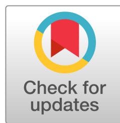

Brunel University London

APPROVED: 20 April 2020

doi:10.2903/sp.efsa.2020.EN-1849

Data collection in support of the Endocrine Disruption (ED) assessment for non-target vertebrates

Olwenn Martin[1], Sibylle Ermler[1], Joanne McPhie[2], Martin Scholze[1], Alice Baynes[1]

[1]Institute for the Environment, Health and Societies, Brunel University London; [2]Information Services, Brunel University London

Abstract

To harmonise vertebrate OECD Test Guidelines for endocrine disruption testing between mammalian and non-mammalian test species, additional Estrogen, Androgen, Thyroid and Steroidogenesis (EATS) modality endpoints in non-mammalian models need to be assessed. These would mean for example the addition of hormonal measurements in fish, birds and amphibians. Furthermore, a better reporting of gross pathology findings for birds would also be considered advantageous for the assessment of endocrine disrupting properties. To facilitate adoption of additional measures, guidance on how to perform, report and evaluate these new endpoints is required. In this report, a variety of methods including a systematic evidence map, an extensive literature review and a survey of ecotoxicology laboratories were adopted to collect data on the topic. The systematic evidence map uncovered a range of methods for measuring sex and thyroid hormones in fish, birds and amphibians, although methods for measuring sex-hormones in fish, were by far, the most frequently encountered in the literature and laboratory survey. However, there are still considerable gaps in knowledge for: optimum sample timing for hormonal measurement (diurnal, developmental stage, etc.), issues with inherent variability, low sample volume (plasma/serum), test species selection, possible impacts of housing/diet/stress. The extensive literature review revealed that although gross pathology and histopathology have been used to investigate the effects of endocrine disrupting chemicals in birds, there are no standardised methods for assessment or interpretation, although relative weight of endocrine organs is frequently used as a gross pathology metric. Reports of how histopathology was assessed varied considerably. From the survey, few contract laboratories are experienced in conducting the avian test guideline and these types of pathology techniques. Recommendations for future work with non-mammalian taxa include: investigating the optimal time (or timings) for measuring hormones, developing non-invasive hormone measuring techniques, gaining knowledge of baseline/control hormonal data, and further developing guidance on conducting and assessing gross pathology and histopathology in birds.

© European Food Safety Authority, 2020

Key words: androgen, estrogen, steroidogenesis, thyroid, non-mammalian vertebrates, hormone measurement, bird pathology

Question number: EFSA-Q-2018-00907

Correspondence: PRAS.Secretariat@efsa.europa.eu

Disclaimer: The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the authors.

Acknowledgements: We are indebted to the internal peer reviewer, Prof Andreas Kortenkamp, for their constructive comments on the results of the systematic evidence map as well as external peer reviewers for their comments on the draft protocol for the same systematic evidence map.

Suggested citation: Martin O, Ermler S, McPhie J, Scholze M, Baynes A, 2020. Data collection in support of the Endocrine Disruption (ED) assessment for non-target vertebrates. EFSA supporting publication 2020:EN-1849. 131 pp. doi:10.2903/sp.efsa.2020.EN-1849

ISSN: 2397-8325

© European Food Safety Authority, 2020

Reproduction is authorised provided the source is acknowledged.

Summary

Within the EU, the approval of pesticides is regulated by Regulation (EC) No 1107/2009. An amendment to this Regulation (Regulation (EU) No 2018/605), which establishes the scientific criteria for the identification of pesticide active substances with endocrine disrupting (ED) properties, was put into place in April 2017. In June 2018, EFSA and the European Chemicals Agency (ECHA) published a guidance document on the implementation of the scientific criteria, this publication primarily addresses the Estrogen, Androgen, Steroidogenesis and Thyroid, known as 'EATS', modalities in vertebrates. It specifically included recommendations on how to perform hormonal measurements in mammalians. However, when it comes to non-mammalian vertebrate test guidelines (TG), there are several 'gaps' which need to be filled to adequately address the assessment of 'EATS' modalities and mirror mammalian TGs. For example, data on EATS hormones are not currently collected in amphibian, fish or bird TG.

To harmonise vertebrate OECD TGs additional endpoints in non-mammalian models need to be collected and therefore some guidance on how to perform, report and evaluate hormonal measurements in fish, birds and amphibians, Additionally information on how to perform, report and evaluate gross pathology investigations in birds, needs to be made available. These issues are the focus of this report, which combines data collected via a systematic evidence map of the literature on hormonal measurements in birds, fish and amphibians, an extensive review of the literature on pathology of endocrine organs in birds and a survey of ecotoxicology laboratories to gain insight into current experience, and best practice concerning these additional endpoints.

Approach

The **systematic evidence map** was used to capture data on methods developed to measure oestrogens, androgen and thyroid hormones in amphibian, fish and bird species. The systematic evidence map protocol was drafted with regard to the PRISMA-P (Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols) 2015 checklist and giving due consideration to the Code of Practice for the Conduct of Systematic Reviews in Toxicology and Environmental Health Research (COSTER).

An **extensive literature review** was conducted to provide data on pathology of endocrine organs in birds. As stipulated in the terms of reference, this review was not conducted according to strict systematic review criteria. Simple search terms were used to identify relevant literature.

In addition to the systematic evidence map (hormones) and extensive literature review (pathology) a **survey of ecotoxicology laboratories** was conducted to gather information on the current experience relating to measuring hormones in fish, amphibians and birds, and conducting pathology assessments in birds, for endocrine disruption assessment. The survey included a structured online questionnaire with multiple-choice closed-frame questions amenable to quantitative analysis and open-ended questions and opportunities for comments.

Hormone measurement

It is essential to recognise that sex-steroids such as oestradiol and testosterone, and thyroid hormones such as thyroxin are structurally conserved across vertebrate taxa, this means methods developed for one vertebrate species can, with some validation steps, be easily modified for a new test species. Whereas, pituitary hormones (e.g. thyroid stimulating hormone) are structurally distinct between species, and therefore species-specific methods need to be developed.

Methods to measure hormones can broadly be split into two groups, those that use biochemical analysis methods employing immune-detection (e.g. radio immune assay (RIA)), and those that use chromatography separation and mass spectrometry (e.g. liquid chromatography–mass spectrometry (LC-MS)). The development of sensitive RIA methods has been reported since the 1970s, and this was reflected in the higher proportion RIA of methods for the target taxa in the literature. As with the RIA, the enzyme-linked immunosorbent assay (ELISA) uses the specificity of the immune system to accurately identify target molecules. Its advantage over the RIA, beyond not employing a radioactive

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

reagent, is that it can directly quantify the target molecule, rather than infer its concentration from the level of bound radiolabelled equivalent. Modern analytical chemistry methods have the benefit of measuring multiple hormones in one sample, however they require specialist skills and instrumentation. Very few of the laboratories surveyed conduct hormone measurements in non-mammalian test species for endocrine disruptor assessment. The majority of respondents suggested this was due hormone measurements not being currently required by the guideline, lack of demand from client, lack of in-house experience or lack of in-house facilities.

Methods developed to measure sex-hormones in fish were the most frequently available. This was also reflected in the experience of the surveyed laboratories. Generally, from the laboratory survey, the addition of hormone measurements was perceived as both an opportunity and a challenge. Hormonal measurements, if informative and reliable, were seen to have the potential, when considered with other endocrine endpoints, to strengthen weight-of-evidence assessments. However, the interpretation of hormonal measurements in fish, birds and amphibians without a baseline or historical controls was considered problematic. There was also a concern that hormone measurements would inherently have greater variability than other TG endpoints and require a larger number of animals.

Although, it is well recognised that hormones cycle over various timeframes (e.g diurnally, development-stage), there was little information in the retrieved literature about the most appropriate sampling timeframes. This issue was also raised as a concern in the laboratory survey. Further research and protocol optimisation are necessary to support high quality, comparable data acquisition.

The small sample volume available for hormone measurements, particularly in small fish species (zebrafish, medaka) was also flagged as a concern in the laboratory survey. Current recommendations for hormone measurement suggest using plasma or serum. However, both the literature and the laboratory survey revealed possible non-invasive sampling matrices for hormones. Non-invasive methods could facilitate a more refined approach for hormone assessment (multiple measures over time, multiple hormones from each individual) and should be further investigated.

Pathology in birds

Globally very few contract laboratories conduct reproductive test on avian species according to OECD TG 206, and their feedback from the laboratory survey was comparably limited in number. Those that did respond considered the TG in need of an overhaul to make it fit for endocrine disruption assessment.

From the literature review, gross pathology in birds was generally used alongside histopathology assessment (and other reproductive function endpoints) to identify endocrine disrupting activity. Histopathology can be sensitive tools for assessing endocrine disruption or toxic modes of action, however, it requires specialist knowledge and training. Further research into this topic and guidance on conducting and assessing gross pathology and histopathology endpoints in birds is needed.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table of contents

Abstract 1
Summary 3
1. Introduction....7
1.1. Background and Terms of Reference as provided by the requestor 7
1.2. Interpretation of the Terms of Reference....7
1.3. Endocrine system background 7
1.3.1. The hypothalamic-pituitary-gonadal (HPG) axis....7
1.3.2. The hypothalamic-pituitary-thyroid (HPT) axis 9
2. Systematic Evidence Map of the Literature on Hormonal Measurements in Birds, Fish and Amphibians....10
2.1. Methodology....10
2.2. Results 11
2.2.1. Literature screening 11
2.2.2. Evidence synthesis 13
3. Extensive review of the literature on pathology of endocrine organs in birds....22
3.1. Extensive review method....22
3.2. Results 22
4. Survey of ecotoxicology laboratories 24
4.1. Methodology....24
4.1.1. Development of questionnaires....24
4.1.2. Identification of laboratories 24
4.2. Results 24
4.2.1. Response rate to online survey 24
4.2.2. Analysis of responses to online survey....25
4.2.3. Summary results of follow-up telephone interviews....27
5. Recommendations....29
5.1. General considerations 29
5.2. Recommendations for hormone analysis in amphibians and fish....29
5.2.1. Thyroid and reproductive hormone analysis in amphibians....31
5.2.2. Thyroid and reproductive hormone analysis in fish 33
5.3. Recommendations for hormone measurements, gross pathology and histopathology in birds 34
5.3.1. General recommendations for the addition of hormone measurements, gross pathology and histopathology in birds 34
5.3.2. Specific recommendation for hormone measurements in birds 35
5.3.3. Specific recommendation for gross pathology assessment in birds 35
5.3.4. Specific recommendation for histopathology assessment in birds 36
6. Future research needs....36
References....38
Appendix A – Protocol for a systematic evidence map of methods to measure hormones related to estrogen, androgen and thyroid pathways in fish, birds and amphibians....55
Appendix B – Analysis of data extracted for the systematic evidence map of methods to measure hormones related to estrogen, androgen and thyroid pathways in fish, birds and amphibians 61
Appendix C – Results of the extensive review of the literature on pathology of endocrine organs in birds 108
Appendix D – Results of online survey of ecotoxicology laboratories 120
Appendix E – Summaries of follow-up telephone interviews....121
Annex A – Protocol development for the systematic evidence map of methods to measure hormones related to estrogen, androgen and thyroid pathways in fish, birds and amphibians 128
Annex B – Data extraction for the systematic evidence map of methods to measure hormones related to estrogen, androgen and thyroid pathways in fish, birds and amphibians....129

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Annex C – Data extraction for the extensive review of the literature on pathology in birds 130
Annex D – Supplementary files related to the online questionnaire 131

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

This contract/grant was awarded by EFSA to: Brunel University London

Contractor/Beneficiary: Brunel University London

Contract/Grant title: Data collection in support of the Endocrine Disruption (ED) assessment for non-target vertebrates

Contract number: NP/EFSA/PRAS/2018/02

1.2. Interpretation of the Terms of Reference

The assignment outlined in the Tender Specifications (NP/EFSA/PRAS/2018/02) is part of EFSA's 2018 Work Programme for grants and operational procurements, specifically this Tender Specification relates to EFSA Pesticides Unit's ongoing program of work relating to the approval of pesticides, linked to the EU's activities in the area of endocrine testing and regulation of chemicals.

Within the EU, the approval of pesticides is regulated by Regulation (EC) No 1107/2009. An amendment to this Regulation (Regulation (EU) No 2018/605), which establishes the scientific criteria for the identification of pesticide active substances with endocrine disrupting (ED) properties, was put into place in April 2017. In June 2018, EFSA and the European Chemicals Agency (ECHA) published a guidance document on the implementation of the scientific criteria, this publication primarily addresses the Estrogen, Androgen, Steroidogenesis and Thyroid, known as 'EATS', modalities in vertebrates. It specifically included recommendations on how to perform hormonal measurements in mammalians. However, when it comes to non-mammalian vertebrate test guidelines (TG) there are a number of 'gaps' which need to be filled to fully address assessment of 'EATS' modalities and mirror mammalian TGs. For example, in the OECD TGs which do address endocrine specific endpoints (i.e. amphibians and fish) there are no specific recommendations on which hormones to measure, how or when to measure hormones or how to interpret the results. In addition, in the current OECD TG 206 avian reproduction test (OECD, 1984), not only is there no recommendation relating to hormonal measurement, but there is also little guidance on gross pathology (i.e. which tissues to investigate, how tissues/organs should be assessed or how the histopathology results should be interpreted). To harmonise vertebrate OECD TGs, these additional endpoints in non-vertebrate models need to be collected and therefore some guidance on how to perform, report and evaluate hormonal measurements in fish, birds and amphibians, and how to perform, report and evaluate gross pathology investigations in birds, needs to be drafted. These issues are the focus of this EFSA Tender.

1.3. Endocrine system background

1.3.1. The hypothalamic-pituitary-gonadal (HPG) axis

Due to the vital role of the endocrine system of vertebrates, the endocrinology of birds, fish and amphibians are broadly similar to those found in mammals. All vertebrates use hypothalamic-pituitary-target organ axes for hormone production and regulation. For example, the hypothalamic-pituitary-gonadal (HPG) axis is a fundamental biological system required in controlling reproduction and sexual behaviours. The HPG axis is present and its function is generally well conserved across vertebrate groups. As with mammals, in lower vertebrates (e.g. fish, amphibians, birds), the axis comprises of endocrine glands and target tissues, with hormones transported via the blood, and is closely regulated by feedback mechanisms (Figure 1) Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus by GnRH-expressing neurons, which in turn stimulates the release of gonadotrophic hormones (e.g. luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) from the anterior pituitary, these travel in the blood stream to their primary target, the gonads. In males, FSH is vital for spermatogenesis and in females, FSH stimulates the maturation of ovarian follicles. LH acts on both the male and the female gonad to produce steroid hormones. In males, LH binds to receptors on Leydig

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

cells in the testes, stimulating the production and secretion of testosterone. Testosterone is required for normal spermatogenesis, and as part of the male HPG feedback loop, inhibits the hypothalamus's secretion of GnRH. In females, LH stimulate Theca cells in the ovaries to produce androgens, which is then converted into oestrogen by nearby granulosa cells. FSH and LH stimulating effects on the ovary, and the ovary's production of oestrogen and progesterone regulate the ovarian and menstrual cycles. Steroid hormones (e.g. testosterone, oestradiol, progesterone), derived from cholesterol, are secreted by the gonads (Figure 1). The production, conversion and breakdown of steroid hormones, in the endocrine glands and target tissues, are carefully controlled by a range of steroidogenic enzymes (Figure 1), many of which belong to the cytochrome P450 family e.g. aromatase/CYP19A1, 17α-hydroxylase/CYP171A.

As many pesticides, herbicides and fungicides target enzyme action in their taxonomic group of interest, it is possible that these types of environmental chemicals can inadvertently disrupt steroidogenic enzymes in non-target organisms (Poulsen et al. 2015). The disruption of steroidogenic enzymes can result in inappropriate steroid concentrations or timing, which in turn can impact an organism by disrupting cellular/organ development, reproductive fitness and behaviour (Panter et al. 2004). One of the best known examples of this is the action of aromatase (CYP19A1) which converts testosterone into oestradiol and controls the fine balance between these two potent sex-steroids (Figure 1).

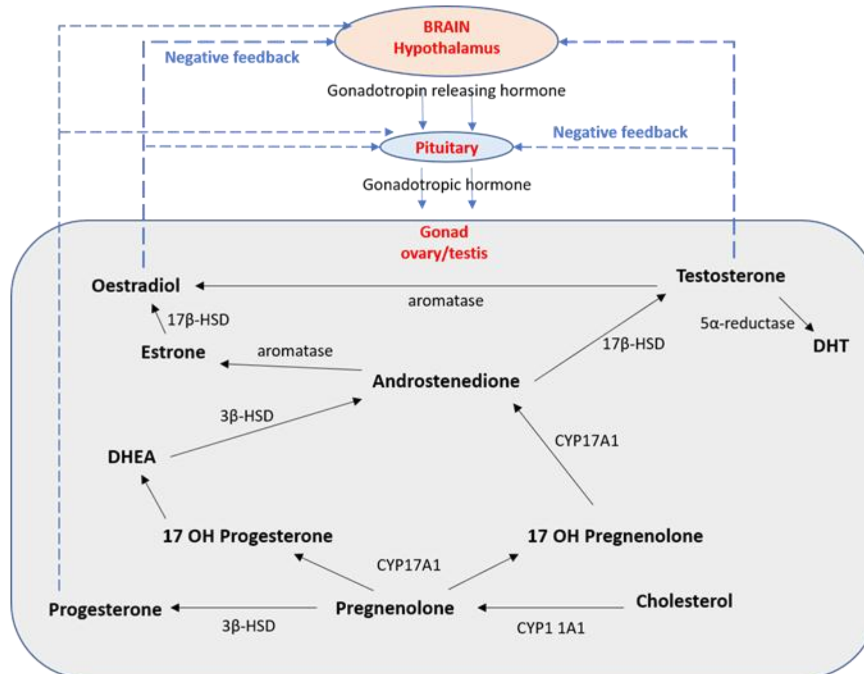

Figure 1: Simplified vertebrate hypothalamic-pituitary-gonadal (HPG) axis and interlinked sex steroidogenesis pathways. Black arrows indicate steroid conversion by steroidogenic enzymes. Blue dotted lines indicate negative feedback which inhibits further release of hormone(s).

Although the structures of vertebrate steroids are conserved among taxa, there are unique physiological and biochemical differences between taxa which are important to consider when attempting to measure and quantify circulating steroids. In fish, 11-Ketotestosterone (11-KT) has long been considered a potent androgen vital in male reproductive development and behaviour. 11-Ketotestosterone (11-KT) is converted from testosterone (T) via the sequential action of steroidogenic enzymes 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase (11β-HSD). More recently 5α-dihydrotestosterone (DHT) has also been shown to be a key androgen in gamete development in both male and female fish (Margiotta-Casaluci et al. 2013; Margiotta-Casaluci and Sumpter 2011).

Differences in steroid binding proteins also occur between taxa. These plasma proteins regulate the non-protein-bound or biologically active 'free' fractions of circulating steroid hormones, which are

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

considered 'available' to target cells/tissues. In mammals, steroids are transported in the blood by albumin, sex hormone-binding globulin (SHBG), and corticosteroid-binding globulin (CBG). SHBG has been identified in all vertebrate groups, except for birds (Wingfield et al. 1984). In birds, it seems albumin alone undertakes the role binding the majority of sex-steroids in circulation. Differences in the binding affinity of SHBG have been investigated in fish, and similar to mammals, can vary considerably between species. With some species binding oestrogens more strongly than androgens, and vice versa. Fish SHBG also differs from the mammalian ortholog in that it binds the androgen precursor, androstenedione, as strongly as testosterone, and with greater affinity than 11-ketotestosterone (Hammond 2011).

1.3.2. The hypothalamic-pituitary-thyroid (HPT) axis

Thyroid endocrinology is also generally well conserved across vertebrate taxa, with similarities in thyroid synthesis, metabolism and mechanism of action. As in mammalian species, thyroid hormone (TH) release from thyroid follicles is regulated by pituitary thyroid stimulating hormone (TSH). Circulating TH negatively influences the activity of the hypothalamus and pituitary and, when TH levels are reduced or "disrupted," the activity of the hypothalamus–pituitary increases to elevate TH production by the thyroid follicles to restore its circulatory set point concentrations, and vice versa. Thus, the activity of the hypothalamic-pituitary-thyroid (HPT) axis is regulated by negative feedback (Carr and Patiño 2011).

In mammals, thyrotropin-releasing hormone (TRH) controls the release of TSH. However, although present in non-mammalian vertebrates, TRH does not always act as a thyrotropin (TSH)-releasing factor, whereas corticotropin-releasing hormone (CRH) appears to be a potent stimulator of TSH secretion (**Error! Reference source not found.**). A recent review suggests additional close interactions between adrenal/interrenal and thyroidal axes, highlighting that corticoids also affect the expression of deiodinases (Dio1) and thyroid hormone receptors (Watanabe et al. 2016).

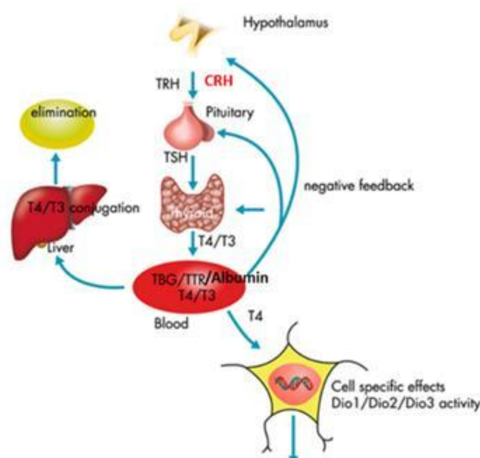

Figure 2: Simplified vertebrate hypothalamic-pituitary-thyroid (HPT) axis and interlinked thyroid hormone pathways. In non-mammalian vertebrates' Corticotrophin-releasing hormone (CRH), not thyrotropin-releasing hormone (TRH) from the paraventricular nucleus (PVN) stimulates synthesis of thyroid-stimulating hormone (TSH) in the pituitary gland. TSH directly stimulates the thyroid gland to produce thyroid hormones (TH; T3 and T4). In turn, circulating TH negatively regulates CRH and TSH synthesis and release as part of a negative feedback loop. In the blood T4 binding protein (TBG), transthyretin (TTR) and albumin carry T3 and T4 to target tissues/cells where deiodinases transform T4 to T3 (Cio1/Dio2) or T4 and T3 to T2 (Dio3). Modified from (Gore et al. 2015).

As with the sex-steroids, only a small fraction of thyroid hormones (T3, T4) are found 'free' in the blood stream, the majority (>99%) being bound to transport proteins. In humans, around 75% of serum T4 is bound to thyroid binding globulin (TBG), 15% to transthyretin (TTR) and <5% to albumin. These three proteins are synthesized by the liver and secreted into the bloodstream, where they distribute

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

thyroid hormones from the thyroid gland to cells throughout the body (Richardson et al. 2005). TBG is not found in all species, with albumin being found most frequently as the binding protein in a survey of 150 species of adult vertebrates (Richardson 2002).

Indeed, in many vertebrate species, the production of certain thyroid binding proteins is transient and related to specific developmental stages. For example, in rats TBG is only present during early development and senescence, while adult birds and many adult mammals have TTR as their main form of thyroid binding transport protein. In fish and amphibians, hepatic TTR synthesis is primarily found during early development, metamorphosis and smoltification (salmonids). More recently, sensitive molecular techniques have detected TTR expression in the liver of adult amphibians (Ishihara et al. 2012) and fish (Li et al. 2011), however, although these levels are still generally much lower than at metamorphic or developmental stages they likely still play a critical role in delivering TH to tissues.

Only one of the transport proteins, TTR, is synthesised in the brain of mammals (specifically choroid plexus) and involved in transporting TH across the blood-brain barrier. TTR is also synthesised in the choroid plexus of non-mammalian terrestrial vertebrates (birds, reptiles) but not aquatic vertebrates (fish, amphibians) (Schreiber 2002). TTR was first described in mammals as thyroxine (T4) binding protein. However, it seems mammals are the exception among vertebrates in respect to the function of TTR, as in teleost fish, amphibians, reptiles and birds TTR preferentially binds triiodothyronine (T3), which is the active form of thyroid hormone (TH) (Richardson 2015). It is also important to note, when comparing results in different test species, that total T4 and total T3 levels in blood vary between classes of vertebrates (Hulbert 2000).

Chemicals which disrupt the thyroid system by targeting thyroid receptors as agonists or antagonist, thyroid hormone transport proteins, thyrotropin releasing hormone (TRH), or alter thyroid synthesis or metabolism could impact a wide range of vertebrate species including non-mammalian species such as birds, fish and amphibians.

2. Systematic Evidence Map of the Literature on Hormonal Measurements in Birds, Fish and Amphibians

2.1. Methodology

Systematic Review (SR) is a rigorous, protocol-driven approach to minimising error and bias in the aggregation and appraisal of evidence initially developed in the fields of psychology, social science and healthcare. The extension of SR techniques to other fields is driven by the common need across disciplines to make the best use of existing evidence when making decisions.

SR methods are best utilised to provide answers to focused, well-defined, closed-frame questions. However, decision-makers and user groups in policy and regulatory practice often ask 'open-framed' questions related to the state of evidence on a topic that do not readily translate into closed-framed questions suitable for systematic review. For example, for this tender, the questions of interest include: What methods are available for hormonal measurements in non-target species? What is their reliability, reproducibility, limitations? How should they be performed, reported and evaluated?

This type of question highlights the need for a means of cataloguing all the available evidence in a comprehensive, transparent and objective manner to describe the state of knowledge and identify sub-sets of topics for further analysis. 'Systematic mapping' methodology was developed in the social sciences in response to this need. It follows the same rigorous, objective and transparent processes as do systematic reviews thus avoiding the potential pitfalls of traditional literature reviews (e.g. reviewer and publication bias) and is particularly valuable for broad, multi-faceted questions.

A systematic evidence map protocol has been drafted with specific regards to the PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) 2015 checklist (Moher et al. 2015) and giving due consideration to the Code of Practice for the Conduct of Systematic Reviews in Toxicology and Environmental Health Research (COSTER) (Whaley et al. 2020). A preliminary draft was published on Zenodo on 10th May 2019 (doi: 10.5281/zenodo.2711704) and comments were invited between

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

13th to 20th May. The revised protocol was published on Zenodo (doi: 10.5281/zenodo.3335993) and can be found in Appendix A –. All supplementary files related to the development of the final protocol can be found in Appendix C –. The agreed PIT statement and revised inclusion and exclusion criteria are given in Table 1:

Table 1: Agreed PIT statement and eligibility criteria

		Inclusion criteria	**Exclusion criteria**
Population	Species of fish, amphibians and birds	Both wild and standard test species of fish, birds or amphibians, *in vivo* and *in vitro.*	All other species that are not classified as fish, birds or amphibians.
Index test	Invasive or non-invasive hormonal measurement methods (related to EATS pathways)	All sampling matrices including faeces or tankwater. Hormones related to the EATS pathways in fish, birds and amphibians. Estrogens, androgens and thyroid hormones are the main target of these searches but retrieved studies investigating CRH, cholesterol, FSH and/or LH will not be excluded.	Hormones not related to EATS pathways in fish, birds and amphibians, e.g. adrenaline and noradrenaline, stanniocalcin. Hormone dependent biomarkers such as vitellogenin.
Target condition	Detection of or supporting evidence for an endocrine disruption mode-of-action or adverse effect.	Studies addressing methodological aspects of hormone measurements, e.g. method development, sample preparation and storage, method validation.	Studies where pre-existing method(s) in the taxonomic group under consideration has been applied to measure hormones[(a)]

(a): As part of data analysis, we will investigate how selected methods of particular interest have been applied using citation searches.

2.2. Results

2.2.1. Literature screening

Literature searches were carried out in full accordance with the published protocol and results are presented in Table 2: .

Whilst screening title and abstracts, it became apparent that defining 'novelty' in relation to the aim of capturing development in analytical methods rather than their routine application was not always a straightforward decision to make on the basis of the information available. Therefore, at this initial stage, a decision was made not to eliminate studies which did specifically mention a relevant method of measuring hormones (i.e. RIA, ELISA or chemical analysis) in their abstract, even if the main objective of the reference was not expressly stated as method development. Further, during full text screening, we recorded the name of any commercial kits employed (when given) and when an existing method was referenced, we made a note of the reference. This list of references was then in turn checked to identify papers with original methods that may have been missed by our search or erroneously eliminated during screening. This process identified a further 21 articles.

A consistency check was carried before each screening stage. The Cadima platform also calculates the kappa value based on the measuring agreement of Cochrane for inter-reviewer agreement. The kappa

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

value was equal to 0.66 which is interpreted as the strength of agreement between reviewers is good. Results of the literature screening process are illustrated by a flow diagram (Figure 3).

Table 2: Result of literature searches

Database or further sources	Results
Web of Science	4940
Science direct	38
Pubmed	2944
Medline	2088
SCOPUS	8847
DART grey search	38
ECETOC	6
EFSA	2
Environar	13
EPA	7
Google Scholar grey search	25
JRC	6
Umweltbundesamt	33
RIVM	7

Table 3: Reasons for exclusion

	Number of exclusions
Taxonomic group	37
Hormone(s)	164
Method	832
Full-text not available	0
Full-text not assessable	11
Full-text duplicate	47
No primary data/summary statistics presented	10

Figure 3 shows the results of the full-text-screening in terms of the recorded reason for exclusion. Notably, we were able to retrieve and check the full text of all 1181 eligible articles after title-abstract screening. A further 47 duplicate articles were identified when examining the full text of articles. Ten review articles were excluded from the data extraction exercise but those covering relevant topics will be considered when drafting guidance.

