C.3.6. Reproduction/Developmental Toxicity Screening Test (OECD TG 421) and Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (OECD TG 422)

Status: Assay validated by the OECD.

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

764. Endpoints: Estrous cyclicity, time to mating, male fertility, female fertility, dystocia, gestation length, number of implantations and corpora lutea, number of live births and post-implantation loss, litter size, sex ratio, litter/pup weight, pup survival index, anogenital distance (PND 0-4) and nipple retention (PND 13) in pups.

Weights of: (parents only) testes, epididymides, prostate and seminal vesicles with coagulating glands (OECD TG 421 and TG 422), plus adrenals (OECD TG 422 only). Optional organ weights could include levator ani plus bulbocavernosus muscle complex, Cowper's glands and glans penis in males, and paired ovaries and uterus (including cervix). Thyroid gland weight could be measured (after fixation) in all adults and PND 13 pups (OECD TG 421 and TG 422).

Vaginal smears at necropsy to determine stage of the estrous cycle and allow correlation with histopathology of ovaries.

Histopathologic changes in: testis, epididymides, ovaries (parents only); thyroid (parents and pups "when necessary") (OECD TG 421 and TG 422). Also adrenals, uterus and cervix, prostate, seminal vesicles plus coagulating glands, in parents only (OECD TG 422 only). Mammary glands and pituitary are optional (OECD TG 422).

Mandatory measurement of serum thyroid hormone (T4) in parental males and pups at PND 13. T4 in dams and PND 4 pups "if relevant". TSH (and other hormones) optional "if relevant".

Background to the assay

765. These assays are designed to provide limited information about the effects of a chemical on the male and female reproductive systems including gonadal function, mating, conception, gestation, development of the conceptus and parturition. The assays are designed for use with the rat. The recommended route of administration is oral, usually via gavage although administration may be via diet or drinking water. Although the titles of the test guidelines (TGs) imply that they are screening tests, they are not screens as given in the definition in Section A, but are apical assays. The TGs have similar experimental schedules, but OECD TG 422 includes a more detailed assessment of repeated dose toxicity and thus more endpoints. The studies were not designed to detect endocrine active substances (EASs), but they have endpoints relevant for the assessment of possible endocrine disruption and provide data on adverse effects related to reproduction and development. The developing fetus is a life stage that may be particularly sensitive to EASs.

The scope of these assays is much smaller than OECD TG 416 and TG 443 (Two-generation Assay and Extended One-Generation Reproductive Toxicity Study), e.g. duration of premating exposure is much shorter, group sizes are generally half and only a relatively short period of postnatal development (13 days) is included. OECD TG 421 was originally adopted in 1995 and TG 422 in 1996. Following a feasibility study (OECD, 2015), both assays were updated in July 2016 to include some endocrine-relevant endpoints as the exposure periods cover some of the sensitive periods during development (pre- or early postnatal periods). It should be noted that if the assays were conducted before 2016, they are unlikely to include these extra endpoints.

766. Anogenital distance (AGD) and nipple retention are included in OECD TG 421 and TG 422 as sensitive endpoints of endocrine effects; however, their utility as apical endpoints or as biological indicators of endocrine action may require further experience in their use. Increased nipple retention and reduced AGD in male offspring are hallmarks of anti-androgenicity. Nevertheless, "retained nipples/areolae" as a qualitative endpoint may have high biological variability (e.g. Melching-Kollmuss et al. [2017]) and alteration of AGD can occur via other modes of action (MOA) (e.g. Miyagawa et al. [2011]; Seifert et al. [2009]). However, current OECD guidance on these endpoints can be found in OECD GD 43 and GD 151 and it is clear that these should be considered as apical endpoints. With regard to AGD, OECD GD 43 (OECD, 2008b) states, "A statistically significant change in [anogenital distance] that cannot be explained by the size of the animal indicates effects of the exposure and should be used for setting the [no observed adverse effect level (NOAEL)]". With regard to nipple retention, OECD GD 151 (OECD, 2013) states "a statistically significant change in nipple retention should be evaluated similarly to an effect on anogenital distance as both endpoints indicate an adverse effect of exposure and should be considered in setting a NOAEL".

767. The feasibility report on OECD TG 421 and TG 422 (OECD, 2015) indicated that the sensitivity for detecting effects based on qualitative nipple retention (i.e. the number of males with or without nipples) was quite low irrespective of the number of litters included. However, nipple retention is a sensitive endpoint if measured quantitatively (i.e. if the number of nipples from 0-12 is recorded). This endpoint of quantitative nipple retention in the male pups was therefore included in these study updates.

