C.2.11. Avian Reproduction Test (OECD TG 206)

Status: Assay validated by the OECD.

439. Modality detected/endpoints: OECD TG 206 does not contain endpoints which solely respond to endocrine disrupters (EDs), and it has not been specifically validated with EDs. However, some of the endpoints in this apical test are nevertheless potentially affected by estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) EDs. Of particular interest in the context of estrogens, androgens and steroidogenesis disrupters are egg production, embryo viability and hatchability, but other endpoints may also be responsive to some EDs (e.g. growth may respond to some thyroid disrupters; percentage of cracked eggs and egg shell thickness may respond to chemicals interfering with the control of shell deposition).

Background to the assay

440. This assay is designed primarily as an apical test for chemicals with suspected reproductive toxicity, but it is not a life cycle test as it only runs from the stage of pre-laying adults to 14-day-old offspring. Furthermore, only the adults are exposed to the test chemical (via the food), and any effects on sexual development would not be detectable. The endpoints are all apical measures of development, growth or reproduction. Key endpoints which might be affected by EDs include egg production, viability and hatchability. Possible test organisms include mallard duck (*Anas platyrhynchos*), bobwhite quail (*Colinus virginiatus*) and Japanese quail (*Coturnix japonica*).

441. Depending on the species and test objectives, endpoints could include *inter alia* sex ratio (phenotypic and/or genotypic), sex hormones, thyroid hormones, reproductive/thyroid organ weights, gonad histopathology and gross pathology, time to first egg laying, and sexual behaviour. These types of endpoint are all included in the Avian Two-Generation Test (ATGT). However, note that the ATGT does not cover all relevant behaviours and is performed in a precocial species which reacts very differently to embryonic exposure to a test material compared with an altricial species. Given the high degree of endocrine system conservation across the vertebrates, adverse endocrine-linked effects in the Avian Reproduction Test may indicate the possibility of related activity in other organisms such as fish, amphibians, reptiles or mammals.

When/why the assay may be used

442. Although OECD TG 206 could, in principle, be used at any stage in the hazard assessment process, the most likely use scenario will be when there are already some data available to suggest possible endocrine disruption properties. In other words, OECD TG 206 will generally be used to investigate whether such properties result in adverse apical effects on development, growth or reproduction over the reproductive part of the avian life cycle. It would be unlikely to be used if other bird reproduction data are already available. OECD TG 206 could not be used as a primary screen for EDs. Another potential limitation of OECD TG 206 is that the effects of test chemicals may not become fully

apparent during the test because the offspring are not directly dosed, and only receive bioaccumulated material which may be passed from their mothers via the egg.

443. 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

444. Existing data available before deployment of OECD TG 206 for ED hazard assessment are likely to include information on possible modes of action (MOA) from quantitative structure activity relationships (QSARs) and/or *in vitro* screens. It would not be advisable to conduct an unmodified OECD TG 206 without mechanistic screening data because it would then not be possible to link any apical effects with endocrine disruption. Given the commonality of endocrine mechanisms in the vertebrates, relevant existing data available before deployment of OECD TG 206 (Avian Reproduction Test) might also include *in vivo* results obtained with other vertebrates (e.g. a positive Uterotrophic Bioassay with rodents; positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies; or positive result in the fish assays OECD TG 229 or TG 230). As the ethical and financial cost of OECD TG 206 is high, it is important to make full use of existing endocrine-related data, both before the test is begun and during data evaluation.

Scenarios: Positive and negative results combined with existing data

445. The scenarios (A to R) presented in Table C.2.11 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.

446. Positive results obtained with one of the OECD TG 206 endpoints which are outside the range of historical controls may result in the conclusion that the test chemical is able to cause adverse effects *in vivo* (Table C.2.11, Scenarios A-I), but not necessarily that it is an

ED. Note that if doubt exists about the test performance (e.g. highly unusual results in controls), a comparison with historical control data with respect to overall test performance might be helpful. However, the nature of these effects and any existing data will require careful consideration. If *in vitro* and/or *in vivo* data already exist which reveal possible endocrine disrupting properties (Scenarios A, B and D), a positive endpoint in OECD TG 206 could lead to a tentative conclusion that the test chemical is an actual ED.