By far the most common reason for exclusion is related to the lack of novelty of the methodology. In some cases, examination of the full text of articles revealed that the hormone(s) measured did not meet the inclusion criteria (primarily progestogens and other steroid) or that the species in which measurements had been carried out did not belong to the taxonomic groups of interest. We could not assess 11 articles as they were published in languages that were neither spoken by members of the team nor could be translated by Google translate (e.g. articles in Farsi or scanned articles that did not allow the copy of text). The sum of reasons for exclusion is greater than the number of articles excluded as at least one but possibly more than one reason to exclude a given study could be recorded.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

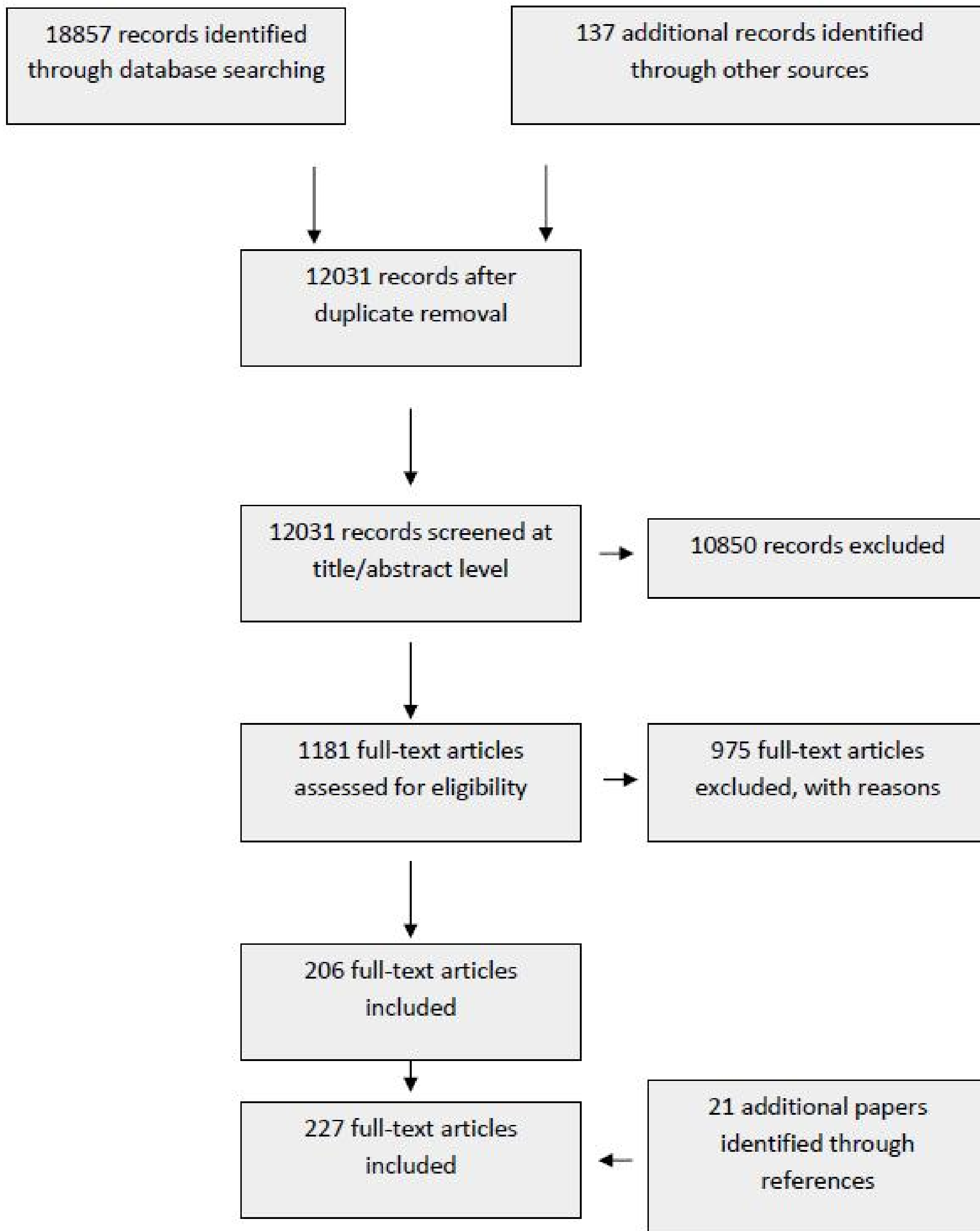

Figure 3: Flow diagram

2.2.2. Evidence synthesis

Data from all 227 eligible studies were extracted using the data extraction template (Annex B –After data were extracted for a given study or methods, it was possible to assess if sufficient data had been reported for inclusion in the evidence synthesis. A detailed narrative summary of the data extracted

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

from eligible studies per type of method, taxonomic group and hormones measured can be found in Appendix B –.

Figure 4 illustrates where the development of methods for the measurements of sex and thyroid hormones in amphibians, fish and birds has focussed to date. The picture that emerges is one where many RIA methods have been published, much more so than ELISA methods. This could be due to the fact that we did not limit our searches in time and retrieved many early RIA method development papers. Chemical analysis in terms of chromatographic methods that do not just allow the separation but also the quantification of hormones tend to be more recent and have primarily focussed on estrogens in fish species. Comparatively, fish are the taxonomic group that has received the most attention regardless of the type of hormone measurement method employed. Birds have also been discussed relatively widely in the literature but this may be due to an interest in stress and corticosteroids in wild birds as part of field studies (see section 2.2.2.2). The hormone levels of amphibians are less studied and for all type of method the main focus in this taxonomic group has tended to be related to thyroid disruption (see section 2.2.2.1). In birds, the focus, probably for reasons mentioned above, has been almost entirely on steroid hormones whilst the interest in using fish models to study disruption of the thyroid axis is relatively recent (see section 2.2.2.3).

	Amphibian				Bird				Fish			
Method type	(Anti-)androgenic	(Anti-)estrogenic	Steroidogenesis	Thyroid disruption	(Anti-)androgenic	(Anti-)estrogenic	Steroidogenesis	Thyroid disruption	(Anti-)androgenic	(Anti-)estrogenic	Steroidogenesis	Thyroid disruption
RIA	4	6	2	8	21	23	4	9	30	21	2	13
ELISA	1	1		1	6	3			9	5		1
Chemical analysis	1	1		3	10	11	1	2	20	31	6	8

Figure 4: Heat map of the number of methods from extracted studies per type of method, taxonomic group and endocrine disrupting modality. More than one method may be reported for a given reference if, for example, the method was applied in more than one species or for different life stages. Not all extracted studies were assessed as suitable for inclusion in the evidence synthesis.

2.2.2.1. Amphibians

Radioimmunoassay (RIA)

After data extraction, no study was found to describe original and detailed methods or report sufficient methodological data for the quantification of estrogens or androgens in amphibian samples using RIA.

Regard et al. (1978) (reference 5934 in Annex B –) validated the use of a commercial T4 RIA kit developed for use with rat or human serum in adult *Bufo marinus* and *Rana pipiens.* They found that amphibians had far lower levels of T4 (in comparison to mammals), such that adaptions to the method were required. They subsequently used the RIA method with plasma from *Rana catesbeiana* tadpoles at a range of metamorphic stages, demonstrating elevated 3,3′,5-triiodothyronine (T3) and thyroxine (T4) plasma concentrations towards metamorphic climax (one of the first publications to show this in spontaneously metamorphosing frogs). The literature search retrieved an additional four studies which gave sufficient details for measuring T3 and T4 in amphibian (*Rana perezi*, *B. Marinus*, *R. catesbeiana*)

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

samples using RIA (Mondou and Kaltenbach 1979, Gancedo et al. 1995, Suzuki and Suzuki 1981, Weber et al. 1994; see references 206, 16071, 6031, 9500, respectively, in Annex B –). Sample matrices described were varied and included plasma, serum, pericardial fluid, whole embryos and thyroid glands. Sample extraction methods differed depending on matrix and publication. The methods described had been previously developed for the measurement off thyroid hormones in fish, human or rodent samples.

Enzyme-linked immunosorbent assay (ELISA)

The literature search found no eligible studies describing original and/or detailed methods or report sufficient methodological data for the quantification of estrogens or androgens in amphibian samples using ELISA methods. Arias Torres et al. (2016) (reference 15412 in Annex B –) validated a competitive chemiluminescence enzyme immunoassay (ECLIA) for ovarian steroid in *Rhinella arenarum* using a method previously developed for human samples.

Korte et al. (2011) (reference 15475 in Annex B –) measured intracellular (within pituitaries), secreted (*ex vivo* pituitary culture), and circulating (serum) TSH in *Xenopus laevis* and *Xenopus tropicalis* using a Sandwich ELISA.

Chemical analysis

Chang et al. (2010) quantified the estrogens (E1, 17β-E2, 17α-E2) in African clawed frogs (*Xenopus laevis*), a standard test species (see reference 1407 in Annex B –) using liquid chromatography–electrospray tandem mass spectrometry (LC–ESI-MS/MS). It required to 0.3-1ml samples of frog plasma and reported a linear calibration range between 0.5–1000 ng/mL.

Three references reported analytical chemistry methods to measure thyroid hormones in amphibians, all based on liquid chromatography. The method by Simon et al. (2002) (reference 8692 in Annex B –) differed from the other two both with regards to the sampling matrix (whole body homogenates of tadpoles of African Clawed frogs) and therefore sample preparation, and the instrumentation (use of the LC-ICP-MS as opposed to the more selective LC-MS/MS methods). Luna et al. (2013) used ultrahigh-performance liquid chromatography and isotope dilution tandem mass spectrometry (UPLC-ID-MS-MS) to quantify total T4 in a small volume (10 µL) of plasma also from tadpoles and adult *Xenopus laevis* (reference 3463 in Annex B –). Hansen et al. (2016) validated a LC-MS/MS method to quantify absolute and total concentrations of eleven thyroid hormones and associated metabolites in both 50 µL plasma of adult *Xenopus* or 50 µL serum of tadpoles (American bullfrog, *Rana (Lithobates) catesbeiana*) (reference 8142 in Annex B –). The LC-MS/MS methods described by Luna et al. (2013) and Hansen et al. (2016) do not only differ in their scope. The method described by Luna et al. (2013) allowed quantification of T4 in smaller plasma sample volumes, that as a result did not need to be pooled even when handling tadpoles. By contrast, Hansen et al. (2016) were able to avoid pooling tadpole serum samples by focusing on a larger species, the American Bullfrog. While the method by Hansen et al. (2016) requires higher sample volumes, overall it was shown to perform better in terms of its absolute recoveries, sensitivity and precision in addition to allow the quantification of a wide range of relevant thyroid hormones and related compounds (see Appendix B.3.1.2).

Comparative performance of different method types

Table 4**:** compiles method performance data extracted, when available and when reported in units that allowed comparison, for hormone measurements in plasma or serum. The accuracy and precision of the different types of analytical investigated were fairly comparable and are not shown. Table 4**:** suggests that for equivalent sampling volumes, modern chromatographic analytical instrumentations can achieve lower limits of detection.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 4: Comparison of the performance of different types of methods to measure thyroid hormones in the plasma of amphibian species (when reported in comparable units).

	RIA		ELISA		Chemical analysis	
	Minimum	**Maximum**	**Minimum**	**Maximum**	**Minimum**	**Maximum**
Sample volume	10µL	500 µL	5µL	20 µL	10µL	50µL
Limit of detection	T4: 0.2 ng/mL T3: 0.15 ng/mL	T4: 50 ng/mL T3: 5 ng/mL	-	-	T4: 70 pg/mL T3: 29 pg/mL	T4: 6.2 ng/mL

–: no information available

The systematic evidence only uncovered one method using a non-invasive sampling matrix in amphibians, specifically faeces (Hogan et al. 2013). The testosterone-to-estrone conjugate metabolite ratios in faeces using ELISA kits have been used in conservation to sex animals. A number of other non-invasive matrices have been investigated and in some cases recently validated for the measurement of stress hormones, including dermal secretions (Scheun et al. 2019) and saliva (Hammond et al. 2018). The use of non-invasive methods permits repeated measurements or measurements at different time points or life-stages and the usefulness and reliability of non-invasive sampling matrices for hormonal measurements in amphibian species should be the focus of further research.

2.2.2.2. Birds

RIA

The literature search retrieved 19 publications which gave sufficient details for measuring estrogens (estrone; E1, and 17β-estradiol; E2) and/or androgens (testosterone: T, dihydrotestosterone: DHT) in avian samples with a RIA. Four of the aforementioned papers measured both estrogens and androgens in birds (see Appendix B.1.2.1). Sample matrices included plasma, serum, droppings/faeces, gonads and brain. Sample extraction methods differed depending on matrix and publication. Estrogens and/or androgens have been measured at different lifestages including the egg yolk, embryo, chicks and adults in wild and standard test species such as the Japanese quail. All published methods required an extraction step (see Appendix B.1.2.1).

Six publications were retrieved which measured sex-steroids in bird droppings/faeces. This sampling matrix has been investigated primarily in conservation biology. However reported extraction efficiencies were low when compared to other sampling matrices such as plasma (see Table 10: for details of the method performance).

The measurement of thyroid hormones in birds has significantly less attention. In total four publications which measured T3 or T4 in avian species gave sufficient details of the RIA methods (see Appendix B.1.2.2). Notably, Wilson and McNabb (1997) (Reference 6630 in Annex B –) investigated maternal T3 and T4 deposition in egg yolk of Japanese quail (*Coturnix japonica*). The EPA Test Guideline (OCSPP 890.2100) (U.S. EPA 2015, Reference 13985 in Annex B –) states that thyroid hormones are lost during organic extraction and should be evaluated in un-extracted plasma samples. It recommends the use of a commercial RIA kits with low cross-reactivity and that any new kit should be tested for sensitivity, parallelism, specificity and intra- and inter-assay variation (U.S. EPA 2015).

ELISA

The literature search retrieved six publications which gave sufficient details for measuring estrogens (estrone; E1, 17β-estradiol; E2, estriol E3) and/or androgens (testosterone: T) in avian samples with ELISA or Enzyme immunoassay (EIA) methods in mostly wild bird species although one study had been carried out in the Japanese quail. The systematic literature did not retrieve any study describing original and/or detailed methods for the detection of thyroid hormones. Sampling matrices described were varied and included plasma, droppings/faeces, saliva and brain. Sample extraction methods differed depending on matrix and publication (see Appendix B.2.2 for details).

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Although the EPA Test Guideline recommends diethyl ether extraction of sex-steroids from plasma and egg yolk, it recommends ethanol for extracting sex-steroids from faeces (U.S.EPA 2015). Hahn et al. (2011) (reference 752 in Annex B –) also investigated the feasibility of measuring testosterone in avian (Cockatiel and Rose-ringed parakeet) saliva (without solvent extraction). However, they reported at present saliva does not appear to be an ideal medium for reliable hormone level measurement. See Table 14: for details of above publications.

Chemical analysis

Androgens and estrogens have been measured in variety of avian species and from different sampling matrices including plasma or serum from blood, brains, egg yolk but also non-invasive sample matrices such as feathers. By contrast with androgens, the systematic literature search retrieved several methods developed to measure estrogens in bird faeces, but none in feather samples. All three methods were developed in China and the motivation for measuring hormone levels in livestock manure appears to be related to both the existence of concentrated animal feeding operation (CAFOs) and the risk that estrogens could be discharged into the aquatic environment via surface runoff from both land application or composting (see Appendix B.3.2.1 for details).

Endogenous male and female hormones are potent growth promoters whose use in farming, including poultry farming, is prohibited in the European Union (The European Parliament and the Council of the European Union 2008). Enforcement has required the use of validated method to ensure compliance to measure hormone levels in meat or muscle tissue but also the serum of farmed animals as described by McDonald et al. (2010) (reference 10281 in Annex B –). The LC-MS/MS method is able to detect both testosterone and epitestosterone and is validated in accordance with Commission Decision 2002/657/EC and ISO/IEC 17025:2005 in poultry in turkey serum. It required a 5ml sample volume which limits its application to smaller bird standard test species in the ecotoxicological regulatory context. Mi et al. (2014) (reference 18009 in Annex B –) describe a method to measure simultaneously a total of 26 steroids, including testosterone and five other androgens in eggs from hen, quail, duck and pigeon for food safety purposes. Mi et al. (2014) usefully compare the limits of detection of analytical methods available at the time of writing with the range of concentrations of steroids, including testosterone, with the naturally occurring ranges reported for hen eggs. It becomes evident that minima for androgens and estrogens overlap with LOQs and raises doubts over the application of these methods in an ecotoxicology testing context.

Koren et al. (2012b) (reference 12969 in Annex B –) and Prior et al. (2016) (reference 8141 in Annex B –) describe LC-MS/MS method developed to measure hormones in much smaller volumes of avian plasma. Koren et al. (2012b) validated sample preparation for simultaneous quantitation of not only testosterone and dehydroepiandrosterone (DHEA) but also E2, corticoids and progestagens in five avian species. The citation search (36 citations in Scopus) revealed that the method has been applied in ecology to investigate wild birds (Crossin et al. 2013) and in aquaculture (Guzmán et al. 2015). Prior et al. (2016) adapted a method used in the clinical setting to avian plasma to examine a total of eight androgens and progestins (see Table 17: in Appendix B.3.2.1 for further details). They present the distinct advantage of allowing simultaneous quantification of both estrogens and androgens.

Liere et al. (2019)(reference 3788 in Annex B –) adapt a GC-MS/MS method to measure steroids in rat brain to quail brains. They measured a wide range of estrogens, androgens and their precursors or conjugated forms in order to investigate brain steroidogenesis in both brains and plasma. An important point to note is that birds were rapidly killed by decapitation without anaesthesia to avoid potential changes in steroid concentrations. They reported detection limits of 0.002 ng/mL for both E2 and T in plasma, which compares favourably to LC-MS/MS methods but the method required a larger sample volume (1mL).

Because obtaining blood samples from wildlife is challenging, and hormones levels can change within a few minutes and is affected by the stress of capture, non-invasive sampling methods have been developed with e.g. faeces and feathers. This systematic evidence map uncovered two LC-MS/MS methods to measure androgens in feathers. Koren et al. (2012a) (reference 2229 in Annex B –) determined testosterone and corticoids in the feathers of wild house sparrows, whereas Bílková et al.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

(2019) (reference 7209 in Annex B –) recently optimised an analytical method that also simultaneously determines testosterone and cortisone from feather samples collected from the flanks of seven different species of passerine birds.

Only two retrieved references were related to chemical methods to measure thyroid hormones in avian samples. Wang et al. (2015) (reference 7269 in Annex B –) present a LC-MS/MS method suitable to determine T4, T3 and rT3 in 5g of chicken meat and chicken egg samples. There is little evidence that the method would be suitable for application in the ecotoxicology setting as opposed to food safety. By contrast, the study of Ruuskanen et al. (2018) (reference 11791 in Annex B –) presents an extremely sensitive nanoflow LC-MS/MS method specifically suited for toxicological research in small animals and small sample volumes. They applied their method in 5-400mg egg yolk samples from both domestic and wild bird species and limits of detection in the attomolar range (10^{-18} mol).

Comparative performance of different method types

Table 5: compiles method performance data extracted, when available and when reported in units that allowed comparison, for hormone measurements in the plasma or serum of avian species. Although similar sensitivities can be achieved by the different method types, analytical chemistry methods tended to require larger sample volumes whilst being able to quantify multiple steroid hormones simultaneously.

Table 5: Comparison of the performance of different types of methods to measure T, E2 or T4 in the plasma or serum of bird species (when reported in comparable units).

	RIA		**ELISA**		**Chemical analysis**	
	Minimum	**Maximum**	**Minimum**	**Maximum**	**Minimum**	**Maximum**
Sample volume						
T	50 µL	500 µL	12 µL	0.5 mL	30 µL	5 mL
E2	25 µL	3 mL	-	-	100 µL	5 mL
T4	20 µL	50 µL	-	-		
Limit of detection						
T	8 pg/mL	-	15 pg/mL	0.1 ng/mL	2 pg/mL	0.1 ng/mL
E2	20 pg/mL	30 pg/mL	-	-	2 pg/mL	0.1 ng/mL
T4	-	-	-	-	-	-

–: no information available

Similarly to amphibians, a number of hormone measurement methods have been developed for non-invasive matrices and their application in the regulatory ecotoxicological testing context deserves further research.

2.2.2.3. Fish

Of all taxonomic group of interest to this study, fish species have received most attention in the retrieved literature. The systematic literature search retrieved methods to measure hormones in a total of 32 fish species, although these tended to be in larger fish.

RIA

The literature search identified 23 publications which describe methods for measurement of estrogens (estrone, E1; 17β-estradiol, E2) and/or androgens (testosterone, T; 11-ketotestosterone, 11-KT; dihydrotestosterone, DHT) in fish using RIA. A summary of the studies can be found in Table 12: in Appendix B.1.3.1.

Depending on sample volumes, most studies include an extraction step for plasma or serum samples to increase assay reproducibility and decrease cross-reactivity. Most commonly, plasma samples are

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

extracted in multiple steps with e.g. ethyl ether, whilst other solvents such as dichloromethane, ethyl acetate and chloroform, or chromatography steps such as thin layer chromatography (TLC) or HPLC fractionation have also been used.

Whereas most studies measured sex steroids in serum or plasma, other matrices were also studied, such as hepatic tissue (Kurobe et al. 2016, ref 16381 in Annex B –), muscle tissue (Prohaska et al. 2013, ref 17351 in Annex B –), incubation medium from *ex vivo* testicular tissue incubations (Schulz et al. 1994, ref 8139 in Annex B –) and sperm (Fostier et al. 1982, ref 283 in Annex B –). Attempts to use non-invasive methods were also made by measuring hormones in tank water (Sebire et al. 2007, ref 1121 in Annex B –).

The literature search identified 11 studies that used RIA to measure triiodothyronine (T3) and/or thyroxine (T4) in fish. One study determined Thyroid-stimulating hormone (TSH) by RIA. Details of the identified studies can be found in Table 13: in Appendix B.1.3.2.

The majority of studies measured T3 and/or T4 in plasma or serum samples. Other matrices included embryos and larvae whole tissue samples of *P. promelas* (Crane et al. 2004, reference 305 in Annex B –), oocytes of *O. mossambicus* (Weber et al. 1992, reference 3207 in Annex B –), various somatic tissues of *S. gairdneri* (Fok et al. 1990, reference 11890 in Annex B –) and whole eggs and fry of *O. keta* (Tagawa and Hirano 1987, reference 13878 in Annex B –).

ELISA

The literature search identified nine studies which describe methods for measurement of estrogens (estrone, E1; 17β-estradiol, E2) and/or androgens (testosterone, T; 11-ketotestosterone, 11-KT) in fish using ELISA. One additional study was identified which used TR-FIA to measure T. A summary of the studies can be found in Table 15: in Appendix B.2.3.1. Five of the studies measured both, estrogens and androgens by ELISA. The majority of studies determined sex steroids in plasma or serum samples. Two studies determined E2, T, or 11-KT in tank water (Felix et al. 2013, Friesen et al. 2012, references 1689 and 6129 respectively in Annex B –).

One study developed a validation protocol of ELISA kits which are used to measure steroid hormones in fish plasma (Metcalfe et al. 2018, reference 5839 in Annex B –). They tested EIA kits for E2, T and 11-KT to measure plasma samples from three different fish species (anemonefish, *A. melanopus*; Barramundi, *L. calcarifer*; Coral trout, *P. leopardus*) and found that assays were not performing well without extraction step. Thus, they included a liquid solvent extraction step using ethyl acetate:N-hexane (50:50), which was repeated three times to extract the steroids. Samples were then measured using a commercial ELISA kit. In addition, the authors recognised the importance of sample stability during long term storage and the detrimental effect of repeated freeze-thaw cycles, thus snap-freezing aliquoted samples immediately after centrifugation, storage at low temperatures and only thawing required aliquots for assay preparation to preserve sample integrity.

Another publication describes an interlaboratory study to determine the variability in measured concentrations of E2, T and 11-KT (Feswick et al. 2014, reference 6607 in Annex B –). Whilst they found large inter-laboratory variability, they noted that the intra-laboratory assay variabilities were generally low. To facilitate better reproducibility of data, they stress the need for publication of CV percentages and an agreement of acceptable CV values. Whilst the variability was high in plasma samples and extracts, the authors also point to the need of standardised extraction procedures to guarantee good assay performance and the reporting of extraction efficiencies. They also found that RIA and ELISA produced different results, but that the results were correlated. For testing of endocrine disrupting chemicals, the authors propose that researchers use fold change to describe changes in hormone levels after treatment rather than absolute values when using steroid measurements as endpoint.

Felix et al. (2013) (reference 6129 in Annex B –) discusses the problem with low plasma sample volumes from small fish species such as *D. rerio* (1-5 µl plasma) which often necessitates whole-body hormone measurements, with no possibility of sequential sampling of the same individual. Therefore, they suggested non-invasive methods and presented a study for 11-KT measurements in holding water. A

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

second study measured E2 and T in tank water of *P. multicolor victoriae* (Friesen et al. 2012, reference 1689 in Annex B –).

The literature search retrieved only one study where T3 and T4 by ELISA in plasma from juvenile fish from two different species, Sea Bass (*Dicentrarchus labrax L.*) and Sea Bream (*Sparus aurata L.*) (CerdaReverter et al. 1996, reference 9522 in Annex B –). The authors concluded that the assay had a comparable degree of sensitivity to RIAs for T3 and T4.

Chemical analysis

Most methods to measure androgens in fish retrieved by our literature search have been developed to measure hormones in the plasma. In the 1980s and 1990s, both HPLC (Huang et al. 1983; Khan et al. 1997) and GC-MS (van Dam et al. 1989) were already in use. They did however require 5-8ml of plasma. Validated test guidelines tend to use small fish such as the fathead minnow, from which usually a maximum of 20 µL plasma can be obtained from an individual fish. For both analytical methods, LODs in the low ng/mL can now be achieved with as little as 10 µL of plasma (Table 19: in Appendix B.3.3).

Analytical and adapted sample preparation methods for the quantitative analysis of androgens in fish have also been described in other sampling matrices. In particular, methods have been developed to enable the enforcement of food safety requirements and residues of natural and synthetic hormones in animal products intended for human consumption, including fish meat.

Zebrafish (*Danio rerio*), for a number of reasons including its relatively short lifecycle, is a prominent animal model in both human toxicology and ecotoxicology. However, its small size can make the measurement of certain endpoints challenging, including the volume of plasma available for the measurement of circulating hormones. For these reasons, methods developed for whole body homogenates are of particular interest. Yang et al. (2015) (reference 3467 in Annex B –) developed a method based on ultrahigh performance LC-MS/MS that allowed the simultaneous determination of 26 EDCs (including five estrogens, eight androgens, and two thyroid hormones) in fish and water was developed and applied to whole body homogenates of zebrafish. The method performed adequately in term of accuracy and precision and achieved limits of detection in the ng/g range. The interpretation of the biological significance of any changes in measured levels of hormones in whole body homogenate may however be complicated by the fact that such measurements do not allow the localisation of hormones either in specific organs or the circulation.

Chemical analysis of steroid estrogens in fish plasma is typically done simultaneously with determination of androgens. Most methods described in the previous section also measure E1 and E2 and sometimes E3. The performance of these methods for estrogens is detailed in Table 20: in Appendix B.3.3.1.

Our search did retrieve a reference describing a novel electrochemical sensor for E2 from molecularly imprinted polymeric microspheres and multi-walled carbon nanotubes grafted with gold nanoparticles (Futra et al. 2016). This ultrasensitive sensor was developed for the aquaculture industry to measure E2 in fish sera in order to determine the sex of the animals. It required very low sample volumes (9µl) and boasted extremely sensitivity (2.5*10-16 M) but little other information about the method performance was reported, e.g. in terms of specificity, accuracy and precision.

Another important difference between the chemical analysis of estrogens and androgens in fish is the number of methods that have focused on sampling matrices other than plasma or serum. Whereas the systematic evidence mapping exercise discovered one or at most two further methods that had considered androgens in bile or whole-body homogenates, there were 5 and 4 additional methods for estrogens in these matrices, respectively (Table 21: in Appendix B.3.3.1). For fish bile samples, the species in which such investigations have been carried out is noteworthy as this is a matrix favoured in wildlife species for eco-epidemiological investigations and there are doubts over the feasibility of using this sampling matrix in smaller species as typically favoured in ecotoxicological investigations. Similar concerns arise in relation to methods measuring estrogens in tissue samples. Such methods have been designed to respond more specifically to the needs of the food industry. An interesting aspect nonetheless of many of these published methods is their focus on efficient and when possible fully automated sample preparation techniques.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Far fewer methods to measure thyroid hormones in fish samples have been published than methods to measure sex steroids. It may be related to the fact that analytical chemistry methods to quantify thyroid hormones have been restricted to liquid chromatography, or that fish was considered a poor model for thyroid disruption until relatively recently. The LC-MS or LC-MS/MS methods identified by our search are summarised in Table 22: in Appendix B.3.3.2. Perhaps, the most significant finding of this literature mapping in the context of developing guidance for the measurement of hormones in certain taxa with regards to the EDC criteria is related to the method described by Bussy et al. (2017b). This very sensitive method which allows the quantification of T3, T4 and rT3 in relatively small samples, from various matrices in the Sea lamprey (plasma, liver, gill and kidneys), has been fully validated according to FDA guidance.

