C.3.13. Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents (OECD TG 409)

Status: Assay validated by the OECD.

921. Modalities detected: (anti)estrogen, (anti)androgen, thyroid, steroidogenesis.

Endpoints: Weight of adrenals, testes, epididymides, uterus, ovaries, thyroid.

Histopathologic changes in pituitary, thyroid gland, gonads, uterus, accessory sex organs, female mammary gland, testes and adrenals.

Background to the assay

922. This assay determines the general toxicity of chemicals in non-rodents after 90 days of oral dosing (by gavage, via the diet, in drinking water or in capsules). The most commonly used non-rodent species is the dog, which should be of a defined breed (beagle usually). It provides information on major toxic effects and target organ toxicity likely to arise from the post-weaning period until well into adulthood. OECD TG 409 was adopted in September 1981 and revised in September 1998.

923. Although it has not been validated for the detection of endocrine active substances (EASs), OECD TG 409 contains many endpoints that are suitable for the determination of endocrine effects. The well-conserved nature of many endocrine receptors and systems means that many endpoints validated in rat studies will have similar responses in non-rodent species used in OECD TG 409. A comparison can be made with validation of the OECD TG 407 (28-Day Oral Toxicity Study) for endocrine endpoints where substances that were moderate and strong endocrine disruptors (EDs) for (anti)estrogenicity and (anti)androgenicity (e.g. ethinylestradiol and flutamide) and weak and strong modulators of thyroid hormone-related effects (e.g. propylthiouracil, T4 and methyl testosterone) were detected (OECD, 2006). Steroidogenesis inhibition was also detected, although only one (potent) chemical was used in the validation study (CGS 18320B). OECD TG 409 may be more sensitive than OECD TG 407 because of the extended dosing period, although the number of animals per group is similar (at least four male and four female, compared with five per sex per group in OECD TG 407). OECD TG 409, however, does not contain some endocrine-sensitive endpoints (e.g. thyroid hormones, estrous cyclicity) that may be included in OECD TG 407. As all the endpoints are apical, it is difficult to discern mechanism of action from this test alone. Information on mechanism of action may need to be obtained from *in vitro* estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) assays or *in vivo* lower tier tests such as the Uterotrophic Bioassay (UT – OECD TG 440) and Hershberger Bioassay (H – OECD TG 441). Possible species differences in response, physiological differences and species differences in test substance metabolism should also be considered.

When/why the assay may be used

924. This assay is likely to be used as part of a pesticide submission package and may form part of the standard information requirements in certain chemical legislations (e.g. Plant Protection Product Regulations in the European Union). At least three dose levels are included so that an estimate of no-adverse-effect-level can be determined and the assay used for hazard identification/characterisation. It should be noted that as this assay is not primarily designed to detect endocrine disruption, a higher degree of systemic toxicity is typically induced than is the case with the other Level 3 and 4 assays. The possibly confounding effect of systemic toxicity on endocrine endpoints therefore needs to be considered.

925. 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 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).

Introduction to the table of scenarios

926. Table C.3.13 gives guidance on a further step to take in the event of a positive (+) or negative (-) result and in the presence of positive (+), negative (-) or equivocal/absent (Eq/0) existing results. “Existing results” are subdivided into “mechanism” and “effects” data (third and fourth columns). The table is divided horizontally into a series of scenarios that represent all the combinations of these events.

927. The results of OECD TG 409 are given in the second column. As OECD TG 409 is not a screening test where a yes/no (qualitative) answer is obtained for the test as a whole, positive results would generally be assessed for individual endpoints. For the purposes of this guidance, however, a positive result is defined as a biologically significant change in any of the endocrine endpoints listed above (e.g. statistically significant reductions in reproductive organ weights). Changes in related endpoints will increase their biological significance (e.g. changes in the weights of testes and epididymides accompanied by histopathological changes). The guidance on histopathologic changes in endocrine tests (OECD, 2009) may be helpful in interpretation. A negative result for OECD TG 409 is taken to be the absence of biologically significant changes in all endocrine endpoints.

928. In the absence of other pertinent lines of evidence, negative results in this test alone cannot be taken as evidence that the substance is not an ED. Further studies will be required as confirmation.