768. Both original (1996) and revised (2017) OECD TG 421 and TG 422 contain endpoints that are suitable for the determination of endocrine effects although the TGs as revised in 2017 are more comprehensive. In addition to reproduction/development, OECD TG 421 and TG 422 may both provide information about potential endocrine effects on male reproductive organs. Information about effects on the thyroid are included in TG 422 and TG 421 as revised in 2017. Female reproductive organs are also examined, but detection of endocrine effects in these organs may be obscured because of pregnancy. Male animals are dosed for a total period of 28 days. A comparison can be made with OECD TG 407 (28-day oral toxicity study) where validation studies (OECD, 2006) demonstrated that 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. Steroidogenesis inhibition was also detected, although only one (potent) chemical was used in the validation study (CGS 18320B).

769. The 2017 revised assays contain many endocrine-sensitive endpoints that are also included in the Conceptual Framework (CF) Level 5 assays – OECD TG 416 and TG 443. Many of these endpoints indicate (anti)estrogenicity, (anti)androgenicity and thyroid

hormone disruption and may help to discern MOA. Information on mode of action (MOA) from *in vitro* estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) assays or *in vivo* lower tier tests such as a Uterotrophic Bioassay (UT) and a Hershberger Bioassay (H) is also helpful. The original (1996) TGs, however, only contained apical endpoints, therefore in assays conducted prior to 2016 it would be difficult to discern MOA from these tests alone.

Thyroid hormone analysis and interpretation in OECD TG 421/422

770. The revised TGs require the determination of serum thyroid hormones in adults and pups. Detection/measurement of thyroid hormones (T4, T3, TSH) in pups can be challenging. In order to provide assurance that thyroid hormone measurements are reliable, laboratories should be able to demonstrate that they are proficient with the assay. Only such a demonstration would enable regulatory authorities to interpret and use the data. A recent workshop on "Practicability of Hormonal Measurements" was organised by the BfR (Germany) and the finding from this workshop will be published (Kucheryavenko et al., 2018). The OECD Expert Group on Reproductive and Developmental Toxicity recommends that to demonstrate proficiency for thyroid hormone measurement, a laboratory should be able to show results from a separate study using a positive control substance. Laboratories may also submit their calibration curves, standard curves, as well as data on the levels of quantification and detection. This group is also establishing a historical control database with thyroid toxicant positive controls.

771. Within OECD TG 421/422, T4 should be measured in adult males and in pups at PND 13, while in pups at PND 4 and in females T4 would be measured "if relevant". Triggers for further measurement may be changes in the initial endpoints, to provide clarity on the time course of thyroid changes. This may also prompt measurement of TSH and possibly T3.

When/why the assay may be used

772. These assays are frequently used for initial hazard assessments for chemicals, as part of the Screening Information Data Set (SIDS) for the assessment of chemicals for which there is little information or for dose setting for more extensive reproduction/developmental assays. These assays are also likely to be used as part of a pesticide submission package and forms part of the standard information requirements in certain chemical legislations (e.g. REACH 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.

773. 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).

Introduction to the table of scenarios

774. Table C.3.6 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 fouth columns). The table is divided horizontally into a series of scenarios that represent all the combinations of these events.

775. The results of OECD TG 421/422 are given in the second column. As these are not tests where a yes/no (qualitative) answer is obtained for the test as a whole, positive results would generally be assessed for individual endpoints and as a whole. It is not possible to provide guidance on all endpoints individually, therefore the endpoints have been pragmatically divided into "apical" and "indicators of hormonal activity". The terminology used has been chosen to be consistent between both the non-mammalian wildlife and mammalian tests. Both groups have similar biological importance, although the "indicators of hormonal activity" in the mammalian assays are serum hormones and are generally, but not always, more variable than "apical endpoints".

776. For this guideline "apical" endpoints are reproductive and developmental parameters (including anogenital distance, presence of nipples, genital abnormalities), estrous cyclicity, weights and histopathologic changes in testes, epididymides, prostate, seminal vesicles (with coagulating glands), ovary, uterus, thyroid. "Indicators of hormonal activity" are hormones (T4, TSH).

777. Three possible outcomes for a positive result are therefore envisaged in Table C.3.6:

1. indicators of hormonal activity and apical endpoints positive
2. indicators of hormonal activity positive and apical endpoints negative
3. indicators of hormonal activity negative and apical endpoints positive.