Comparative performance of different method types

Table 6: compiles method performance data extracted, when available and when reported in units that allowed comparison, for hormone measurements in the plasma or serum of fish species. This compilation would suggest that although analytical chemistry method have been reported for lower sample volumes, immunoassays (RIA or ELISA) may be more sensitive for steroid estrogens and androgens. Chromatography may be the most sensitive method to measure thyroid hormones.

Table 6: Comparison of the performance of different types of methods to measure E2, T, 11-KT or T4 in the plasma or serum of fish species (when reported in comparable units).

	RIA		**ELISA**		**Chemical analysis**	
	Minimum	**Maximum**	**Minimum**	**Maximum**	**Minimum**	**Maximum**
Sample volume						
Estrogens	50 µL	6 mL	50 µL	1mL	10 µL	1 mL
Androgens	5 µL	6 mL	50 µL	1mL	10 µL	5 mL
Thyroid hormones	10 µL	0.1 mL	100 µL	-	50 µL	100 µL
Limit of detection						
Estrogens	0.03 ng/mL	0.3 ng/mL	6.7 pg/mL	12 pg/mL	0.01 ng/mL	1 ng/mL
Androgens	4 pg/mL	0.4 ng/mL	0.7 pg/mL	9.5 pg/mL	0.05 ng/mL	1.25 ng/mL
Thyroid hormones	0.095 ng/mL	0.27 ng/mL	0.02 ng/mL	-	< 1 pg/mL	

–: no information available

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

3. Extensive review of the literature on pathology of endocrine organs in birds

3.1. Extensive review method

As stipulated in the terms of reference, the avian gross pathology and histopathology review was not conducted according to strict systematic review criteria. The following search terms were used to identify relevant literature: pathology, histopathology, endocrine, thyroid, avian, bird. Short search strings were used to focus the search e.g. "histopathology AND thyroid AND avian". Using these terms Web of Science, PubMed central and Scopus resulted in over a thousand (1118) 'hits'. Duplicates from different databases and searches were removed. Thirty-three papers fulfilled the inclusion criteria (below) from the title and abstract sifting.

Inclusion criteria:

1. Laboratory based studies in birds which investigate endocrine disruption by chemicals (not infections or husbandry) AND include gross pathological and/or histopathological endpoints of reproductive tissue (gonad, accessory glands) and/or thyroid tissues (thyroid gland),

2. Reviews focusing on pathology/histopathology of birds which include reproductive tissue (gonad, accessory glands) and/or thyroid tissues (thyroid gland).

3.2. Results

After extracting the selected papers, 25 articles were included on the basis of inclusion criteria 1. Two paper were included under criteria 2, for background information relating to bird pathology and endocrinology. In addition, the EPA Avian Two-generation Toxicity Test OCSPP 890.2100 (U.S. EPA, 2015) and OECD Avian Reproduction TG 206 (OECD, 1984) were included as these are the current regulatory test guidance documents. However, it is important to note that 1) OECD TG 206 has not been specifically validated for the detection of endocrine disrupters, rather is a reproductive toxicity test and 2) the U.S. EPA two generation test guideline is not required for chemicals testing at EU level.

A broad range of tissues and endocrine endpoints were reported in the articles retrieved in the literature searches. Summary of included studies can be found in Appendix C –.

The effect of chemicals (or mixtures) on avian thyroid tissues were assessed in eight publications in the review. Four papers were from the same group (Mohanty, Pandey, & Tsutsui, 2017; Pandey & Mohanty, 2015, 2017; Pandey, Tsutsui, & Mohanty, 2017) investigating the effect of pesticides mancozeb (MCZ) and imidacloprid (IMI) and their mixture in adult male red avadavat (*Amandava amandava*) thyroid and gonads, tissue size/weight and histopathology were assessed (Table 26:). In addition, this group also measured hormones in the bird via ELISA. Two other papers investigated effects of chemical mixtures (PCBs: Fowles et al. 1997; crude oil: Harr et al. 2017) on adult birds (mallard ducks (*Anas platyrhynchos*) and double-crested cormorants (*Phalacrocorax auritus*) respectively). Gross pathology (weight) and histopathology of thyroid glands and other organs (e.g. liver, spleen) were examined (Fowles et al. 1997, Harr et al. 2017). A final paper assessing thyroid specific endpoints exposed Japanese quail (*Coturnix japonica*) *in ovo* to flame retardants (Jacobsen et al. 2017), histopathology of both the thyroid and liver were assessed in the 14-day-old chicks.

Literature search identified papers investigating the possible effects of a range of compounds (insecticides, fungicides, antibiotics, natural and synthetic steroids, and xenoestrogens) on reproductive organs. As with the group of papers above in red avadavat, one other publication focused possible disruption to both thyroid and testis (Razia et al. 2006). Razia et al. (2006) exposed Japanese quail *in ovo* to the steroid oestrogen oestradiol (E2), and the alkylphenol nonylphenol (NP) assessing thyroid and testes development in the 15-day-old chicks via histopathology. Three other papers investigated the effects of xenoestrogens (Bisphenol A, NP) on Japanese quail either *in ovo* (Oshima et al. 2012) or in adults (Cheng et al. 2017, 2019). Male and female gonad development was assed via gross pathology

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

and histopathology (Oshima et al. 2012), whereas Cheng et al. (Cheng et al. 2017, 2019) primarily used histopathology and reproductive endpoint as assessment criteria.

Two comprehensive studies, outlining possible additional endpoints for inclusion to OECD TG 206, used E2 to assess reproductive pathologies in Japanese quail (Shibuya et al. 2005; Yamashita et al. 2011). Both adult (F1) and 14-day-old chicks (F0) were included in the gross pathology and histopathology assessments (male and female reproductive organs, accessory organs, as well as liver, kidney, etc.). In addition to the gross and histopathological endpoints, this group also measured the female yolk protein, vitellogenin, and E2 hormone concentrations in males and females (F0) (Shibuya et al. 2005; Yamashita et al. 2011).

Synthetic steroid androgens (trenbolone) have also been employed to investigate reproductive pathologies in Japanese quail in both adult birds and *in ovo* exposures (Henry et al. 2012; Quinn et al. 2007). Reproductive outputs/fertility were investigated alongside gross pathology and histopathology of reproductive organs (Henry et al. 2012; Quinn et al. 2007) and testosterone concentrations (Henry et al. 2012).

A further six papers investigated the possible endocrine disrupting effects of a number of insecticides, fungicides and antibiotics, primarily on Japanese quail or chickens. Two studies injected DDT or DDE *in ovo* (Quinnet al. 2008, Swartz 1984) to investigate subsequent effects on reproductive organ development in males and females at 5 or 12 days exposure (Swartz 1984), or 18-weeks-old (Quinn et al. 2008). Swartz (1984) primarily used histopathology of the developing chickens, whereas Quinn et al. (2008) assessed female organs via gross pathology, and male organs via both gross pathology and histopathology. The other four papers focus on possible endocrine disruption in adult male Japanese quail. Grote et al. (2008) investigated effects of epoxiconazole on gross pathology and histopathology of the testes as well as hormone levels, fertility, and reproductive outcome. Aire (2005) investigated the gross pathological and histopathological effects of carbendazim (fungicide) on the testis. Tokumoto et al. (2013) investigated effects of clothianidin exposure on the gross and histopathology of the testes, and pathology of the spleen and liver, of male Japanese quail. Singh et al. (2013) investigated the effect of an antibiotic (norfloxacin) on a range of endpoints including serum testosterone, gonad gross pathology and histopathology, sperm parameters and molecular assessments (Androgen receptor expression) in male tissues, and reproductive performance when paired with non-exposed females (Singh et al. 2013). In addition, Ahmed et al. (2015) also focused on possible endocrine disrupting effects effect of P-Nitrophenol in male Japanese quail. Male reproductive tissues were weighed and the testes were assessed via histopathology and hormones were measured via RIA (Ahmed et al. 2015).

Yang et al. (2012) focused primarily on female specific endpoints relating to cadmium exposure in chickens. The ovary was assessed using standard histopathology method plus electron microscopy and apoptosis assay (TUNEL). Oestradiol and progesterone were measured in the bird's serum (via RIA) and oxidative stress was assessed.

It is important to note that none of the papers relied on gross pathology assessment of endocrine organs/tissues alone (Table 26:), the majority of papers used histopathological results alongside gross pathology and/or other reproductive or endocrine endpoints (e.g. hormone measures, reproductive outputs).

Standardised methods for assessing gross pathology and histopathology of endocrine organs were not apparent from the literature search, although relative weight of endocrine organs was frequently used as a gross pathology metric. Only four papers (Ahmed et al. 2015, Harr et al. 2017, Oshima et al. 2012, Razia et al. 2006) provide any type of criteria in the methods for how histomorphic variations were characterised and measured. The majority of articles only reported which endpoint were considered different between treatment and control e.g. 'reduced spermatogenesis' but without specifying how these were qualified or quantified (e.g. scoring system, image analysis). In addition, most of the articles did not consider general toxicity in their assessment, and how general toxicity may affect the interpretation of findings.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

4. Survey of ecotoxicology laboratories

4.1. Methodology

4.1.1. Development of questionnaires

The results of the systematic literature review on hormonal measurements in fish, amphibians and birds informed the development of a structured questionnaire including some multiple-choice closed-frame questions amenable to quantitative analysis and some open-ended questions and opportunities for comments. For gross pathology of endocrine relevant organs in birds, the design of the questionnaire was informed by the EDSP test guideline OCSPP 890.2100: Avian Two-generation Toxicity Test in the Japanese Quail developed by the USEPA and a preliminary scoping of the literature.

The range of questions covers information about the tests carried out in those laboratories, hormonal measurements in non-target vertebrate species, gross pathology in birds, analytical methods employed, analytical capabilities, method preferences, and perceived opportunities and barriers (e.g. equipment, training) to carry out hormonal measurements in non-target vertebrate species and gross pathology of endocrine organs in birds. Participants were also asked about their willingness to take part in follow-up telephone interviews to seek further insights and clarifications if necessary.

The questionnaire was build using the Jisc online platform 'Online Surveys'. The survey methods and questionnaire were subject to review and approved by Brunel University's ethics committee and included a participant's information sheet and consent form. All project team members have completed General Data Protection Regulation (GDPR) compliance training. A draft version of the questionnaire was piloted by ecotoxicological experts within the Institute of Environment, Health and Society at Brunel University London.

The information sheet given to potential participants, consent form and online questionnaire can be found in Annex D –.

4.1.2. Identification of laboratories

A two-strand approach was applied to identify Good Laboratory Practice (GLP) certified laboratories that routinely perform the ecotoxicological guideline studies of interest. The first used internet searches to discover companies that advertise their services. The websites of such companies were checked for GLP accreditation and experience in carrying out guideline studies with fish, amphibians and/or birds. An alternative approach was to systematically check the National websites on Good Laboratory Practice as listed on the OECD pages and use registers of National GLP-accredited laboratories and/or capabilities to search such registers to identify those that provide ecotoxicology testing services.

An initial list of laboratories thus discovered was completed in consultation with EFSA. This yielded a final list of 40 laboratories to be contacted.

4.2. Results

4.2.1. Response rate to online survey

All 40 laboratories were contacted. Where no specific contact responsible for ecotoxicological testing had been identified online, laboratories were contacted by phone to forward the questionnaire to identify an appropriate expert to complete the questionnaire. Due to commercial links between contract research organisations (CROs), four of the listed laboratories were identified as duplicates for other institutions. A further two laboratories were excluded because they either measured hormones in environmental media for environmental exposure assessment or carried out field studies only.

Up to three attempts to contact relevant persons within the remaining 34 laboratories were made during the duration of the online questionnaire. The link to the online questionnaire was forwarded to 31

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

ecotoxicology experts within 20 different institutions. By the closing date of 31st January 2020, a total of 12 responses had been received online. This corresponds to an overall 35% response rate for all qualifying laboratories and 60% response rate from organisations that had responded to initial contact and been sent the link to the survey. A likely reason for non-response is that hormonal measurements and gross pathology of endocrine in birds are not currently mandatory in current TGs and therefore not routinely carried out by CROs.

4.2.2. Analysis of responses to online survey

Individual responses to the questionnaire were collated and full summary results are available in Appendix D –.

4.2.2.1. Experience with hormone measurements in mammalian assays

Of the 12 responses, 4 indicated that their company also performed tests for the identification of endocrine disrupters in mammals and all 4 had capacity to measure hormones (oestrogens, androgens and/or thyroid hormones) in mammalian test species. The methods used by these laboratories for hormone analysis in mammalian tests are illustrated in Figure 5 and show that for both steroids and thyroid hormones analysis preferred methods are ELISA or LC-MS. The respondent who had indicated 'Other' specified that they were unsure of the method used by their company.

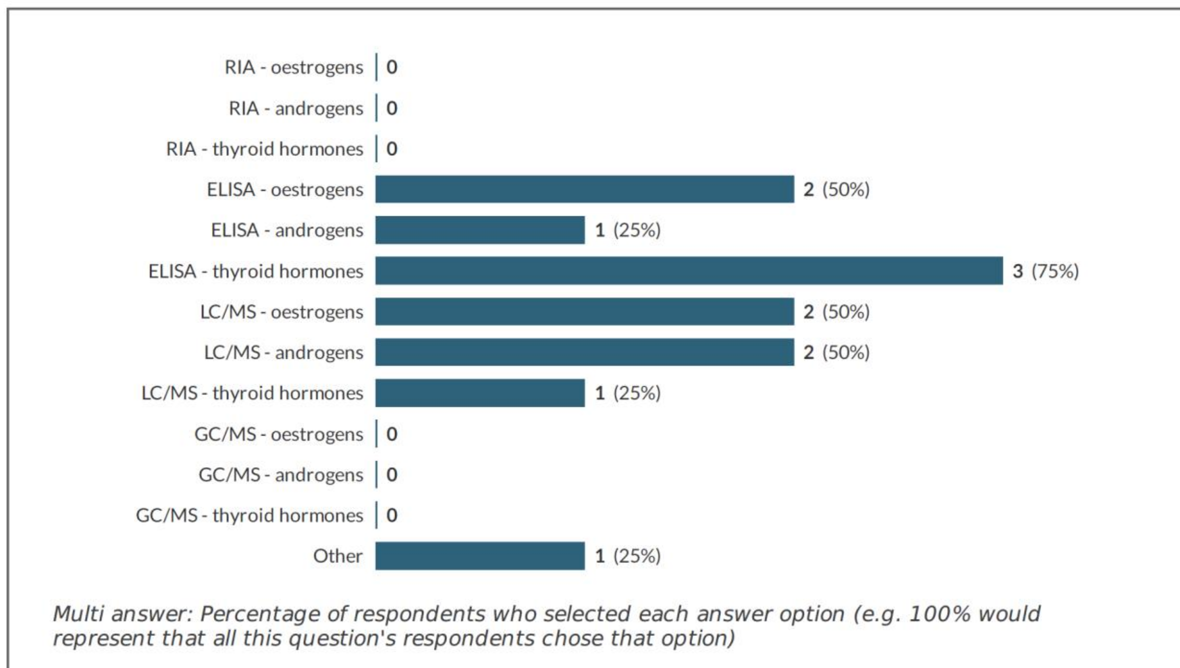

Figure 5: Methods of hormone analysis used by companies also conducting mammalian testing

The same 4 respondents indicated that their company also conducts the steroidogenesis H295R *in vitro* assay and a similar picture emerged in terms of methods for analysis of hormones in culture medium (Figure 6) with a preference for ELISA and LC-MS. The 'other' response was explained by the respondent as their lack of knowledge regarding the method used by their company.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

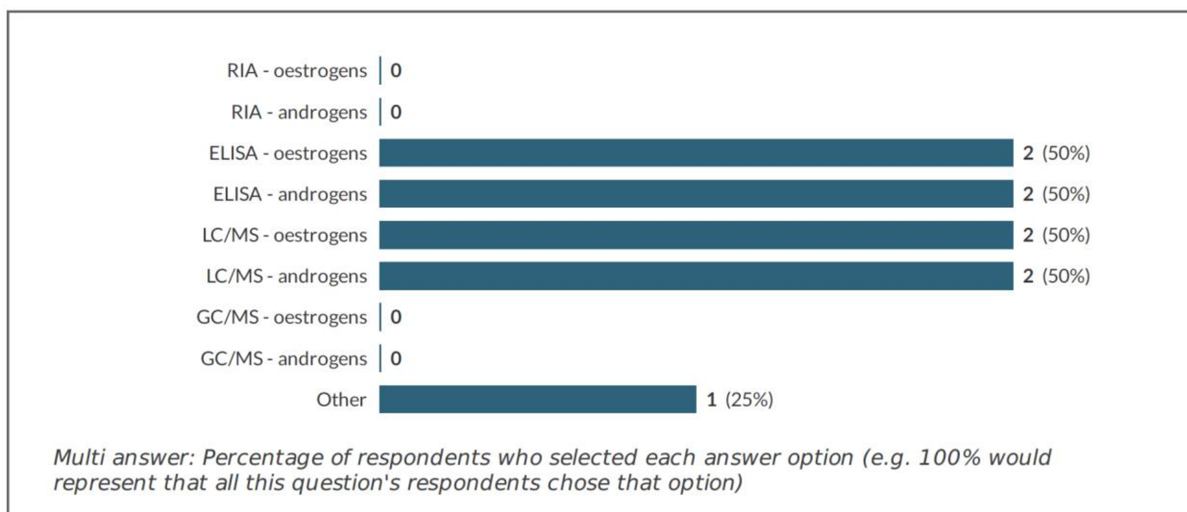

Figure 6: Methods of analysis for steroids in the culture medium for the H295R steroidogenesis *in-vitro* assay

4.2.2.2. Experience with non-mammalian ecotoxicological tests for the identification of endocrine disrupters

Three quarters of respondents (9/12) affirmed their company did perform test for the identification of endocrine disrupters in non-mammalian ecotoxicological models. The majority of those work with fish models (8/9), and a third work with amphibian models (3/9). None of the respondents indicated that their company worked with avian models.

The most commonly used test species of fish is the fathead minnow (5/8), although the zebrafish (2/8), medaka (1/8) and three-spined stickleback (1/8) are also used.

4.2.2.3. Hormones measurements

Only 2 of the 9 respondents who affirmed they carried out identification of endocrine disrupters conduct hormone analyses, solely in fish species, both for estrogens and only one for androgens. No survey participants declared measuring thyroid hormones in any bird, fish or amphibian test species.

One of these two laboratories measures total estrogens in plasma and liver samples of juvenile and adult fathead minnows using ELISA kits. The plasma is analysed directly without further sample preparation steps. The reasons cited for preferring ELISA method was that it was driven by client preference as well as ease of use and in-house experience. They declared having standard operating procedures for animal care and social grouping, anaesthesia and/or euthanasia, time of day the samples are collected, maximum/minimum sample volume, sample pooling and storage requirements. These procedures follow guidance documents, are agreed case-by-case with the client and documented in the study plan.

The other laboratory measuring hormones in fish measures both total and free fractions of estrogens and androgens in plasma samples of three-spined stickleback embryos as well as juvenile and adult fish using either RIA or LC-MS. Plasma samples are analysed after liquid-liquid extraction with diethyl ether. Method recovery is evaluated using labelled analytical standards (deuterated, titriated). A preference for the RIA method was indicated due to its high specificity and reliability. This respondent further specified that they developed a non-invasive method using tank water. They declared having standard operating procedures for animal care and social grouping, time of day the samples are collected, maximum/minimum sample volume and storage requirements. They keep a detailed record of which animals the samples came from, how they were housed, what time of the day the sample was taken and sample volume to calculate dilution, freezers are all alarmed. When using a new hormone method

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

or an established method on a new species/taxa or sample matrix, the assay is validated by assessing its specificity and sensitivity using positive (spiked) and negative samples.

Neither respondents measure follicle stimulating hormone (FSH) or luteinising hormone (LH) when conducting hormone measurements but one respondent generally measures further steroid hormones, particularly progestogens.

Low sample volume was identified by both these laboratories as an issue they commonly face. One of the respondents affirms that regardless of low volume, they consider pooling samples poor practice and never do so. The other highlighted the lack of information on best practice.

The reasons cited for not carrying out hormone measurements were lack of demand from client (42.9%), lack of in-house experience or lack of in-house facilities (both 28.6%). Other reasons given were the fact that it is not currently required by the guideline (4 out of 7 or 57.1%), with 2 respondents specifying that they do currently measure vitellogenin. A further respondent indicated that the measurement of hormones in fish is still in its validation phase.

4.2.2.4. Gross pathology in avian species

None of the respondents carry out tests in avian species. As they do not have in-house facilities (aquatic ecotoxicology only). One respondent specified that in the studies conducted in CROs, gross pathology is investigated (no histopathology) consistent to OECD TG 206 (OECD 1984).

4.2.3. Summary results of follow-up telephone interviews

Six of the 12 respondents agreed to take part in a follow-up telephone interview. One of these respondents further recommended experts able to comment on avian tests. A total of seven interviews with 8 participants were scheduled for which written consent was obtained. When possible, interviews were recorded and transcribed using online Artificial Intelligence (AI) software. These transcripts were used to draft summary reports of the conversation which respondents were given opportunity to amend, correct and approve. These summaries can be found in Appendix E –.

As for the online questionnaire, the sample was small and respondents themselves recognised that only a limited number of laboratories are currently equipped and experienced for the investigation of endocrine disruption endpoints. Respondents nonetheless represented a qualitatively diverse range of set-ups, specialist expertise and capacities to conduct hormone measurements, primarily in fish test species. As these measurements are currently optional in guideline studies, they are typically not routinely carried out by commercial entities.

Because of their size, larger laboratories indicate that they would be in a position to draw upon existing capacity held in departments conducting rodent studies. Others may face logistical challenges related to either sourcing GLP-compliant laboratories that can carry out hormone analyses or shipping samples due to geographical constraints. A few respondents mention the fact that they have recent experience validating ELISA methods for the measurement of vitellogenin, in fish and/or frogs. This method is therefore favoured by these respondents due to its relative ease of use. One research institution with extensive experience of developing methods for hormone measurements however in fish favour RIA methods in terms of their reliability and specificity over ELISA.

All respondents expressed concerns related to the appropriate sample size. Guidance of sample sizes needs to account for the likely sampling volumes in fish test species of differing sizes as well as take into account pairs potentially lost due to unsuccessful social grouping (in both fish and birds). Larger sample sizes than those recommended in guideline studies will be necessary to study effects on steroid levels, as hormone levels are inherently more variable that other OECD test endpoints. In terms of variability, it is also important to bear in mind the importance of the source for the test animals and the fact that they exhibit greater inherent genetic variability than strains of rodent test species. Several respondents expressed specific concerns related to the zebrafish model for endocrine disruption testing due to the lack of genetic sex and the rather peculiar type of sexual differentiation they display (first they all become females and then some develop as males).

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Guidance on the timing of sampling either in terms of life-cycle stage or time of day to account for diurnal variations would also be welcome.

Two interviewees were able to offer specific insights into the amphibian and avian assays, respectively. For amphibians, the Larval Amphibian Growth and Development Assay (LAGDA) (OECD 2015b) was not validated with thyroid disrupting substances and is thought to be less suitable than an extended Amphibian Metamorphosis Assay (AMA) (OECD 2009). The Avian Reproduction Test OECD TG 206 is recognised to be an old test guideline (OECD 1984) that was never designed for endocrine disruption testing whose limitations are widely acknowledged.

As for the value of hormone measurements for the interpretation of test results, it is perceived as both an opportunity and a challenge. Hormonal measurements, if informative and reliable, have the potential, when considered with other endocrine endpoints, to strengthen weight-of-evidence assessments. However interpreting hormonal measurements in fish, birds and amphibians may present a significant challenge related to the lack of as solid knowledge base of normal hormonal levels in those taxonomic groups as for rodents including the lack of historical controls, as well as the previously mentioned inherent greater variability of these measurements for these taxa.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

5. Recommendations

5.1. General considerations

Inclusion of hormonal measurements in toxicity testing has been generally suggested for investigation of the effects of chemical treatments on endocrine functions. Recommendations for hormonal studies, more specifically measurements of thyroid and reproductive hormones, in mammalian (usually rodent) test systems have previously been described (European Chemicals Agency (ECHA) and European Food Safety Authority (EFSA) with support from the Joint Research Centre (JRC). 2018; Kucheryavenko et al. 2019). Using non-mammalian vertebrates to study thyroid or reproductive toxicity of chemicals can provide important insights for wildlife species. Due to the high degree of conservation of the thyroid system and the similarities in reproductive hormone function across taxa, these assays may also be more generally applicable and be predictive for adverse endocrine effects in humans (OECD 2002; OECD 2006; OECD 2008). The purpose of hormonal measurements in toxicity testing is to compare the baseline conditions (in untreated controls) to changes in hormone levels (decrease or elevation) upon treatment with test chemicals.

Integrating hormone measurement for thyroid and reproductive toxicity in existing guidelines for endocrine disruptor testing allows to provide additional information without the use of additional animals. Alternatively, changes in hormone levels may be assessed in separate studies. It is however recommended to integrate hormone measurements in OECD non-mammalian TGs, within Level 4 and 5 tests of the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters, where in vivo assays also provide data on adverse effects on endocrine relevant endpoints. For fish tests this includes the "Fish sexual development test (FSTD)" (OECD TG 234, OECD 2011) in level 4 and the "Medaka Extended One Generation Reproduction Toxicity Study Test (MEOGRT)" (OECD TG 240, OECD 2015c) in level 5. Inclusion of hormone measurements in assays currently under validation i.e. the "Fish Reproduction Partial Lifecycle Test" in level 4; and Fish Life Cycle Toxicity Test (FLCTT) and Zebrafish extended one-generation reproduction test (ZEOGRT) (draft OECD TGs) in level 5 should be considered if/when the TGs become available. For amphibians, there is currently only a level 4 TG available, the "Larval Amphibian Growth & Development Assay (LAGDA)" (OECD TG 241, OECD 2015b). Level 3 tests are not recommended for inclusion of hormone measurements since they have lower power to detect changes in hormone levels and are less informative with regards to adverse effects.

Since hormone measurements are currently not included in guideline tests, specific recommendations on the measurements of thyroid and reproductive hormones are not yet available. If hormonal studies are incorporated into existing guidelines, it has to be ensured that treatment schedules and sampling times are appropriate to detect changes in hormone levels.

5.2. Recommendations for hormone analysis in amphibians and fish

The recommendations below cover EATS pathways and are based on currently available, published methodologies. For some taxa and hormones, the information is scarce whereas for others a range of experimental conditions and measurement methods have been described by and validated in test laboratories. The recommendations are therefore not prescriptive but intended to indicate best practice for use and validation of assays for hormone measurement.

Recommendations for hormone analysis which are applicable to both taxa (amphibians and fish) are listed below. The recommendations which are specific for amphibians and fish are described in 5.2.1 and 5.2.2, respectively.

Thyroid hormones. Ideally, all three thyroid hormones, T3, T4 and TSH, should be measured in non-target vertebrates (amphibians and fish). Measurement of rT3 has also been included in hormonal studies. However, methods for measurements of all T3, T4 and TSH are not available for all taxa. Due to the identical structure of T3 and T4 in all taxa, assays developed for other species (i.e. rodent or human) may be used but require validation. Measurement of the free fraction of T3 and T4 in blood samples is more meaningful than total hormone because it indicates the amount of active hormone that

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

is available. However, measurement of the free hormones is technically more challenging and frequently hampered by the relatively large volume of sample needed for evaluation of the free fraction. Sufficiently sensitive techniques such as chromatography or, if available, RIA or ELISA methods should be employed to measure free hormones. Furthermore, pooling of samples may be required.

Reproductive hormones. Measurements of estrogens and androgens, particularly E2, Testosterone and DHT should be integrated into amphibian and fish tests for reproductive toxicity. In addition, taxa/species specific hormones such as 11-KT in fish should be included in measurements.

Sample collection and storage. Blood samples should be collected in appropriate containers depending on whether serum or plasma is used for hormone analysis. Serum or plasma should be prepared immediately. Depending on sample volume, it may be split into aliquots for different analyses. Sample aliquots should be stored immediately below -20 °C until further processing and analysis. The storage conditions have to be validated (e.g. stability at storage temperature, during freeze-thawing and length of storage until analysis).

Serum/plasma samples should not be extracted for measurement of *thyroid hormones*, because they may be lost during organic extraction and should be evaluated in unextracted samples.

From available results for measurement of *reproductive hormones*, it is recommended that serum/plasma samples should be organically extracted before analysis. Different extraction methods have been reported and all protocols should be validated before use.

One challenge of measuring hormone levels in different species can be the size of the animals and depending on species, serum/plasma volumes are often limiting. In very small animals such as amphibian larvae or fish embryos it may not be practical to measure hormone levels in blood. Therefore, other matrices such as whole-body homogenates may be used. Other methods have been described to measure hormones in other tissues, as well as non-invasive methods such as tank water, faeces, saliva or dermal secretions. Other matrices should be sampled appropriately and may need extraction or further processing before measuring hormones.