929. Equivocal results for the guideline are not considered in the table, partly for brevity but also because equivocal results are by nature uncertain. A decision must eventually be reached about whether the endocrine endpoints tend to be positive or negative or whether the result must be put to one side and the test repeated (using the same or a different test guideline). Factors which may have interfered with the result (e.g. composition of the diet used, environmental influences) should be considered.

Existing data to be considered

930. Existing "mechanism" *in vitro* data are assumed to be available from estrogen receptor (ER-), androgen receptor (AR-) and steroidogenesis-based assays (Level 2). Assays may also be available for interference with thyroid modalities. In practice, it is possible that data from all of these assays may not be available, so judgement will need to be used to decide which assays to perform. Although the current *in vitro* test guidelines do not incorporate metabolic activation, published information on use of metabolic activation systems is available in Jacobs et al. (2008; 2013) and OECD (2008). These methods, however, have not yet been validated.

931. Existing "effects" data refer to *in vivo* effects that may come from Level 3 or 4 tests in the Conceptual Framework (e.g. UT or H assays). In these cases, it should be remembered that these assays are specifically designed to be sensitive to EDs. As mentioned above, the results of the study may be interpreted as part of a battery or group of tests carried out for regulatory purposes. Data may also be available on effects in mammalian and non-mammalian wildlife species, although caution should be used when extrapolating between taxa. A chemical causing endocrine effects in non-mammalian environmental species (fish, for example) may also have endocrine effects in mammals, but the physiological consequences of the effects are likely to be different.

932. When considering the results of the OECD TG 409 assay, all available data should be used in order to reach a conclusion and a weight of evidence approach taken. This may include high throughput screening data, read-across data from structural analogues and quantitative structure activity relationship (QSAR). Several QSAR models for estrogen and androgen binding/activation are now available (see Sections B.1.1.1 and B.1.1.2).

Scenarios: Positive and negative results combined with existing data

933. A series of scenarios (A to R) are presented in Table C.3.13 and 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. Although the OECD TG 409 assay uses mammals, the well-conserved nature of the hormonal pathways across taxa indicate that results on endocrine endpoints in this assay may be relevant to other vertebrate species. Effects in laboratory mammal tests are also highly relevant for environmental mammalian species. 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. The sensitivity and physiological function of the hormone under investigation in the test species should also be considered. As OECD TG 409 is a non-rodent assay, it provides insight into endocrine effects across species. In general, lower level tests should be conducted before higher level tests in order to avoid unnecessary animal usage, unless it is apparent that a Level 5 test will be required anyway or will be needed to establish the

evidence to conclude on ED properties. Information on some endocrine-related tumours may be detected more comprehensively in carcinogenicity studies (OECD TG 451/453) (Level 4); for example, detection of certain types of thyroid tumors in the absence of reproductive or developmental effects, as well as substances causing tumors in other endocrine-sensitive tissues. At Level 5, the Extended One-Generation Reproduction Toxiticy Study (EOGRTS – OECD TG 443) is the most sensitive reproduction assay for detecting endocrine disruption because it includes evaluation of a number of endocrine endpoints not included in the two-generation study (OECD TG 416) adopted in 2001. It is recognised, however, that some jurisdictions may require a two-generation study. Further considerations specific to each scenario are given in the table.

934. Scenarios A to C represent positive results in the OECD TG 409 assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in the *in vitro* assays in combination with a positive OECD TG 409 assay is moderate or strong evidence for E,A,T,S-mediated activity that may or may not be supported by the *in vivo* effects data. OECD TG 409 uses non-rodents and therefore a positive result in non-rodent species in combination with positive rodent data indicates a higher level of concern for human health. In the absence of robust upper-level data, the next step may be to conduct an upper-level test. In the presence of robust *in vivo* data, there may be sufficient evidence to conclude concern for endocrine disruption and therefore no need for further testing. Positive results in the OECD TG 409 assay may also indicate the potential for endocrine mediated effects in lower vertebrates. These could be followed up with partial life cycle tests such as the Fish Sexual Development Test (FSDT), the Larval Amphibian Growth and Development Assay (LAGDA) or the Medaka Extended One-Generation Reproduction Test (MEOGRT) if the evidence were strong enough. *In vivo* assays/tests with negative results should be interpreted with caution as they may either indicate that the tests used do not have sufficient power to detect weak effects or, alternatively, that the effects do not present a concern for endocrine disruption. There may also be species differences in the effects on the endocrine system. The possibility of other (non-E,A,T,S) mechanisms should also not be overlooked (e.g. involving other receptors or endocrine axes).

