens are able to cause changes in growth rates (Knacker et al., 2010). In this example, an effect solely on growth or survival, while potentially of concern from the viewpoint of environmental hazard identification/characterisation, would not on its own lead to a conclusion that the chemical is an ED in fish.

598. If a plausible link of a responding FLCTT endpoint with previously identified endocrine activity can be made, regulatory authorities may conclude that sufficient evidence is available to categorise the chemical as an ED (i.e. interference with the endocrine system has caused adverse effects *in vivo*), and no further information might then be required. Of course, if the intention is to conduct an environmental hazard identification/characterisation, it may also be necessary to consider whether or not effects observed are relevant at the population level (e.g. reproduction, growth, development). On the other hand, if data from prior endocrine screens and tests are negative (Scenario E), a positive response in the FLCTT would not, in general, support the hypothesis that the chemical is an ED in fish (although it could be argued that a change in sex ratio is likely to have been caused by an ED). It could, of course, still be subjected to an environmental hazard identification/characterisation.

599. The scenarios in which the FLCTT gives a negative result (Table C.2.21, Scenarios J-R) lead to a tentative conclusion that the test chemical is not an ED in fish, and this conclusion is strengthened considerably if prior screens have failed to reveal endocrine activity (Scenario N). In the latter circumstances, regulatory authorities would be justified in concluding that no further action is needed. On the other hand, if one or more of those screens was positive (Scenarios J-M and P), the probable reasons for lack of effects in the FLCTT might be metabolism to an inactive chemical, or failure to reach the active site, and no further action would be indicated.

600. In each of the above scenarios, it is possible that existing data will be equivocal (Table C.2.21, Scenarios C, F-I, L and O-R), or there may be no existing data. This will weaken the conclusions which can be drawn about a positive FLCTT, and this is reflected in Table C.2.21. However, as indicated above, it would be undesirable to proceed with an FLCTT if prior data on endocrine activity are equivocal or absent, and if there are no other effect- or exposure-related reasons for considering such a comprehensive test. On the other hand, if the FLCTT is positive, it would be essential to obtain some reliable mechanistic data before reaching a conclusion about whether or not the chemical is an ED in fish. 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.

601. The scenario in which the results of the FLCTT are themselves equivocal has not been dealt with in Table C.2.21, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. However, if a comprehensive set of prior screens are all negative, it is doubtful whether further action is needed, because the chemical is unlikely to be an ED. If an endocrine screen is positive, some types of equivocal FLCTT results would have to be taken more seriously. For example, a non-monotonic concentration-response would not necessarily rule out the test chemical as an ED in fish. An example of this would be a chemical like ethinylestradiol which causes adverse effects on fish reproduction at low doses, but reduced reproductive success at very high doses, thus potentially giving a U-shaped response curve. Ideally, concentrations causing systemic toxicity of this type should not be tested in an FLCTT, but such toxicity may have been missed in earlier screens.

602. In summary, positive results in the FLCTT indicate that a chemical is a probable ED if they can be plausibly linked to an endocrine MOA established on the basis of prior mechanistic screening or concurrent observation of mechanistic effects or their biochemical/physiological manifestations. If such screening data are unavailable or negative, it should not be concluded that a positive FLCTT is the result of endocrine disruption (although it is likely that biased sex ratio will be the result of ED). On the other hand, a negative FLCTT combined with a sufficiently comprehensive set of negative screening data could lead to a firm conclusion that a chemical is not an ED in fish. A negative FLCTT set against a background of a positive screen might, however, raise concerns (e.g. if the chemical is known to be involved in epigenesist).

References

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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.2.21. **Fish Life Cycle Toxicity Test (FLCTT) (US EPA OPPTS 850.1500): 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.

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.

Note that although this assay has been used for many years to assess the chronic effects of chemicals, no attempt has been made to validate it for use with potential endocrine disruptors, and it has not been published as an OECD test guideline.

Scenario	Result of FLCTT	Existing results		Possible conclusions	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	+	+	+	The test chemical is probably an endocrine disruptor (ED) if the modality identified in existing screens/tests can be plausibly linked to the affected endpoint.	Further evidence is probably not required.	If the affected endpoint in the FLCTT cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED. The FLCTT is unlikely to detect epigenetic effects.
B	+	+	–	The test chemical is probably an ED in fish if the modality identified in existing screens/tests can be plausibly linked to the affected endpoint.	Further evidence is probably not required.	If the affected endpoint in the FLCTT cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED. The FLCTT is unlikely to detect epigenetic effects.
C	+	+	Eq/0**	The test chemical is probably an ED in fish if the modality identified in existing screens can be plausibly linked to the affected endpoint.	Further evidence is probably not required.	If the affected endpoint in the FLCTT cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED. The FLCTT is unlikely to detect epigenetic effects. 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.
D	+	–	+	The test chemical may be an ED, but the negative mechanistic data reduce the confidence in this conclusion. However, if the endocrine disruption effects in existing *in vivo* tests can be plausibly linked to the FLCTT responses, this increases the probability that the chemical is an ED.	Further evidence is probably not required.	If the affected endpoint in the FLCTT cannot be plausibly linked to the endocrine effects in existing *in vivo* tests, the test chemical is unlikely to be an ED. The FLCTT is unlikely to detect epigenetic effects.
E	+	–	–	The test chemical is unlikely to be an ED.[1]	Further evidence is probably not required.	It is possible that the effects observed in the FLCTT have been caused by an unknown endocrine mechanism. This would not, however, prevent the chemical being subjected to hazard identification/characterisation. The FLCTT is unlikely to detect epigenetic effects.
F	+	–	Eq/0	The test chemical is unlikely to be an ED, but the relevance of any equivocal existing *in vivo* data to the FLCTT results should be examined.	Further evidence is probably not required.	It is possible that the effects observed in the FLCTT have been caused by an unknown endocrine mechanism – equivocal existing *in vivo* data may throw some light on this. The absence of data on a possible endocrine mechanism would, however, not prevent the chemical being subjected to hazard identification/characterisation. The FLCTT is unlikely to detect epigenetic effects. 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.

Note: 1. However, note that if biased sex ratio is observed, it is likely to have been caused by an endocrine disrupting chemical.