Quantitation Methods. Several quantitation methods for measurement of *thyroid* and/or *reproductive hormones* have been described and all of them may be suitable. Available methods include RIA, ELISA and chromatography techniques. Many of the applied assays are adapted from methods developed for other taxa/species. RIA or ELISA methods for measurement of T3 and T4 may in general be adaptable for different species since they are chemically identical in all vertebrates. Similarly, the structure of steroid hormones is conserved amongst vertebrates, therefore, methods developed for other taxa may be adapted and validated for non-target vertebrates. However, serum components may differ between taxa and species. Therefore, mammalian (human or rodent) kits may not be directly applicable for non-target vertebrates and all methods have to be validated. Anti-body-based methods for measurement of TSH rely on species (or closely related) specific anti-bodies and almost no immunoassays are available to measure TSH in amphibians or fish. In addition, 11-KT is a vital androgen in fish for which no methods from other taxa exist. Thus, methods specific for TSH in selected test species should be developed for inclusion in TG for thyroid toxicity, and measurement of 11-KT in fish should be included and validated for use in TGs that measure reproductive hormones.

Chromatography may be the most sensitive method, with the additional advantage that several hormones can be measured in one sample. If the use of chromatography is limited by the availability of equipment, equally sensitive and validated methods should be used. Overall it is important that any quantitation method used is adapted and validated in the employed species before use in regulatory testing.

Assay validation. No standard methods for most of the *thyroid* and *reproductive hormones* and species exists. Therefore, no strict recommendations which assays to use can be made. However, some methods using RIAs, ELISAs or analytical chemistry (chromatography) have been described for some amphibian and fish species as well as for other taxa. If assays are adapted from other species (e.g. from humans or rat) or newly developed, they should be validated and meet certain quality criteria (ECHA/EFSA, 2018, Kucheryavenko et al. 2019):

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

- Laboratory proficiency: assay validation study with a positive control (reference compound), using different doses levels.
- Validation of hormone measurement methods for each species separately.
- Determination of the lower limit of quantification (LLoQ) and upper limit of quantification (ULoQ) of the chosen method.
- Measurement range: Linearity of the whole measurement range should be proved and the upper limit set.
- Assessment of data reproducibility: calculation of intra- and inter-assay variation (in case of commercial kits, these should be similar to the limits established for that particular kit).
- Reproducibility across control groups: meeting of criteria for coefficient of variation (CV) for T3 and T4 measurements in untreated controls (< 25%), and for TSH (< 35%).
- Evidence of assay repeatability within a day and across several days.
- Recording of all quality controls: spiked samples, biological control samples, reference ranges, sample extract dilution curve etc.
- Determination of assay performance with the particular sample matrix (should be assessed for all matrices used to determine matrix effects).
- Validation of sample stability under the chosen storage conditions.
- If immunoassays are used: establish cross-reactivity of antibodies
- Determination of lot-to-lot variation of reagents such as antibodies (if possible).

Use of historical data. Historical data are not required for the evaluation of test results from *thyroid* or *reproductive hormone* measurements. Effects should be evaluated by comparison to concurrent, untreated controls. However, historical data may be used to confirm assay reliability and demonstrate that the method meets acceptability criteria i.e. if doubts exist about test performance. In case historical data is intended to be used, its validity has to be demonstrated (i.e. identical assay methodology, sampling times, same strains and age groups, kept under the same housing and feeding conditions).

Statistical analysis. There are no specific recommendations for specific statistical analysis of *thyroid* or *reproductive hormone* measurements. The methods should be appropriate to identify statistically significant differences in hormone levels. In case of high data variability, outlier statistics should be performed. Exclusion of outliers should be justified for each datapoint based on physiological reasons or known technical failures in individual samples.

5.2.1. Thyroid and reproductive hormone analysis in amphibians

Hormones. Preferably, all three *thyroid hormones*, T3, T4 and TSH, should be measured in amphibians. Methods for measurement of T3, T4 and rT3 are relatively easily adaptable from established assays (e.g. for human or rat hormones). Anti-body-based methods for amphibian TSH require specific antibodies. Similarly, all *relevant reproductive* hormones should be measured within amphibian tests. However, not many methods have been described. Therefore, development and validation of suitable methods for measurements of estrogens and androgens in amphibians are urgently needed. Previous attempts to include hormone measurements in TG assays were not successful, possibly due to lack of proper validation of assays adapted from other species. Thus, development or adaption of methods in combination with detailed validation for the test species is strongly recommended.

Species. It is recommended to incorporate *thyroid* and *reproductive hormone* measurements into existing guideline tests at Level 4 of the OECD Conceptual Framework that assess effects attributable to an endocrine mode of action, such as the Larval Amphibian Growth and Development Assay (LAGDA) (OECD TG 241). The standard species used for amphibian assays is *Xenopus laevis* (e.g. OECD TG 231,

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

OECD TG 241). *X. laevis* is routinely cultured in laboratories worldwide and is easily obtainable through commercial suppliers. Furthermore, it can have its genetic sex determined.

Methods for other, non-guideline anuran species have been described (e.g. Bufo and Rana)(Mondou and Kaltenbach 1979, Gancedo et al. 1995, Suzuki and Suzuki 1981, Weber et al. 1994, Regard et al. 1978, Hansen et al. 2016). These are usually not recommended for toxicological studies, but if they are used, the assays would have to be adapted and validated accordingly.

Age/life stage. *Thyroid hormones* may be measured at various life stages in amphibians, including in tadpoles, juveniles and adults. Measurement of *reproductive hormones* is recommended in serum or plasma samples from adult amphibians (frogs). If hormone measurements are performed within OECD TG 241 (OECD 2015) in terminal samples (serum or plasma), treatment begins at NF stage 8-10 and the sampling is done at 10 weeks after the median time to NF stage 62 (controls), with an interim sample at NF stage 62. The suitability of these timepoints should be validated and additional samples may have to be included.

Sex. Both sexes may be used for measurement of *thyroid hormones*. Information from both sexes is informative in the assessment of *reproductive hormones*. Therefore, separate measurements in males and females should be included in the study design. This has to be taken into account in the power analysis to ensure enough male and female animals are analysed.

Number of animals. To determine the number of animals, a power analysis should be conducted, taking into account the sensitivity of the detection method used for hormone measurements. OECD TG pre-define a minimum number of animals to be used, but a power analysis is necessary to determine whether these minimum numbers are sufficient to detect changes in hormone levels. If *thyroid* and/or *reproductive hormone* levels are determined within existing guideline test, the final number of animals will also depend on which tissues are used and whether enough sample can be obtained from the animals in the study.

Animal Care. General animal care and housing of amphibians should be provided following current EU legislation (Directive 2010/63/EU on the protection of animals used for scientific purposes). If hormone measurements are incorporated into existing guideline tests, the housing and feeding conditions of these tests should be followed. Appropriate care and breeding of *X. laevis* is further described by a standardised guideline (ASTM 2004).

Euthanasia. General recommendations for euthanasia of amphibians in accordance with current EU legislation (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 On The Protection Of Animals Used For Scientific Purposes) should be followed if sampling is performed at termination of the test. It is recommended to use an anaesthetic overdose of a suitable anaesthetic solution (such as MS-222) at life stage (larvae/adults) appropriate concentrations to euthanise amphibians. Depending on life stage this may be done by immersion or injection. The anaesthetic overdoes is followed by confirmation of death (e.g. by permanent cessation of the circulation or destruction of the brain).

Considerations on timing of measurements. Timing of sampling for *thyroid hormone* measurements should be considered carefully. The reliability of outcomes is dependent on factors like hormone physiology, circadian rhythm, developmental stage amongst others. Similarly, taking into account the effects of the circadian rhythm on *sex hormone* levels, sampling of animals has to be kept in a relatively short time window. Other influences, such as seasonal changes in hormone levels should also be taken into consideration. It is recommended to establish a sampling protocol which keeps variability of hormone levels due to timing at a minimum. If possible, sampling should occur at approximately the same time of day and the time needed for sampling should be kept at a minimum.

Matrices. The recommended sample matrices for measurement of *thyroid* and *reproductive hormones* in amphibians are serum or plasma. If sample volume is limiting, the hormones should be measured in plasma due the greater volume recovered from whole blood compared to serum. Use of other matrices, such as thyroid glands, pericardial fluid, or whole-body homogenates of tadpoles for measurement of thyroid hormones have been described. For measurement of reproductive hormone levels, methods for

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

brain or ovarian tissue have been reported. If matrices other than serum or plasma are used, these have to be justified and all the extraction and measurement procedures have to be validated. Non-invasive methods such as analysis of faeces (Hogan et al. 2013), dermal secretions (Scheun et al. 2019) and saliva (Hammond et al. 2018) have also been explored for reproductive and other steroids in amphibians. Non-invasive methods allow measurements at different time points or life-stages und their use for thyroid and reproductive hormones should be explored in the future.

Tissue sampling. Apart from non-invasive methods, animals are anaesthetised before blood sampling. If enough blood can be obtained from the animals, *thyroid* and *reproductive hormones* should be assessed in serum or plasma samples. After blood sampling death has to be confirmed (e.g. by permanent cessation of the circulation or destruction of the brain). If protocols for whole body homogenates or other tissues are used, the sampling procedures need to be validated.

5.2.2. Thyroid and reproductive hormone analysis in fish

Hormones. Ideally, all three *thyroid hormones*, T3, T4 and TSH, should be measured in fish tests. Methods for measurement of T3, T4 and rT3 are relatively easily adaptable from established assays (e.g. for human or rat hormones). Anti-body-based methods for fish TSH require specific antibodies. Similarly, all relevant *reproductive hormones* should be measured within fish assays. In addition to E2, testosterone and DHT, 11-KT should be assessed in fish.

Species. It is recommended to incorporate *thyroid* and/or *reproductive hormone* measurements into existing guideline tests at levels 4 and 5 of the OECD Conceptual Framework such as the Fish Sexual Development Test (FSDT) (OECD TG 234) or the Medaka Extended One-Generation Reproduction Test (MEOGRT) (OECD TG 240). Incorporation of hormone measurement into currently developed level 4 and 5 guideline assays such as the Fish Reproduction Partial Lifecycle Test and the Fish LifeCycle Toxicity Test (FLCTT) should also be considered. Fresh water species that are commonly employed in testing are Japanese medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), three-spined Stickleback (Gasterostreus aculeatus) or rainbow trout (*Oncorhynchus mykiss*). Fish species for which the genetic sex can be determined may be preferable for measurement of *sex hormones*.

If other, non-guideline species are used for determination of *thyroid* or *reproductive hormone* levels, the detection methods have to be adapted and validated.

Age/life stage. *Thyroid hormone* measurements in fish may be conducted at various life stages. Methods for plasma/serum analysis in adult fish have been described, as well as for other tissues. In addition, embryo/larvae can be used for *thyroid hormone* analysis. It is recommended to measure *reproductive hormones* in serum or plasma samples from adult and juvenile fish.

Sex. Both sexes may be used for measurement of *thyroid hormones* in fish assays. Information from both sexes is informative in the assessment of *reproductive hormones*. Therefore, measurements in males and females separately should be included in the study design. This has to be taken into account in the power analysis to ensure enough male and female animals are analysed.

Number of animals. To determine the required number of animals, a power analysis should be conducted, taking into account the sensitivity of the detection method used for hormone measurements. OECD TGs pre-define a minimum number of animals to be used, but a power analysis is necessary to determine whether these minimum numbers are sufficient to detect changed in hormone levels. If *thyroid* and/or *reproductive hormone* levels are determined within existing guideline test, the final number of animals will also depend on which tissues are used and whether enough sample can be obtained from the animals in the study.

Animal Care. General animal care and housing of fish should be provided following current EU legislation (Directive 2010/63/EU on the protection of animals used for scientific purposes). If hormone measurements are incorporated into existing guideline tests, the housing and feeding conditions of these tests should be followed.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Euthanasia. General recommendations for euthanasia of fish in accordance with current EU legislation (Directive 2010/63/EU) should be followed if sampling is performed at termination of the test. It is recommended to use an anaesthetic overdose of an anaesthetic solution (e.g. MS-222 or FA-100) at life stage appropriate concentrations to euthanise the fish, followed by confirmation of death (e.g. by permanent cessation of the circulation or destruction of the brain).

Considerations on timing of measurements. Timing of sampling for *thyroid hormone* measurements should be considered carefully. The reliability of outcomes is dependent on factors like hormone physiology, circadian rhythm, developmental stage amongst others. Taking into account the effects of the circadian rhythm on *reproductive hormone* levels, sampling of animals has to be kept in a relatively short time window. Other influences, such as seasonal changes in hormone levels should also be taken into consideration. It is recommended to establish a sampling protocol which keeps variability of all hormone levels due to timing at a minimum. If possible, sampling should occur at approximately the same time of day and the time needed for sampling should be kept at a minimum.

Matrices. The recommended matrix for *thyroid* and *reproductive hormone* measurement in fish is serum or plasma. If sample volume is limited, homogenates of other tissues or whole-body homogenates of adult fish may be used. For determination of *thyroid levels* during development, fish eggs or embryo-/larvae homogenates can be analysed. For *sex hormone* measurements, the use of matrices, such as testicular, muscle or hepatic tissue, bile or sperm has been described. If matrices other than serum or plasma are used, these have to be justified and the extraction and measurement procedures have to be validated. Non-invasive methods for *thyroid* or *reproductive hormone* analysis in tank water have also been described (Sebire et al. 2007, Felix et al. 2013; Friesen et al. 2012). Non-invasive methods allow measurements at different time points or life-stages and could be a useful measure when serum or plasma volumes are to small due to the size of the fish. Thus, their use for *thyroid* and r*eproductive hormones* should be explored in the future.

Tissue sampling. Apart from non-invasive methods, fish must be anaesthetised before blood sampling. If enough blood can be obtained from the animals, *thyroid* and *reproductive hormones* should be assessed in serum or plasma samples. After blood sampling death has to be confirmed (e.g. by permanent cessation of the circulation or destruction of the brain). Furthermore, protocols for whole body homogenates or other tissues may be used and need to be validated.

5.3. Recommendations for hormone measurements, gross pathology and histopathology in birds

Under the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters, the Avian Reproduction Assay TG 206 (OECD 1984) is considered a Level 4 test used to gain data on adverse effects specifically on birds reproduction (OECD 2012). Although proposals have previously been made for an OECD two-generation guideline (to include endpoints to analyse possible endocrine disrupting effects in birds) there is no avian Level 5 test under the current OECD Test Guideline framework. In the context of the ED assessment, data from TG 206 is generally considered as supporting information. However, when TG 206 is conducted to provide data on potential endocrine disruption, the following guidance should be considered when incorporating hormone measurements, gross pathology and histopathology.

5.3.1. General recommendations for the addition of hormone measurements, gross pathology and histopathology in birds

Species: The current recommendations suggest incorporating hormone measurements, gross pathology and histopathological assessment into the existing avian Level 4 test guideline. The OECD Avian Reproduction Test TG 206 (OECD 1984) recommends the use of Japanese quail (*Coturnix japonica*), bobwhite quail (*Colinus virginianus*) and/or mallard duck (*Anas platyrhynchos*).

Age/life stage and sex: TG 206 (OECD 1984) primarily focuses on effects in adult birds. Hormones, gross pathology and histopathology can be assessed in adult birds of both sexes. In addition, egg yolk

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

samples could be used to measure hormones if disruption of maternal transfer of hormones is considered relevant.

Animal Care and Euthanasia: General animal care and housing of birds should follow current EU legislation (Directive 2010/63/EU on the protection of animals used for scientific purposes). Within the EU euthanasia methods should adhere to those specified in Directive 2010/63/EU. Some methods for euthanasia can cause artefacts in gross pathology/histopathology therefore, within the regulations, impacts of different euthanasia techniques should also be considered and the most appropriate technique selected for the endpoint(s) of interest.

Number of animals: To determine the number of animals, a power analysis should be conducted, taking into account the sensitivity of the detection method used for hormone measurements, and the variability and sensitivity of the gross pathology and/or histopathology endpoint to be considered. OECD TG pre-define a minimum number of animals to be used, but a power analysis is necessary to determine whether these minimum numbers are sufficient to detect changed in hormone levels or changes to gross or histopathology.

Statistical analysis. Continuous quantitative data can be analysed using commonly used appropriate methods, for example ANOVA (one-way, or two-way) or Kruskal-Wallis (for non-parametric data) followed by appropriate post-hoc test (e.g. Dunnetts, Least Significant Difference, Tukey's). It is recommended that abnormality frequency or incidence data (e.g. gross pathology/histopathology) should be analysed using tested developed for this type of data (e.g. Fishers exact test/Chi squared). It is important to recognise that histopathology severity score data is non-quantitative. Statistical analysis method which take into account non-quantitative scores and combine severity and frequency data have recently been developed for histopathological assessments (e.g. Rao Scott Cochran-Armitage by slices, RSCABS) and could be considered for these endpoints (Green et al. 2014; OECD 2015a; U.S. EPA 2015).

5.3.2. Specific recommendation for hormone measurements in birds

Hormones such as triiodothyronine (T3), thyroxine (T4), testosterone (T) and oestradiol (E2) are structurally the same across all vertebrate species. Therefore, immune-assay (RIA, ELISA) and chromatography methods (LC-MS, GC-MS) developed for mammals can be adapted for use with bird samples. Whereas, the structures of pituitary gland hormones, e.g. thyroid-stimulating hormone (TSH), are species specific and as such requires specific antibodies to be developed for each species.

Ideally, all three thyroid hormones, T3, T4 and TSH, and all relevant reproductive hormones should be measured if endocrine activity is suspected. Methods exist for analysis of T3, T4, E1, E2, testosterone and DHT in bird plasma/serum (Table 5:). No specific immunoassay or chromatography methods for avian species TSH were identified in the literature search for the evidence map.

Considerations on timing and environmental conditions when sampling for hormones. Temperature, photoperiod, stress, food intake and fasting have all been observed to alter thyroid hormone concentrations in birds (reviewed in Schmidt and Reavill 2008, reference 32 in Annex C –). Sex hormones also cycle. Therefore, taking into account the effects of the circadian rhythm on hormone levels, sampling of animals should be kept in a relatively short time window. It is recommended to establish a sampling protocol which keeps variability of hormone levels due to timing at a minimum.

Matrices: It is recommended to use serum or plasma samples to measure hormones in birds. Non-invasive methods such as analysis of feathers, faeces/droppings may be an alternative in future, but further work is required to validate the use of them in a regulatory setting.

5.3.3. Specific recommendation for gross pathology assessment in birds

Gross pathological assessments involve examination of organs and tissues macroscopically. These can be observations or descriptions of morphologic abnormalities (e.g. lesions, unusual shape or colouration, etc.) as well as relative size or weight of organs. The OECD Avian Reproduction Test TG 206 (OECD

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

1984) recommends conducting gross pathology assessment on all adult birds, however it does not currently specify which tissues to examine for endocrine modes of action.

Tissues collection and assessment: Samplers should be familiar with avian dissection, physiology and gross pathology assessment with regard toxicological pathologies in the target tissues. The U.S. EPA avian two-generation guideline (U.S. EPA, 2015) has a useful photographic atlas to support dissection of Japanese quail. When conducting OECD TG 206 (OECD 1984) to provide data on potential endocrine disruption, gross pathology assessments should be conducted on the reproductive organs (ovary, testes), thyroid, kidney, adrenals and liver.

Use of historical data. Historical data are not required for the evaluation of test results. However, historical data of control gross pathology could support the evaluation, provided that further guidance is available on the use and interpretation of historical control data.

5.3.4. Specific recommendation for histopathology assessment in birds

Histopathological assessments involve looking at thin slices of preserved (fixed) and stained tissue under the microscope. These observations can relate to occurrence of specific cells, relative cell numbers or cell sizes, or disruptions to cellular and tissue organisation. The OECD Avian Reproduction Test TG 206 (OECD 1984) currently has no recommendations or guidance for histopathological assessment of organs. However, it is clear from the literature search that many researchers are employing histopathology endpoints to investigate endocrine disrupting chemicals in birds (Table 26:). Histopathology of endocrine organs (e.g. thyroid, gonad) is currently employed in other endocrine disruptor TG for non-target species such as in the Larval Amphibian Growth & Development Assay OECD TG 241 (OECD 2015b). OECD guidelines for histopathology in fish (Johnson et al. 2009), amphibians (OECD 2015a) and mammals (OECD 2008) have all been developed to aid assessment of these endpoints.

Tissues: When conducting OECD TG 206 (OECD 1984) to provide data on potential endocrine disruption, histopathology should be conducted on the reproductive organs (ovary, testes), thyroid gland and liver.

Sample collection, fixation and **storage.** Samplers should be familiar with avian dissection and physiology. Tissues should be fixed in appropriate volumes of histological fixative (e.g. Davison's, Bouin's or a suitable alternative) for the required time (depending on tissue type and thickness). N.B. tissues should not be stored in Davison's or Bouin's long-term, tissues need to be rinsed in 70% alcohol before being stored in 70% alcohol or 10% NBF and stored at room temperature prior to histological processing.

Analysis and Quantitation. It is recommended that at the outset of histopathological analysis, criteria and scoring guidelines should be put in place. The assessor should be familiar with bird physiology, histopathology and toxicological pathologies in the target tissues. If severity scores are used, photographic examples of different score levels can be used for reference purposes. Final analysis should be conducted blinded to treatment to prevent bias.

6. Future research needs

Research including hormone measurement in non-target vertebrates has led to the description of various methods for different species, hormones and purposes. However, there is a need to harmonise methods for inclusion into (eco)toxicological tests. The majority of methods have been described for fish species and less information is available on hormone measurements birds and amphibians. Protocols have to be adapted and validated for use in standard species.

A lot of existing protocols use RIA methods for hormone measurement. These methods are still in use due to their reliability and sensitivity. However, a general move away from radioactive methods towards alternatives such as ELISA methods has been observed. These still rely on specific antibodies and only measure one hormone at a time. Chromatography is commonly the most sensitive method and has the advantage that several hormones can be measured at the same time. However, more specialised

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

equipment and expertise are required which are not always readily available. Therefore, there is an urgent need to develop sensitive, selective and easy to use methods to measure thyroid and reproductive hormones in non-target vertebrate species.

If hormone measurements are to be conducted at different life stages, e.g. in TGs using amphibians, there is an urgent requirement to determine and understand the most relevant life stages. Whilst hormone measurement may be incorporated into the sampling procedures within existing TGs, it is of utmost importance to ensure that these endpoints produce meaningful data. Therefore, research is needed to establish the best timepoints for sampling of non-target vertebrates to measure hormone levels.

One of the main issues arising from the recommendation to add hormone measurement to OECD TGs is that hormones cycle can be markedly different concentrations depending on the time of day, stage of development, or reproductive state (e.g. ovulation). Therefore, there is a pressing need to identify optimum the time window for hormone collection in amphibians, birds and fish to provide scientifically robust guidelines.

There is also a need to investigate the use of more non-invasive techniques to assess hormone disruption in non-target vertebrates. In amphibians, faeces, saliva or dermal secretions could be used for non-invasive hormone measurement. These would allow repeat measurements in the same animal at different developmental stages. Not much information is available for non-invasive methods in amphibians. One study using faeces to measure reproductive hormones has been described (Hogan et al. 2013). Other studies have used dermal secretions (Scheun et al. 2019) and saliva (Hammond et al. 2018) to measure stress hormones. Future research could investigate whether these techniques can be adapted for measurement of thyroid and/or reproductive hormones.

In fish, non-invasive techniques use tank water to measure hormone levels. If the methods are sensitive enough, this allows to investigate more time points and exposure periods and different life stages. These methods have been described for measurement of reproductive hormones using immuno-assays (RIA: Table 12: , ELISA: Table 15:).

In birds, feathers and droppings could be investigated, as these could provide repeated assessment of the same animal, possibly giving a more nuanced and refined measure of an animals hormonal state over the test period (rather than one 'spot sample' measurement at the end). Investigators have developed these types of methods for both immuno-assays (RIA: Table 11, ELISA: Table 14) and chromatograph (Table 17 and Table 18) for steroid hormones. Studies describing RIA and ELISA for measuring E2 or T in dropping used limited extraction methods (dissolving, liquid-liquid) resulted in low steroid recoveries (Table 11, Table 14). However, more recent publications using ultrasonic extraction and solid phase extraction (SPE), with HPLC analysis gave much high E2 recoveries (Fu et al. 2013). Before adapting these types of hormone measures the use of these non-invasive measures in OECD TGs setting would need to be determined and validated.

If non-mammalian TG are to mirror mammalian ones, there is also a need to investigate which pituitary gland hormones (e.g. TSH) will provide suitable data to support endocrine disruptor assessment in non-mammal test species. If these hormones are deemed useful endpoints, species-specific methods for assessing pituitary gland hormones in each species need to be developed, as for example, no avian specific TSH methods were found in the literature search.

From the literature review on bird pathology, none of the papers relied on gross pathology alone to assess reproductive organ disruption (Table 26) and possible endocrine effects. Organ histopathological assessment alongside gross pathology were the preferred methods (sometime alongside reproductive output, hormones measures, or molecular or protein markers). Therefore, an OECD guidance on histopathology in birds is needed, to complement those developed for other Test Guidelines (Johnson et al., 2009; OECD, 2008, 2015a).

Currently for TG 206 gross pathology is only recommended in adult birds (F0). However, as F1 exposed animals are often considered more sensitive to endocrine disruption, due to exposure during critical developmental windows. Shibuya et al. (2005) and Yamashita et al. (2011) provide some evidence that

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

additional endpoints could be integrated into OECD TG 206. Therefore, the possibility to add endpoints such as gross pathology, histopathology, hormone measurements to F1 14-day-old chicks in OECD TG 206 could also be investigated.

References

Ahmed E, Nagaoka K, Fayez M, Abdel-Daim MM, Samir H & Watanabe G. 2015. Suppressive effects of long-term exposure to P-nitrophenol on gonadal development, hormonal profile with disruption of tissue integrity, and activation of caspase-3 in male Japanese quail Coturnix japonica. Environmental Science and Pollution Research, 2214: 10930–10942. https://doi.org/10.1007/s11356-015-4245-9

Aire TA. 2005. Short-term effects of carbendazim on the gross and microscopic features of the testes of Japanese quails Coturnix coturnix japonica. In Anatomy and Embryology. 210(1): pp. 43–49. DOI: 10.1007/s00429-005-0001-0.

Al-Ansari AM, Kimpe SA, Trudeau SE, Blais JM. 2011. The development of an optimized sample preparation for trace level detection of 17 alpha-ethinylestradiol and estrone in whole fish tissue. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences. 879(30): 3649–3652. DOI: 10.1016/j.jchromb.2011.09.033.

Arias Torres, Ana Josefina Bernardino Paez, Jose Isabel Zelarayan, Liliana 2016.: Validation of Electrochemiluminiscence Immunoassay for Ovarian Steroid Determination in Rhinella arenarum. In JOURNAL OF EXPERIMENTAL ZOOLOGY PART A-ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY 325 4., pp. 265–273. DOI: 10.1002/jez.2013.

Arukwe, Augustine Cangialosi, Maria V. Letcher, Robert J. Rocha, Eduardo Mortensen, Anne S. 2013.: Changes in morphometry and association between whole-body fatty acids and steroid hormone profiles in relation to bioaccumulation patterns in salmon larvae exposed to perfluorooctane sulfonic or perfluorooctane carboxylic acids. In Aquatic toxicology Amsterdam, Netherlands. 130-131, pp. 219–230. DOI: 10.1016/j.aquatox.2012.12.026.

ASTM 2004. Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus Fetax. In ASTM E1439 - 98, Philadelphia, PA, USA.

Azzouz, Abdelmonaim Ballesteros, Evaristo 2015.: Multiresidue method for the determination of pharmacologically active substances in egg and honey using a continuous solid-phase extraction system and gas chromatography-mass spectrometry. In Food chemistry 178, pp. 63–69. DOI: 10.1016/j.foodchem.2015.01.044.

Bautista, L. M. Silván, G. Cáceres, S. Martínez-Fernández, L. Bravo, C. Illera, J. C. et al. 2013.: Faecal sexual steroids in sex typing and endocrine status of great bustards. In European Journal of Wildlife Research 59 6., pp. 815–822. DOI: 10.1007/s10344-013-0735-6.

Bieniarz, K. Sokolowska, M. Fostier, A. Epler, P. 1986.: Daily changes in blood serum levels of 17 beta-estradiol and 11-ketotestosterone in the mature carp Cyprinus carpio L. In Chronobiologia 13 1., pp. 23–27. Available online at https://www.scopus.com/inward/record.uri?eid=2-s2.0-0022568488&partnerID=40&md5=5c80b01c0efe50731e36b73f0c32ac4b.

Bílková, Zuzana Adámková, Marie Albrecht, Tomáš Šimek, Zdeněk 2019.: Determination of testosterone and corticosterone in feathers using liquid chromatography-mass spectrometry. In Journal of Chromatography A 1590, pp. 96–103. DOI: 10.1016/j.chroma.2018.12.069.

Blasco, M. Carriquiriborde, P. d. Marino Ronco, A. E. Somoza, G. M. 2009.: A quantitative HPLC-MS method for the simultaneous determination of testosterone, 11-ketotestosterone and 11-β hydroxyandrostenedione in fish serum. In Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences 877 14-15., pp. 1509–1515. DOI: 10.1016/j.jchromb.2009.03.028.

Brown, S. Eales, J. G. 1977.: Measurement of L-thyroxine and 3,5,3'-triiodo-L-thyronine levels in fish plasma by radioimmunoassay. In Canadian journal of zoology 55 2., pp. 293–299.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Buchanan, K. L. Goldsmith, A. R. Hinde, C. A. Griffith, S. C. Kilner, R. M. 2007.: Does testosterone mediate the trade-off between nestling begging and growth in the canary Serinus canaria.? In Hormones and Behavior 52 5., pp. 664–671. DOI: 10.1016/j.yhbeh.2007.08.009.