935. Scenarios D to F represent positive results in the OECD TG 409 assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Negative results in the *in vitro* assays should be viewed with caution in case a metabolite is responsible for the positive OECD TG 409 assay. Unless the metabolic profile of the test substance is known, one option may be to conduct these assays with an added metabolising system. If the metabolic profile is known, then a higher level *in vivo* test may be advisable. The choice of tests will depend on the available *in vivo* effects data. Positive results in the OECD TG 408 assay may also indicate the potential for endocrine mediated effects in lower vertebrates. As in Scenarios A to C, *in vivo* assays/tests with negative results should be interpreted with caution as they may either indicate that the tests used do not have sufficient power to detect weak effects or, alternatively, that the effects do not present a concern for endocrine disruption. There may also be species differences in the effects on the endocrine system.

936. Scenarios G to I represent positive results in the OECD TG 409 assay in the presence of various combinations of missing or equivocal data. Positive results in the OECD TG 409 assay may also indicate the potential for endocrine mediated effects in lower vertebrates. The next step to take in these eventualities will depend on the nature of the other available data and the jurisdiction in which it is being used. In some cases, equivocal data may be viewed as positive whilst in others it may or may not contribute to the weight

of evidence. The interpretation may also depend on the mode of action (MOA) in question and why the data are considered equivocal, e.g. a study that is equivocal for thyroid effects may still be of value in evaluating (anti)androgenic effects. In all three scenarios, the recommended first step is to obtain reliable mechanistic (*in vitro*) data rather than proceed further with *in vivo* testing. Equivocal and missing data are alternative scenarios and two possibilities for the next step are given in most cases, but the nature of equivocal data means that decisions need to be taken on a case-by-case basis. As OECD TG 409 is a non-rodent assay, the possibility of species differences in response should be considered. In all cases, the role of metabolism, route of exposure and data from structural analogues should be considered before deciding on the next step.

937. Scenarios J to L represent negative results in the OECD TG 409 assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Negative outcomes in OECD TG 409 should be viewed with caution because of the power of the assay to detect (anti)estrogens and androgens may be limited. All three scenarios could also arise from a chemical that is positive in *in vitro* assays, but is metabolised to a non-active metabolite leading to negative results in the OECD TG 409 assay. This should be considered first when investigating the next step. Endocrine active potency may also explain differences between *in vitro* and *in vivo* results (e.g. a weak chemical may give a positive result *in vitro* but may be negative *in vivo*). Species differences may also account for a negative result in the non-rodent assay and positive *in vivo* effects data in rodents. Positive *in vivo* effects data may involve other E,A,T,S, non-E,A,T,S mechanisms (e.g. involving other receptors or endocrine axes), more sensitive endpoints, greater statistical power or life stages that are more sensitive to the substance than the young adult exposed animals in OECD TG 408.

938. Scenarios M to O represent negative results in the OECD TG 409 assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Negative results for all tests (Scenario N) may be sufficient to enable a conclusion of no concern for endocrine disruption. OECD TG 409 uses non-rodents and therefore a negative result in non-rodent species in combination with negative rodent data may allow more confidence in a conclusion of no concern for human health. This will depend on the weight of evidence and may not be possible. Where there are positive *in vivo* effects data, there could still be an E,A,T,S-related mechanism, the effects may be related to length of exposure, route of exposure, exposure at different life stages or species differences. Other E,A,T,S or non-E,A,T,S mechanisms may also be involved.

939. Scenarios P to R represent negative results in the OECD TG 409 assay in the presence of various combinations of missing or equivocal data. As with the positive result scenarios above (see Paragraph 937), the next step to take in these eventualities will have to be decided on a case-by-case basis. However, the recommended first step is generally to obtain reliable mechanistic (*in vitro*) data rather than proceed further with *in vivo* testing. In all cases, the role of metabolism, route of exposure, species differences and data from structural analogues should be considered before deciding on the next step.

940. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. The table is meant to provide a succinct guide and may not cover all circumstances or possibilities. The scenarios may also suggest that chemicals have simple or single MOA, when in practice they may have multiple endocrine and non-endocrine MOA. In some cases, for example, two opposite modes of simultaneous action (e.g. estrogenic and anti-estrogenic) 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. Endocrine pathways interact, mixed effects are common and there are many pathways that cannot be distinguished with currently available TGs. If multiple MOA are suspected, either from the existing results or based on QSAR/read-across/integrated approaches, this should be investigated further if needed for regulatory decision making.