Scenario	Result of FLCTT	Existing results		Possible conclusions	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)**			
G	+	Eq/0	+	The test chemical may be an ED, but the equivocal or absent mechanistic data reduce the confidence in this conclusion. However, if the endocrine disruption effects in existing *in vivo* tests can be plausibly linked to the FLCTT responses, this increases the probability that the chemical is an ED.	If reliable mechanistic data are not available, it would be desirable to obtain some.	The test chemical is probably an ED if a modality identified in the newly commissioned mechanistic screens, or in the existing *in vivo* data, can be plausibly linked to the affected endpoint. The FLCTT is unlikely to detect epigenetic effects. 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.
H	+	Eq/0	–	The test chemical may be an ED, but the equivocal or absent mechanistic data reduce the confidence in this conclusion.	If reliable mechanistic data are not available, it would be desirable to obtain some.	The test chemical is probably an ED if a modality identified in the newly commissioned mechanistic screens can be plausibly linked to the affected endpoint. The FLCTT is unlikely to detect epigenetic effects. 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	The test chemical may be an ED, but the equivocal or absent mechanistic and *in vivo* data reduce the confidence in this conclusion.	If reliable mechanistic data are not available, it would be desirable to obtain some.	The test chemical is probably an ED if a modality identified in the newly commissioned mechanistic screens can be plausibly linked to the affected endpoint. The FLCTT is unlikely to detect epigenetic effects. 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.
J	–	+	+	The chemical is probably not an ED in fish, unless this conclusion is contradicted by existing *in vivo* data.	No further action.	–
K	–	+	–	The chemical is probably not an ED in fish.	No further action.	–
L	–	+	Eq/0	The chemical is probably not an ED in fish.	No further action.	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
M	–	–	+	The chemical is probably not an ED in fish.	No further action.	–
N	–	–	–	The chemical is probably not an ED.	Further evidence is probably not required.	–

Scenario	Result of FLCTT	Existing results		Possible conclusions	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)**			
O	–	–	Eq/0	The chemical is probably not an ED in fish.	Further evidence is probably not required.	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.
P	–	Eq/0	+	The chemical is probably not an ED in fish.	If reliable mechanistic data are not available, it would be desirable to obtain some.	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, but confidence in this conclusion is reduced by the lack of clear mechanistic data.	Further evidence is probably not required, but confidence in the conclusion would be increased by the provision of reliable negative mechanistic data.	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, but confidence in this conclusion is reduced by the lack of clear mechanistic and existing *in vivo* data.	Further evidence is probably not required, but confidence in the conclusion would be increased by the provision of reliable negative mechanistic data.	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.

C.2.22. Zebrafish Extended One-Generation Reproduction Test (ZEOGRT) (draft OECD TG 240)

Status: Assay being validated by the OECD.

603. Modality detected/endpoints: This draft fish life cycle test was specifically designed to investigate the apical effects of endocrine disrupters, and has several endpoints which can be considered diagnostic of some types of estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) activity. This gives it a potential advantage over other currently standardised life cycle tests apart from the Medaka Extended One-Generation Reproduction Test (MEOGRT). If it is successfully validated, its use for evaluating endocrine disruptors (EDs) is to be preferred to the Fish Life Cycle Toxicity Test (FLCTT; see Section C.2.21) which, although sensitive to the apical effects of some EDs, contains no endocrine-sensitive endpoints. In view of the inclusion of certain ED-specific endpoints, the ZEOGRT can contribute useful evidence about the probable causality of apical effects, which is a key issue in the definition of EDs.

Background to the assay

604. This assay is a comprehensive test using zebrafish (*Danio rerio*) exposed continuously from the adult stage of the first generation (F0) to the newly hatched stage of the third generation (F2). In other words, it includes two phases of reproductive activity, and two phases of embryonic development and hatching, separated by a full phase of growth and sexual development. The test differs from the MEOGRT in that zebrafish are group spawners, whereas medaka are pair spawners. It begins with spawning groups of 5 male and 5 female sexually mature F0 fish (approximately 15 weeks post-fertilisation, or wpf) reproducing for 3 weeks, brings their F1 offspring to sexual maturity (13-20 weeks), then allows the F1 adults to breed, and finally follows their offspring (F2) to hatching (up to 14 days post-fertilisation, or dpf). The main emphasis of the assay concerns population-relevant apical endpoints (e.g. survival, development, growth, sex ratio and reproduction). However, in order to obtain mechanistic information, additional endpoints include measurements of vitellogenin (either as protein – VTG, or as mRNA coding for vitellogenin – *vtg*), phenotypic sex ratio and gonadal histopathology. Histopathology of liver and kidney may also be measured in order to distinguish between endocrine effects and possible systemic or other toxicity. Unlike the MEOGRT, the assay may be able to distinguish relatively small changes in the sex ratio of the F1 generation as it includes a large number of F1 fish (36 per replicate). On the other hand, it does not have a genetic sex endpoint, which may diminish the power to measure changes in sex ratio. It is also worth noting that because sex determination in zebrafish is polygenic (i.e. it is not driven by a single gene as in medaka), a range of environmental influences such as crowding, hypoxia and temperature fluctuations can have an influence on sex ratio. Finally, as zebrafish are not sexually dimorphic, this assay is not able to measure secondary sexual characteristics.

605. It should be noted that the ZEOGRT has only just begun validation by the OECD (Standard Project Submission Form approved by the OECD in September 2015) and has

not yet been widely used. It is expected that there will be a significant risk of test failure because of its length and difficulty. Currently, however, few testing laboratories have experience with the ZEOGRT.

606. Only zebrafish are recommended for use in this test design. The related assay using medaka (*Oryzias latipes*) – the MEOGRT – has been an OECD test guideline since 2015 (OECD TG 240).

When/why the assay may be used

607. Although the ZEOGRT 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 data available to suggest possible endocrine disruption properties. In other words, the ZEOGRT will generally be used to investigate whether such potential properties result in adverse apical effects on development, growth or reproduction over an entire life cycle. It is unlikely (and undesirable) that the ZEOGRT will be the first ED-responsive test procedure to be applied to a chemical. Furthermore, the conduct of a ZEOGRT **in addition to** a MEOGRT is not likely to be necessary (for example, to address perceived sensitivity differences). Before either assay is initiated, careful thought should be given to which is more appropriate in the circumstances. For example, if previous data are available with zebrafish and the ZEOGRT is sufficiently powerful for the expected endpoint of concern, then conducting a ZEOGRT may be the correct choice. However, if a genetic sex marker or secondary sexual characters are desired, it may be more beneficial to consider a MEOGRT.

608. This is a comprehensive test which examines a range of potentially adverse apical effects, but also considers several ED-specific endpoints. It is therefore suitable for helping to define whether a test chemical is an ED, and the results could be used in an environmental risk assessment for fish. Given the high degree of endocrine system conservation across the vertebrates, adverse endocrine-linked effects in the ZEOGRT may also indicate the possibility of related activity in other organisms such as amphibians, reptiles, birds or mammals.

609. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an 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

610. Existing data available before deployment of the ZEOGRT for endocrine disruption hazard assessment are likely to include information on possible modes of action (MOA) from quantitative structure activity relationships (QSARs), adverse outcome pathways and/or *in vitro* screens. These may be accompanied by *in vivo* fish assay data from EASZY, the Juvenile Medaka Anti-Androgen Screening Assay, OECD TG 229 and/or OECD TG 230, and may also include data from the Fish Sexual Development Test (FSDT – TG 234). In addition, existing information on endocrine-related effects from other vertebrates (up to and including mammals, e.g. positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies) should also be considered, given the commonality of endocrine mechanisms in these taxa. It would not be advisable or ethically desirable to conduct a ZEOGRT without mechanistic or *in vivo* screening data because it would then be less straightforward to link any apical effects with endocrine disruption. Furthermore, data from OECD TG 229 and/or TG 234 (FSDT), especially if obtained with zebrafish, could be of use in focusing attention in the ZEOGRT on particularly vulnerable parts of the life cycle. Given the high ethical and financial cost of the ZEOGRT, it is important to make full use of existing endocrine-related data, both before the test is begun and during data evaluation.