447. If a plausible link of a responding OECD TG 206 endpoint with previously identified endocrine activity can be made, regulatory authorities may conclude that sufficient evidence is available to categorise the chemical as an ED (i.e. interference with the endocrine system has caused adverse effects *in vivo*), and no further information might then be required. However, if a more robust link between adverse effects and an endocrine modality is required (bearing in mind that none of the existing data are likely to have been generated in avian systems), or if possible effects during the sexual development part of the life cycle are suspected, or if the chemical is suspected to cause epigenetic effects, it would be desirable to run an ATGT. Furthermore, if data on hazard are required for an environmental hazard identification/characterisation, an ATGT may also be needed unless the precision of the data from OECD TG 206 (which only uses three test concentrations) are considered adequate for such an assessment. On the other hand, if data from prior endocrine screens and tests are negative (Scenario E), a positive response in OECD TG 206 would not support the hypothesis that the chemical is an ED in birds. It could, of course, still be subjected to an environmental hazard identification/characterisation, but only if sufficient concentrations have been tested to allow derivation of an adequately precise lowest-observed-effect-concentration no-observed-effect-concentration (LOEC/NOEC).

448. The scenarios in which OECD TG 206 gives a negative result (Table C.2.11, Scenarios J-R) lead to a tentative conclusion that the test chemical is not an ED in birds, and this conclusion is strengthened considerably if prior screens have failed to reveal endocrine activity (Scenario N). In the latter circumstances, regulatory authorities may be justified in concluding that no further action is needed. However, if it is thought possible that the sexual development part of the life cycle is sensitive, then conducting an ATGT should be considered. Also, if one or more of those screens was positive (Scenarios J-M and P), the bioconcentration factor of the chemical should be checked. If the bioconcentration factor indicates that the chemical is strongly bioaccumulative, it would also be worth considering conducting an ATGT. If a chemical which screened positive is not bioaccumulative, the probable reasons for lack of effects in OECD TG 206 might be metabolism to an inactive chemical, or failure to reach the active site, and no further action would be indicated.

449. In each of the above scenarios, it is possible that existing data will be equivocal (Table C.2.117, Scenarios C, F-I, L and O-R), or there may be no existing data. This will weaken the conclusions which can be drawn about a positive OECD TG 206, and this is reflected in Table C.2.11. However, as indicated above, it would be undesirable to proceed with OECD TG 206 if prior data on endocrine activity are equivocal or absent. On the other hand, if OECD TG 206 is positive, it would be essential to obtain some reliable mechanistic data before reaching a conclusion about whether or not the chemical is an ED in birds. There is also the possibility that equivocal mechanistic data may be the result of multiple modes of endocrine action. Under some circumstances, two opposite modes of simultaneous action (e.g. estrogenic and anti-estrogenic) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA (e.g. estrogenic and anti-androgenic) could potentially reinforce effects on certain apical endpoints. If multiple MOA are suspected, either from the existing results or based on

QSAR/read-across/integrated approaches, this situation should be investigated further if needed for regulatory decision making.

450. The scenario in which the results of OECD TG 206 are themselves equivocal has not been dealt with in Table C.2.11, for reasons of brevity. In this context, an equivocal result might be an inconsistent concentration-response (e.g. no effect at a high concentration but effects at a lower concentration), or a result which borders on statistical significance. Without knowing the exact circumstances, reliable advice cannot be given, but the opinions of an experienced ecotoxicologist should be sought. However, if prior screens are negative, it is doubtful if further action is needed, because the chemical is unlikely to be an ED. If an endocrine screen is positive, some types of equivocal OECD TG 206 results would have to be taken more seriously. For example, an inconsistent concentration-response would not necessarily rule out the test chemical as an ED in birds. An example of this would be a chemical which causes adverse effects on reproduction at low doses, but reduced reproductive success and ultimately mortality at very high doses, thus potentially giving a U-shaped response curve. Ideally, concentrations causing systemic toxicity of this type should not be tested in OECD TG 206, but such toxicity may have been missed in earlier screens.