Budzinski, H. Devier, M. H. Labadie, P. Togola, A. 2006.: Analysis of hormonal steroids in fish plasma and bile by coupling solid-phase extraction to GC/MS. In Analytical and bioanalytical chemistry 386 5., pp. 1429–1439. DOI: 10.1007/s00216-006-0686-9.

Bussy, Ugo Chung-Davidson, Yu-Wen Buchinger, Tyler J. Li, Ke Li, Weiming 2017a.: High-sensitivity determination of estrogens in fish plasma using chemical derivatization upstream UHPLC–MSMS. In Steroids 123, pp. 13–19. DOI: 10.1016/j.steroids.2017.04.003.

Bussy, Ugo Chung-Davidson, Yu-Wen Li, Ke Fissette, Skye D. Buchinger, Ethan G. Li, Weiming 2017b.: A validated LC–MS/MS method for thyroid hormone determination in sea lamprey Petromyzon marinus. plasma, gill, kidney and liver. In Journal of Chromatography B 1041-1042, pp. 77–84. DOI: 10.1016/j.jchromb.2016.12.024.

Bykova, Liliya Archer-Hartmann, Stephanie A. Holland, Lisa A. Iwanowicz, Luke R. Blazer, Vicki S. 2010.: Steroid determination in fish plasma using capillary electrophoresis. In Environmental Toxicology and Chemistry 29 9., pp. 1950–1956. DOI: 10.1002/etc.252.

Carr, James A. Patiño, Reynaldo 2011.: The hypothalamus-pituitary-thyroid axis in teleosts and amphibians: endocrine disruption and its consequences to natural populations. In General and Comparative Endocrinology 170 2., pp. 299–312. DOI: 10.1016/j.ygcen.2010.06.001.

CerdaReverter, J. M. Zanuy, S. Carrillo, M. Kah, O. 1996.: Development of enzyme immunoassays for 3,5,3'-triiodo-L-thyronine and L-thyroxine: Time-course studies on the effect of food deprivation on plasma thyroid hormones in two marine teleosts, sea bass Dicentrarchus labrax L. and sea bream Sparus aurata L. In General and Comparative Endocrinology 103 3., pp. 290–300. DOI: 10.1006/gcen.1996.0124.

Chan, S. T. Yeung, W. S. 1986.: A new method for the simultaneous determination of androstenedione, testosterone, 11-oxotestosterone and 11 beta-OH-testosterone in fish plasma using combined techniques of Celite chromatography and radioimmunoassay. In Journal of Steroid Biochemistry 25 6., pp. 1013–1021.

Chang, H. Wan, Y. Naile, J. Zhang, X. Wiseman, S. Hecker, M. et al. 2010.: Simultaneous quantification of multiple classes of phenolic compounds in blood plasma by liquid chromatography-electrospray tandem mass spectrometry. In Journal of Chromatography A 1217 4., pp. 506–513. DOI: 10.1016/j.chroma.2009.11.076.

Chao, A. Schlinger, B. A. Remage-Healey, L. 2011.: Combined liquid and solid-phase extraction improves quantification of brain estrogen content. In Frontiers in Neuroanatomy SEP. DOI: 10.3389/fnana.2011.00057.

Charlier, T. D. Po, K.W.L. Newman, A.E.M. Shah, A. H. Saldanha, C. J. Soma, K. K. 2010.: 17β-Estradiol levels in male zebra finch brain: Combining Palkovits punch and an ultrasensitive radioimmunoassay. In General and Comparative Endocrinology 167 1., pp. 18–26. DOI: 10.1016/j.ygcen.2010.02.002.

Chen, Xiaopeng Walter, Kyla M. Miller, Galen W. Lein, Pamela J. Puschner, Birgit 2018.: Simultaneous quantification of T4, T3, rT3, 3,5-T2 and 3,3'-T2 in larval zebrafish Danio rerio. as a model to study exposure to polychlorinated biphenyls. In Biomedical chromatography : BMC 32 6., e4185-e4185. DOI: 10.1002/bmc.4185.

Cheng, Yan Shan, Zhengjun Zhou, Junying Bu, Yuanqing Li, Pengfu Lu, Shan 2017.: Effects of 4-nonylphenol in drinking water on the reproductive capacity of Japanese quails Coturnix japonica. In Chemosphere 175, pp. 219–227. DOI: 10.1016/j.chemosphere.2017.02.041.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Cheng, Yan Shan, Zhengjun Zhou, Junying Bu, Yuanqing Li, Pengfu Lu, Shan 2019.: Effects of long-term 4-nonylphenol dietary exposure on reproductive ability of Japanese quails Coturnix japonica. In Environmental Science and Pollution Research 26 3., pp. 2908–2917. DOI: 10.1007/s11356-018-3820-2.

Clark, T. D. Donaldson, M. R. Drenner, S. M. Hinch, S. G. Patterson, D. A. Hills, J. et al. 2011.: The efficacy of field techniques for obtaining and storing blood samples from fishes. In Journal of Fish Biology 79 5., pp. 1322–1333. DOI: 10.1111/j.1095-8649.2011.03118.x.

Cockrem, J. F. Rounce, J. R. 1994.: Faecal measurements of oestradiol and testosterone allow the non-invasive estimation of plasma steroid concentrations in the domestic fowl. In British Poultry Science 35 3., pp. 433–443. DOI: 10.1080/00071669408417708.

Cohen, Alejandro Ross, Neil W. Smith, Paul M. Fawcett, James P. 2017.: Analysis of 17 beta-estradiol, estriol and estrone in American eel Anguilla rostrata. tissue samples using liquid chromatography coupled to electrospray differential ion mobility tandem mass spectrometry. In RAPID COMMUNICATIONS IN MASS SPECTROMETRY 31 10., pp. 842–850. DOI: 10.1002/rcm.7853.

Craig, J. M. Papoulias, D. M. Thomas, M. V. Annis, M. L. Boase, J. 2009.: Sex assignment of lake sturgeon Acipenser fluvescens. based on plasma sex hormone and vitellogenin levels. In Journal of Applied Ichthyology 25 SUPPL. 2., pp. 60–67. DOI: 10.1111/j.1439-0426.2009.01289.x.

Crane, Helen M. Pickford, Daniel B. Hutchinson, Thomas H. Brown, J. Anne 2004.: Developmental changes of thyroid hormones in the fathead minnow, Pimephales promelas. In General and Comparative Endocrinology 139 1., pp. 55–60. DOI: 10.1016/j.ygcen.2004.07.004.

Crossin, Glenn T. Phillips, Richard A. Wynne-Edwards, Katherine E. Williams, Tony D. 2013.: Postmigratory Body Condition and Ovarian Steroid Production Predict Breeding Decisions by Female Gray-Headed Albatrosses. In Physiological and Biochemical Zoology 86 6., pp. 761–768. DOI: 10.1086/673755.

Cuisset, B. Pradelles, P. Kime, D. E. Kühn, E. R. Babin, P. Davail, S. Le Menn, F. 1994.: Enzyme immunoassay for 11-ketotestosterone using acetylcholinesterase as laberl: application to the measurement of 11-ketotestosterone in plasma of Siberian sturgeon. In Comparative Biochemistry and Physiology. Part C: Pharmacology 108 2., pp. 229–241. DOI: 10.1016/1367-828094.90035-3.

Culbert, J. 1973.: Method for the extraction and purification of steroid hormones from blood plasma of the domestic fowl. In Laboratory practice 22 5., p. 348.

da Silva, Denis A M Buzitis, Jon Reichert, William L. West, James E. O'Neill, Sandra M. Johnson, Lyndal L. et al. 2013.: Endocrine disrupting chemicals in fish bile: A rapid method of analysis using English sole Parophrys vetulus. from Puget Sound, WA, USA. In Chemosphere 92 11., pp. 1550–1556. DOI: 10.1016/j.chemosphere.2013.04.027.

Dashow, L. Katz, Y. Trachtman, M. S. Epple, A. 1984.: Plasma steroids in the ammocoete of Petromyzon marinus. In General and Comparative Endocrinology 55 3., pp. 361–366. DOI: 10.1016/0016-648084.90004-2.

Delville, Y. Sulon, J. Balthazart, J. 1985.: Hormonal Correlates of Gonadal Regression and Spontaneous Recovery in Japanese Quail Exposed to Short Day-Lengths. In Archives Internationales de Physiologie et de Biochimie 93 2., pp. 123–133. DOI: 10.3109/13813458509079598.

Denver, Robert J. 1993.: Acceleration of Anuran Amphibian Metamorphosis by Corticotropin-Releasing Hormone-like Peptides. In General and Comparative Endocrinology 91 1., pp. 38–51. DOI: 10.1006/gcen.1993.1102.

Dickens, M. J. Boumonville, C. de Balthazart, J. Cornil, C. A. 2014.: Relationships between rapid changes in local aromatase activity and estradiol concentrations in male and female quail brain. In Hormones and Behavior 65 2., pp. 154–164. DOI: 10.1016/j.yhbeh.2013.12.011.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Dickhoff, W. W. Folmar, L. C. Gorbman, A. 1978.: Changes in plasma thyroxine during smoltification of coho salmon, Oncorhynchus kisutch. In General and Comparative Endocrinology 36 2., pp. 229–232. DOI: 10.1016/0016-648078.90027-8.

DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance. Available online at http://data.europa.eu/eli/dir/2010/63/oj.

Dong, Xiang-Zhi Zhao, Li-Xia Guo, Guang-Sheng Lin, Jin-Ming 2009.: Development and Application of Dispersion Solid-Phase Extraction for Estrogens in Aquatic Animal Samples. In Analytical Letters 42 1., pp. 29–44. DOI: 10.1080/00032710802568622.

Driot, F. J. Garnier, D. H. Terqui, M. 1978.: Development and validation of a "direct" radioimmunoassay for plasma testosterone in the fowl Gallus domesticus. In General and Comparative Endocrinology 36 2., pp. 244–249.

ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the Joint Research Centre (JRC), Andersson N, Arena M, Auteri D, Barmaz S, Grignard E, Kienzler A, Lepper P, Lostia AM, Munn S, Parra Morte JM, Pellizzato F, Tarazona J, Terron A and Van der Linden S, 2018. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. EFSA Journal 2018;16(6):5311, 135 pp. https://doi.org/10.2903/j.efsa.2018.5311. ECHA-18-G-01-EN..

Feder, H. H. Storey, A. d. Goodwin 1977.: Testosterone and '5α-dihydrotestosterone' levels in peripheral plasma of male and female ring doves Streptopelia risoria. during the reproductive cycle. In Biology of Reproduction 16 5., pp. 666–677. DOI: 10.1095/biolreprod16.5.666.

Felix, Ana S. Faustino, Ana I. Cabral, Eduarda M. Oliveira, Rui F. 2013.: Noninvasive measurement of steroid hormones in zebrafish holding-water. In Zebrafish 10 1., pp. 110–115. DOI: 10.1089/zeb.2012.0792.

Feswick, A. Ankley, G. T. Denslow, N. Ellestad, L. E. Fuzzen, M. Jensen, K. M. et al. 2014.: An inter-laboratory study on the variability in measured concentrations of 17β-estradiol, testosterone, and 11-ketotestosterone in white sucker: Implications and recommendations. In Environmental Toxicology and Chemistry 33 4., pp. 847–857. DOI: 10.1002/etc.2503.

Fok, P. Eales, J. G. Brown, S. B. 1990.: Determination of 3,5,3'-triiodo-L-thyronine T3. levels in tissues of rainbow trout Salmo gairdneri. and the effect of low ambient pH and aluminum. In Fish Physiology and Biochemistry 8 4., pp. 281–290. DOI: 10.1007/BF00003423.

Fokidis, H. B. Ma, C. Radin, B. Prior, N. H. Adomat, H. H. Guns, E. S. Soma, K. K. 2019.: Neuropeptide Y and orexin immunoreactivity in the sparrow brain coincide with seasonal changes in energy balance and steroids. In Journal of Comparative Neurology 527 2., pp. 347–361. DOI: 10.1002/cne.24535.

Fostier, A. Billard, R. Breton, B. Legendre, M. Marlot, S. 1982.: Plasma 11-oxotestosterone and gonadotropin during the beginning of spermiation in rainbow trout Salmo gairdneri R.. In General and Comparative Endocrinology 46 4., pp. 428–434. DOI: 10.1016/0016-648082.90096-X.

Fowles, Jefferson R. Fairbrother, Anne Trust, Kimberly A. Kerkvliet, Nancy I. 1997.: Effects of aroclor 1254 on the thyroid gland, immune function, and hepatic cytochrome P450 activity in mallards. In Environmental Research 75 2., pp. 119–129. DOI: 10.1006/enrs.1997.3776.

Friesen, Caitlin N. Chapman, Lauren J. Aubin-Horth, Nadia 2012.: Holding water steroid hormones in the African cichlid fish Pseudocrenilabrus multicolor victoriae. In General and Comparative Endocrinology 179 3., pp. 400–405. DOI: 10.1016/j.ygcen.2012.07.031.

Fu, Yin-Jie Ling, Wan-Ting Dong, Chang-Xun Liu, Juan Gao, Yan-Zheng Pan, Yu-Lan 2013.: [Estrogens determination of livestock dung based on UE-SPE-HPLC/FLD]. In Ying yong sheng tai xue bao = The journal of applied ecology 24 11., pp. 3280–3288.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Futra, D Heng, L. Y. Jaapar, M. Z. Ulianas, A. Saeedfar, K. Ling, T. L. 2016.: A novel electrochemical sensor for 17β-estradiol from molecularly imprinted polymeric microspheres and multi-walled carbon nanotubes grafted with gold nanoparticles. In Analytical Methods 8 6., pp. 1381–1389. DOI: 10.1039/c5ay02796a

Gancedo, B. Alonso-Gomez, A. L. Pedro, N. de Corpas, I. Delgado, M. J. Alonso-Bedate, M. 1995.: Seasonal changes in thyroid activity in male and female frog, Rana perezi. In General and Comparative Endocrinology 97 1., pp. 66–75. DOI: 10.1006/gcen.1995.1007.

Gibson, R. Smith, M. D. Spary, C. J. Tyler, C. R. Hill, E. M. 2005.: Mixtures of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents. In Environmental science & technology 39 8., pp. 2461–2471.

Gore, A. C. Chappell, V. A. Fenton, S. E. Flaws, J. A. Nadal, A. Prins, G. S. et al. 2015.: EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. In Endocrine Reviews 36 6., E1-E150. DOI: 10.1210/er.2015-1010.

Goymann, Wolfgang Mostl, Erich Gwinner, Eberhard 2002.: Non-invasive methods to measure androgen metabolites in excrements of European stonechats, Saxicola torquata rubicola. In General and Comparative Endocrinology 129 2., pp. 80–87.

Goymann, Wolfgang Trappschuh, Monika Jensen, Willi Schwabl, Ingrid 2006.: Low ambient temperature increases food intake and dropping production, leading to incorrect estimates of hormone metabolite concentrations in European stonechats. In Hormones and Behavior 49 5., pp. 644–653. DOI: 10.1016/j.yhbeh.2005.12.006.

Grange, R. D. Thompson, J. P. Lambert, D. G. 2014.: Radioimmunoassay, enzyme and non-enzyme-based immunoassays. In Br J Anaesth 112 2., pp. 213–216. DOI: 10.1093/bja/aet293.

Green, John W. Springer, Timothy A. Saulnier, Amy N. Swintek, Joe 2014.: Statistical analysis of histopathological endpoints. In Environmental Toxicology and Chemistry 33 5., pp. 1108–1116. DOI: 10.1002/etc.2530.

Greenacre, C. B. Young, D. W. Behrend, E. N. Heather Wilson, G. 2001.: Validation of a novel high-sensitivity radioimmunoassay procedure for measurement of total thyroxine concentration in psittacine birds and snakes. In American Journal of Veterinary Research 62 11., pp. 1750–1754. DOI: 10.2460/ajvr.2001.62.1750.

Grimm, H. 1979.: FINNEY, D. J.: Statistical Method in Biological Assay. 3. ed. Charles Griffin & Co., London and High Wycombe 1978. VII, 508 S., £ 19 net. In Biom. J. 21 7., pp. 689–690. DOI: 10.1002/bimj.4710210714.

Grote, Konstanze Niemann, Lars Selzsam, Britta Haider, Wolfgang Gericke, Christine Herzler, Matthias Chahoud, Ibrahim 2008.: Epoxiconazole causes changes in testicular histology and sperm production in the Japanese quail Coturnix coturnix japonica. In Environmental Toxicology and Chemistry 27 11., pp. 2368–2374. DOI: 10.1897/08-048.1.

Guioli, Silvana Nandi, Sunil Zhao, Debiao Burgess-Shannon, Jessica Lovell-Badge, Robin Clinton, Michael 2014.: Gonadal asymmetry and sex determination in birds. In Sexual Development 8 5., pp. 227–242. DOI: 10.1159/000358406.

Guzmán, José M. Luckenbach, J. Adam da Silva, Denis A M Ylitalo, Gina M. Swanson, Penny 2015.: Development of approaches to induce puberty in cultured female sablefish Anoplopoma fimbria. In General and Comparative Endocrinology 221, pp. 101–113. DOI: 10.1016/j.ygcen.2015.02.024.

Hahn, A. Reitemeier, S. Gottschalk, J. Haense, M. v. Schmidt Steinbach-Sobiraj, K. et al. 2011.: Assessment of the male reproductive status in psittaciformes by endocrine analysis. In Tierarztliche Praxis Ausgabe K: Kleintiere - Heimtiere 39 4., pp. 249–257. Available online at https://www.scopus.com/inward/record.uri?eid=2-s2.0-80052408245&partnerID=40&md5=79763b10ce4146f63266453611f42fec.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Hala, D. Overturf, M. D. Petersen, L. H. Huggett, D. B. 2011.: Quantification of 2-hydrazinopyridine derivatized steroid hormones in fathead minnow Pimephales promelas. blood plasma using LC-ESI+/MS/MS. In Journal of chromatography. B, Analytical technologies in the biomedical and life sciences 879 9-10., pp. 591–598. DOI: 10.1016/j.jchromb.2011.01.020.

Hammond, G. L. 2011.: Diverse Roles for Sex Hormone-Binding Globulin in Reproduction. In Biology of Reproduction 85 3., pp. 431–441. DOI: 10.1095/biolreprod.111.092593.

Hammond, Talisin T. Au, Zoe A. Hartman, Allison C. Richards-Zawacki, Corinne L. 2018.: Assay validation and interspecific comparison of salivary glucocorticoids in three amphibian species. In Conservation Physiology 6 1., coy055. DOI: 10.1093/conphys/coy055.

Hansen, Martin Luong, Xuan Sedlak, David L. Helbing, Caren C. Hayes, Tyrone 2016.: Quantification of 11 thyroid hormones and associated metabolites in blood using isotope-dilution liquid chromatography tandem mass spectrometry. In Analytical and bioanalytical chemistry 408 20., pp. 5429–5442. DOI: 10.1007/s00216-016-9614-9.

Harr, Kendal E. Reavill, Drury R. Bursian, Steven J. Cacela, Dave Cunningham, Fred L. Dean, Karen M. et al. 2017.: Organ weights and histopathology of double-crested cormorants Phalacrocorax auritus. dosed orally or dermally with artificially weathered Mississippi Canyon 252 crude oil. In Ecotoxicology and Environmental Safety 146 SI., pp. 52–61. DOI: 10.1016/j.ecoenv.2017.07.011.

Hartmann, Sonja Lacorn, Markus Steinhart, Hans 1998.: Natural occurrence of steroid hormones in food. In Food chemistry 62 1., pp. 7–20. DOI: 10.1016/S0308-814697.00150-7.

Hartmann, Sonja Steinhart, Hans 1997.: Simultaneous determination of anabolic and catabolic steroid hormones in meat by gas chromatography–mass spectrometry. In Journal of Chromatography B: Biomedical Sciences and Applications 704 1-2., pp. 105–117. DOI: 10.1016/S0378-434797.00460-X.

Heimovics, S. A. Prior, N. H. Ma, C. Soma, K. K. 2016.: Rapid Effects of an Aggressive Interaction on Dehydroepiandrosterone, Testosterone and Oestradiol Levels in the Male Song Sparrow Brain: a Seasonal Comparison. In Journal of neuroendocrinology 28 2. DOI: 10.1111/jne.12345.

Henry, Paula F.P. P. Akuffo, Valorie G. Chen, Yu Karouna-Renier, Natalie K. Sprague, Daniel T. Bakst, Murray R. 2012.: Effect of 17β-trenbolone on male and female reproduction in Japanese quail Coturnix japonica. In Avian Biology Research 5 2., pp. 61–68. DOI: 10.3184/175815512X13350167598421.

Hogan, L. A. Lisle, A. T. Johnston, S. D. Goad, T. Robertston, H. 2013.: Adult and juvenile sex identification in threatened monomorphic geocrinia frogs using fecal steroid analysis. In Journal of Herpetology 47 1., pp. 112–118. DOI: 10.1670/11-290.

Hornung, Michael W. Kosian, Patricia A. Haselman, Jonathan T. Korte, Joseph J. Challis, Katie Macherla, Chitralekha et al. 2015.: In Vitro, Ex Vivo, and In Vivo Determination of Thyroid Hormone Modulating Activity of Benzothiazoles. In TOXICOLOGICAL SCIENCES 146 2., pp. 254–264. DOI: 10.1093/toxsci/kfv090.

Hu, Yuling Wang, Yangyang Chen, Xiaoguang Hu, Yufei Li, Gongke 2010.: A novel molecularly imprinted solid-phase microextraction fiber coupled with high performance liquid chromatography for analysis of trace estrogens in fishery samples. In Talanta 80 5., pp. 2099–2105. DOI: 10.1016/j.talanta.2009.11.015.

Huang, F.-L. Ke, F.-C. Hwang, J.-J. Lo, T.-B. 1983.: High-pressure liquid chromatographic separation of a mixture of corticosteroids, androgens, and progestins. In Archives of Biochemistry and Biophysics 225 2., pp. 512–517. DOI: 10.1016/0003-986183.90061-9.

Hulbert, A. J. 2000.: Thyroid hormones and their effects: a new perspective. In Biological reviews of the Cambridge Philosophical Society 75 4., pp. 519–631. Available online at http://www.ncbi.nlm.nih.gov/pubmed/11117200.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ishihara, Akinori Nishiyama, Norihito Makita, Yu Yamauchi, Kiyoshi 2012.: The genomic structure and the expression profile of the Xenopus laevis transthyretin gene. In Gene 510 2., pp. 126–132. DOI: 10.1016/j.gene.2012.09.002.

Jacobsen, Mona L. Jaspers, Veerle L. B. Ciesielski, Tomasz M. Jenssen, Bjorn M. Loseth, Mari E. Briels, Nathalie et al. 2017.: Japanese quail Coturnix japonica. liver and thyroid gland histopathology as a result of in ovo exposure to the flame retardants tris1,3-dichloro-2-propyl. phosphate and Dechlorane Plus. In Journal of Toxicology and Environmental ealth-Part a current Issues 80 9., pp. 525–531. DOI: 10.1080/15287394.2017.1336414.

Jallageas, M. Attal, J. 1968.: [Determination by gas phase chromatography of non-conjugated plasma testosterone in ducks, quail, and pigeons]. In Comptes rendus hebdomadaires des seances de l'Academie des sciences. Serie D: Sciences naturelles 267 3., pp. 341–343.

Johnson, Rodney Wolf, Jeffrey Braunbeck, Thomas 2009.: OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads. In Oecd, pp. 1–42.

Kellam, J. S. Wingfield, J. C. Lucas, J. R. 2004.: Nonbreeding season pairing behavior and the annual cycle of testosterone in male and female downy woodpeckers, Picoides pubescens. In Hormones and Behavior 46 5., pp. 703–714. DOI: 10.1016/j.yhbeh.2004.06.014.

Khan, I. A. Lopez, E. Leloup-Hâtey, J. 1986.: Effects of hypophysectomy on the testis of the European eel Anguilla anguilla L.. In General and Comparative Endocrinology 62 3., pp. 411–418. DOI: 10.1016/0016-648086.90051-1.

Khan, M. N. Reddy, P. K. Renaud, R. L. Leatherland, J. F. 1997.: Application of HPLC methods to identify plasma profiles of 11-oxygenated androgens and other steroids in Arctic Charr Salvelinus alpinus. during gonadal recrudescence. In Comparative Biochemistry and Physiology - C Pharmacology Toxicology and Endocrinology 118 2., pp. 221–227. DOI: 10.1016/S0742-841397.00131-X.

Kindler, P. M. Philipp, D. P. Gross, M. R. Bahr, J. M. 1989.: Serum 11-ketotestosterone and testosterone concentrations associated with reproduction in male bluegill Lepomis macrochirus: Centrarchidae. In General and Comparative Endocrinology 75 3., pp. 446–453.

Koren, Lee Nakagawa, Shinichi Burke, Terry Soma, Kiran K. Wynne-Edwards, Katherine E. Geffen, Eli 2012a.: Non-breeding feather concentrations of testosterone, corticosterone and cortisol are associated with subsequent survival in wild house sparrows. In Proceedings. Biological sciences 279 1733., pp. 1560–1566. DOI: 10.1098/rspb.2011.2062.

Koren, Lee Ng, Ella S. M. Soma, Kiran K. Wynne-Edwards, Katherine E. 2012b.: Sample preparation and liquid chromatography-tandem mass spectrometry for multiple steroids in mammalian and avian circulation. In PLoS ONE 7 2., e32496-e32496. DOI: 10.1371/journal.pone.0032496.

Korte, Joseph J. Sternberg, Robin M. Serrano, Jose A. Thoemke, Kara R. Moen, Scott M. Lillegard, Kathryn E. et al. 2011.: Thyroid-stimulating hormone TSH.: Measurement of intracellular, secreted, and circulating hormone in Xenopus laevis and Xenopus tropicalis. In General and Comparative Endocrinology 171 3., pp. 319–325. DOI: 10.1016/j.ygcen.2011.02.017.

Kucheryavenko, Olena Lurman, Glenn Lehmann, Anja Braz, Juliana Niemann, Lars Chahoud, Ibrahim et al. 2019.: Report from the BfR expert hearing on practicability of hormonal measurements: recommendations for experimental design of toxicological studies with integrated hormonal end points. In Archives of Toxicology. DOI: 10.1007/s00204-019-02436-3.

Kurobe, Tomofumi Park, Micheal O. Javidmehr, Alireza Teh, Foo-Ching Acuna, Shawn C. Corbin, Cynthia J. et al. 2016.: Assessing oocyte development and maturation in the threatened Delta Smelt, Hypomesus transpacificus. In Environmental Biology of Fishes 99 4., pp. 423–432. DOI: 10.1007/s10641-016-0483-z.

Labadie, P. Budzinski, H. 2006.: Alteration of steroid hormone profile in juvenile turbot Psetta maxima. as a consequence of short-term exposure to 17α-ethynylestradiol. In Chemosphere 64 8., pp. 1274–1286. DOI: 10.1016/j.chemosphere.2005.12.065.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Lamba, V. J. Goswami, S. V. Sundararaj, B. I. 1982.: Radioimmunoassay for plasma cortisol, testosterone, estradiol-17β, and estrone in the catfish Heteropneustes fossilis Bloch.: Development and validation. In General and Comparative Endocrinology 47 2., pp. 170–181. DOI: 10.1016/0016-648082.90218-0.

Lan, Hangzhen Gan, Ning Pan, Daodong Hu, Futao Li, Tianhua Long, Nengbing et al. 2014.: Development of a novel magnetic molecularly imprinted polymer coating using porous zeolite imidazolate framework-8 coated magnetic iron oxide as carrier for automated solid phase microextraction of estrogens in fish and pork samples. In Journal of Chromatography A 1365, pp. 35–44. DOI: 10.1016/j.chroma.2014.08.096.

Larsen, P. R. 1972.: Direct immunoassay of triiodothyronine in human serum. In Journal of Clinical Investigation 51 8., pp. 1939–1949. DOI: 10.1172/JCI107000.

Larsen, P. R. Dockalova, J. Sipula, D. Wu, F. M. 1973.: Immunoassay of Thyroxine in Unextracted Human Serum. In The Journal of Clinical Endocrinology & Metabolism 37 2., pp. 177–182. DOI: 10.1210/jcem-37-2-177.

Larsson, D. G. Joakim Mayer, Ian Hyllner, Sven Johan Forlin, Lars 2002.: Seasonal variations of vitelline envelope proteins, vitellogenin, and sex steroids in male and female eelpout Zoarces viviparus. In General and Comparative Endocrinology 125 2., pp. 184–196. DOI: 10.1006/gcen.2001.7740.

Leary, Steven Underwood, Wendy Anthony, Raymond Cartner, Samuel Grandin, Temple Greenacre, Cheryl et al. 2020.: AVMA Guidelines for the Euthanasia of Animals: 2020 Edition.

Lee, J. V. Whaling, C. S. Lasley, B. L. Marler, P. 1995.: Validation of an enzyme immunoassay for measurement of excreted estrogen and testosterone metabolites in the white-crowned sparrow Zonotrichia leucophrys oriantha. In Zoo Biology 14 2., pp. 97–106. DOI: 10.1002/zoo.1430140203.