Scenarios: Positive and negative results combined with existing data

611. The advice given for the following scenarios is largely based on experience gained with the MEOGRT, and so should be treated with caution.

612. The scenarios (A to R) presented in Table C.2.22 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.

613. Positive results obtained with one of the ZEOGRT apical endpoints result in the conclusion that the test chemical is able to cause adverse effects *in vivo* (Table C.2.22, Scenarios A-I), but not necessarily that it is an ED. Note that if doubt exists about the test performance (e.g. highly unusual results in controls), a comparison with historical control data with respect to overall test performance might be helpful. However, the nature of these effects and any existing data will require careful consideration. If *in vitro* and/or *in vivo* data already exist which reveal possible endocrine disrupting properties (Scenarios A, B and D), a positive apical endpoint in the ZEOGRT could lead to a conclusion that the test chemical is an actual ED if adverse population effects are expected as a consequence. This conclusion will, of course, be reinforced if mechanistic endpoints in the ZEOGRT itself also respond. The probability that the test chemical is an ED will also be strengthened considerably if the endocrine modality identified in the present or earlier tests is plausibly linked to the responding endpoint. For example, if the chemical has estrogenic properties (such as the induction of VTG in males) and there is observed to be reduced fecundity of the F0 or F1 adults in the ZEOGRT, this gives added confidence in this conclusion. On the other hand, it may be harder to argue a plausible link between estrogenic properties on the one hand, and an endpoint such as growth or survival on the other, although it is known that some estrogens are able to cause changes in growth rates (Knacker et al., 2010). In this example, an effect

solely on growth or survival, while potentially of concern from the viewpoint of environmental hazard identification/characterisation, would not on its own lead to a conclusion that the chemical is an ED in fish.

614. If a plausible link of a responding ZEOGRT apical endpoint with identified endocrine activity can be made, regulatory authorities may conclude that sufficient evidence is available to categorise the chemical as an ED (i.e. interference with the endocrine system has caused adverse effects *in vivo*), and no further information might then be required. It may also be necessary to consider whether or not effects observed are relevant at the population level (e.g. reproduction, growth, development). On the other hand, if data from prior endocrine screens and tests are negative, including negative mechanistic data from the ZEOGRT itself (Scenario E), a positive apical response in the ZEOGRT would not in general support the hypothesis that the chemical is an ED in fish (although a change in sex ratio may have been caused by an ED). The chemical could, of course, still be subjected to an environmental hazard identification/characterisation.

615. The scenarios in which the ZEOGRT gives a negative apical result (Table C.2.22, Scenarios J-R) lead to a tentative conclusion that the test chemical is not an ED in fish, and this conclusion is strengthened considerably if prior screens, or the ZEOGRT itself, have failed to reveal endocrine activity (Scenario N). In the latter circumstances, regulatory authorities would be justified in concluding that no further action is needed. On the other hand, if one or more of those screens was positive (Scenarios J-M and P), the bioconcentration factor (BCF) of the chemical should be checked. If the BCF indicates that the chemical is strongly bioaccumulative and equilibrium is reached slowly, it would be worth considering the conduct of an extended ZEOGRT (but no TG is available for this), although as indicated above, there is little evidence at present that EDs with a high BCF would be consistently more potent in such a test. If a chemical which screened positive is not bioaccumulative, the probable reasons for lack of effects in the ZEOGRT might be metabolism to an inactive chemical, or failure to reach the active site, and no further action would be indicated.

616. In each of the above scenarios, it is possible that existing data will be equivocal (Table C.2.22, Scenarios C, F-I, L and O-R), or there may be no existing data. This will weaken the conclusions which can be drawn about a positive apical endpoint in the ZEOGRT, and this is reflected in Table C.2.22. However, as indicated above, it would be undesirable to proceed with a ZEOGRT if prior data on endocrine activity are equivocal or absent, and if there are no other effect- or exposure-related reasons for considering such a comprehensive test. On the other hand, if the ZEOGRT shows a positive apical endpoint, it would be essential to obtain some reliable mechanistic data before reaching a conclusion about whether or not the chemical is an ED in fish. 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.

617. The scenario in which the results of the ZEOGRT are themselves equivocal has not been dealt with in Table C.2.22, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. However, if a comprehensive set of prior screens are all negative, it is doubtful whether further action is needed, because the chemical is unlikely to be an ED. If an endocrine screen is positive, some types of equivocal ZEOGRT apical results would have to be taken more seriously. For example, a non-monotonic concentration-response would not necessarily rule out the test chemical as an ED in fish. An example of this would be a chemical like ethinylestradiol which causes adverse effects (elevated fecundity) on fish reproduction at low doses, but reduced reproductive success at very high doses, thus potentially giving an inverted U-shaped response curve (e.g. Jobling et al. [2004]). Ideally, concentrations causing systemic toxicity of this type should not be tested in ZEOGRT, but such toxicity may have been missed in earlier screens.

618. In summary, positive apical results in the ZEOGRT indicate that a chemical is a probable ED if they can be plausibly linked to an endocrine MOA established on the basis of prior mechanistic screening or concurrent observation of mechanistic effects or their biochemical/physiological manifestations. If such screening data are unavailable or negative, it should not be concluded that a positive ZEOGRT is the result of endocrine disruption (although a biased sex ratio may have been the result of ED). On the other hand, a negative ZEOGRT combined with a sufficiently comprehensive set of negative screening data could lead to a firm conclusion that a chemical is not an ED in fish. A negative ZEOGRT set against a background of a positive screen might, however, raise concerns (e.g. if the chemical is strongly bioaccumulative or known to be involved in epigenesist). In this case, an extended ZEOGRT should be considered, although this is not expected to be covered by a ZEOGRT test guideline.

References

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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.2.22. **Zebrafish Extended One-Generation Reproduction Test (ZEOGRT) (draft OECD TG): Guidance for scenarios of combinations of results with existing data**

It should be noted that this assay has not yet been validated, so the advice given in the table is provisional and may change. 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.

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 conclusions are grouped into a series of scenarios (A-R), each scenario representing a different combination of assay results, existing mechanistic data and existing *in vivo* effects 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.

Results of the ZEOGRT: * Apical results of the ZEOGRT include effects on survival, growth, development and reproduction. The other ZEOGRT endpoints, including VTG, sex ratio and gonadal histopathology, can be indicative of endocrine mechanisms which may have caused the apical effect.