451. In summary, positive results in OECD TG 206 indicate that a chemical may be an ED if they can be plausibly linked to an endocrine MOA established on the basis of prior screening. However, more conclusive data in this regard would be obtainable from an ATGT. If screening data are unavailable or negative, it should not be concluded that a positive OECD TG 206 is the result of endocrine disruption. On the other hand, a negative OECD TG 206 combined with negative screening data should lead to a conclusion that a chemical is probably not an ED in birds. A negative OECD TG 206 set against a background of a positive screen might, however, raise concerns if the chemical is strongly bioaccumulative, known to be involved in epigenesis, or suspected of having effects on sexual development, when an ATGT should be considered.

Reference

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

Table C.2.11. **Avian Reproduction Test (OECD TG 206): Guidance for scenarios of combinations of results with existing data**

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

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

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

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

Note that although this assay has been used for many years to assess the sub-acute effects of chemicals, and no formal attempt has been made to validate it for use with potential endocrine disruptors (EDs), the United States Environmental Protection Agency (US EPA) has shown that reproduction is a part of the avian life cycle which can be responsive to EDs (https://www.regulations.gov/document?D=EPA-HQ-OPPT-2014-0766-0019). Furthermore, the US EPA has published the Avian Two-Generation Test (ATGT) protocol which contains several ED-specific endpoints, although it has not been internationally validated or harmonised with OECD guidelines.

Scenario	Result of OECD TG 206	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
A	+	+	+	The test chemical is probably an endocrine disruptor (ED) if the modality identified in existing screens/tests can be plausibly linked to the affected endpoint.	Further evidence is probably not required.	If the affected endpoint in OECD TG 206 cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED in birds. OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an Avian Two-Generation Reproduction Test (ATGT) may reveal them.
B	+	+	–	The test chemical is probably an ED in birds if the modality identified in existing screens/tests can be plausibly linked to the affected endpoint.	Further evidence is probably not required.	If the affected endpoint in OECD TG 206 cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED in birds. OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them.
C	+	+	Eq/0**	The test chemical is probably an ED in birds if the modality identified in existing screens/tests can be plausibly linked to the affected endpoint.	Further evidence is probably not required.	If the affected endpoint in OECD TG 206 cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED in birds. OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple modes of action (MOA). If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
D	+	–	+	The test chemical may be an ED, but the negative mechanistic data reduce the confidence in this conclusion. However, if the endocrine disruption effects in existing *in vivo* tests can be plausibly linked to the OECD TG 206 responses, this increases the probability that the chemical is an ED in birds.	Further evidence is probably not required.	If the affected endpoint in OECD TG 206 cannot be plausibly linked to the known modality, the test chemical is unlikely to be an ED in birds. OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them.
E	+	–	–	The test chemical is unlikely to be an ED.	Further evidence is probably not required.	It is possible that the effects observed in OECD TG 206 have been caused by an unknown endocrine mechanism. This would not, however, prevent the chemical being subjected to hazard identification/characterisation. OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them.