References

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Table C.3.13. **Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents (OECD TG 409): 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.

Existing results: ** "Effects (*in vivo* effects of concern)" assumes various information, such as data from repeat dose oral toxicity studies, reproduction/developmental toxicity screen tests, read-across from analogues, will be available.

*** *Note:* a positive result is defined as a biologically significant change in any of the endocrine endpoints.

Scenarios	Result of OECD TG 409 (non-rodent 90-day 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	+ ***	+	+	Increased evidence of (anti)-E,A,T,S activity in multiple species.	Perform assay from Level 5 (e.g. Extended One-Generation Reproduction Toxiticy Study [EOGRTS] or two-generation assay).	If existing data are from a Level 5 assay, there may be sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for endocrine disrupting chemicals [EDCs] with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. Effects on apical endpoints alone may indicate estrogen/androgen/ thyroid/steroidogenesis (E,A,T,S) modalities or other mechanisms. Consider route of exposures for effects data and possible implications of absorption, distribution, metabolism and excretion (ADME) characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing a Fish Sexual Development Test (FSDT), Larval Amphibian Growth and Development Assay (LAGDA) or Medaka Extended One-Generation Reproduction Test (MEOGRT).
B	+	+	–	Evidence of (anti)-E,A,T,S activity in non-rodent species. (Anti)-E,A,T,S activity. Route of exposure or species differences may account for the differences between OECD TG 409 and existing data.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, question why there are differences. If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures and possible implications of ADME characteristics of the chemical in different species. Consider species differences in physiology and response. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.
C	+	+	Eq/0	Evidence of (anti)-E,A,T,S activity in non-rodent species. (Anti)-E,A,T,S activity. Route of exposure or species differences may account for the differences between OECD TG 409 and existing data.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Check data on chemical analogues. Consider species and route of exposure for OECD TG 409 and follow-up assay. Possible implications of ADME characteristics of the chemical in different species. Consider species differences in physiology and response. Equivocal results may indicate chemical has multiple modes of action (MOA). Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.

Scenarios	Result of OECD TG 409 (non-rodent 90-day 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)**			
D	+	–	+	Increased evidence of (anti)-E,A,T,S activity in multiple species. Acts via non-estrogen receptor (ER-),androgen receptor (AR-), thyroid hormone receptor (TR), steroidogenesis (S) mechanism or may require metabolic activation for activity.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for endocrine disrupting chemicals [EDCs] with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical in different species. Further mechanistic studies may help determine MOA. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.
E	+	–	–	Evidence of (anti)-E,A,T,S activity in non-rodent species. Acts via non-ER, AR, TR, S mechanism or may require metabolic activation for activity. Route of exposure or species differences may account for the differences between OECD TG 409 and existing data.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, question why there are differences. If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures and possible implications of ADME characteristics of the chemical in different species. Consider species differences in physiology and response. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.
F	+	–	Eq/0	Evidence of (anti)-E,A,T,S activity in non-rodent species. Acts via non-ER, AR, TR, S mechanism or may require metabolic activation for activity. Route of exposure or species differences may account for the differences between OECD TG 409 and existing data.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Check data on chemical analogues. Further mechanistic studies may help determine MOA. If existing data are from an adequate Level 5 assay, question why there are differences. If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures and possible implications of ADME characteristics of the chemical in different species. Consider species differences in physiology and response. Equivocal results may indicate chemical has multiple MOA. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.