Existing results: ** "Mechanism (*in vitro* and/or *in vivo* 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 also be available. 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	Apical result of ZEOGRT*	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* and/or *in vivo* 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 they do not appear 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 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.	Further evidence is probably not required.	If the affected apical endpoint in the ZEOGRT cannot be plausibly linked to the known modality, the test chemical is unlikely to be an endocrine disruptor (ED). The ZEOGRT is unlikely to detect epigenetic effects. If these are suspected, extending the test beyond F2 hatching could be considered, although this would depart from the expected test guideline (TG).
B	+	+	–	1) Strong evidence for adverse effects in fish by an endocrine mechanism. 2) Strong evidence for endocrine effects in fish, but they do not appear 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.	Further evidence is probably not required.	If the affected apical endpoint in the ZEOGRT cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED. The ZEOGRT is unlikely to detect epigenetic effects. If these are suspected, extending the test beyond F2 hatching could be considered, although this would depart from the expected TG.
C	+	+	Eq/0	1) Strong evidence for adverse effects in fish by an endocrine mechanism. 2) Strong evidence for endocrine effects in fish, but they do not appear 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.	Further evidence is probably not required.	If the affected apical endpoint in the ZEOGRT cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED. The ZEOGRT is unlikely to detect epigenetic effects. If these are suspected, extending the test beyond F2 hatching could be considered, although this would depart from the expected TG. 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	Apical result of ZEOGRT*	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* and/or *in vivo* 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.	Further evidence is probably not required.	If the affected apical endpoint in the ZEOGRT cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED. The ZEOGRT is unlikely to detect epigenetic effects. If these are suspected, extending the test beyond F2 hatching could be considered, although this would depart from the expected TG.
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.	Further evidence is probably not required.	It is possible that the effects observed in the ZEOGRT have been caused by an unknown endocrine mechanism. This would not, however, prevent the chemical being subjected to hazard identification/characterisation. The ZEOGRT is unlikely to detect epigenetic effects. If these are suspected, extending the test beyond F2 hatching could be considered, although this would depart from the expected TG.

Scenario	Apical result of ZEOGRT*	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* and/or *in vivo* mechanistic data)**	Effects (*in vivo* effects of concern)***			
F	+	–	Eq/0	1) Moderate 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.	Further evidence is probably not required.	It is possible that the effects observed in the ZEOGRT have been caused by an unknown endocrine mechanism or not by an endocrine mechanism at all – equivocal existing *in vivo* data may throw some light on this. The absence of data on a possible endocrine mechanism would, however, not prevent the chemical being subjected to hazard identification/characterisation. The ZEOGRT is unlikely to detect epigenetic effects. If these are suspected, extending the test beyond F2 hatching could be considered, although this would depart from the expected TG. 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.	If reliable mechanistic data are not available, it would be desirable to obtain some.	The test chemical is probably an ED if a modality identified in the newly commissioned mechanistic screens, or in the existing *in vivo* data, can be plausibly linked to the affected endpoint. The ZEOGRT is unlikely to detect epigenetic effects. If these are suspected, extending the test beyond F2 hatching could be considered, although this would depart from the expected TG. 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	Apical result of ZEOGRT*	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* and/or *in vivo* 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.	If reliable mechanistic data are not available, it would be desirable to obtain some.	The test chemical is probably an ED if a modality identified in the newly commissioned mechanistic screens can be plausibly linked to the affected endpoint. The ZEOGRT is unlikely to detect epigenetic effects. If these are suspected, extending the test beyond F2 hatching could be considered, although this would depart from the expected TG. 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.	If reliable mechanistic data are not available, it would be desirable to obtain some.	The test chemical is probably an ED if a modality identified in the newly commissioned mechanistic screens can be plausibly linked to the affected endpoint. The ZEOGRT is unlikely to detect epigenetic effects. If these are suspected, extending the test beyond F2 hatching could be considered, although this would depart from the expected TG. 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.
J	–	+	+	The chemical is probably not an ED in fish, unless this conclusion is contradicted by existing *in vivo* data.	If the chemical is strongly bioaccumulative, or if epigenetic effects are suspected, an extended version of the ZEOGRT could be considered, although this would depart from the expected TG.	If any effects in an extended ZEOGRT can be plausibly linked with mechanistic data, the test chemical is probably an ED.

Scenario	Apical result of ZEOGRT*	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* and/or *in vivo* mechanistic data)**	Effects (*in vivo* effects of concern)***			
K	–	+	–	The chemical is probably not an ED in fish.	If the chemical is strongly bioaccumulative, or if epigenetic effects are suspected, an extended version of the ZEOGRT could be considered, although this would depart from the expected TG.	If any effects in an extended ZEOGRT can be plausibly linked with mechanistic data, the test chemical is probably an ED.
L	–	+	Eq/0	The chemical is probably not an ED in fish.	If the chemical is strongly bioaccumulative, or if epigenetic effects are suspected, an extended version of the ZEOGRT could be considered, although this would depart from the expected TG.	If any effects in an extended ZEOGRT can be plausibly linked with mechanistic data, the test chemical is probably an ED. 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.
M	–	–	+	The chemical is probably not an ED in fish.	If the chemical is strongly bioaccumulative, or if epigenetic effects are suspected, an extended version of the ZEOGRT could be considered, although this would depart from the expected TG.	If any effects in an extended ZEOGRT can be plausibly linked with *in vivo* data which provide information on ED properties, the test chemical is probably an ED, but likely not by a mechanism covered by the existing *in vitro* screens.
N	–	–	–	The chemical is probably not an ED.	Further evidence is probably not required.	–
O	–	–	Eq/0	The chemical is probably not an ED in fish.	Further evidence is probably not required.	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 any mechanistic information.

Scenario	Apical result of ZEOGRT*	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* and/or *in vivo* mechanistic data)**	Effects (*in vivo* effects of concern)***			
P	–	Eq/0	+	The chemical is probably not an ED in fish.	If reliable mechanistic data are not available, it would be desirable to obtain some.	If the newly commissioned mechanistic data are positive and the chemical is strongly bioaccumulative, or if epigenetic effects are suspected, consider conducting an extended ZEOGRT, although this would depart from the expected TG. 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, but confidence in this conclusion is reduced by the lack of clear mechanistic data.	Further evidence is probably not required, but confidence in the conclusion would be increased by the provision of reliable negative mechanistic data.	If the newly commissioned mechanistic data are positive and the chemical is strongly bioaccumulative, or if epigenetic effects are suspected, consider conducting an extended ZEOGRT, although this would depart from the expected TG. 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, but confidence in this conclusion is reduced by the lack of clear mechanistic and existing *in vivo* data.	Further evidence is probably not required, but confidence in the conclusion would be increased by the provision of reliable negative mechanistic data.	If the newly commissioned mechanistic data are positive and the chemical is strongly bioaccumulative, or if epigenetic effects are suspected, consider conducting an extended ZEOGRT, although this would depart from the expected TG. 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.

C.2.23. Avian Two-Generation Toxicity Test in the Japanese Quail (ATGT) (US EPA OCSPP 890.2100/740-C-15-003)

Status: Assay validated at national level.

619. Modality detected/endpoints: This avian multigeneration test was specifically designed to investigate the apical effects of endocrine disrupters, and has several endpoints which can be considered diagnostic of some types of estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) activity. In view of the inclusion of certain endocrine disruptor (ED-) specific endpoints, the ATGT can contribute useful evidence about the probable causality of apical effects, which is a key issue in the definition of EDs.

Background to the assay

620. The assay is a comprehensive test using Japanese quail (*Coturnix japonica*). The F0 generation are exposed to a range of test chemical concentrations in their food for 49 days from 28 days post-hatch (dph). The F1 generation is exposed via the egg and orally from hatch to 70 dph. The F2 generation is only exposed via the egg, not via the food, and is followed to 14 dph (although there is an option to continue the test to F2 sexual maturity at 42 dph). The complete test therefore takes a minimum of 19 weeks.