778. A positive result for apical endpoints could be biologically significant changes in pup AGD, accompanied by treatment-related histopathologic changes in parental reproductive organs. A positive result for indicators of hormonal activity could be biologically significant changes in hormone profiles. A positive result for indicators of hormonal activity alone should be considered with caution, although it is possible that these endpoints may have detected weak effects that were not detected by the apical endpoints.

779. A negative result for OECD TG 421/422 is taken to be the absence of biologically significant changes in all endocrine endpoints.

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

781. 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

782. 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, therefore 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 (2008a). These methods, however, have not yet been validated.

783. 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 EASs. Given the usage of these assays for general chemical testing, it is possible that an OECD TG 407 (28-day test) is available. It is unlikely that OECD TG 421/422 will be performed if higher tier reproduction/developmental toxicity data are already available, as they offer no advantage over these assays. The results of the study may also 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.

784. When considering the results of OECD TG 421/422, 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 ER and AR 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

785. A series of scenarios (A to R) are presented in Table C.3.6 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 rats are the preferred species for TG 421/422, the well-conserved nature of the hormonal pathways across taxa should be a strong indication that results 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, exposure route and species-specific metabolism should always be considered. The sensitivity and physiological function of the hormone under investigation in the test species should also be considered. 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 Toxicity 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.

786. Scenarios A to C represent positive results in OECD TG 421/422 in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Each positive result scenario is divided into the three possible outcomes given above. A positive result in the *in vitro* assays in combination with a positive OECD TG 421/422 is strong evidence of adverse effects on reproduction/development and/or endocrine organs via E,A,T,S mechanisms. Effects on only apical endpoints or only indicators of hormonal activity may assist with interpretation. Positive results in the OECD TG 421/422 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 is 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. The possibility of other (non-E,A,T,S) mechanisms should also not be overlooked (e.g. involving other receptors or endocrine axes).

787. Scenarios D to F represent positive results in OECD TG 421/422 in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal in vivo effects data. Each positive result scenario is divided into the three possible outcomes given above. A positive result is strong evidence of adverse effects on reproduction/development and/or endocrine organs. Effects on the different apical endpoints or indicators of hormonal activity may assist with interpretation. Negative results in the *in vitro* assays should be viewed with caution in case a metabolite is responsible for the positive OECD TG 421/422. If the metabolic profile of the test substance is not known, performing the *in vitro* assays with addition of a metabolising system may help to understand mechanism. Positive results in the OECD TG 421/422 assay may also indicate the potential for endocrine mediated effects in lower vertebrates.

788. Scenarios G to I represent positive results in OECD TG 421/422 in the presence of various combinations of missing or equivocal data. Positive results in the OECD TG 421/422 assay may also indicate the potential for endocrine mediated effects in lower vertebrates. Each positive result scenario is divided into the three possible outcomes given above. 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 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. In all cases, the role of metabolism, route of exposure and data from structural analogues should be considered before deciding on the next step.

789. Scenarios J to L represent negative results in OECD TG 421/422 in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A negative result is taken to be negative findings for both indicators of hormonal activity and apical endpoints (unlike the situation with positive outcomes), therefore there is only one possible negative outcome. All three scenarios could fit a chemical that is positive in *in vitro* assays but is metabolised to a non-active metabolite, leading to negative results in OECD TG 421/422. This possibility may be investigated to help understand mechanism. Endocrine active potency may also explain differences between *in vitro* and *in vivo* results (e.g. a a chemical with weak endocrine activity may give a positive result *in vitro* but may be negative *in vivo*). Positive *in vivo* effects data may involve E,A,T,S or non-E,A,T,S mechanisms (e.g. involving other receptors or endocrine axes), more sensitive endpoints, or greater statistical power, but knowledge of absorption, distribution, metabolism and excretion (ADME) may help to explain differences from the OECD TG 421/422 data.

790. Scenarios M to O represent negative results in the OECD TG 421/422 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) indicate an absence of concern for reproductive toxicity via an endocrine disruption mechanism. If the study is not considered to be robust, supplemental testing could be considered. Positive *in vivo* effects data may involve E,A,T,S or non-E,A,T,S mechanisms (e.g. involving other receptors or endocrine axes), but knowledge of ADME may help to explain differences from the OECD TG 421/422 data.

791. Scenarios P to R represent negative results in OECD TG 421/422 in the presence of various combinations of missing or equivocal data. As with the positive result scenarios, the next step to take in these eventualities will have to be decided on a case-by-case basis. In all cases, the role of metabolism, route of exposure and data from structural analogues should be considered before deciding on the next step.

792. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.3.6 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

Jacobs, M. et al. (2013), "*In vitro* metabolism and bioavailability tests for endocrine active substances: What is needed next for regulatory purposes?", *ALTEX – Alternatives to Animal Experimentation*, Vol. 30/3, pp. 331-351.