Scenarios	Result of OECD TG 409 (non-rodent 90-day 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)**			
G	+	Eq/0	+	Increased evidence of (anti)-E,A,T,S activity in multiple species. May act via ER, AR, TR, S mechanism (metabolic activation may be needed).	Perform *in vitro* ER, AR, TR, S assays with added metabolising system (for the "0" scenario, otherwise Eq result available).	If existing data are from a Level 5 assay, there may be sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Check data on chemical analogues. Further mechanistic studies may help determine MOA. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical in different species. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
H	+	Eq/0	–	Evidence of (anti)-E,A,T,S activity in non-rodent species. Acts via unknown mechanism or may require metabolic activation for activity. Route of exposure may account for the differences between OECD TG 409 and existing data. Unknown potential for adverse effects.	For the "0" scenario, perform *in vitro* ER, AR, TR, S assays with added metabolising system (otherwise Eq result available).	If existing data are from an adequate Level 5 assay, question why there are differences. Consider route of exposures and possible implications of ADME characteristics of the chemical. Consider species differences in physiology and response. If existing data are from a less sensitive assay, a higher level test may be required. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
I	+	Eq/0	Eq/0	Evidence of (anti)-E,A,T,S activity in non-rodent species. Acts via unknown mechanism or requires metabolic activation for activity. Route of exposure may account for the differences between OECD TG 409 and existing data.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Check data on chemical analogues. Further mechanistic studies may help determine MOA. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
J	–	+	+	No evidence for (anti)-E,A,T,S activity in non-rodent species. Weak (anti)-E,A,S activity may not be detected by this assay. Route of exposure or species differences may account for the differences between OECD TG 409 and existing data. Metabolism or potency may explain *in vitro*/*in vivo* differences.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, question why there are differences. Effects seen in existing studies may be in a more sensitive species or life stage. Consider route of exposures and possible implications of ADME characteristics of the chemical in different species. Consider species differences in physiology and response. Further mechanistic studies may help determine MOA.

Scenarios	Result of OECD TG 409 (non-rodent 90-day 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)**			
K	–	+	–	No evidence for (anti)-E,A,T,S activity in multiple species. Weak (anti)-E,A,S activity may not be detected by this assay. Metabolism or potency may explain *in vitro*/*in vivo* differences. Unknown potential for adverse effects.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Further mechanistic studies with metabolism may help determine MOA. Consider species differences in physiology and response.
L	–	+	Eq/0	No evidence for (anti)-E,A,T,S activity in non-rodent species. Weak (anti)-E,A,S activity may not be detected by this assay.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system.	Metabolic deactivation of chemical may occur *in vivo* so that possible *in vitro* activity is not realised. Consider route of exposures and possible implications of ADME characteristics of the chemical in different species. Consider species differences in physiology and response. Equivocal results may indicate chemical has multiple MOA.
M	–	–	+	No evidence for (anti)-E,A,T,S activity in non-rodent species. Weak (anti)-E,A,S activity may not be detected by this assay. Effects seen in existing studies are via non-E,A,T,S mechanism.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, question why there are differences. Effects seen in existing studies may be in a more sensitive species or life stage. Consider route of exposures and possible implications of ADME characteristics of the chemical in different species. Consider species differences in physiology and response.
N	–	–	–	No evidence for (anti)-E,A,T,S activity in multiple species. Weak (anti)-E,A,S activity may not be detected by this assay. No evidence for (anti)-E,A,T,S activity *in vitro*. No evidence of adverse effects.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive).
O	–	–	Eq/0	No evidence for (anti)-E,A,T,S activity in non-rodent species. Weak (anti)-E,A,S activity may not be detected by this assay. No evidence for (anti)-E,A,T,S activity *in vitro*. Unknown potential for adverse effects.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Consider route of exposures and possible implications of ADME characteristics of the chemical in different species.

Scenarios	Result of OECD TG 409 (non-rodent 90-day 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)**			
P	–	Eq/0	+	No evidence for (anti)-E,A,T,S activity in non-rodent species. Weak (anti)-E,A,S activity may not be detected by this assay. Potential for adverse effects via unknown mechanism.	Perform *in vitro* ER, AR,TR, S assays with added metabolising system.	Consider route of exposures and possible implications of ADME characteristics of the chemical in different species. Effects seen in existing studies may be in a more sensitive species or life stage. Further mechanistic may would strengthen weight of evidence. Equivocal results may indicate chemical has multiple MOA.
Q	–	Eq/0	–	No evidence for (anti)-E,A,T,S activity in multiple species. Weak (anti)-E,A,S activity may not be detected by this assay. No evidence of adverse effects.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Further mechanistic studies may strengthen weight of evidence.
R	–	Eq/0	Eq/0	No evidence for (anti)-E,A,T,S activity in non-rodent species. Weak (anti)-E,A,S activity may not be detected by this assay. Unknown potential for adverse effects.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system, otherwise Eq result available.	Further mechanistic studies may strengthen weight of evidence. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.