621. A large range of endpoints is measured, including growth; development; reproduction; histopathology of multiple organs including gonads; phenotypic and genotypic sex; and various hormone titres including thyroid hormone (T4), estradiol (E2) and testosterone (T). Gonadal histopathology, hormone titres and sex ratio can all be used to provide information about possible endocrine modes of action (MOA).

622. It should be noted that the ATGT is a relatively new test (adopted by the United States Environmental Protection Agency in 2015) which has not yet been widely used and has not been validated by the OECD because it is time-consuming and technically challenging, and requires considerable resources. There is a significant risk of test failure because of its length and difficulty. Currently, few testing laboratories have experience with the ATGT.

When/why the assay may be used

623. Although the ATGT 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 data available to suggest possible endocrine disruption properties *in vitro* and/or *in vivo*. In other words, the ATGT will generally be used to investigate whether such potential properties result in adverse apical effects on development, growth or reproduction over two generations. It is unlikely (and undesirable) that the ATGT will be the first ED-responsive test procedure to be applied to a chemical.

624. This is a comprehensive test which examines a range of potentially adverse apical effects, but also considers several ED-specific endpoints. It is therefore suitable for helping to define whether a test chemical is an ED, and the results could be used in an

environmental hazard identification/characterisation for birds. Given the high degree of endocrine system conservation across the vertebrates, adverse endocrine-linked effects in the ATGT may also indicate the possibility of related activity in other organisms such as fish, amphibians, reptiles or mammals.

625. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an 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

626. Existing data available before deployment of the ATGT for endocrine disruption hazard assessment are likely to include information on possible MOA from quantitative structure activity relationships (QSARs), adverse outcome pathways and/or *in vitro* screens. These may be accompanied by *in vivo* bird assay data from the Avian Reproduction Test (OECD TG 206). In addition, existing information on endocrine-related effects from other vertebrates (up to and including mammals, e.g. positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies) should also be considered, given the commonality of endocrine mechanisms in these taxa. It would not be advisable or ethically desirable to conduct an ATGT without mechanistic or *in vivo* screening data because it would then be less straightforward to link any apical effects with endocrine disruption. Furthermore, data from OECD TG 206 could be of use in focusing attention in the ATGT on particularly vulnerable parts of the life cycle. Given the high ethical and financial cost of the ATGT, it is important to make full use of existing endocrine-related data, both before the test is begun and during data evaluation.

Scenarios: Positive and negative results combined with existing data

627. The scenarios (A to R) presented in Table C.2.23 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.

628. Positive results obtained with one of the ATGT apical endpoints result in the conclusion that the test chemical is able to cause adverse effects *in vivo* (Table C.2.23, Scenarios A-I), but not necessarily that it is an ED. Note that if doubt exists about the test performance (e.g. highly unusual results in controls), a comparison with historical control data with respect to overall test performance might be helpful. However, the nature of these effects and any existing data will require careful consideration. If *in vitro* and/or *in vivo* data already exist which reveal possible endocrine disrupting properties (Scenarios A, B and D), a positive apical endpoint in the ATGT could lead to a conclusion that the test chemical is an actual ED if adverse population effects are expected as a consequence. This conclusion will, of course, be reinforced if mechanistic endpoints in the ATGT itself also respond. The probability that the test chemical is an ED will also be strengthened considerably if the endocrine modality identified in the present or earlier tests is plausibly linked to the responding endpoint. For example, if the chemical has estrogenic properties and there is observed to be reduced fecundity of the F0 or F1 adults in the ATGT, this gives added confidence in this conclusion. On the other hand, it may be harder to argue a plausible link between estrogenic properties on the one hand, and an endpoint such as growth or survival on the other, although it is known that some estrogens are able to cause changes in growth rates. In this example, an effect solely on growth or survival, while potentially of concern from the viewpoint of environmental hazard identification/characterisation, would not on its own lead to a conclusion that the chemical is an ED in birds.

629. If a plausible link of a responding ATGT apical endpoint with identified endocrine activity can be made, regulatory authorities may conclude that sufficient evidence is available to categorise the chemical as an ED (i.e. interference with the endocrine system has caused adverse effects *in vivo*), and no further information might then be required. Of course, if the intention is to conduct an environmental hazard identification/characterisation, it may also be necessary to consider whether or not effects observed are relevant at the population level (e.g. reproduction, growth, development). On the other hand, if data from prior endocrine screens and tests are negative, including negative mechanistic data from the ATGT itself (Scenario E), a positive apical response in the ATGT would not, in general, support the hypothesis that the chemical is an ED in birds (although it could be argued that a change in sex ratio is likely to have been caused by an ED). The chemical could, of course, still be subjected to an environmental hazard identification/characterisation.

630. The scenarios in which the ATGT gives a negative apical result (Table C.2.23, Scenarios J-R) lead to a tentative conclusion that the test chemical is not an ED in birds, and this conclusion is strengthened considerably if prior screens, or the ATGT itself, have failed to reveal endocrine activity (Scenario N). In the latter circumstances, regulatory authorities would be justified in concluding that no further action is needed. On the other hand, if one or more of those screens was positive (Scenarios J-M and P), the test chemical may simply be inactive in *Coturnix japonica*. If a chemical screened positive, the probable reasons for lack of effects in the ATGT might be metabolism to an inactive chemical, or failure to reach the active site, and no further action would be indicated.

631. In each of the above scenarios, it is possible that existing data will be equivocal (Table C.2.23, Scenarios C, F-I, L and O-R), or there may be no existing data. This will weaken the conclusions which can be drawn about a positive apical endpoint in the ATGT, and this is reflected in Table C.2.23. However, as indicated above, it would be undesirable to proceed with an ATGT if prior data on endocrine activity are equivocal or absent, and if there are no other effect- or exposure-related reasons for considering such a comprehensive test. On the other hand, if the ATGT shows a positive apical endpoint, it would be essential

to obtain some reliable mechanistic data before reaching a conclusion about whether or not the chemical is an ED in birds. 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.

632. The scenario in which the results of the ATGT are themselves equivocal has not been dealt with in Table C.2.23, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. However, if a comprehensive set of prior screens are all negative, it is doubtful whether further action is needed, because the chemical is unlikely to be an ED. If an endocrine screen is positive, some types of equivocal ATGT apical results would have to be taken more seriously. For example, a non-monotonic concentration-response would not necessarily rule out the test chemical as an ED in birds. Ideally, concentrations causing systemic toxicity should not be tested in the ATGT, but such toxicity may have been missed in earlier screens.

633. In summary, positive apical results in the ATGT indicate that a chemical is a probable ED if they can be plausibly linked to an endocrine MOA established on the basis of prior mechanistic screening or concurrent observation of mechanistic effects or their biochemical/physiological manifestations. If such screening data are unavailable or negative, it should not be concluded that a positive ATGT is the result of endocrine disruption (although it is likely that biased sex ratio will be the result of ED). On the other hand, a negative ATGT combined with a sufficiently comprehensive set of negative screening data could lead to a firm conclusion that a chemical is not an ED in birds. A negative ATGT set against a background of a positive screen might, however, raise concerns.

Reference

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.2.23. **Avian Two-Generation Toxicity Test in the Japanese Quail (ATGT) (US EPA OCSPP 890.2100/740-C-15-003): 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.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from endocrine 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 birds 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.