Li, Wei Zha, Jinmiao Yang, Lihua Li, Zhaoli Wang, Zijian 2011.: Regulation of thyroid hormone related genes mRNA expression by exogenous T_3 in larvae and adult Chinese rare minnow Gobiocypris rarus. In Environmental toxicology and pharmacology 31 1., pp. 189–197. DOI: 10.1016/j.etap.2010.10.007.

Liere, Philippe Cornil, Charlotte A. Bournonville, Marie Pierre de Pianos, Antoine Keller, Matthieu Schumacher, Michael Balthazart, Jacques 2019.: Steroid profiles in quail brain and serum: Sex and regional differences and effects of castration with steroid replacement. In Journal of neuroendocrinology 31 2., e12681-e12681. DOI: 10.1111/jne.12681.

Lorenzi, Varenka Earley, Ryan L. Grober, Matthew S. 2012.: Differential Responses of Brain, Gonad and Muscle Steroid Levels to Changes in Social Status and Sex in a Sequential and Bidirectional Hermaphroditic Fish. In PLoS ONE 7 12. DOI: 10.1371/journal.pone.0051158.

LU, Jie FENG, Xiao-Mei SUI, Xiao CHEN, Man-Hua HAN, Yu-Qian XUE, Chang-Hu 2013.: Determination of 6 Kinds of Sex Hormones in Fish Using Subcritical 1,1,1,2-Tetrafluoroethane Extraction-Gas Chromatography-Tandem Mass Spectrometry. In Chinese Journal of Analytical Chemistry 41 10., pp. 1487–1492. DOI: 10.1016/S1872-204013.60686-6.

Lu, Junhe Kong, Deyang Zhao, Li Zhou, Quansuo 2014.: Analysis of oestrogenic hormones in chicken litter by HPLC with fluorescence detection. In International Journal of Environmental Analytical Chemistry 94 8., pp. 783–790. DOI: 10.1080/03067319.2014.891108.

Luna, L. G. Coady, K. McFadden, J. R. Markham, D. A. Bartels, M. J. 2013.: Quantification of total thyroxine in plasma from Xenopus laevis. In Journal of analytical toxicology 37 6., pp. 326–336. DOI: 10.1093/jat/bkt029.

Lyons, K. Wynne-Edwards, K. E. 2019.: Steroid concentrations in maternal serum and uterine histotroph in round stingrays Urobatis halleri. In General and Comparative Endocrinology 274, pp. 8–16. DOI: 10.1016/j.ygcen.2018.12.009.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Margiotta-Casaluci, L. Courant, F. Antignac, J.-P. Le Bizec, B. Sumpter, J. P. 2013.: Identification and quantification of 5α-dihydrotestosterone in the teleost fathead minnow Pimephales promelas. by gas chromatography-tandem mass spectrometry. In General and Comparative Endocrinology 191, pp. 202–209. DOI: 10.1016/j.ygcen.2013.06.017.

Margiotta-Casaluci, Luigi Sumpter, John P. 2011.: 5α-Dihydrotestosterone is a potent androgen in the fathead minnow Pimephales promelas. In General and Comparative Endocrinology 171 3., pp. 309–318. DOI: 10.1016/j.ygcen.2011.02.012.

May, J. D. 1978.: A radioimmunoassay for 3,5,3'-triiodothyronine in chicken serum. In Poultry science 57 6., pp. 1740–1745. DOI: 10.3382/ps.0571740.

McDonald, Mark Malone, Edward McBride, John 2010.: Confirmation of hormones in animal serum by liquid chromatography/tandem mass spectrometry according to European Commission Decision 2002/657. In Journal of AOAC International 93 1., pp. 343–349.

McNabb, F. M. Hughes, T. E. 1983.: The role of serum binding proteins in determining free thyroid hormone concentrations during development in quail. In Endocrinology 113 3., pp. 957–963. DOI: 10.1210/endo-113-3-957.

Medina, M. B. Sherman, J. T. 1986.: High performance liquid chromatographic separation of anabolic oestrogens and ultraviolet detection of 17β-oestradiol, zeranol, diethylstilboestrol or zearalenone in avian muscle tissue extracts. In Food Additives and Contaminants 3 3., pp. 263–272. DOI: 10.1080/02652038609373590.

Mensah-Nyagan, A. G. Do-Rego, J. L. Feuilloley, M. Marcual, A. Lange, C. Pelletier, G. Vaudry, H. 1996.: In vivo and in vitro evidence for the biosynthesis of testosterone in the telencephalon of the female frog. In Journal of neurochemistry 67 1., pp. 413–422.

Merrill, L. Chiavacci, S. J. Paitz, R. T. Benson, T. J. 2019.: Quantification of 27 yolk steroid hormones in seven shrubland bird species: interspecific patterns of hormone deposition and links to life history, development, and predation risk. In Canadian journal of zoology 97 1., pp. 1–12. DOI: 10.1139/cjz-2017-0351.

Metcalfe, Suzanne S. Kroon, Frederieke J. Beale, David J. Miller, Gabrielle 2018.: Development of a validation protocol of enzyme immunoassay kits used for the analysis of steroid hormones in fish plasma. In Journal of Experimental Marine Biology and Ecology 499, pp. 26–34. DOI: 10.1016/j.jembe.2017.12.001.

Mi, Xiaoxia Li, Sicong Li, Yanhua Wang, Kaigiang Zhu, Dan Chen, Gang 2014.: Quantitative determination of 26 steroids in eggs from various species using liquid chromatography-triple quadrupole-mass spectrometry. In Journal of Chromatography A 1356, pp. 54–63. DOI: 10.1016/j.chroma.2014.05.084.

Mohanty, Banalata Pandey, Surya Prakash Tsutsui, Kazuyoshi 2017.: Thyroid disrupting pesticides impair the hypothalamic-pituitary-testicular axis of a wildlife bird, Amandava amandava. In Reproductive Toxicology 71, pp. 32–41. DOI: 10.1016/j.reprotox.2017.04.006.

Moher, David Shamseer, Larissa Clarke, Mike Ghersi, Davina Liberati, Alessandro Petticrew, Mark et al. 2015.: Preferred reporting items for systematic review and meta-analysis protocols PRISMA-P. 2015 statement. In Systematic Reviews 4 1., p. 1. DOI: 10.1186/2046-4053-4-1.

Mondou, P. M. Kaltenbach, J. C. 1979.: Thyroxine concentrations in blood serum and pericardial fluid of metamorphosing tadpoles and of adult frogs. In General and Comparative Endocrinology 39 3., pp. 343–349. DOI: 10.1016/0016-648079.90131-X.

Moriyama, S. Swanson, P. Larsen, D. A. Miwa, S. Kawauchi, H. Dickhoff, W. W. 1997.: Salmon thyroid-stimulating hormone: isolation, characterization, and development of a radioimmunoassay. In General and Comparative Endocrinology 108 3., pp. 457–471. DOI: 10.1006/gcen.1997.7004.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Nash, J. P. Cuisset, B. D. Bhattacharyya, S. Suter, H. C. Le Menn, F. Kime, D. E. 2000.: An enzyme linked immunosorbant assay ELISA. for testosterone, estradiol, and 17,20 beta-dihydroxy-4-pregenen-3-one using acetylcholinesterase as tracer: application to measurement of diel patterns in rainbow trout Oncorhynchus mykiss. In Fish Physiology and Biochemistry 22 4., pp. 355–363. DOI: 10.1023/A:1007850014021.

Navarro, P. Bustamante, J. Vallejo, A. Prieto, A. Usobiaga, A. Arrasate, S. et al. 2010.: Determination of alkylphenols and 17β-estradiol in fish homogenate. Extraction and clean-up strategies. In Journal of Chromatography A 1217 38., pp. 5890–5895. DOI: 10.1016/j.chroma.2010.07.049.

Newman, A. E. M. Chin, E. H. Schmidt, K. L. Bond, L. Wynne-Edwards, K. E. Soma, K. K. 2008.: Analysis of steroids in songbird plasma and brain by coupling solid phase extraction to radioimmunoassay. In General and Comparative Endocrinology 155 3., pp. 503–510. DOI: 10.1016/j.ygcen.2007.08.007.

Niemann, Lars Haider, Wolfram Grote, Konstanze Chahoud, Ibrahim 2010.: Histopathology of the testis as a sensitive tool to detect potentially adverse effects of environmental chemicals on male fertility in Japanese quail. In Avian Biology Research 3 1., pp. 35–38. DOI: 10.3184/175815510X12630495813906.

Noaksson, E. Gustavsson, B. Linderoth, M. Zebuhr, Y. Broman, D. Balk, L. 2004.: Gonad development and plasma steroid profiles by HRGC/HRMS during one reproductive cycle in reference and leachate-exposed female perch Perca fluviatilis. In Toxicology and Applied Pharmacology 195 2., pp. 247–261. DOI: 10.1016/j.taap.2003.11.017.

Noyes, Pamela D. Lema, Sean C. Roberts, Simon C. Cooper, Ellen M. Stapleton, Heather M. 2014.: Rapid method for the measurement of circulating thyroid hormones in low volumes of teleost fish plasma by LC-ESI/MS/MS. In Analytical and bioanalytical chemistry 406 3., pp. 715–726. DOI: 10.1007/s00216-013-7528-3.

Obregon, Maria Jesus Roelfsema, Ferdinand Escobar, Gabriella Morreale de Escobar Del Rey, Francisco Querido, Andries 1979.: Exchange Of Triiodothyronine Derived From Thyroxine With Circulating Triiodothyronine As Studied In The Rat. In Clinical Endocrinology 10 3., pp. 305–315. DOI: 10.1111/j.1365-2265.1979.tb02085.x.

OECD 2008.: Endocrine disruption: a guidance document for histologic evaluation of endocrine and reproductive tests. Organization for Economic Development and Co-operation. Paris.

OECD 2009.: Test No. 231: Amphibian Metamorphosis Assay. Available online at https://www.oecd-ilibrary.org/content/publication/9789264076242-en.

OECD 2011.: Test No. 234: Fish Sexual Development Test: OECD OECD Guidelines for the Testing of Chemicals, Section 2. Available online at https://www.oecd-ilibrary.org/environment/test-no-234-fish-sexual-development-test_9789264122369-en.

OECD 2015a.: Guidance document on histopathology techniques and evaluation part 2. for The Larval Amphibian Growth and Development Assay LAGDA.

OECD 2015b.: OECD Guideline for the testing of chemicals 241 The Larval Amphibian Growth and Development Assay LAGDA. July., pp. 1–38.

OECD 2015c.: Test No. 240: Medaka Extended One Generation Reproduction Test MEOGRT.: OECD OECD Guidelines for the Testing of Chemicals, Section 2. Available online at https://www.oecd-ilibrary.org/environment/test-no-240-medaka-extended-one-generation-reproduction-test-meogrt_9789264242258-en.

OECD 2015d.: Test No. 241: The Larval Amphibian Growth and Development Assay LAGDA.: OECD OECD Guidelines for the Testing of Chemicals, Section 2. Available online at https://www.oecd-ilibrary.org/environment/test-no-241-the-larval-amphibian-growth-and-development-assay-lagda_9789264242340-en.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

OECD: OECD Series on Testing and Assement No. 21 Detailed Review Paper Appraisal of Test Methods for Sex Hormone Disrupting Chemicals.

OECD: OECD SERIES ON TESTING AND ASSESSMENT Number 46 DETAILED REVIEW PAPER ON AMPHIBIAN METAMORPHOSIS ASSAY FOR THE DETECTION OF THYROID ACTIVE SUBSTANCES.

OECD: OECD SERIES ON TESTING AND ASSESSMENT Number 57 DETAILED REVIEW PAPER ON THYROID HORMONE DISRUPTION ASSAYS.

OECD 1984. Test No.206: Avian Reproduction Test, OECD Guidelines for the Testing of Chemicals, Section 2. Paris. Available online at https://doi.org/10.1787/ 9789264070028-en.

Ohta, K. Mine, T. Yamaguchi, A. Matsuyama, M. 2001.: Steroidogenic pathway to estradiol-17β synthesis in the ovarian follicles of the protogynous wrasse, Pseudolabrus sieboldi. In Zoological Science 18 7., pp. 937–945. DOI: 10.2108/zsj.18.937.

Omeljaniuk, R. J. COOK, R. F. Eales, J. G. 1984.: SIMULTANEOUS MEASUREMENT OF THYROXINE AND TRIIODOTHYRONINE IN TROUT PLASMA USING A SOLID-PHASE RADIOIMMUNOASSAY. In CANADIAN JOURNAL OF ZOOLOGY-REVUE CANADIENNE DE ZOOLOGIE 62 8., pp. 1451–1453. DOI: 10.1139/z84-208.

Orozco, A. Ruiz, A. Valverde, C. 1992.: The importance of employing homologous serum free of thyronines in radioimmunoassays to assess circulating thyroid hormones in rainbow trout. In Boletin de estudios medicos y biologicos 40 1-4., pp. 41–47.

Oshima, Atsushi Yamashita, Ryo Nakamura, Keigo Wada, Masaru Shibuya, Kazumoto 2012.: In ovo exposure to nonylphenol and bisphenol A resulted in dose-independent feminization of male gonads in Japanese quail Coturnix japonica. embryos. In Environmental Toxicology and Chemistry 31 5., pp. 1091–1097. DOI: 10.1002/etc.1787.

Ottinger, M. A. Bakst, M. R. 1981.: Peripheral androgen concentrations and testicular morphology in embryonic and young male Japanese quail. In General and Comparative Endocrinology 43 2., pp. 170–177. DOI: 10.1016/0016-648081.90309-9.

Overk, C. R. Perez, S. E. Ma, C. Taves, M. D. Soma, K. K. Mufson, E. J. 2013.: Sex Steroid Levels and AD-Like Pathology in 3xTgAD Mice. In Journal of neuroendocrinology 25 2., pp. 131–144. DOI: 10.1111/j.1365-2826.2012.02374.x.

Pandey, Surya Prakash Mohanty, Banalata 2015.: The neonicotinoid pesticide imidacloprid and the dithiocarbamate fungicide mancozeb disrupt the pituitary-thyroid axis of a wildlife bird. In Chemosphere 122, pp. 227–234. DOI: 10.1016/j.chemosphere.2014.11.061.

Pandey, Surya Prakash Mohanty, Banalata 2017.: Disruption of the hypothalamic-pituitary-thyroid axis on co-exposures to dithiocarbamate and neonicotinoid pesticides: Study in a wildlife bird, Amandava amandava. In NeuroToxicology 60, pp. 16–22. DOI: 10.1016/j.neuro.2017.02.010.

Pandey, Surya Prakash Tsutsui, Kazuyoshi Mohanty, Banalata 2017.: Endocrine disrupting pesticides impair the neuroendocrine regulation of reproductive behaviors and secondary sexual characters of red munia Amandava amandava. 173. In Physiology and Behavior.

Panter, G. H. Hutchinson, T. H. Hurd, K. S. Sherren, A. Stanley, R. D. Tyler, C. R. 2004.: Successful detection of anti-.androgenic and aromatase inhibitors in pre-spawning adult fathead minnows Pimephales promelas. using easily measured endpoints of sexual development. In Aquatic Toxicology 70 1., pp. 11–21. DOI: 10.1016/j.aquatox.2004.06.007.

Pedernera, E. Solis, L. Peralta, I. Velazquez, P. N. 1999.: Proliferative and steroidogenic effects of follicle-stimulating hormone during chick embryo gonadal development. In General and Comparative Endocrinology 116 2., pp. 213–220. DOI: 10.1006/gcen.1999.7352.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Picard-Aitken, Michelle Fournier, Henri Pariseau, Richard Marcogliese, David J. Cyr, Daniel G. 2007.: Thyroid disruption in walleye Sander vitreus. exposed to environmental contaminants: cloning and use of iodothyronine deiodinases as molecular biomarkers. In Aquatic toxicology Amsterdam, Netherlands. 83 3., pp. 200–211. DOI: 10.1016/j.aquatox.2007.04.004.

Poulsen, Rikke Luong, Xuan Hansen, Martin Styrishave, Bjarne Hayes, Tyrone 2015.: Tebuconazole disrupts steroidogenesis in Xenopus laevis. In Aquatic toxicology Amsterdam, Netherlands. 168, pp. 28–37. DOI: 10.1016/j.aquatox.2015.09.008.

Prior, Nora H. Yap, Kang Nian Adomat, Hans H. Mainwaring, Mark C. Fokidis, H. Bobby Guns, Emma S. et al. 2016.: Sex steroid profiles and pair-maintenance behavior of captive wild-caught zebra finches Taeniopygia guttata. In Journal of comparative physiology. A, Neuroethology, sensory, neural, and behavioral physiology 202 1., pp. 35–44. DOI: 10.1007/s00359-015-1050-3.

Prior, Nora H. Yap, Kang Nian Mainwaring, Mark C. Adomat, Hans H. Crino, Ondi L. Ma, Chunqi et al. 2017.: Sex steroid profiles in zebra finches: Effects of reproductive state and domestication. In General and Comparative Endocrinology 244, pp. 108–117. DOI: 10.1016/j.ygcen.2016.02.018.

Prohaska, B. K. Tsang, P.C.W. Driggers III, W. B. Hoffmayer, E. R. Sulikowski, J. A. 2013.: Development of a Nonlethal and Minimally Invasive Protocol to Study Elasmobranch Reproduction. In Marine and Coastal Fisheries 5 1., pp. 181–188. DOI: 10.1080/19425120.2013.788590.

Quérat, B. Hardy, A. Leloup-Hâtey, J. 1985.: Niveaux plasmatiques, vitesses de clairance métabolique, et vitesses de sécrétion de la testostérone et de l'oestradiol-17β chez l'anguille Anguilla anguilla L. argentée. In General and Comparative Endocrinology 59 3., pp. 482–493. DOI: 10.1016/0016-648085.90408-3.

Quinn, Michael J. Lavoie, Emma T. Ottinger, Mary Ann 2007.: Reproductive toxicity of trenbolone acetate in embryonically exposed Japanese quail. In Chemosphere 66 7., pp. 1191–1196. DOI: 10.1016/j.chemosphere.2006.07.085.

Quinn, Michael J. Summitt, Cassie L. Ottinger, Mary Ann 2008.: Consequences of in ovo exposure to p,p′-DDE on reproductive development and function in Japanese quail. In Hormones and Behavior 53 1., pp. 249–253. DOI: 10.1016/j.yhbeh.2007.10.004.

Razia, Sultana Maegawa, Yukari Tamotsu, Satoshi Oishi, Tadashi 2006.: Histological changes in immune and endocrine organs of quail embryos: Exposure to estrogen and nonylphenol. In Ecotoxicology and Environmental Safety 65 3., pp. 364–371. DOI: 10.1016/j.ecoenv.2005.07.026.

Regard, E. Taurog, A. Nakashima, T. 1978.: Plasma thyroxine and triiodothyronine levels in spontaneously metamorphosing Rana catesbeiana tadpoles and in adult anuran amphibia. In Endocrinology 102 3., pp. 674–684. DOI: 10.1210/endo-102-3-674.

Rensel, Michelle A. Ellis, Jesse M. S. Harvey, Brigit Schlinger, Barney A. 2015.: Sex, estradiol, and spatial memory in a food-caching corvid. In Hormones and Behavior 75, pp. 45–54. DOI: 10.1016/j.yhbeh.2015.07.022.

Richardson, Samantha J. 2002.: The evolution of transthyretin synthesis in vertebrate liver, in primitive eukaryotes and in bacteria. In Clinical chemistry and laboratory medicine 40 12., pp. 1191–1199. DOI: 10.1515/CCLM.2002.209.

Richardson, Samantha J. 2015.: Tweaking the Structure to Radically Change the Function: The Evolution of Transthyretin from 5-Hydroxyisourate Hydrolase to Triiodothyronine Distributor to Thyroxine Distributor. In Front. Endocrinol. 5, p. 245. DOI: 10.3389/fendo.2014.00245.

Richardson, Samantha J. Monk, Julie A. Shepherdley, Caroline A. Ebbesson, Lars O. E. Sin, Frank Power, Deborah M. et al. 2005.: Developmentally regulated thyroid hormone distributor proteins in marsupials, a reptile, and fish. In American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 288 5., R1264-R1272. DOI: 10.1152/ajpregu.00793.2004.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Rodríguez, L. Begtashi, I. Zanuy, S. Carrillo, M. 2000.: Development and validation of an enzyme immunoassay for testosterone: Effects of photoperiod on plasma testosterone levels and gonadal development in male sea bass Dicentrarchus labrax, L. at puberty. In Fish Physiology and Biochemistry 23 2., pp. 141–150. DOI: 10.1023/A:1007871604795.

Ros, Oihana Izaguirre, Jon Kepa Olivares, Maitane Bizarro, Cristina Ortiz-Zarragoitia, Maren Cajaraville, Miren Pilar et al. 2015.: Determination of endocrine disrupting compounds and their metabolites in fish bile. In Science of The Total Environment 536, pp. 261–267. DOI: 10.1016/j.scitotenv.2015.07.074.

Rosenblum, P. M. Yamada, L. Callard, I. P. Callard, G. V. 1985.: Validation of radioimmunoassay systems for the measurement of 11-keto- and 11 beta-hydroxytestosterone in teleost blood. In Comparative biochemistry and physiology. B, Comparative biochemistry 82 4., pp. 659–665.

Ruuskanen, Suvi Hsu, Bin-Yan Heinonen, Arttu Vainio, Minna Darras, Veerle M. Sarraude, Tom Rokka, Anne 2018.: A new method for measuring thyroid hormones using nano-LC-MS/MS. In Journal of Chromatography B 1093-1094, pp. 24–30. DOI: 10.1016/j.jchromb.2018.06.052.

Rzasa, Janusz Sechman, Andrzej Paczoska-Eliasiewicz, Helena Hrabia, Anna 2009.: EFFECT OF TAMOXIFEN ON SEX STEROID CONCENTRATIONS IN CHICKEN OVARIAN FOLLICLES. In Acta veterinaria Hungarica 57 1., pp. 85–97. DOI: 10.1556/AVet.57.2009.1.9.

Sangalang, G. B. Freeman, H. C. 1977.: A new sensitive and precise method for determining testosterone and 11-ketotestosterone in fish plasma by radioimmunoassay. In General and Comparative Endocrinology 32 4., pp. 432–439.

Scheun, Juan Greeff, Dominique Medger, Katarina Ganswindt, Andre 2019.: Validating the use of dermal secretion as a matrix for monitoring glucocorticoid concentrations in African amphibian species. In Conservation Physiology 7 1., coz022. DOI: 10.1093/conphys/coz022.

Schmidt, Robert E. Reavill, Drury R. 2008.: The Avian Thyroid Gland. In Veterinary Clinics of North America - Exotic Animal Practice 11 1., pp. 15–23. DOI: 10.1016/j.cvex.2007.09.008.

Schreck, C. B. Flickinger, S. A. Hopwood, M. L. 1972.: Plasma androgen levels in intact and castrate rainbow trout, Salmo gairdneri. In Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine New York, N.Y. 140 3., pp. 1009–1011.

Schreiber, G. 2002.: The evolutionary and integrative roles of transthyretin in thyroid hormone homeostasis. In Journal of Endocrinology 175 1., pp. 61–73. DOI: 10.1677/joe.0.1750061.

Schulz, R. 1984.: Serum levels of 11-oxotestosterone in male and 17β-estradiol in female rainbow trout Salmo gairdneri. during the first reproductive cycle. In General and Comparative Endocrinology 56 1., pp. 111–120. DOI: 10.1016/0016-648084.90067-4.

Schulz, R. 1985.: Measurement of five androgens in the blood of immature and maturing male rainbow trout, Salmo gairdneri Richardson. In Steroids 46 2-3., pp. 717–726.

Schulz, R. W. van der Corput, L. Janssen-Dommerholt, J. Goos, H.J.Th. 1994.: Sexual steroids during puberty in male African catfish Clarias gariepinus.: serum levels and gonadotropin-stimulated testicular secretion in vitro. In Journal of Comparative Physiology B 164 3., pp. 195–205. DOI: 10.1007/BF00354080.

Sebire, Marion Katsiadaki, Ioanna Scott, Alexander P. 2007.: Non-invasive measurement of 11-ketotestosterone, cortisol and androstenedione in male three-spined stickleback Gasterosteus aculeatus. In General and Comparative Endocrinology 152 1., pp. 30–38. DOI: 10.1016/j.ygcen.2007.02.009.

Senior, B. E. 1974.: Oestradiol concentration in the peripheral plasma of the domestic hen from 7 weeks of age until the time of sexual maturity. In Journal of Reproduction and Fertility 41 1., pp. 107–112. DOI: 10.1530/jrf.0.0410107.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Shah, Amit H. Chin, Eunice H. Schmidt, Kim L. Soma, Kiran K. 2011.: DHEA and estradiol levels in brain, gonads, adrenal glands, and plasma of developing male and female European starlings. In JOURNAL OF COMPARATIVE PHYSIOLOGY A-NEUROETHOLOGY SENSORY NEURAL AND BEHAVIORAL PHYSIOLOGY 197 10., pp. 949–958. DOI: 10.1007/s00359-011-0655-4.

Shibuya, Kazumoto Wada, Masaru Mizutani, Makoto Sato, Kazuo Itabashi, Masafumi Sakamoto, Tamami 2005.: Vitellogenin detection and chick pathology are useful endpoints to evaluate endocrine-disrupting effects in avian one-generation reproduction study. In Environmental Toxicology and Chemistry 24 7., pp. 1654–1666. DOI: 10.1897/04-420R.1.

Simon, R. Tietge, J. E. Michalke, B. Degitz, S. Schramm, K. W. 2002.: Iodine species and the endocrine system: thyroid hormone levels in adult Danio rerio and developing Xenopus laevis. In Analytical and bioanalytical chemistry 372 3., pp. 481–485. DOI: 10.1007/s00216-001-1211-9.

Singh, Ram P. Sastry, Kochiganti V.H. Dubey, Pawan K. Agrawal, Radha Singh, Renu Pandey, Nitin Kumar Mohan, Jag 2013.: Norfloxacin drug induces reproductive toxicity and alters androgen receptor gene expression in testes and cloacal gland of male Japanese quail Coturnix Japonica. In Environmental Toxicology and Chemistry 32 9., pp. 2134–2138. DOI: 10.1002/etc.2291.

Sun, Jianxian Wang, Chen Peng, Hui Zheng, Guomao Zhang, Shiyi Hu, Jianying 2016.: p,p'-DDE Induces Gonadal Intersex in Japanese Medaka Oryzias latipes. at Environmentally Relevant Concentrations: Comparison with o,p'-DDT. In Environmental science & technology 50 1., pp. 462–469. DOI: 10.1021/acs.est.5b05042.

Suzuki, S. Suzuki, M. 1981.: Changes in thyroidal and plasma lodine compounds during and after metamorphosis of the bullfrog, Rana catesbeiana. In General and Comparative Endocrinology 45 1., pp. 74–81. DOI: 10.1016/0016-648081.90171-4.

Swartz, William J. 1984.: Effects of 1,1-bis p-chlorophenyl.-2,2,2-trichloroethane DDT. on gonadal development in the chick embryo: A histological and histochemical study. In Environmental Research 35 2., pp. 333–345. DOI: 10.1016/0013-935184.90141-5.

Tagawa, M. Hirano, T. 1987.: Presence of thyroxine in eggs and changes in its content during early development of chum salmon, Oncorhynchus keta. In General and Comparative Endocrinology 68 1., pp. 129–135.

Taves, Matthew D. Schmidt, Kim L. Ruhr, Ilan M. Kapusta, Katarzyna Prior, Nora H. Soma, Kiran K. 2010.: Steroid Concentrations in Plasma, Whole Blood and Brain: Effects of Saline Perfusion to Remove Blood Contamination from Brain. In PLoS ONE 5 12. DOI: 10.1371/journal.pone.0015727.

Temple, Diana Springer, Timothy Gallagher, Sean Wheeler, James R. 2020.: The value of avian gross pathology in identifying endocrine disrupting properties. In Regulatory Toxicology and Pharmacology 112 April 2020., p. 104577. DOI: 10.1016/j.yrtph.2020.104577.

Tokumoto, Junko Danjo, Megumi Kobayashi, Yoshihiro Kinoshita, Kyoko Omotehara, Takuya Tatsumi, Atsutoshi et al. 2013.: Effects of exposure to clothianidin on the reproductive system of male quails. In Journal of Veterinary Medical Science 75 6., pp. 755–760. DOI: 10.1292/jvms.12-0544.

U.S. EPA 2015.: Endocrine Disruptor Screening Program Test Guidelines 890.2100: Avian Two-Generation Toxicity Test in the Japanese Quail [EPA No. 740-C-15-003].

Union, The European Parliament and the Council of the European 2008.: Directive 2008/105/EC of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/ECC, 86/280/ECC and amending Directive 2000/60/EC. In Official Journal of the European Union L348, pp. 84–97.

Vallejo, Asier Usobiaga, Aresatz Ortiz-Zarragoitia, Maren Cajaraville, Miren P. Fernandez, Luis A. Zuloaga, Olatz 2010.: Focused ultrasound-assisted acceleration of enzymatic hydrolysis of alkylphenols and 17 beta-oestradiol glucuronide in fish bile. In Analytical and bioanalytical chemistry 398 5., pp. 2307–2314. DOI: 10.1007/s00216-010-4156-z.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

van Dam, G. H. Schoonen, W.G.E.J. Lambert, J.G.D. van Oordt, P.G.W.J. 1989.: Plasma profiles of fourteen ovarian steroids before, during and after ovulation in African catfish, Clarias gariepinus, determined by gas chromatography and mass spectrometry. In Fish Physiology and Biochemistry 6 2., pp. 79–89. DOI: 10.1007/BF01875481.