Jacobs, M.N. et al. (2008), "The use of metabolising systems for *in vitro* testing of endocrine disrupters", *Current Drug Metabolism*, Vol. 9/8, pp. 796-826.

Kucheryavenko, O. et al. (2018), "Report from the BfR Expert Hearing on Practicability of Hormonal Measurements", *Archives of Toxicology* (in prep.).

Melching-Kollmuss, S. et al. (2017), "Comparing effect levels of regulatory studies with endpoints derived in targeted anti-androgenic studies: Example prochloraz", *Archives of Toxicology*, Vol. 91/1, pp. 143-162, https://doi.org/10.1007/s00204-016-1678-y.

Miyagawa, S. et al. (2011), "The role of sonic hedgehog-Gli2 pathway in the masculinization of external genitalia", *Endocrinology*, Vol. 152/7, pp. 2894-2903, https://doi.org/10.1210/en.2011-0263.

OECD (2015), "Feasibility study for minor enhancements of TG 421/422 with ED relevant endpoints", OECD Series on Testing and Assessment, No. 217, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2015)24&doclanguage=en.

OECD (2013), "Guidance document supporting of OECD test guideline on the extended one-generation reproductive toxicity study", OECD Series on Testing and Assessment, No. 151, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2013)10&doclanguage=en.

OECD (2008a), *Detailed Review Paper on the Use of Metabolising Systems for* In Vitro *Testing of Endocrine Disrupters*, OECD Series on Testing and Assessment, No. 97, OECD Publishing, Paris, https://doi.org/10.1787/9789264085497-en.

OECD (2008b), "Guidance document on mammalian reproductive toxicity testing and assessment", OECD Series on Testing and Assessment, No. 43, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2008)16&doclanguage=en.

OECD (2006), "Report of the validation of the updated test guideline 407: Repeat dose 28-day oral toxicity study in laboratory rats", OECD Series on Testing and Assessment, No. 59, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2006)26&doclanguage=en.

Seifert, A.W. et al. (2009), "Multiphasic and tissue-specific roles of sonic hedgehog in cloacal septation and external genitalia development", *Development*, Vol. 136/23, pp. 3949-3957, http://dx.doi.org/10.1242/dev.042291.

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.3.6. **Reproduction/Developmental Toxicity Screening Test (OECD TG 421) and Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (OECD TG 422): 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 effects have been observed in other *in vivo* screens/tests which give rise to concern that the test chemical may be an endocrine disrupter. These may be other repeated dose toxicity tests, UT and H assays or read-across from chemical analogues.

*** *Note:* three possible outcomes for a positive result are given:

1. indicators of hormonal activity and apical endpoints positive
2. indicators of hormonal activity positive and apical endpoints negative
3. indicators of hormonal activity negative and apical endpoints positive.

"Apical endpoints" are reproductive and developmental parameters (including anogenital distance, presence of nipples, genital abnormalities), estrous cyclicity, weights and histopathologic changes in testes, epididymides, prostate, seminal vesicles (with coagulating glands), ovary, uterus, thyroid.

"Indicators of hormonal activity" are hormones (T4, TSH).

Scenarios	Result of OECD TG 421/422	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal 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) Evidence of adverse effects on endocrine/apical endpoints via estrogen/androgen/thyroid/ steroidogenesis (E,A,T,S) mechanism. 2) Evidence of effects on hormonal endpoints via E,A,T,S mechanism. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects via E,A,T,S mechanism. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. Extended One-Generation Reproduction Toxicity Study [EOGRTS] or two-generation assay).	May be sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for endocrine disrupting chemicals (EDCs) with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider potency of effects for existing results and whether E,A,T,S mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine 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	+	+	–	1) Evidence of adverse effects on endocrine/apical endpoints via E,A,T,S mechanism. 2) Evidence of effects on hormonal endpoints via E,A,T,S mechanism. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects via E,A,T,S mechanism. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	May be sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider potency of effects for existing results and whether E,A,T,S mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.