The assay under discussion could either be positive for both apical endpoints and indicators of endocrine activity (e.g. mechanistic endpoints such as hormone titres and gonad histopathology), or positive just for apical endpoints, or positive just for indicators of endocrine activity. For each scenario, each of these three possibilities is addressed separately in the possible conclusions column.

Scenario	Result of ATGT	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 birds and other organisms by an endocrine mechanism. 2) Strong evidence for endocrine effects, but they do not appear adverse in birds. 3) Strong evidence for adverse effects in more than one organism, but mechanism may not be via direct interaction with endocrine receptor (ER) or androgen receptor (AR), or by aromatase inhibition or thyroid disruption.	Probably no need for additional data.	–
B	+	+	–	1) Strong evidence for adverse effects in birds by an endocrine mechanism. 2) Strong evidence for endocrine effects in birds, but they do not appear adverse. 3) Strong evidence for adverse effects in birds, but mechanism may not be via direct interaction with ER or AR, or by aromatase inhibition or thyroid disruption.	Probably no need for additional data.	–
C	+	+	Eq/0**	1) Strong evidence for adverse effects in birds by an endocrine mechanism. 2) Strong evidence for endocrine effects in birds, but they do not appear adverse. 3) Strong evidence for adverse effects in birds, but mechanism may not be via direct interaction with ER or AR, or by aromatase inhibition or thyroid disruption.	Probably no need for additional data.	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.
D	+	–	+	1) Strong evidence for adverse effects in birds and other organisms, possibly by an unknown endocrine mechanism. 2) Medium-strong evidence for endocrine effects in birds, but they do not appear to be adverse. 3) Strong evidence for adverse effects in more than one organism, but mechanism may not be by endocrine disruption.	Probably no need for additional data, but see right-hand column.	Negative *in vitro* mechanistic data combined with positive endocrine-specific *in vivo* endpoints suggests that an unknown endocrine MOA is causing any adverse effects. Some regulatory authorities may consider that the MOA needs further investigation before it can be concluded that the chemical in this scenario (sub-section 1) is an ED.
E	+	–	–	1) Strong evidence for adverse effects in birds, possibly by an unknown endocrine mechanism. 2) Medium-strong evidence for endocrine effects in birds, but they do not appear to be adverse. 3) Strong evidence for adverse effects in birds, but mechanism may not be by endocrine disruption.	Probably no need for additional data, but see right-hand column.	Negative *in vitro* mechanistic data combined with positive endocrine-specific *in vivo* endpoints suggests that an unknown endocrine MOA is causing any adverse effects. Some regulatory authorities may consider that the MOA needs further investigation before it can be concluded that the chemical in this scenario (sub-section 1) is an ED, especially as existing *in vivo* data are negative.

Scenario	Result of ATGT	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 birds, possibly by an unknown endocrine mechanism. 2) Medium-strong evidence for endocrine effects in birds, but they do not appear to be adverse. 3) Strong evidence for adverse effects in birds, but mechanism may not be by endocrine disruption.	Probably no need for additional data, but see right-hand column.	Negative *in vitro* mechanistic data combined with positive endocrine-specific *in vivo* endpoints suggests that an unknown endocrine MOA is causing any adverse effects. Some regulatory authorities may consider that the MOA needs further investigation before it can be concluded that the chemical in this scenario (sub-section 1) is an ED, especially as existing *in vivo* data are equivocal or absent. However, such a conclusion appears likely on the basis of the positive endocrine-sensitive endpoints *in vivo*. 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 birds. 3) Strong evidence for adverse effects in more than one organism, but mechanism may not be by endocrine disruption.	It would be desirable to obtain some clear mechanistic data before concluding that the chemical is an ED. See right-hand column.	Some regulatory authorities may consider that the MOA needs further investigation before it can be concluded that the chemical in sub-section 1 is an ED. However, such a conclusion appears likely on the basis of the positive endocrine-sensitive endpoints *in vivo*. 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.
H	+	Eq/0	–	1) Strong evidence for adverse effects in birds, possibly by an unknown endocrine mechanism. 2) Medium-strong evidence for endocrine effects in birds, but they do not appear to be adverse. 3) Strong evidence for adverse effects in birds, but mechanism may not be by endocrine disruption.	It would be desirable to obtain some clear mechanistic data before concluding whether the chemical is an ED. See right-hand column.	Some regulatory authorities may consider that the MOA needs further investigation before it can be concluded that the chemical in sub-section 1 is an ED. However, such a conclusion appears likely on the basis of the positive endocrine-sensitive endpoints *in vivo*. 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 ATGT	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)**			
I	+	Eq/0	Eq/0	1) Strong evidence for adverse effects in birds, possibly by an unknown endocrine mechanism. 2) Moderate-strong evidence for endocrine effects in birds, but they do not appear to be adverse. 3) Strong evidence for adverse effects in birds, but mechanism may not be by endocrine disruption.	It would be desirable to obtain some clear mechanistic data before concluding whether the chemical is an ED. See right-hand column.	Some regulatory authorities may consider that the MOA needs further investigation before it can be concluded that the chemical in sub-section 1 is an ED. However, such a conclusion appears likely on the basis of the positive endocrine-sensitive endpoints *in vivo*. 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.
J	–	+	+	The chemical is an ED *in vivo* in other species but does not appear to act on growth, sexual development or reproduction in birds. If any other bird tests are also negative, birds may not be responsive at all to the test chemical.	Regulatory authorities may consider that further evidence is not required.	The fact that the chemical has endocrine properties *in vitro* and in other species *in vivo* suggests that it may be an ED, but probably not in birds. If the existing positive *in vivo* data are from a lower tier bird assay, note that it is generally considered that a negative higher tier test trumps a positive lower tier test.
K	–	+	–	Despite the *in vitro* mechanistic data for possible endocrine activity, there is no evidence for endocrine disruption *in vivo*. This may be because the chemical is degraded to an inactive metabolite, or because it only interacts very weakly with endocrine receptors.	Regulatory authorities may consider that further evidence is not required.	–
L	–	+	Eq/0	The chemical is not an ED in birds, but it may be active in other species as there is only one unequivocal *in vivo* test result (a negative).	Regulatory authorities may consider that further evidence is not required.	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.
M	–	–	+	The chemical is probably not an ED acting on growth, sexual development or reproduction in birds, 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 bird species other than that tested.	Regulatory authorities may consider that further evidence is not required.	The fact that the chemical has endocrine properties in other species *in vivo* suggests that it may be an ED, but probably not in birds. If the existing positive *in vivo* data are from a lower tier bird assay, note that it is generally considered that a negative higher tier test trumps a positive lower tier test.
N	–	–	–	The chemical is probably not an ED in birds or other species.	Regulatory authorities may consider that further evidence is not required.	–
O	–	–	Eq/0	The chemical is probably not an ED in birds.	Regulatory authorities may consider that further evidence is not required.	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 ATGT	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 growth, sexual development or reproduction in birds, 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 bird species other than that tested.	Regulatory authorities may consider that further evidence is not required.	The fact that the chemical has endocrine properties in other species *in vivo* suggests that it may be an ED, but probably not in birds. If the existing positive *in vivo* data are from a lower tier bird assay, note that it is generally considered that a negative higher tier test trumps a positive lower tier test. 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 growth, sexual development or reproduction in birds, or *in vivo* on other species.	Regulatory authorities may consider that further evidence is not required, although negative *in vitro* data would strengthen the conclusion that the chemical is probably not an ED.	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 is probably not an ED acting on growth, sexual development or reproduction in birds.	Regulatory authorities may consider that further evidence is not required, although negative *in vitro* data would strengthen the conclusion that the chemical is probably not an ED.	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.

