C.2.10. Larval Amphibian Growth and Development Assay (LAGDA) (OECD TG 241)

Status: Assay validated by the OECD.

422. Modality detected/endpoints: OECD TG 241 has three endpoints indicating generalised toxicity (mortality, abnormal behaviour and growth), and several providing specific information about endocrine disruption or impaired reproduction (histopathology of thyroid, gonads, kidney and liver, time to metamorphosis [NF stage 62]; secondary sex characteristics (nuptial pads); vitellogenin (optional); genetic and phenotypic sex ratio). Most of these specific endocrine endpoints are likely to respond to interference with the hypothalamic/pituitary/gonadal (HPG) axis, while thyroid histopathology and time to metamorphosis may respond to interference with the hypothalamic/pituitary/thyroid axis (as may the "generalised toxicity" indicator, growth).

Background to the assay

423. This assay is a partial life cycle test with the clawed frog *Xenopus laevis*. The LAGDA was performed adequately to evaluate apical effects of chronic exposure to two endocrine-active compounds (Haselman et al., 2016a; 2016b). It starts with NF stage 8 F0 larvae and ends 10 weeks after the median time that controls take to reach NF stage 62 F0 juveniles (typically a total of 16 weeks). In essence, therefore, it covers the stages of larval/juvenile growth and sexual development, but not those of reproduction and embryonic development. It could therefore be thought of as the amphibian near-equivalent of the Fish Sexual Development Test (FSDT – OECD TG 234), although it also includes endpoints that are specifically responsive to thyroid disrupters. It does not include all processes which may respond to estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) endocrine disruptors (EDs) (especially reproduction), and it is currently unknown whether the LAGDA is therefore less responsive to some of these chemicals than an amphibian life cycle test (a standardised protocol which is not available). It may ultimately be concluded that a fish life cycle test may be a more suitable test than the LAGDA or an amphibian life cycle test for long-term evaluation of chemicals with E,A,S properties despite the fact that the LAGDA can detect certain estrogenic active substances. Due to the LAGDA's higher sensitivity towards thyroid (T) properties, this test may be the preferred testing choice for confirming T properties.

424. OECD TG 241 provides a table of endpoints (test guidelines Table 1), some of which are "apical", while others should more properly be considered as indicators of hormonal activity. Probably the only true apical endpoints which could be used for hazard identification/characterisation (because they can be related directly to adverse effects on populations) are mortality, growth and phenotypic/genotypic sex ratio. The latter two are likely to be responsive to some EDs, but growth may also respond to certain other chemicals. On the other hand, indicators of hormonal activity of use in diagnosing the effects of EDs include gonad and thyroid histopathology, liver-somatic index, time to metamorphosis, and vitellogenin (VTG). Time to metamorphosis can also arguably be

considered as an apical endpoint with potential implications at the population level. The endpoints will be grouped in this way for the purposes of this document.

425. Consequently, if the assay gives a positive result, this may be due to a combination of a positive indicator of hormonal activity (gonad and thyroid histopathology, liver-somatic index, time to metamorphosis, and vitellogenin) and a positive apical endpoint (sex ratio and possibly growth), or a positive for an indicator of hormonal activity alone, or for an apical endpoint alone. Each of these possible combinations of positive response should be considered separately (although the distinctions between indicators of hormonal activity and apical effects are not always clear), so they have been listed individually as points 1, 2 and 3 in the possible conclusions column of Table C.2.10. Given the high degree of endocrine system conservation across the vertebrates, adverse endocrine-linked effects in the LAGDA may also indicate the possibility of related activity in other organisms such as fish, reptiles, birds or mammals.

When/why the assay may be used

426. Although the LAGDA could, in principle, be used at any stage in the hazard assessment process, the most likely use scenario will be when there are some data available about the possible thyroid disrupting properties of a chemical, or if the chemical is suspected of having (anti)estrogenic or (anti)androgenic properties. Thus, there are likely to be data available from *in vitro* mechanistic screens, as well as *in vivo* non-mammalian wildlife screens such as OECD TG 229, TG 230, TG 231, *Xenopus* Embryonic Thyroid Signalling Assay (XETA) or EASZY. Furthermore, a number of mammalian (rat) assays (which may have been performed before any non-mammalian wildlife testing) are sensitive to thyroid disruption, including the pubertal assay (male or female), the enhanced repeat dose assay (OECD TG 407), and the intact male screening assay. Rodent screening assays (e.g. the Hershberger or Uterotrophic Bioassays) with responsiveness to other EDs (e.g. androgens or estrogens) may also have been conducted.