Scenarios	Result of OECD TG 421/422	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal 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)**			
C	+	+	Eq/0	1) Evidence of adverse effects on endocrine/apical endpoints via E,A,T,S mechanism. 2) Evidence of effects on hormonal endpoints via E,A,T,S mechanism. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects via E,A,T,S mechanism. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider potency of effects for existing results and whether E,A,T,S mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple modes of action (MOA).
D	+	–	+	1) Evidence of adverse effects on endocrine/apical endpoints but not via E,A,T,S mechanism or requires metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, possibly requires metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects on apical endpoints but not via E,A,T,S mechanism or requires metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* estrogen receptor (ER-), androgen receptor (AR-), thyroid hormone receptor (TR), steroidogenesis (S) assays with added metabolising system.	May be sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.
E	+	–	–	1) Evidence of adverse effects on apical endpoints but not via E,A,T,S mechanism or requires metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, possibly requires metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects on apical endpoints but not via E,A,T,S mechanism or requires metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	May be sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical.

Scenarios	Result of OECD TG 421/422	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal 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) Evidence of adverse effects on apical endpoints via non-E,A,T,S/non-endocrine mechanism or requires metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, possibly requires metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects on apical endpoints, via non-E,A,T,S/non-endocrine disruption mechanism or requires metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	May be sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
G	+	Eq/0	+	1) Evidence of adverse effects on apical endpoints, may act via E,A,T,S mechanism and may require metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, may act via E,A,T,S mechanism and may require metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects on apical endpoints, may act via E,A,T,S mechanism and may require metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	May be sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of OECD TG 421/422	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
H	+	Eq/0	–	1) Evidence of adverse effects on apical endpoints via non-E,A,T,S/non-endocrine disruption mechanism or requires metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, possibly requires metabolic activation for activity. 3) Evidence of adverse effects on apical endpoints via non-E,A,T,S/non-endocrine disruption mechanism or requires metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	May be sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. 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	1) Evidence of adverse effects on apical endpoints via unknown mechanism. 2) Evidence of effects on hormonal endpoints via unknown mechanism. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects on apical endpoints via unknown mechanism. Hormonal endpoints may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	May be sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
J	–	+	+	No evidence of adverse effects in OECD TG 421/422. Effects seen in existing (lower level) studies do not lead to adverse outcome in Level 5 assay. Metabolism or potency explains the difference from existing *in vitro* and *in vivo* 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).	Consider route of exposures and possible implications for ADME characteristics of the chemical. Effects seen in existing studies may be in a more sensitive life stage. Further mechanistic studies with metabolism may help determine MOA.

Scenarios	Result of OECD TG 421/422	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal 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)**			
K	–	+	–	No evidence of adverse effects in OECD TG 421/422. Metabolism or potency explains *in vitro*/*in vivo* differences.	To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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). Metabolic deactivation of chemical may occur *in vivo* so that possible *in vitro* activity is not realised. Further mechanistic studies with metabolism may help determine MOA. Equivocal results may indicate chemical has multiple MOA.
L	–	+	Eq/0	No evidence of adverse effects in OECD TG 421/422. Metabolism or potency explains *in vitro*/*in vivo* differences. Effects seen in existing (lower level) studies do not lead to adverse outcome in this assay.	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).	Metabolic deactivation of chemical may occur *in vivo* so that possible *in vitro* activity is not realised. Further mechanistic studies with metabolism may help determine MOA. Consider route of exposures and possible implications for ADME characteristics of the chemical. Equivocal results may indicate chemical has multiple MOA.
M	–	–	+	No evidence of adverse effects in OECD TG 421/422. Effects seen in existing (lower level) studies do not lead to adverse outcome in this assay.	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 life stage. Consider route of exposures and possible implications of ADME characteristics of the chemical.
N	–	–	–	No evidence of adverse effects in OECD TG 421/422.	Consider existing data, there may be no need for further testing.	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 of adverse effects in OECD TG 421/422. No evidence for (anti)-E,A,T,S activity *in vitro*.	Consider existing data, there may be no need for further testing. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	There may be sufficient information to conclude absence of concern for endocrine disruption. Further mechanistic studies may strengthen weight of evidence. Consider route of exposures and possible implications for ADME characteristics of the chemical. Check data on chemical analogues.

Scenarios	Result of OECD TG 421/422	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal 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	+	No evidence of adverse effects in OECD TG 421/422. Effects seen in existing (lower level) studies do not lead to adverse outcome in this assay. Effects seen in existing studies are via unknown mechanism.	Perform *in vitro* ER, AR,TR, S assays.	Further mechanistic studies may strengthen weight of evidence. Consider route of exposures and possible implications for ADME characteristics of the chemical. Effects seen in existing studies may be in a more sensitive life stage. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.
Q	–	Eq/0	–	No evidence of adverse effects in OECD TG 421/422.	To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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. Check data on chemical analogues.
R	–	Eq/0	Eq/0	No evidence of adverse effects in OECD TG 421/422.	To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	Further mechanistic studies may strengthen weight of evidence. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.