Venkatesh, B. Tan, C. H. Lam, T. J. 1989.: Blood steroid levels in the goldfish: Measurement of six ovarian steroids in small volumes of serum by reverse-phase high-performance liquid chromatography and radioimmunoassay. In General and Comparative Endocrinology 76 3., pp. 398–407. DOI: 10.1016/0016-648089.90135-4.

Wang, Hui Zhou, Xiuqing Zhang, Yiqun Chen, Haiyan Li, Guijie Xu, Yang et al. 2012.: Dynamic microwave-assisted extraction coupled with salting-out liquid-liquid extraction for determination of steroid hormones in fish tissues. In Journal of agricultural and food chemistry 60 41., pp. 10343–10351. DOI: 10.1021/jf303124c.

Wang, Huiyong Bussy, Ugo Chung-Davidson, Yu-Wen Li, Weiming 2016.: Ultra-performance liquid chromatography tandem mass spectrometry for simultaneous determination of natural steroid hormones in sea lamprey Petromyzon marinus. plasma and tissues. In Journal of Chromatography B 1009-1010, pp. 170–178. DOI: 10.1016/j.jchromb.2015.12.030.

Wang, Quan-lin Zhang, Ai-zhi Pan, Xu Chen, Li-ren 2010.: Simultaneous determination of sex hormones in egg products by ZnCl2 depositing lipid, solid-phase extraction and ultra performance liquid chromatography/electrospray ionization tandem mass spectrometry. In ANALYTICA CHIMICA ACTA 678 1., pp. 108–116. DOI: 10.1016/j.aca.2010.08.014.

Wang, Shu Li, Yun Wu, Xiaoli Ding, Meijuan Yuan, Lihua Wang, Ruoyu et al. 2011.: Construction of uniformly sized pseudo template imprinted polymers coupled with HPLC-UV for the selective extraction and determination of trace estrogens in chicken tissue samples. In JOURNAL OF HAZARDOUS MATERIALS 186 2-3., pp. 1513–1519. DOI: 10.1016/j.jhazmat.2010.12.026.

Wang, Zongyi Wang, Yulu Lv, Fangfang Liu, Xiaoyu Jia, Changxi Chen, Xiangning Han, Tao 2015.: An Effective Method for Monitoring Three Iodothyronines in Meat Tissue and Eggs by Liquid Chromatography-Tandem Mass Spectrometry. In Food Analytical Methods 8 8., pp. 2069–2074. DOI: 10.1007/s12161-015-0091-0.

Washburn, B. E. Millspaugh, J. J. Morris, D. L. Schulz, J. H. Faaborg, J. 2007.: Using a commercially available enzyme immunoassay to quantify testosterone in avian plasma. In Condor 109 1., pp. 181–186. DOI: 10.1650/0010-54222007.109[181:UACAEI]2.0.CO2.

Watanabe, Yugo Grommen, Sylvia V. H. Groef, Bert de 2016.: Corticotropin-releasing hormone: Mediator of vertebrate life stage transitions? In General and Comparative Endocrinology 228, pp. 60–68. DOI: 10.1016/j.ygcen.2016.02.012.

Weber, G. M. Farrar, E. S. Tom, C.K.F. Grau, E. G. 1994.: Changes in Whole-Body Thyroxine and Triiodothyronine Concentrations and Total Content during Early Development and Metamorphosis of the Toad Bufo marinus. In General and Comparative Endocrinology 94 1., pp. 62–71. DOI: 10.1006/gcen.1994.1060.

Weber, G. M. Okimoto, D. K. Richman, N. H. 3rd Grau, E. G. 1992.: Patterns of thyroxine and triiodothyronine in serum and follicle-bound oocytes of the tilapia, Oreochromis mossambicus, during oogenesis. In General and Comparative Endocrinology 85 3., pp. 392–404.

Wei Tian Lanteng Wang Hongtao Lei Yuanming Sun Zhili Xiao 2018.: Antibody production and application for immunoassay development of environmental hormones: a review. In Chem. Biol. Technol. Agric. 5 1., pp. 1–12. DOI: 10.1186/s40538-018-0117-0.

Whaley, Paul Aiassa, Elisa Beausoleil, Claire Beronius, Anna Bilotta, Gary Boobis, Alan et al.2020.: A code of practice for the Conduct Of Systematic reviews in Toxicology and Environmental health Research COSTER. submitted.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Widell, B. Noaksson, E. Balk, L. Zebühr, Y. 2011.: Simultaneous determination of several natural steroids in blood plasma from perch Perca fluviatilis. by GC-HRMS. In International Journal of Environmental Analytical Chemistry 91 4., pp. 303–318. DOI: 10.1080/03067319.2010.484884.

Wilson, C. M. McNabb, F. M. 1997.: Maternal thyroid hormones in Japanese quail eggs and their influence on embryonic development. In General and Comparative Endocrinology 107 2., pp. 153–165. DOI: 10.1006/gcen.1997.6906.

Wingfield, John C. Matt, Kathleen S. Farner, Donald S. 1984.: Physiologic properties of steroid hormone-binding proteins in avian blood. In General and Comparative Endocrinology 53 2., pp. 281–292. DOI: 10.1016/0016-648084.90254-5.

Wiseman, Steve Thomas, Jith K. Higley, Eric Hursky, Olesya Pietrock, Michael Raine, Jason C. et al. 2011.: Chronic exposure to dietary selenomethionine increases gonadal steroidogenesis in female rainbow trout. In Aquatic Toxicology 105 3-4., pp. 218–226. DOI: 10.1016/j.aquatox.2011.06.012.

Woods, J. E. Brazzill, D. M. 1981.: Plasma 17β-estradiol levels in the chick embryo. In General and Comparative Endocrinology 44 1., pp. 37–43. DOI: 10.1016/0016-648081.90353-1.

Yamada, H. Satoh, R. Yamashita, T. Kambegawa, A. Iwata, M. 1997.: Development of a time-resolved fluoroimmunoassay TR-FIA. for testosterone: measurement of serum testosterone concentrations after testosterone treatment in the rainbow trout Oncorhynchus mykiss. In General and Comparative Endocrinology 106 2., pp. 181–188.

Yamashita, Ryo Oshima, Atsushi Hasegawa-Baba, Yasuko Wada, Masaru Shibuya, Kazumoto 2011.: Endocrine disrupting effects of low dose 17 β-estradiol E 2. on the Japanese quail Coturnix japonica. were detected by modified one-generation reproduction study. In Journal of Toxicological Sciences 36 1., pp. 43–54. DOI: 10.2131/jts.36.43.

Yang, Lihua Lan, Chongyu Liu, Hongtao Dong, Jun Luan, Tiangang 2006a.: Full automation of solid-phase microextraction/on-fiber derivatization for simultaneous determination of endocrine-disrupting chemicals and steroid hormones by gas chromatography-mass spectrometry. In Analytical and bioanalytical chemistry 386 2., pp. 391–397. DOI: 10.1007/s00216-006-0631-y.

Yang, Lihua Luan, Tiangang Lan, Chongyu 2006b.: Solid-phase microextraction with on-fiber silylation for simultaneous determinations of endocrine disrupting chemicals and steroid hormones by gas chromatography–mass spectrometry. In Journal of Chromatography A 1104 1., pp. 23–32. DOI: 10.1016/j.chroma.2005.11.108.

Yang, M. Ma, Y. Gui, W. Ren, Y. Zhu, G. Liu, Y. 2015.: Determination of 26 endocrine disrupting chemicals in fish and water using modified QuEChERS combined with solid-phase extraction and UHPLC-MS/MS. In Analytical Methods 7 19., pp. 8418–8431. DOI: 10.1039/c5ay01082a.

Yang, Shuhua Zhang, Ziwei He, Jianbin Li, Jinlong Zhang, Jiuli Xing, Houjuan Xu, Shiwen 2012.: Ovarian toxicity induced by dietary cadmium in hen. In Biological Trace Element Research 148 1., pp. 53–60. DOI: 10.1007/s12011-012-9343-7.

Zena, Lucas A. Dillon, Danielle Hunt, Kathleen E. Navas, Carlos A. Bicego, Kenia C. Buck, C. Loren 2019.: Seasonal changes in plasma concentrations of the thyroid, glucocorticoid and reproductive hormones in the tegu lizard Salvator merianae. In General and Comparative Endocrinology 273 SI., pp. 134–143. DOI: 10.1016/j.ygcen.2018.06.006.

Zhang, Fagen Bartels, Michael J. Geter, David R. Carr, Melissa S. McClymount, Lynn E. Marino, Troy A. Klecka, Gary M. 2009.: Simultaneous quantitation of testosterone, estradiol, ethinyl estradiol, and 11-ketotestosterone in fathead minnow fish plasma by liquid chromatography/positive atmospheric pressure photoionization tandem mass spectrometry. In Rapid communications in mass spectrometry : RCM 23 23., pp. 3637–3646. DOI: 10.1002/rcm.4298.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Zhang, Hui Shi, Jianghong Liu, Xiaowei Zhan, Xinmin Dang, Jinhua Bo, Ting 2014.: Occurrence of free estrogens, conjugated estrogens, and bisphenol A in fresh livestock excreta and their removal by composting in North China. In Environmental science and pollution research international 21 16., pp. 9939–9947. DOI: 10.1007/s11356-014-3002-9.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Appendix A – Protocol for a systematic evidence map of methods to measure hormones related to estrogen, androgen and thyroid pathways in fish, birds and amphibians

A.1. Registration:

Final protocol uploaded to Zenodo and attributed the following digital object identifier,

doi: 10.5281/zenodo.3335993

A.2. Authors:

Dr Olwenn V Martin*, Department of Life Sciences, Brunel University London, olwenn.martin@brunel.ac.uk

Dr Alice Baynes, Institute for the Environment, Health and Societies, Brunel University London, Alice.baynes@brunel.ac.uk

Dr Sibylle Ermler, Institute for the Environment, Health and Societies, Brunel University London, Sibylle.ermler@brunel.ac.uk

Joanne McPhie, Information Services, Brunel University London, Joanne.Mcphie@brunel.ac.uk

Ines Carvalho De Azevedo Moreira, Department of Life Sciences, Brunel University London, ines.moreira@brunel.ac.uk

Georgina Collins, Department of Life Sciences, Brunel University London, Georgina.Collins@brunel.ac.uk

Stefania Oliverio, Department of Life Sciences, Brunel University London, Stefania.Oliverio@brunel.ac.uk

Martin Scholze, Institute for the Environment, Health and Societies, Brunel University London, martin.scholze@brunel.ac.uk

Maria Arena, European Food Safety Authority, Parma, Maria.Arena@efsa.europe.eu

Stefania Barmaz, European Food Safety Authority, Parma, Stefania.BARMAZ@efsa.europa.eu

Laura Villamar-Bouza, European Food Safety Authority, Parma, Laura.VILLAMARBOUZA@efsa.europa.eu

A.3. Authors' contributions

Brunel University submitted a tender that met the technical specifications of the invitation to tender developed by European Food Safety Authority (EFSA). The successful proposal was drafted collaboratively by AB, SE and OVM with the input of JMcP and MS. AB contributed her specialist expertise in ecotoxicology and endocrine disruption in wildlife. SE contributed her specialist expertise in endocrine disruption and steroidogenesis *in vitro*. JMcP contributed her expert knowledge on information systems and literature databases. OVM contributed her knowledge of endocrine disruption and good practice of systematic review methods. MS contributed his expertise in the mathematical and statistical assessment of validated test guidelines. IM, GC and SO carried out pilot literature searches and assisted with piloting the data extraction template.

A.4. Funding and sources of support

The time spent on this project by Olwenn V Martin, Alice Baynes, Martin Scholze, Sibylle Ermler, Joanne McPhie and research assistants Ines Moreira, Georgina Collins and Stefania Oliverio is funded by EFSA. Additionally, Stefania Barmaz, Simona Affani, Maria Arena, Laura Villamar-Bouza and Cristina Martin, all employed by EFSA, contributed their time and expertise in kind.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

A.5. Role of the funder:

The protocol for this systematic evidence map was developed in response to an invitation to tender (Procedure reference number: NP/EFSA/PRAS/2018/02) and EFSA reviewed all documents at all stages.

A.6. Competing interests

Before the contract was awarded, all team members employed by Brunel University London duly completed declarations of interests. The authors have no competing interests to declare.

A.7. Introduction

A.7.1. Regulatory rationale

Within the EU, the approval of pesticides is regulated by Regulation (EC) No 1107/2009. An amendment to this Regulation (Regulation (EU) No 2018/605), which establishes the scientific criteria for the identification of pesticide active substances with endocrine disrupting (ED) properties, was published in April 2018. In June 2018, European Food Safety Authority, the European Chemicals Agency (ECHA) and the Joint Research Centre (JRC) published a guidance document on the implementation of the scientific criteria, this publication primarily addresses the Estrogen, Androgen, Steroidogenesis and Thyroid, known as 'EATS', modalities in vertebrates. It specifically included recommendations on how to perform hormonal measurements in mammalians. However, when it comes to non-mammalian vertebrate test guidelines (TG), there are a number of 'gaps' which need to be filled to fully address assessment of 'EATS' modalities and mirror mammalian TGs. For example, in the OECD test models which do address endocrine specific endpoints (i.e. amphibians and fish) there are no specific recommendations on which hormones to measure, how or when to measure hormones, or how to interpret the results. To harmonise vertebrate OECD TGs, these additional endpoints need to be investigated and therefore some guidance on how to perform, report and evaluate hormonal measurements in fish, birds and amphibians needs to be prepared.

A.8. Aim and objectives

The overall aim of this systematic evidence map is to collect and analyse data in support of the ED assessment (Estrogen, Androgen, Steroidogenesis and Thyroid) for non-target vertebrates in order to develop recommendations on how to perform, report and interpret hormonal measurements in fish, amphibians and birds in toxicity studies.

To support this aim, the specific objective of this evidence map is to collate literature that addresses the development, optimisation and/or validation of hormone measurement methods in fish, amphibian and bird species.

A.9. Methods

This protocol has been drafted with specific regards to the PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) 2015 checklist ((Moher et al. 2015) and is giving due consideration to the Code of Practice for the Conduct of Systematic Reviews in Toxicology and Environmental Health Research (COSTER) (Whaley et al. 2020). A preliminary draft was published on Zenodo on 10th May 2019 (doi: 10.5281/zenodo.2711704) and comments were invited between 13th to 20th May. The protocol was revised in response to these comments and the google form, responses collated and responses to individual comments can be found in Appendix C –.

A.10. Eligibility criteria

Early decisions made during protocol development have significant impact on the scope and form of the systematic map. Protocol development is underpinned by imparting a common understanding of the context and motivation for the review. Well-formulated statements have a critical impact on other

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

components of the review – including the literature search strategy, data extraction, synthesis and presentation of findings.

Question formulation follows a similar procedure as that for systematic reviews, i.e. using PI/ECO (population, intervention/exposure, comparator, outcome), PIT (population, index test, target condition) or PO (population, outcome) statements. For this project, research questions are related to the reliability and relevance of test methods for detection or diagnosis, in which case the population (P), index test (I) and target condition (T) must be specified. The initial PIT statement for is illustrated in Table 7: .

Table 7: PIT statement

	How do available methods to measure hormones related to the estrogen, androgen, steroidogenesis and thyroid hormones pathways in non-mammalian vertebrate species (birds, fish and amphibians) perform?
Population	Species of fish, amphibians and birds
Index test	Invasive or non-invasive hormonal measurement methods (related to EATS pathways)
Target condition	Detection of or supporting evidence for an endocrine disruption mode-of-action or adverse effect.

This PIT statement is operationalised as inclusion and exclusion criteria as described in Table 8: overleaf.

Table 8: Eligibility criteria

		Inclusion criteria	**Exclusion criteria**
Population	Species of fish, amphibians and birds	Both wild and standard test species of fish, birds or amphibians, *in vivo* and *in vitro.*	All other species that are not classified as fish, birds or amphibians.
Index test	Invasive or non-invasive hormonal measurement methods (related to EATS pathways)	All sampling matrices including faeces or tankwater. Hormones related to the EATS pathways in fish, birds and amphibians. Estrogens, androgens and thyroid hormones are the main target of these searches but retrieved studies investigating CRH, cholesterol, FSH and/or LH will not be excluded.	Hormones not related to EATS pathways in fish, birds and amphibians, e.g. adrenaline and noradrenaline, stanniocalcin. Hormone dependent biomarkers such as vitellogenin.
Target condition	Detection of or supporting evidence for an endocrine disruption mode-of-action or adverse effect.	Studies addressing methodological aspects of hormone measurements, e.g. method development, sample preparation and storage, method validation.	Studies where pre-existing method(s) in the taxonomic group under consideration has been applied to measure hormones[(a)]

(a): As part of data analysis, we will investigate how selected methods of particular interest have been applied using citation searches.

A.11. Information sources

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Searches for peer-reviewed articles will be conducted in the following bibliographic databases:

- PubMed
- Web of Science - All databases (selecting BioSIS Citation Index and SciELO in addition to the Core collection in the drop-down menu)
- MEDLINE
- Scopus
- Full text databases such as ScienceDirect

Further, in order to identify ongoing research, we will examine conference papers via resources like the British Library service **Zetoc** (http://zetoc.jisc.ac.uk/).

To identify grey literature not listed in databases, manual searches will be carried out using topic focused search engines such as **Mednar** (http://mednar.com/mednar/desktop/en/search.html) and **Environar** (https://environar.com/environar/desktop/en/search.html). Additional searches will be carried out in open access bibliographical databases such as **OpenGrey** (http://www.opengrey.eu/) which searches grey literature across Europe by interrogating open access items in institutional repositories.

Manual searches of the bibliography and citations of eligible studies will be carried out.

Finally, this can be complemented by targeted manual searches of open repositories on the website of European institutions such as EChA, EFSA, JRC, American institutions such as National Institute of Environmental Health Sciences (NIEHS), National Institute of Health (NIH) or the Food and Drug Administration (FDA) and, European national institutions including Bundesinstitut für Risikobewertung (BfR), Swedish Chemical Agency (KEMI), the Dutch National Institute for Public Health and the Environment (RIVM), Umwelt BundesAmt (UBA), the French National Institute for Industrial Environment and Risks (Ineris), the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) as well as those of interest groups such as the industry funded European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) or European Chemical Industry Council (CEFIC).

Language capabilities within the project team include German, Portuguese, Italian and French in addition to English. For studies in languages not covered by the project team and for which no English version can be located, the full text will be interrogated through the use of online tools such as Google Translate. It is not proposed to limit the timespan of literature searches.

A.12. Search strategy

Constructing search strategies for complex review questions can be challenging and has led to the use of multi-stranded strategies instead of a single combination of key elements, e.g. such as PI or IT. An initial series of four groups of search terms targeting either species, sampling matrix, hormones or method elements has been developed to be run sequentially and then combined using the Boolean operators 'AND' and 'OR'[1]. The result of pilot searches listing a total of 93 search terms and their combination in the three literature databases Web of Science, Scopus and Pubmed can be found in Appendix C –. The search strategy was piloted in PubMed. However, following peer review, it was decided to carry out the search in Medline. The search strategy will be revised after review by EFSA and public invitation for comments. The quality of the revised literature search strategy will be assessed by testing for its ability to retrieve pre-identified key papers and by using the EFSA critical appraisal tool (CAT) for systematic/extensive literature searches[2]. The CAT focuses on both the search strategy; and the completeness of the information sources used.

A.13. Data management

[1] EFSA (2017). EFSA supporting publications 2017:EN-1207. 48 pp. doi:10.2903/sp.efsa.2017.EN-1207

[2] EFSA (2015) EFSA supporting publication 2015:EN-836. 65 pp. Appendix D.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Literature and all systematic review processes will be managed and coordinated with the support of the freely available online tool CADIMA established in a close collaboration between the Julius Kühn-Institut and the Collaboration for Environmental Evidence (https://www.cadima.info/index.php/area/evidenceSynthesisDatabase).

A.14. Relevance screening

The list of eligibility criteria will be applied to the merged reference list in duplicate, i.e. by two people working independently, and in two stages. In the first stage, only titles and abstracts will be checked for relevance to the study question. Clearly irrelevant studies will be excluded. The full text of the resulting list of included references after title/abstract screening will then be examined for inclusion. The reason for exclusion of studies after assessment of the full text will be recorded.

Multiple reports of the same research (e.g. multiple publications, conference abstracts etc.) will not be excluded but instead the methodological information from each of the reports shall be collated as part of the data extraction process as one unit of evidence.

The CADIMA online tool facilitates the process of consistency check by identifying disagreement between the two evaluators. Disputes will be arbitrated and resolved by a third party, the project manager and most senior ecotoxicology expert. For quality control purposes, the percentage agreement between the two independent evaluators and kappa statistic will be reported.

The inter-rater reliability (level of agreement between two or more assessors) of clearly formulated inclusion and exclusion criteria will be also piloted and eligibility criteria shall be clarified and amended accordingly, if necessary.

A.15. Data extraction

As this project is concerned with the validation of methods for hormonal measurements, data related to the critical appraisal of methods is central to this exercise and forms part of the data extraction and coding itself. There is therefore an overlap between data extraction and risk-of-bias. Furthermore, the latter step is not automatically required in systematic evidence mapping. In this specific instance, it was found that this would duplicate data extraction and risk-of-bias was therefore not included. An initial data extraction template has been created comprising of elements relevant for different types of studies, e.g.:

- Meta-data (authors, date, journal name or report number)
- Information about the test system, specifically:
    - Species name(s)
    - Taxonomic group (fish, bird or amphibian)
    - Life stage and gender of the organisms
    - Name of the specific bioassay, if relevant
    - Information about the study design, including:
    - Whether it investigated a specific intervention or exposure, and if so details of any chemical exposure(s)
- Hormones measured and associated endocrine modality(ies)
- Details of the sampling for hormone measurements, particularly;
    - Whether the sampling method was invasive
    - The matrix sampled
    - The amount (e.g. volume or weight) of sample required for analysis
    - Whether samples needed to be pooled across several individual
- Details of the hormone measurement method including;
    - The type of method and method name
    - Whether a standard operating procedure exists (SOP)
    - The use of internal standards
    - Sensitivity (limit of detection and/or limit of quantification)
    - Any reported information relevant to specificity

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

At this stage, the aim of this data extraction template is to capture essential information that can be used to categorise different methods for further data analysis rather than attempt to capture every relevant detail reported in each article. This is achieved by the use of drop-down menus restricting possible answers to lists of categories whenever possible.

This template was piloted by extracting data from four representative articles by four evaluators in parallel duplicates. As a result of this exercise, the data extraction template was amended and corrected. The following items were added;

- The aim of the hormonal measurements,
- Whether sample extraction step(s) was/were necessary and brief details about methods used,
- Brief information about the accuracy and precision of the method.

This amended version of the data extraction template will be further revised following review by EFSA colleagues and public invitation for comments. The revised version will again be piloted with a different set of relevant studies and a set of guidelines for evaluators as to what is considered a 'unit of evidence', or how information is collated when an article reports information about different species, methods, hormones etc. The amended data extraction temple can be found in Appendix C –.

A.16. Data analysis

A description of the volume and characteristics of the evidence base collated in the previous step, such as the availability of analytical methods for different hormones and taxonomic groups and if data permits indicators of performance (e.g. accuracy, precision, sensitivity, selectivity) of these different methods will be visually summarised and presented using the capabilities of the data visualisation software Tableau.

An in-depth qualitative analysis will also be carried out by combining results by taxonomic group, method type and endocrine modality or hormone measured. This may require additional searches to identify the papers that have cited an article about a given method to gauge how widely and reliably it has been applied in the context of chemical testing.

This analysis will guide the design of a questionnaire addressed to laboratories that routinely carry out regulatory ecotoxicity studies. This questionnaire will seek opinions on technical and scientific aspects as well practical feasibility aspects that are rarely addressed in the scientific literature such as availability of equipment, technical expertise required and costs involved.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Appendix B – Analysis of data extracted for the systematic evidence map of methods to measure hormones related to estrogen, androgen and thyroid pathways in fish, birds and amphibians

B.1. Radioimmunoassays (RIA) for measuring hormones

Harnessing the high specificity of antibodies to detect and attach to a target molecule, radioimmunoassays (RIA) have been widely used in endocrine disruption assessment. By stimulating the immune system of an individual from a different species (Wei Tian et al. 2018), antibodies are raised against target molecules of interest. For example, researchers investigating a target molecule concentration in a frog would inject the molecule into a rabbit. The rabbit's immune system would identify the frog molecule as extraneous, an antigen in immune terminology, and produce an antibody that tags it for remediation by the rabbit's immune system. This antibody will continue to be produced by the rabbit's immune system in case of further 'infection' and can be harvested by the research team for their RIA. Hypothalamus and pituitary hormones (e.g. Thyrotropin-releasing hormone (TRH), Gonadotropin-releasing hormone (GnRH), or Luteinising hormone (LH), Follicle-stimulating hormone (FSH), Thyroid-stimulating hormone (TSH)) are both large molecular weight and species specific molecules, therefore they would induce an immune reaction in the way described above. However, in the case of steroid hormones such as testosterone (T) and oestradiol (E2), and thyroid hormones such as thyroxine (T4), the chemical structure is exactly the same across vertebrate species and therefore the target molecule wouldn't be 'seen' by the host species as 'foreign' and mount a reaction. In these instances, the hormone has first to be bound to macromolecular carrier, such as keyhole limpet haemocyanin, to both increase its molecular mass and immunogenicity. Therefore, a thyroxine-conjugate would need to be injected into a rabbit to make antibodies to measure T4 in frogs.

The other key component is a radio labelled version of the target molecule. Iodine isotope [^{125}I] have often been used in RIA, chosen for its emission of easily detectable low energy photons and its relatively long half-life (and hence shelf life) of ~60 days. However, tritium (^{3}H, a weak beta emitter with a half-life of 12.3 years) labelled antigens have frequently been used for steroid RIA.

The RIA is conducted by incubating the antibody, known as the primary antibody, with a sample from the target species, allowing the primary antibody to bind to the hormone (antigen) it was raised against (Grange et al. 2014). This sample can take the form of blood plasma or serum. This sampling matrix carries relatively high concentrations of hormones which, although harvested invasively, can be done so without killing larger animals. Other sampling matrices such as mucus, feathers or faeces can also be used, although they have much lower concentrations of hormones, they can be collected non-invasively. The majority of RIA protocols include an extraction step to clean the sample and concentrate the hormone, particularly important in low concentration matrix such as faeces. A known amount of radiolabelled hormone is then added. This competes with the endogenous hormone, also binding with the primary antibody. The more of the endogenous hormone present, the less of the radiolabelled version can bind with the antibody. A secondary antibody is added that binds to the primary antibody causing it to flocculate and allowing it to be separated by centrifugation. The radioactivity of the resulting pellet is measured. The higher the radioactivity of the pellet, the lower the concentration of the target hormone in the sample. The concentration is quantified by comparing the radioactivity value with that on a standard curve .

The first RIA was developed in 1959 and this assay was a key method for the next 2-3 decades. This method is still favoured in the clinical setting. Its critics points to the fact that the competition of endogenous and radiolabelled antigen with the antibody is not directly quantifiable, leading to a loss of resolution. Combined with the health and safety risk of using a radioactive reagent and the development of a safer assay (ELISA), the latter method has been favoured by many researchers in the environmental field since the late 1990s.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

B.1.1. Measuring Hormones in Amphibians

B.1.1.1. Hypothalamic–pituitary–gonadal axis hormones

The literature search resulted in zero papers describing original and detailed methods for the detection of estrogens or androgens in amphibian samples using RIA.

B.1.1.2. Hypothalamic–pituitary–thyroid axis hormones

One of the earliest papers retrieved for measuring thyroid hormones in amphibian plasma by RIA used a commercial T4 RIA kit developed for use with rat or human serum (Regard et al. 1978), reference 5934 in Annex B –). Whilst validating the RIA for amphibians they found adult *Bufo marinus* and *Rana pipiens* had far lower levels of T4 (in comparison to mammals), such that adaptions to the method were required for use with amphibians. They subsequently used the RIA method with plasma from *Rana catesbeiana* tadpoles at a range of metamorphic stages, demonstrating elevated 3,3',5-triiodothyronine (T3) and thyroxine (T4) plasma concentrations towards metamorphic climax (one of the first publications to show this in spontaneously metamorphosing frogs). The modifications employed by Regard et al. (1978) were as follows: the antibody supplied in the kit and the radiolabelled T4 were diluted further, incubation was lengthen from 1 hour at room temperature to 18-25 hours at 4°C, T4 standards were prepared by dilution of a standard solution of crystalline T4 with plasma from *B. marinus*, and samples were read for 2 minutes not 1 minute. Similarly Regard et al. (1978) modified a T3 RIA method (Larsen 1972) developed for human serum for use with amphibians by preparing T3 standards of crystalline T3 with plasma from *B. marinus* (instead of T3-free rat serum), they also slightly modified the method for the separation of free and bound ^{125}I-T3 by shacking freshly prepared dextran-coated charcoal solution for 10 min at 4°C instead of allowing to stand at 4 °C without shaking, for 30 min. For both T4 and T4 plasma, samples (25-50 µl and 10-20 µl, respectively) were assayed directly in the RIA (without extraction). Limit of detection (LOD) in these assays were reported as 0.05 µg/mL for T4 and 5 ng/mL for T3. T3 was shown to cross-react with T4 antisera, however the authors felt that T3 plasma concentrations were much lower than T4, so didn't take it into account when calculating values.