Scenario	Result of OECD TG 206	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
F	+	–	Eq/0	The test chemical is unlikely to be an ED, but the relevance of any equivocal existing *in vivo* data to the OECD TG 206 results should be examined.	Further evidence is probably not required.	It is possible that the effects observed in OECD TG 206 have been caused by an unknown endocrine mechanism – equivocal existing *in vivo* data may throw some light on this. The absence of data on a possible endocrine mechanism would, however, not prevent the chemical being subjected to hazard identification/characterisation. OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them. 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	+	The test chemical may be an ED, but the equivocal or absent mechanistic data reduce the confidence in this conclusion. However, if the endocrine disruption effects in existing *in vivo* tests can be plausibly linked to the OECD TG 206 responses, this increases the probability that the chemical is an ED.	If reliable mechanistic data are not available, it would be desirable to obtain some.	The test chemical is probably an ED in birds if a modality identified in the newly commissioned mechanistic screens (see left-hand column), or in the existing *in vivo* data, can be plausibly linked to the affected endpoint. OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
H	+	Eq/0	–	The test chemical may be an ED, but the equivocal or absent mechanistic data reduce the confidence in this conclusion.	If reliable mechanistic data are not available, it would be desirable to obtain some.	The test chemical is probably an ED in birds if a modality identified in the newly commissioned mechanistic screens (see left-hand column) can be plausibly linked to the affected endpoint. OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenario	Result of OECD TG 206	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
I	+	Eq/0	Eq/0	The test chemical may be an ED, but the equivocal or absent mechanistic and *in vivo* data reduce the confidence in this conclusion. Final conclusions about whether a chemical is a potential ED cannot be drawn from the results of this test alone.	If reliable mechanistic data are not available, it would be desirable to obtain some.	The test chemical is probably an ED in birds if a modality identified in the newly commissioned mechanistic screens (see left-hand column) can be plausibly linked to the affected endpoint. OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
J	–	+	+	The chemical is probably not an ED in birds **that acts through the mechanisms tested in the available *in vitro* and *in vivo* studies**.	If the chemical is strongly bioaccumulative, is suspected to affect sexual development or cause epigenetic effects, consider conducting an ATGT.	If any effects in an ATGT can be plausibly linked with mechanistic data, the test chemical is probably an ED in birds.
K	–	+	–	The chemical is probably not an ED **in birds that acts through the mechanisms tested in the available *in vitro* and *in vivo* studies.**	If the chemical is strongly bioaccumulative, is suspected to affect sexual development or cause epigenetic effects, consider conducting an ATGT.	If any effects in an ATGT can be plausibly linked with mechanistic data, the test chemical is probably an ED in birds.
L	–	+	Eq/0	The chemical is probably not an ED in birds **that acts through the mechanisms tested in the available *in vitro* and *in vivo* studies**.	If the chemical is strongly bioaccumulative, is suspected to affect sexual development or cause epigenetic effects, consider conducting an ATGT.	If any effects in an ATGT can be plausibly linked with mechanistic data, the test chemical is probably an ED in birds. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
M	–	–	+	The chemical is probably not an ED in birds **that acts through the mechanisms tested in the available *in vitro* and *in vivo* studies**.	If the chemical is strongly bioaccumulative, is suspected to affect sexual development or cause epigenetic effects, consider conducting an ATGT.	If any effects in an ATGT can be plausibly linked with *in vivo* data which provide information on endocrine disruption properties, the test chemical is probably an ED in birds, but likely not by a mechanism covered by the existing *in vitro* screens.
N	–	–	–	The chemical is probably not an ED in birds **that acts through the mechanisms tested in the available *in vitro* and *in vivo* studies**.	Further evidence is probably not required.	OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them.

Scenario	Result of OECD TG 206	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
O	–	–	Eq/0	The chemical is probably not an ED in birds **that acts through the mechanisms tested in the available *in vitro* and *in vivo* studies**.	Further evidence is probably not required.	OECD TG 206 cannot detect effects on sexual development and is unlikely to detect effects from long-term bioaccumulation. If these are suspected, an ATGT may reveal them. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
P	–	Eq/0	+	The chemical is probably not an ED in birds **that acts through the mechanisms tested in the available *in vitro* and *in vivo* studies**.	If reliable mechanistic data are not available, it would be desirable to obtain some.	If the newly commissioned mechanistic data are positive and the chemical is strongly bioaccumulative, or if developmental effects are suspected, consider conducting an ATGT. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
Q	–	Eq/0	–	The chemical is probably not an ED in birds, but confidence in this conclusion is reduced by the lack of clear mechanistic data.	Further evidence is probably not required, but confidence in the conclusion would be increased by the provision of reliable negative mechanistic data.	If any newly commissioned mechanistic data are positive and the chemical is strongly bioaccumulative, or if developmental effects are suspected, consider conducting an ATGT. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
R	–	Eq/0	Eq/0	The chemical may not be an ED in birds, but confidence in this conclusion is reduced by the lack of clear mechanistic and existing *in vivo* data.	Further evidence is probably not required, but confidence in the conclusion would be increased by the provision of reliable negative mechanistic data.	If any newly commissioned mechanistic data are positive and the chemical is strongly bioaccumulative, or if developmental effects are suspected, consider conducting an ATGT. 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.