427. It is unlikely that no endocrine-relevant data will available before the LAGDA is deployed (i.e. the LAGDA has been used as a primary screen), but in that case a positive result in the LAGDA could be followed up with relevant *in vitro* assays to investigate the suspected mode of action (MOA). However, it should be noted that while *in vitro* assays are available for estrogens, androgens and steroidogenesis inhibitors, they additionally exist only 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), while thyroid disruption can occur at other points in the endocrine system for which *in vitro* assays do not exist, or are still at the research stage (e.g. FRTL-5 rat cell lines sensitive to iodide uptake inhibitors). Furthermore, none of these *in vitro* thyroid assays have yet been validated and standardised at the international level, although several are in development.

428. 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

429. Given the commonality of endocrine mechanisms in the vertebrates, relevant existing data available before deployment of the LAGDA might include *in vivo* results obtained with other vertebrates (e.g. a positive *in vivo* assay with rats or fish – see above), or one or more of a range of *in silico* or *in vitro* results which suggest that estrogenic, androgenic or thyroid disruption may occur *in vivo* (but note the limitations of this approach for thyroid disrupters, as indicated above). Such indicators of possible endocrine activity might include quantitative structure activity relationship (QSAR) predictions, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen. Further strong indication of *in vivo* estrogenic activity may also be available from an EASZY Assay with transgenic zebrafish embryos, and evidence for thyroid activity could additionally be available from a *Xenopus* Embryonic Thyroid Signalling Assay (XETA).

Scenarios: Positive and negative results combined with existing data

430. The scenarios (A to R) presented in Table C.2.10 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.

431. Positive results obtained with an indicator of hormonal activity in the LAGDA but not with apical endpoints (Table C.2.10, Scenarios A-I, sub-section 2) result in the conclusion that the test chemical is probably a potential ED *in vivo*. If both an indicator of hormonal activity and an apical endpoint give a response (Table C.2.10, Scenarios A-I, sub-section 1), this provides evidence that one is dealing with an actual ED with adverse effects *in vivo* if adverse population effects are expected as a consequence. If only an apical endpoint responds (Table C.2.10, Scenarios A-I, sub-section 3), it suggests that the chemical is harmful to growth or sexual development, but is not necessarily an ED (although existing positive *in vitro* data, or positive *in vivo* data from other species, would have to be weighed against this conclusion).

432. The situation in which a LAGDA gives a negative result (Table C.2.10, 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 LAGDA is simply insufficiently sensitive (perhaps because it does not include reproduction).

Depending on the robustness of the existing data, it might therefore be appropriate to conduct an amphibian life cycle test, although a protocol for one has not been standardised or validated.

433. If the LAGDA 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 effects *in vivo* in amphibians or other organisms, or it may be rapidly metabolised. In such a situation, further testing is probably not necessary.

434. On the other hand, if the LAGDA 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 probably not an ED acting on amphibian growth or development, but it may act via MOA not covered by the *in vitro* screens, or it may be more potent in species or life stages that have not been tested. In this situation, the existing *in vivo* data should be used to guide decisions about whether to conduct any further testing.

435. Finally, a negative LAGDA, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is not a possible E,A,T,S ED, and further action is unnecessary.

436. 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 LAGDA, and this is reflected in Table C.2.10. However, a lack of *in vitro* mechanistic data should ideally be rectified before any further *in vivo* testing is finally rejected, although as indicated above, many thyroid modalities are not detectable in *in vitro* screens. On the other hand, if the LAGDA is positive, further *in vivo* testing would not generally be needed unless it is suspected that the chemical acts primarily on reproduction. Again, however, it may be useful to obtain some mechanistic information before conducting further *in vivo* testing, although note that a validated amphibian life cycle protocol is unavailable. A possible substitute for the latter might be a fish life cycle test (either the MEOGRT or ZEOGRT), although the responsiveness of such a procedure to thyroid disrupters is unknown. 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.

437. The scenario in which the results of a LAGDA are themselves equivocal has not been dealt with in Table C.2.10, 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, thyroid histopathology at a high concentration might be masked by any systemic toxicity, while growth measurements might just fail to reach a statistically significant level due to unexpectedly high variability. If these or other possible reasons for false negatives are suspected with good reason, the test could be repeated (e.g. conduct it at lower

concentrations which avoid systemic toxicity), or a more appropriate version of it (e.g. more larvae per replicate) could be designed and conducted.