C.2.24. RADAR: Rapid Androgen Disruption Adverse Outcome Reporter Assay (draft OECD TG)

Status: Assay being validated by the OECD.

634. Modality detected/endpoints: This draft *in vivo* transfected medaka assay is sensitive to androgen receptor agonists and androgen receptor antagonists and to chemicals interfering with androgen biosynthesis. In principle, it can also be used to identify estrogen agonists and antagonists, as well as aromatase inhibitors. However, this guidance will restrict itself to the detection of receptor-mediated androgenicity and anti-androgenicity alone, as data on responses to the other modalities are not yet available.

Background to the assay

635. This assay is started validation by the OECD in June 2017 for possible approval as a test guideline (TG), a Standard Project Submission Form (SPSF) having been approved by the Working Group of National Coordinators of the Test Guidelines Programme in April 2017. No validation data have yet been produced, but some published data on development and use of the assay are available (Sébillot et al., 2014). It is planned to have a TG ready by 2020 at the earliest. The assay is based on freshly hatched embryonic medaka (*Oryzias latipes*), stably transfected with the *spiggin1* promoter cloned upstream of a green fluorescent protein coding sequence. The presence of the spiggin promoter linked to androgen receptor alpha (ARα) means that the transparent transgenic fish fry will fluoresce green when exposed to an androgen for up to six days. The presence of an anti-androgen can be detected by exposing the fish in combination with an androgen such as 17-methyl testosterone (17MT) and measuring the decrease in expected fluorescence. The assay is relatively cheap to operate by comparison with *in vivo* screening assays using juvenile or adult fish. Furthermore, its sensitivity to anti-androgens is expected to be broadly similar to the Androgenised Female Stickleback Screen (AFSS – OECD GD 148) (Sébillot et al., 2014), and it is expected that the metabolic capability of medaka embryos, while limited by comparison with adult fish, will allow the detection of some metabolically activated endocrine active substances (EASs). It can be run in multiwell plates and is potentially suitable for use in a robotic screening programme.

When/why the assay may be used

636. Although data from RADAR could, in principle, be available at any stage in the hazard assessment process, the most likely scenario will be when there are relatively few data available about the possible endocrine disrupting properties of a chemical. The assay is most likely to be used either as part of a battery of *in vitro* and *in vivo* screens, or to follow up on existing data which suggest possible endocrine disruption activity. Given the high degree of endocrine system conservation across the vertebrates, adverse endocrine-linked effects in the RADAR assay may also indicate the possibility of related activity in other organisms such as amphibians, reptiles, birds or mammals. It is also possible that no

existing endocrine-relevant data are available (i.e. RADAR has been used as a primary screen), but in that case a positive result in the screen should ideally be followed up with relevant *in vitro* screening in an attempt to confirm the precise mode of action (MOA). Furthermore, a positive RADAR result would also need to be followed up with an additional *in vivo* fish test such as the Fish Short-Term Reproduction Assay (FSTRA – OECD TG 229) or Fish Sexual Development Test (FSDT – OECD TG 234), which will give some indication of any adverse apical effects. Possible conclusions to be derived from the results of RADAR, and guidance about potential additional studies to strengthen weight of evidence, are summarised in Table C.2.24.

637. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an endocrine disruptor (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 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 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

638. Existing information on endocrine-related effects from other vertebrates (up to and including mammals, e.g. positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies) should always be considered, given the commonality of endocrine mechanisms in these taxa. Existing data available before deployment of RADAR might include *in vivo* results obtained with other vertebrates (e.g. a Hershberger Bioassay with rodents – OECD TG 441), or one or more of a range of *in silico* or *in vitro* results which suggest that androgenicity or anti-androgenicity 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 data, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen for androgen receptor-mediated activity, or for effects on androgen biosynthesis.

639. It should be noted that a sensitive *in vivo* assay for anti-androgenicity is already available, the AFSS (OECD GD 148). This is longer than RADAR (21 day), and relies on the pre-treatment of adult female sticklebacks (*Gasterosteus aculeatus*) with an androgen before measuring anti-androgenic effects of the test chemical (reduction in induced spiggin glue protein).

Scenarios: Positive and negative results combined with existing data

640. The scenarios (A to R) presented in Table C.2.24 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.

641. Positive results obtained with the fluorescence endpoint (Table C.2.24, Scenarios A-I) result in the conclusion that the test chemical is a possible androgen or anti-androgen *in vivo*. This would ideally need to be followed up with more comprehensive testing to show whether adverse apical effects related to endocrine impacts occur at any part of the life cycle (and hence to discover whether the chemical is an ED acting through certain estrogen/androgen/thyroid/steroidogenesis [E,A,T,S] pathways). In other words, a positive result in the RADAR assay may trigger OECD TG 234 (FSDT) at Level 4 or fish life cycle testing (e.g. Medaka Extended One-Generation Reproduction Test [MEOGRT] – OECD TG 240) at Level 5. Existing data suggesting androgenic or anti-androgenic activity will strengthen the case for additional testing.

642. The situation in which the RADAR assay gives a negative result (Table C.2.24, Scenarios J-R) needs careful consideration of any existing data. If the weight of evidence of these data suggests that the chemical is endocrine active both *in vitro* and *in vivo* in other species (Scenario J), then the probability is that RADAR may simply be insufficiently responsive in that case, or fish in general may be unresponsive. For example, this might be the case if the medaka embryos have not transformed a chemical to an active metabolite. In some of these circumstances, it might be appropriate to conduct an FSDT (OECD TG 234), or alternatively, a fish life cycle test (e.g. MEOGRT OECD TG 240) to confirm that there is no endocrine activity in fish.

643. If the RADAR 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 may or may not be necessary. If the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests are not of sufficient duration, in which case longer term testing might be justified.

644. On the other hand, if the RADAR and the *in vitro* tests are negative, but there are positive existing *in vivo* data (Scenario M), the chemical is probably not a potential ED with androgenic or anti-androgenic activity, but it may act via androgen-related 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 relevant existing *in vitro* and *in vivo* data should be used to guide decisions about whether to conduct any further testing, including life stages represented in OECD TG 234 (FSDT) or TG 240 (MEOGRT).

645. Finally, a negative RADAR, set against a background of negative *in vitro* and *in vivo* data (Scenario N) that includes relevant *in vivo* data for fish, suggests that the test chemical is not a potential ED in fish or other vertebrates, and no further testing for androgenic or anti-androgenic MOA will generally be necessary.