438. In summary, positive indicators of hormonal activity in the LAGDA indicate that a chemical is a potential ED via one of several modalities, while a combination of positive indicators of hormonal activity and positive apical results suggest that it is an actual ED (especially if the two types of response are causally related). However, if an apical endpoint alone responds, the chemical may not be an ED (although existing data may help to inform this decision). Negative results in the LAGDA do not necessarily mean that the chemical is not an ED – a judgement about possible endocrine disruption and the possible need for additional testing will have to be made in the light of existing *in vitro* and *in vivo* data.

References

Haselman, J.T. et al. (2016a), "Development of the Larval Amphibian Growth and Development Assay: Effects of chronic 4-tert-octylphenol or 17β-trenbolone exposure in *Xenopus laevis* from embryo to juvenile", *Journal of Applied Toxicology*, Vol. 36/12, pp. 1639-1650, https://doi.org/10.1002/jat.3330.

Haselman, J.T. et al. (2016b), "Development of the Larval Amphibian Growth and Development Assay: Effects of benzophenone-2 exposure in *Xenopus laevis* from embryo to juvenile", *Journal of Applied Toxicology*, Vol. 36/12, pp. 1651-1661, https://doi.org/10.1002/jat.3336.

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.2.10. **Larval Amphibian Growth and Development Assay (LAGDA) (OECD TG 241): 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.

The assay under discussion could either be positive for both apical endpoints and indicators of endocrine activity, or positive just for an apical endpoints or indicators of endocrine activity. For each scenario, each of these three possibilities is addressed separately in the possible conclusions column.

Scenarios	Result of LAGDA	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive 3) Apical endpoint 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 *in vivo* endocrine activity with adverse effects (on growth or sexual development) in amphibians, and effects in other species. 2) Strong evidence for *in vivo* endocrine activity in amphibians and other species. 3) Strong evidence for adverse effects on growth or sexual development in amphibians, and effects in other species, but possibly not via an endocrine mechanism in the case of growth.	Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The Larval Amphibian Growth and Development Assay (LAGDA) does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information.
B	+	+	–	1) Strong evidence for *in vivo* endocrine activity with adverse effects (on growth or sexual development) in amphibians. 2) Strong evidence for *in vivo* endocrine activity in amphibians. 3) Strong evidence for adverse effects on growth or sexual development in amphibians, but possibly not via an endocrine mechanism in the case of growth.	Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information.
C	+	+	Eq/0	1) Strong evidence for *in vivo* endocrine activity with adverse effects (on growth or sexual development) in amphibians. 2) Strong evidence for *in vivo* endocrine activity in amphibians. 3) Strong evidence for adverse effects on growth or sexual development in amphibians, but possibly not via an endocrine mechanism in the case of growth.	Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information. 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) Moderate evidence for *in vivo* endocrine activity with adverse effects (on growth or sexual development) in amphibians and other species, but possibly not via an estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) mechanism. 2) Moderate evidence for *in vivo* endocrine activity in amphibians and other species, but possibly not via an E,A,T,S mechanism. 3) Strong evidence for adverse effects on growth or sexual development in amphibians and other species, but probably not via an endocrine mechanism in the case of growth.	Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information.

Scenarios	Result of LAGDA	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive 3) Apical endpoint 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)**			
E	+	–	–	1) Strong evidence for *in vivo* endocrine activity with adverse effects (on growth or sexual development) in amphibians, but possibly not via an E,A,T,S mechanism. 2) Strong evidence for *in vivo* endocrine activity in amphibians, but possibly not via an E,A,T,S mechanism. 3) Strong evidence for adverse effects on growth or sexual development in amphibians, but probably not via an endocrine mechanism in the case of growth.	Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column..	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information.
F	+	–	Eq/0	1) Strong evidence for *in vivo* endocrine activity with adverse effects (on growth or sexual development) in amphibians, but possibly not via an E,A,T,S mechanism. 2) Strong evidence for *in vivo* endocrine activity in amphibians, but possibly not via an E,A,T,S mechanism. 3) Strong evidence for adverse effects on growth or sexual development in amphibians, but probably not via an endocrine mechanism in the case of growth.	Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information.. 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 *in vivo* endocrine activity with adverse effects (on growth or sexual development) in amphibians and other species, but possibly not via an E,A,T,S mechanism. 2) Strong evidence for *in vivo* endocrine activity in amphibians and other species, but possibly not via an E,A,T,S mechanism. 3) Strong evidence for adverse effects on growth or sexual development in amphibians and other species, but probably not via an endocrine mechanism in the case of growth.	It would be desirable to obtain some unequivocal mechanistic data to confirm whether or not an E,A,T,S mechanism is operating. Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information. 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 LAGDA	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive 3) Apical endpoint positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
H	+	Eq/0	–	1) Strong evidence for *in vivo* endocrine activity with adverse effects (on growth or sexual development) in amphibians, but possibly not via an E,A,T,S mechanism. 2) Strong evidence for *in vivo* endocrine activity in amphibians, but possibly not via an E,A,T,S mechanism. 3) Strong evidence for adverse effects on growth or sexual development in amphibians, but possibly not via an endocrine mechanism in the case of growth.	It would be desirable to obtain some unequivocal mechanistic data to confirm whether or not an E,A,T,S mechanism is operating. Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information. 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 *in vivo* endocrine activity with adverse effects (on growth or sexual development) in amphibians, but possibly not via an E,A,T,S mechanism. 2) Strong evidence for *in vivo* endocrine activity in amphibians, but possibly not via an E,A,T,S mechanism. 3) Strong evidence for adverse effects on growth or sexual development in amphibians, but possibly not via an endocrine mechanism in the case of growth.	It would be desirable to obtain some unequivocal mechanistic data to confirm whether or not an E,A,T,S mechanism is operating. Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information. 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 has E,A,T,S activity in other species but not apparently in amphibians, although it is possible that *Xenopus laevis* has responded atypically in this case (e.g. if *X. laevis* responded positively in OECD TG 231).	Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available, although a fish life cycle test (MEOGRT) could provide useful information.
K	–	+	–	The test chemical has E,A,T,S activity *in vitro*, but no apparent activity *in vivo* in amphibians or other species, possibly due to quick degradation/metabolism or failure to reach the active site.	Regulatory authorities may consider that further testing is unnecessary.	–

Scenarios	Result of LAGDA	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive 3) Apical endpoint 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)**			
L	–	+	Eq/0	The test chemical has E,A,T,S activity *in vitro*, but no apparent activity *in vivo* in amphibians, possibly due to quick degradation/metabolism or failure to reach the active site.	Regulatory authorities may consider that further testing is unnecessary, but see right-hand column.	Given the presence of E,A,T,S activity *in vitro*, and the absence of reliable *in vivo* data from other species, it might be desirable to run an *in vivo* endocrine screen with fish or mammals. 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 does not apparently have E,A,T,S activity in amphibians, but endocrine activity is present in other species.	Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently available.
N	–	–	–	The test chemical does not have E,A,T,S activity in amphibians or other species.	No further action is necessary.	–
O	–	–	Eq/0	The test chemical does not have E,A,T,S activity in amphibians.	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.
P	–	Eq/0	+	The test chemical probably does not have E,A,T,S activity in amphibians, but the uncertain mechanistic data and the presence of endocrine activity in other species reduces confidence in this conclusion. It is possible that *Xenopus laevis* has responded atypically in this case (e.g. if *X. laevis* responded positively in OECD TG 231).	Regulatory authorities may consider that further data from amphibians are not required. However, see right-hand column. Also, if clear *in vitro* mechanistic data are missing, it might be desirable to obtain some.	The LAGDA does not cover the reproductive phase of the life cycle, but a life cycle test which could be used to address any concerns about reproduction is not currently 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.

Scenarios	Result of LAGDA	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive 3) Apical endpoint 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)**			
Q	–	Eq/0	–	The test chemical is probably without endocrine activity in amphibians or other taxa, but this conclusion is tentative given the lack of supporting mechanistic data.	If clear *in vitro* mechanistic data are missing, it might 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.
R	–	Eq/0	Eq/0	The test chemical is probably without endocrine activity in amphibians, but this conclusion is tentative given the lack of supporting data.	Some regulatory authorities may conclude that no further evidence is required, but see right-hand column.	If clear *in vitro* mechanistic data are missing, it may be desirable to obtain some. If these data reveal E,A,T,S activity, it might then be desirable to conduct a fish or rodent screen. 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.