646. 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 RADAR, and this is reflected in Table C.2.24. However, a lack of mechanistic data on endocrine activity should usually be rectified before any further *in vivo* testing is finally decided on. Indeed, as a general principle, it is desirable to obtain mechanistic data before any *in vivo* testing. On the other hand, if RADAR is positive, further *in vivo* testing is generally indicated, particularly when existing data are equivocal, or if there are no existing data. 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. androgenic and anti-androgenic) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA (e.g. anti-steroidogenic and androgenic) could potentially reinforce effects on the RADAR. 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.

647. The scenario in which the results of the RADAR are themselves equivocal has not been dealt with in Table C.2.24, for reasons of brevity. In this context, an equivocal result might be an inconsistent 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. If false negatives (e.g. systemic toxicity) are suspected with good reason, the screen could be repeated if none of the test concentrations have given reliable data (e.g. conduct it at lower concentrations which avoid systemic toxicity). However, note that a repeat test in the event of systemic toxicity would not be needed providing at least one tested concentration was not subject to such effects.

648. In summary, positive results in the RADAR assay indicate that a chemical is a possible endocrine disrupter. More predictive *in vivo* testing would then be necessary to produce a long-term no-observed-effect-concentration/x% effect concentration (NOEC/ECx) and/or to confirm whether or not the chemical is an actual endocrine disrupter with adverse effects *in vivo*. Negative results in the RADAR do not necessarily mean that the chemical is not a potential ED – a judgement about its endocrine disruption potential and the possible need for additional testing will have to be made based on a weight of evidence evaluation of existing *in vitro* and *in vivo* data.

References

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Table C.2.24. **RADAR: Rapid Androgen Disruption Adverse Outcome Reporter Assay (draft OECD TG): 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. Note that there are no apical endpoints in this assay considered to be diagnostic of an estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modality.

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.

Scenarios	Result of RADAR	Existing results		Possible conclusions	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	+	+	+	Strong evidence for *in vivo* androgenic or anti-androgenic activity in fish and other organisms.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental no-observed-effect-concentration/x% effect concentration (NOEC/ECx).	An alternative approach would be to deploy OECD TG 234 (Fish Sexual Development Test), especially if sexual development is expected to give a response at lower concentrations than reproduction.
B	+	+	–	Strong evidence for *in vivo* androgenic or anti-androgenic activity in fish, despite lack of *in vivo* effects in existing tests.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	An alternative approach would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.
C	+	+	Eq/0**	Strong evidence for *in vivo* androgenic or anti-androgenic activity in fish, despite equivocal or absent *in vivo* data in other species.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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.
D	+	–	+	Strong evidence for *in vivo* androgenic or anti-androgenic activity in fish and other species, but confidence about MOA is reduced by negative mechanistic data.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo*, or it may operate via mechanisms not covered by the *in vitro* screens. An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction.
E	+	–	–	Moderate-strong evidence for *in vivo* androgenic or anti-androgenic activity in fish, but confidence is reduced by negative *in vitro* data and negative *in vivo* activity in other species.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo*, or it may operate via mechanisms not covered by the *in vitro* screens. An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.

Scenarios	Result of RADAR	Existing results		Possible conclusions	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	Moderate-strong evidence for *in vivo* androgenic or anti-androgenic activity in fish, but confidence is reduced by negative *in vitro* data and equivocal or absent *in vivo* activity in other species.	Consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo*, or it may operate via mechanisms not covered by the *in vitro* screens. If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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	+	Strong evidence for *in vivo* androgenic or anti-androgenic activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240).	An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. 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.
H	+	Eq/0	–	Strong-moderate evidence for *in vivo* androgenic or anti-androgenic activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240).	An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before possibly conducting a life cycle test. 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	Moderate evidence for *in vivo* endocrine activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (e.g. MEOGRT – OECD TG 240).	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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.

Scenarios	Result of RADAR	Existing results		Possible conclusions	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	–	+	+	Based on the existing data, the chemical has androgenic or anti-androgenic activity *in vivo*. The lack of response in RADAR suggests that fish are not responsive, unless the existing data are from fish.	Consider performing OECD TG 234 (FSDT).	It is possible that the failure to give a positive result in RADAR was caused by the relatively short exposure time (up to six days). If this is suspected (e.g. the chemical only bioaccumulates slowly), or if the existing *in vivo* data are from a fish, OECD TG 234 (FSDT) or potentially a life cycle test (e.g. OECD TG 240 – MEOGRT) would be able to study the effects of longer exposure and confirm whether there is a hazard to fish. Choice of test should be guided by the existing *in vivo* data.
K	–	+	–	There is no evidence that the chemical is a possible androgenic or anti-androgenic ED *in vivo*, probably because it is very weakly acting or rapidly metabolised.	Probably no further action, but see comments in right-hand column.	It is possible that EDs which bioaccumulate slowly may only cause effects *in vivo* after exposure times longer than 28 days. If this is suspected, and depending on which part of the life cycle is suspected of being the most sensitive, consider performing OECD TG 234 (FSDT).
L	–	+	Eq/0	The chemical may not be an androgenic or anti-androgenic ED *in vivo*, but the confidence in this conclusion is relatively low as there is only one unequivocal *in vivo* test result (a negative).	Probably no further action, but see comments in right-hand column.	If the equivocal existing data are from a fish assay, consider performing a fish assay (e.g. OECD TG 229 or TG 230) with a different species, or a longer term test (e.g. OECD TG 234 [FSDT] or life cycle test [MEOGRT – TG 240]) if the chemical is a slow bioaccumulator. 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.
M	–	–	+	The chemical is apparently not a possible androgenic or anti-androgenic ED in fish but it does have activity in another species.	Use the existing *in vivo* data to help decide whether a longer term test with an appropriate fish species is indicated.	Use the existing *in vivo* data to guide any further testing.
N	–	–	–	The chemical is probably not a possible androgenic or anti-androgenic ED *in vivo*.	No further action with respect to androgenic or anti-androgenic MOA.	
O	–	–	Eq/0	The chemical is probably not a possible androgenic or anti-androgenic ED in fish.	Probably no further action. However, see comments in right-hand column.	If the paucity of *in vivo* data is a concern, performance of a screening test (OECD TG 229 or TG 230) with a different species, or a longer term test (i.e. OECD TG 234 [FSDT]) or life cycle test [MEOGRT]) could be considered. 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.

Scenarios	Result of RADAR	Existing results		Possible conclusions	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 a possible androgenic or anti-androgenic ED in fish, but confidence in this conclusion is low given the lack of mechanistic *in vitro* data and the availability of positive existing *in vivo* data.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential androgenic or anti-androgenic action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (OECD TG 234 [FSDT] or life cycle test [MEOGRT]). Use the existing *in vivo* data as a guide to test choice. 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 a possible androgenic or anti-androgenic ED in fish, but the lack of mechanistic *in vitro* data are a concern, even though the existing *in vivo* data are negative.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential endocrine action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (OECD TG 234 [FSDT] or life cycle test [MEOGRT]). Use the existing *in vivo* data as a guide to test choice. 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 is probably not a possible androgenic or anti-androgenic ED in fish, but confidence in this conclusion is low given the lack of mechanistic *in vitro* and existing *in vivo* data.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential androgenic or anti-androgenic action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (OECD TG 234 [FSDT] or life cycle test [MEOGRT]). Use the existing *in vivo* data as a guide to test choice. 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.