In addition to the above paper the literature search retrieved another three papers which gave sufficient details for measuring T3 andT4 in amphibian (*Rana perezi, B. Marinus, R. catesbeiana*) samples using RIA (Gancedo et al. 1995; Suzuki and Suzuki 1981; Weber et al. 1994, see references 16071, 6031, 9500 in Annex B –). One additional paper was retrieved, specifically stating that total T4 measured (Mondou and Kaltenbach 1979, reference 260 in Annex B –) in amphibian (*R. catesbeiana*) samples via RIA.

Sample matrices described were varied and included plasma, serum, pericardial fluid, whole embryos and thyroid glands. Sample extraction methods differed depending on matrix and publication.

Whole embryos (0.1-0.6 g tissue consisting of pooled individuals, *B. Marinus*) were frozen (-80°C), then homogenised and sonicated with an extraction solution of 6-n-propylthiouracil/sodium hydroxide (99:1), the supernatant decanted and further extracted with a mixture of chloroform and ammonium hydroxide, dried and reconstituted in chloroform and sodium hydroxide, centrifuged and the RIA performed on the supernatant. Embryos spiked with ^{125}I-T3 (radiolabelled tracer) which underwent the same extraction procedure showed recovery of 87.3% ± 0.6 (mean ± SEM). Tissues spiked with non-radio labelled standard showed recoveries of 104 ± 2% (T3) and 107 ± 2% (T4). The RIA method employed by these authors was modified from previous publications with fish (Brown and Eales 1977; Weber et al. 1992). The modified version had a limit of detection (LOD) of 0.02 ng/mL, with an inter-assay coefficient of variation (CV) of 9.0% and 10.7% and intra-assay CV of 8.1% and 8.7% for T3 and T4, respectively (Weber et al. 1994).

One study measured both T3 and T4 in thyroid tissue (*R. perezi*). Both free and bound T3 and T4 were extracted and measured separately. Free T3 and T4 were extracted in methanol by sonication and centrifugation. The pellet was further extracted to access the bound T3 and T4 by overnight proteolytic digestion followed by another methanol extraction (Gancedo et al. 1995). The same paper also describes the extraction and quantification of T3 and T4 in plasma samples. Plasma samples were extracted with

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

a chloroform-methanol mixture followed by purification using an anion exchange resin. Plasma samples were spiked with radio-labelled T3 and T4, with extraction recoveries of 60-85% and 50-70%, respectively. For both, the thyroid and plasma extract the RIA method was one previously described by Obregon et al. (1979) in rat samples. The method had a LOD of 0.78 pg/tube for T3 and 1.5 pg/tube for T4, intra-assay CV was 3.23-8.05% for T3 and 3.9-5.95% for T4, inter-assay CV were 4.16-11.01% for T3 and 5.91-10.88% for T4.

T3 and T4 were also measured in plasma (Suzuki and Suzuki 1981, *R. catesbeiana*) using previously published RIA methods used for human serum Larsen 1972; Larsen et al. 1973. *R. Catesbeiana* plasma samples were extracted with methanol, extraction efficiencies for T4 and T3 were given as more than 60% and 80%, cross-reactivity for T4 antiserum to T3 was 0.1% and T3 antiserum to T4 was 0.14%. The RIA method LOD was given as 15 ng/100mL and 20 ng/100mL for T3 and T4, respectively.

Another method (Mondou and Kaltenbach 1979, *R. catesbeiana*) with serum or pericardial fluid samples used no extraction step prior to the RIA. However, this commercial RIA kit included I-anilino-l-napthalene-sulfonic acid to inhibit T4 binding to proteins in the assay, thereby allowing measurement of total T4 (i.e. both free and unbound fractions) within the samples. However, spiked samples for recovery efficacy were not tested. This kit only required 10 µl of plasma per sample, so no pooling was required. Cross-reactivity of the T4 antiserum with T3 was 0.45%. RIA assay LOD was calculated as 0.2 µg/100ml with intra-assay and inter-assay CVs of 8% and <15% respectively. See Table 9: for details of the method performance for the above studies.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 9: Publications retrieved involving analysis of thyroid hormones using RIA in amphibians

Ref no.	Reference	Species	Sample matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
5934	Regard et al. 1978	Bufo marinus Rana pipiens Rana catesbeiana	plasma	T3	-	5ng/100ml	-	no appreciable cross-reactivity of T4 with the T3 antibody
			plasma	T4	-	0.05ug/100ml	-	T3 does interact
16071	Gancedo et al. 1995	Rana perezi	Plasma	T3	60-85%	0.78 pg/tube	intra-assay CV was 3.23-8.05% inter-assay CV were 4.16-11.01%	-
			Thyroid gland	T3	-			
			plasma	T4	50-70%	1.5 pg/tube	intra-assay CV was 3.9-5.95% inter-assay CV were 5.91-10.88%	-
			Thyroid gland	T4	-			
6031	Weber et al. 1994	Bufo Marinus	Whole embryos	T3	125I-T3 87.3% ± 0.6 (mean ± SEM) T3 104 ± 2%	< 0.02 ng/ml	inter-assay CV 9.0% intra-assay CV of 8.1%	-
				T4	T4 107 ± 2%	< 0.02 ng/ml	inter-assay CV 10.7% intra-assay CV of 8.7%	-
9500	Suzuki and Suzuki 1981	Rana catesbeiana	plasma	T3	80%	15 ng/100mL	-	T3 antiserum to T4 was 0.14%
				T4	60%	20 ng/100mL	-	T4 antiserum to T3 was 0.1%
260	Mondou and Kaltenbach 1979	Rana catesbeiana	pericardial fluid	Total T4	-	0.2 µg/100ml	intra-assay CV 8% inter-assay CV <15%	T4 antiserum with T3 was 0.45%

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

B.1.2. Measuring Hormones in Birds

B.1.2.1. Hypothalamic–pituitary–gonadal axis hormones

The literature search retrieved 19 publications which gave sufficient details for measuring estrogens (estrone; E1, and 17β-estradiol; E2) and/or androgens (testosterone: T, dihydrotestosterone: DHT) in avian samples with a RIA. Four of the aforementioned papers measured both estrogens and androgens in birds.

Sample matrices described were varied and included plasma, serum, droppings/faeces, gonads and brain. Sample extraction methods differed depending on matrix and publication.

Three publications were retrieved which reported RIA methods for DHT and T in the plasma: Ring dove (*Streptopelia risoria*, (Feder et al. 1977, reference 18047 in Annex B –), chicken (*Gallus domesticus*, Driot et al. 1978, reference 1744 Annex B –) and Japanese quail (*Coturnix japonica*, Delville et al. 1985, Reference 18651a Annex B –). These relatively old methods all used solvent extraction followed by Celite chromatography to separate hormones and reduce cross-reactivity issues e.g. between T and DHT. Driot et al. (1978) employed cyclohexane-ethyl acetate, with freezing to separate the aqueous phase (50 µl plasma), whereas Delville et al. (1985) and Feder et al. (1977) used diethyl-ether (plasma volume not specified and 200-500 µl plasma, respectively). One other publication focused primarily on measuring T in the plasma of embryos and chicks of Japanese quail (*Coturnix japonica*, Ottinger and Bakst 1981, reference 9660 Annex B –), these authors also used solvent extraction hexane-benzene, but due to the small sample volume didn't use chromatography to separate closely related hormones (T and DHT). Not all the studies reported extraction efficiency but those that did were above 60%. See Table 10: for details of the method performance for the above studies.

Eight records were retrieved which measured estrogens in bird (chicken or turkey) plasma or serum via RIA. All of the methods used some kind of solvent extraction prior to conducting the RIA. Two of the publications used ether to extract estrogens from avian plasma without further separation by chromatography (Woods and Brazzill 1981, Reference 5357, 25 µL plasma per replicate; Shodona et al. 1975, Reference 9648, 20 µL plasma). Three publications used ether (Peterson and Common 1972, reference 162 Annex B –, 0.5 mL plasma; Senior 1974, reference 8165, 0.5-1 mL plasma; Wineland and Wentworth 1975, reference 10913 Annex B –, 1-3 mL plasma) followed by chromatography (Sephadex LH-20 separation separates E1 from E2, but not 17β-E2 from 17α-E2) to extract and separate hormones, and one paper used benzene followed by chromatography (Sephadex LH-20) ((Opel and Arcos 1978), 0.5-1 mL plasma). Not all the studies reported extraction efficiency but those that did were above 60%. See Table 10: for details of the method performance for the above studies. However, the majority of these methods are at least 40 years old.

More recently diethyl ether extraction had also been recommended as the most suitable solvent for extraction of steroid hormones (both T and E2) from avian plasma, by the US EPA (U.S. EPA 2015, reference 13985). The EPA Test Guideline (OCSPP 890.2100) does not stipulate additional chromatograph steps to separate hormones, this is likely due to modern commercial kits high specificity (recommended E2 kit 0.2% cross-reactivity with T, LOD 2.5 pg/mL, T kit 0.1% cross-reactivity with E2, 3.9 pg/mL).

Another method widely used in chemical analysis is the use of solid phase extraction (SPE) to extract and clean-up plasma samples. The main focus of the paper by Newman et al. (2008) (Reference 663 Annex B –) was to improve extraction, recovery and clean-up of steroid from plasma samples. Newman et al. reported that using C18 SPE reduces lipids and interfering compounds in the RIA, and that eluting the steroids from C18 columns with 90% HPLC-grade methanol in deionized water yielded the highest recovery of radio-labelled steroids, whilst drying under nitrogen at 38 °C was better than under air. The addition of absolute ethanol in the resuspension, 5-10%, was also reported to improve recovery of radio-labelled steroids (Newman et al. 2008).

Another publication which used SPE as an extraction and clean-up method for steroid estrogens was by Charlier et al. (2010). In this publication steroid levels were measured in brain tissue of Zebra finch

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

(*Taeniopygia guttata*) using a commercially available double antibody ^{125}I-E2 radioimmunoassay with a LOD of 0.1875 pg E2 per tube. The E2 antibody from the kit had a low cross-reactivity with estrone (2.4%), estriol (0.64%), 17β-estradiol (0.21%), 17β-estradiol-3-glucuronide (2.56%), 17β-estradiol-17-glucuronide (<0.01%), estradiol-3-SO4 (0.17%), testosterone (<0.01%) and DHEA (<0.01%) (Charlier et al. 2010, reference 10034 in Annex B –).

One publication was retrieved which measured estrogens and androgens in chicken ovarian tissue (Rzasa et al. 2009, Reference 9987 Annex B –). In this paper chicken ovarian follicles were homogenised in liquid nitrogen and kept at –20 °C until steroid assay. The resulting powdered was added to assay buffer and added directly to the RIA. Radio-labelled spiked hormone recovery was reported as T - 97% and E2 - 95%, with LODs of 12 pg/mL (T) and 5.4 pg/mL (E2), and intra- and inter-assay coefficients of variation reported as 4.9%, 5.2% and 5.3% and 6.4%, respectively.

Six publications were retrieved which measured sex-steroids in bird droppings/faeces. Early methods assaying faecal material for testosterone and 17β-estradiol used limited sample preparation, such that droppings were directly mixed with assay buffer, left overnight before centrifuging and decanting the supernatant for dilution and analysis (Cockrem and Rounce 1994, reference 13074 Annex B –). However, extraction efficiency using this method resulted in incredibly low percentage recovery of radio-labelled standard (<0.5% of spiked hormone), see Table 10: for details. Later publications have used a range of solvent extraction methods employing methanol (Goymann et al. 2002; Goymann et al. 2006; Kellam et al. 2004), ethanol and dichloromethane (Buchanan et al. 2007), or ethanol alone (Staley et al. 2007). Following solvent extraction Goymann et al. (2002, 2006) also deconjugated their extracts (β-glucuronidase/arylsulfatase), whereas Kellam et al. (2004) used HPLC to separate hormones prior to RIA. Kellam et al. (2004) reported their extraction efficiency, 38.6%, which is considerably higher than for Cockrem & Ronce's method, but still low when compared to other sampling matrices such as plasma. See Table 10: for details of the method performance for the above studies.

B.1.2.2. Hypothalamic–pituitary–thyroid axis hormones

In total four publications were retrieved which measured T3 or T4 in avian species and gave sufficient details of the RIA methods. An early study by May (1978) describes the development of a T3 RIA assay for chickens. They discuss issues such as T3 binding to proteins and the fat that accumulates in serum. May (1978) found that the addition of 8-Anilino-l-Naphthalene Sulfonic Acid (ANS) could inhibit binding of T3 to binding proteins and recommended high speed centrifugation to minimise the fat issue (May 1978, reference 12117 in Annex B –).

A study by Greenacre et al. (2001) investigated the use of a T4 RIA developed for humans to measure thyroid hormones in a range of bird species (including macaws, parrots and cockatoos, (Greenacre et al. 2001, Reference 136003 in Annex B –). They report on their modifications which combined the use of proteolytic enzyme from a thermophilic bacterium, which allowed heat treatment of serum samples without coagulation and precipitation with ethanol to remove interfering thyroid-hormone-binding proteins. These modifications resulted in extraction recoveries of spiked serum of 75.2-91.1 % with total T4 LOD of 0.24 nmol in the RIA.

Wilson and McNabb (1997) investigated maternal T3 and T4 deposition in egg yolk of Japanese quail (*Coturnix japonica*, Wilson and McNabb 1997, Reference 6630 in Annex B –). Thyroid hormones were extracted from egg yolk (0.5 g) using a modification of a methanol/chloroform extraction procedure previously used for amphibian larvae by Denver (1993). Methanol containing propylthiouracil was used in the extraction process, the methanol supernatant was then extracted further with multiple applications of chloroform and ammonium hydroxide, with the resulting extract dried with nitrogen before being assayed in double antibody RIA by the method of McNabb and Hughes (1983). Thyroid extraction rates were reported as 61 ± 3% for ^{125}I-T3 spiked yolk and 63 ± 1 ^{125}I-T4 spiked yolk. T3 LOD was reported as 0.125 ng/mL and T4 1.25 ng/mL. See Table 11: for details of above studies measuring thyroid hormones.

The final publication is the EPA Test Guideline (OCSPP 890.2100) (U.S.EPA 2015), Reference 13985 in Annex B –). Here it states that thyroid hormones are lost during organic extraction and should be

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

evaluated in un-extracted plasma samples. It recommends the use of a commercial RIA kits with low cross-reactivity (1.4% T4 in T3 RIA, 3.3% T3 in TA RIA) and LOD T3 0.03 ng/mL, T4 1.6 ng/mL and that any new kit should be tested for sensitivity, parallelism, specificity and intra- and inter-assay variation (EPA 2015).

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 10: Publications retrieved involving analysis of androgens, estrogens and thyroid hormones in birds using RIA

Ref no.	Reference	Species	Sample matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
18047	Feder et al. 1977	Ring dove (*Streptopelia risoria*)	Plasma	T	89.89 ± 1.75%	5-10 pg/tube	11.20%	No significant cross reaction with steroids DHT is observed
			Plasma	DHT	81.55 ± 2.56%	15-20 pg/tube	15.79%	No significant cross reaction with steroids other than T is observed
18651a	Delville et al. 1985	Japanese quail (*Coturnix japonica*)	Plasma	T	-	3.9 pg/tube	Intra-assay ≤10%, inter-assay 5%	
			Plasma	DHT	-	1.6 pg/tube	-	T (22%), 5α-androstanedione (17%), and androstenedione (2%)
1744	Driot et al. 1978	Chicken (*Gallus domesticus*)	Plasma	T	-	100 pg/100mL	Intra-assay 3%, inter-assay 5-9%	
			Plasma	DHT	68%	25 pg/mL	Intra-assay 7%, inter-assay 4%	T 12%, 5αDHT 100%, 5βDHT 48%, 5αAndrostan-3β-17β-diol 18%, 3α-Hydroxy-5α-androstan-17-one 3.9%
9660	Ottinger & Bakst, 1981	Japanese quail (*Coturnix japonica*)	Plasma	T	-	8 pg/mL	Intra-assay CV 8.66% and inter-assay CV 14%	DHT 30%
162	Peterson & Common, 1972	Chicken (*Gallus domesticus*)	Plasma	E1	75-77.2	14 pg/tube	-	-
			Plasma	17β-E2	66.7-73.8%	13.1 pg/tube	-	-
5357	Wood et al. 1981	Chicken (*Gallus domesticus*)	Plasma	E2	98.5 ± 2.2%	20 pg/mL	Intra-assay 4.9% inter-assay 8.4%	Estrone: 1.0%
9648	Shodono et al. 1975	Chicken (*Gallus domesticus*)	Plasma	E2	-	-	Intraassay CV: 3.7-6.9%; interassay CV: 4.5-9.1%	Anti-oestradiol-17β-6-CMO-BSA serum showed very little crossreactions with steroids other than oestradiol-17β
10913	Wineland & Wentworth, 1975	Broad Breasted Bronze turkeys (Meleagris gallopavo)	Plasma	E2	98.8-101.5%	5 pg/tube	-	0.1% with 17α estradiol
17904	Opel & Arcos, 1978	Turkey (Orlopp White) *Meleagris gallopavo*	Plasma	E2	-	13 pg/tube	interassay cv: 12.3% intraassay cv: 9.1%	-

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ref no.	Reference	Species	Sample matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
8165	Senior, 1974	chicken (*Gallus domesticus*)	Plasma	E2	-	20 to 30 pg/ml	Within-assay CV: 9.7%	-
13603	Greenacre et al. 2001	*Ara ararauna, Cacatua alba, Cacatua moluccensis, Psittacus erithacus erithacus, Amazona ochrocephala, Amazona aestiva*	serum	T4	75.2-91.1 %	0.24 nmol	Intra-assay 3.8%, Inter-day 7.8% CV of samples freeze thawed x7 times were 7.8%	-
6630	Wilson & McNabb 1997	*Coturnix japonica*	egg yolk	T3	61 ± 3% for 125I-T3	0.125 ng/mL	-	-
				T4	63 ± 1% 125I-T4	1.25 ng/mL	-	-

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 11: Publications retrieved involving analysis of bird droppings for estrogens and androgens using RIA

Ref no.	Reference	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
13074	Cockrem & Ronce 1994	Chicken (*Gallus domesticus*)	droppings	T	0.260-.0269	0.595 ng/mL	Intra-assay < 14-0% Inter-assay 12.2-17.6%	dihydrotestosterone (34%), 5β-androstan-3α, 17β-diol (3.8%), 11-hydroxytestosterone (3.3%), three other androgens at 2 to 3% and < 1% for other steroids.
			droppings	E2	0.190-0.213	70 pg/mL	Intra-assay < 14-0% Inter-assay 12.2-17.6%	oestrone (1.3%), oestriol (0.24%), and testosterone, progesterone and androstenedione (all < 0-004%).
7942	Buchanan et al. 2007	Canary *(Serinus canaria*)	droppings	T	-	0.027 ng/mL	intra-assay CV was 6.49%, inter-assay CV was 7.02%	-
4546	Goymann et al. 2002	European stonechats (*Saxicola torquata rubicola*)	droppings	T	-	0.03 ng/mL	Intra-assay CV: 1.0%; inter-assay CV: 10.9%	Cross-reactivity: 5α-dihydrotestosterone (44%), d-1-testosterone (41%), d-1-dihydrotestosterone (18%), 5α-androstan-3β, 17β-diol (3%), 4-androsten-3β, 17β-diol (2.5%), d-4-androstenedione (2%), 5β-androstan-3β, 17β-diol (1.5%), estradiol (0.5%), and less than 0.2% with 23 other steroids tested
3092	Kellam et al. 2004	Downy woodpeckers (*Picoides pubescens*)	droppings	T	38.6%	0.85 ng/g	Intra-assay 1.6%, Inter-assay 8.6%	-
7541	Goymann et al. 2006	European stonechats (*Saxicola torquata rubicola*)	droppings	T	-	0.65-0.72 pg per tube	Intra-assay 1.3 and 4.5%, inter-assay was 4.8%.	-
14950	Staley et al. 2007	Golden eagle (*Aquila chrysaetos*)	droppings	T	Surrogate hormone CORT 88.7%	0.03 ng/mL	intra <10%, inter 6-14.2%	-
		Peregrine falcon (*Falco peregrinus*)	droppings	E2		0.31 pg/tube	intra <10%, inter 9.1-10%	-

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

B.1.3. Measuring Hormones in Fish

B.1.3.1. Hypothalamic–pituitary–gonadal axis hormones

The literature search identified 23 publications which describe methods for measurement of estrogens (estrone, E1; 17β-estradiol, E2) and/or androgens (testosterone, T; 11-ketotestosterone, 11-KT; dihydrotestosterone, DHT) in fish using RIA. A summary of the studies can be found in Table 12: .

The earliest reports of RIA for sex steroid hormones were from the 1970s and describe measurements of T and 11-KT in fish plasma samples. Schreck et al. (1972) recognised the need for methods to determine hormones in fish without lengthy analytical procedures and for limited sample volumes. Their study describes the modification of a competitive protein binding assay for androgens for use in fish, i.e. they measured T in plasma of *S. gairdneri* (reference 16266 in Annex B –). They used plasma samples (0.1 ml) which were extracted twice with chloroform before measured in the RIA.

Another early protocol measured plasma T and 11-KT in Brook trout (*Salvenilus fontinalis*) and Cod (*Gadus morhua*) (Sangalang and Freeman 1977, reference 11238 in Annex B –). There, plasma samples (250-500 µl) were equilibrated with radioactively labelled steroid markers before being extracted with dichloromethane and dichloromethane:methanol (4:1) and subsequent purification by thin layer chromatography to separate T from 11-KT. Both steroids were then measured by RIA.

Depending on sample volumes, most studies include an extraction step for plasma or serum samples to increase assay reproducibility and decrease cross-reactivity. Most commonly, plasma samples are extracted in multiple steps with e.g. ethyl ether in the majority of studies, whilst other solvents such as dichloromethane, ethyl acetate and chloroform were also used. Chromatography steps such as thin layer chromatography (TLC) or HPLC fractionation were also used. One study compared simple heat treatment of eelpout plasma with plasma samples subjected to a TLC clean-up before E2, T and 11-KT RIA and concluded that the clean-up step was not necessary (Larsson et al. 2002, reference 8980 in Annex B –). Another publication investigating the daily changes in blood serum levels of E2 and 11-KT in *C. carpio* described the need for a chromatographic step before the E2 RIA, which was not necessary for the 11-KT assay due to the high specificity of the antibody that was used (Bieniarz et al. 1986, reference 3399 in Annex B –). With the advent of more specific antisera for target hormones, the need for clean-up and separation procedures to increase specificity prior to RIA has become less important, which is especially important for smaller sample volumes which are obtained from species such as zebrafish or fathead minnow.

A study measuring T and 11-KT in plasma samples of *M. albus* and *T. mossambicu* used several extraction steps with dichloromethane followed by diethyl ether before separating the steroids on Celite columns (Chan and Yeung 1986, reference 29 in Annex B –). This method was described to be especially useful for small sample sizes and low steroid content. Another protocol for measuring T and 11-KT in plasma of *A. anguilla* also used diethyl ether extraction but used chromatographic separation over Celite columns only prior to T RIA, due to the low specificity of the T-antibody (Khan et al. 1986, reference 11119 in Annex B –). The antibody employed in the 11-KT RIA was highly specific, thus no chromatographic step was needed.

For samples with low sample volumes and measurement of multiple hormones, it was also suggested to include HPLC fractionation before RIA (Venkatesh et al. 1989, reference 8006 in Annex B –). This method was suggested to replace the used of Celite columns, which are more labour intensive and not easy to automate. It was applied to measure T amongst other steroids in goldfish serum samples. The required sample volume was only 50 µl, which were extracted using Sep-Pak C18 cartridges before fractionation via HPLC and hormone measurement in a T RIA. E2 was directly measured after Sep-Pak purification since the HPLC step was not required.

Another study protocol for measuring T and 11-KT by RIA in rainbow trout extracted the plasma samples with diethyl ether before conducting thin layer chromatography to separate the steroids and increase specificity (Schulz 1985, reference 2959 in Annex B –). A similar protocol was described earlier for

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

analysis of E2 and 11-KT in rainbow trout plasma (Schulz 1984, reference 9602 in Annex B –). The RIA assays were conducted following standard procedures.

A validation study of RIA systems for 11-KT (and 11β-hydroxytestosterone) employed diethyl-ether extraction of plasma from *I. nebulosus* followed by column chromatography (Rosenblum et al. 1985, reference 17737 in Annex B –).

Plasma samples from *P. flavescence* were analysed for circulating steroid hormones after ethyl acetate extraction, followed by further processing with anion exchange and hydrophobic solid phase extraction cartridges (Bykova et al. 2010, reference 18954 in Annex B –). This allowed removal of fatty acids which were also extracted with ethyl acetate. Samples were then analysed by E2 and T RIA.

Other protocols using simple liquid solvent extractions of plasma samples with diethyl ether were used for measurement of E2 and T in *A. flavescence* (Craig et al. 2009, reference 15062 in Annex B –); E1, E2 and T in *H. fossilis* (Lamba et al. 1982, reference 18081 in Annex B –); E2 and T in *A. anguilla* (Quérat et al. 1985, reference 18250 in Annex B –) and T in *L. macrochirus* (Kindler et al. 1989, reference 13293 in Annex B –).

Whereas most studies measured sex steroids in serum or plasma, other matrices were also studied, such as hepatic tissue (Kurobe et al. 2016, reference 16381 in Annex B –), muscle tissue (Prohaska et al. 2013, reference 17351 in Annex B –), incubation medium from *ex vivo* testicular tissue incubations (Schulz et al. 1994, reference 8139 in Annex B –) and sperm (Fostier et al. 1982, reference 283 in Annex B –). Attempts to use non-invasive methods were also made by measuring hormones in tank water (Sebire et al. 2007, reference 1121 in Annex B –).

Measurement of E2 in liver extracts from *H. transpacificus* was conducted after tissue homogenisation of hepatic tissue by sonication followed by diethyl ether extraction of the steroids. E2 was then quantified by competitive E2 RIA. (Kurobe et al. 2016, reference 16381 in Annex B –). Measurements of E2 and T in muscle tissue of *R. terranovae* and *S. acanthias* was suggested as non-lethal, minimal invasive method for hormone analysis (Prohaska et al. 2013, reference 17351 in Annex B –). Muscle tissue was homogenised and extracted by 2:1 chloroform:methanol extraction prior to analysis by E2 and T RIA. Analysis of T and 11-KT in incubation medium from *C. geriepinus* testicular tissue ex vivo was performed after heat treatment of the medium and standard T and 11-KT RIAs (Sebire et al. 2007, reference 1121 in Annex B –). Details on assay performance for these studies can be found Table 12: .

Overall, the reported sensitivities of RIA measurements for androgenic steroid hormones ranged from 5 pg in catfish plasma (ref 18081) to 299 pg/ml in lake sturgeon plasma (ref 15062) for T; 0.2ng/fish/hour in tank water for DHT; 12 pg in brook trout plasma (reference 11238) to 1000 pg/tube in rainbow trout blood and sperm samples (reference 283) for 11-KT. For estrogenic hormones, the sensitivities for E2 ranged from 6.2 pg/tube in silver eel plasma (reference 18250) and for E1 it was 10 pg in catfish plasma (reference 18081).

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 12: Publications retrieved involving analysis of fish samples for sex steroid hormones using RIA

Ref no.	Author/date	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
29	Chan and Yeung 1986	*Monopterus albus*	plasma	T	-	LOD: 4 pg/ml LOQ: 22 pg/ml	intra-assay cv: 15.2%, inter-assay cv: 12.2%	5α-dihydro-testosterone (26%)
				11-KT	-	LOD:16 pg/ml LOQ: 98 pg/ml	intra-assay cv: 8.6%, inter-assay cv: 12.7%	androstenetrione 'slightly'
		Tilapia mossambicu	plasma	T	-	LOD: 4 pg/ml LOQ: 22 pg/ml	intra-assay cv: 15.2%, inter-assay cv: 12.2%	5a-dihydro-testosterone (26%)
				11-KT	-	LOD:16 pg/ml LOQ: 98 pg/ml	intra-assay cv: 8.6%, inter-assay cv: 12.7%	androstenetrione 'slightly'
283	Fostier et al. 1982	Rainbow Trout	blood	11-KT	-	1000 pg/tube	CV: 9%	Cross reactivity: 9% for testosterone, 6% for 11 B-hydroxytestosterone, 10% for adrenosterone. No effect with other steroids
			sperm	11-KT	-	1000 pg/tube	CV: 9%	Cross reactivity: 9% for testosterone, 6% for 11 B-hydroxytestosterone, 10% for adrenosterone. No effect with other steroids
1121	Sebire et al. 2007	Three-spined stickleback (Gasterosteus aculeatus)	tank water	T	Recovery 95-105%	0.2 ng/fish/hr		-
				DHT	Recovery 95-105%	0.2 ng/fish/hr		-
				11-KT	Recovery 95-105%	0.2 ng/fish/hr		-
			plasma	11-KT	-	-	-	-
2959	Schulz 1985	rainbow trout	plasma	T	Recovery 71.5±8.7& for each androgen	-	inter-assay cv: 3.8-14.4, intra-assay cv: 2.8-7.3	T 100%, DHT 111.4%, 11-KT 4.69%
				11-KT	Recovery 71.5±8.7& for each androgen	-	inter-assay cv: 4.6-9.1, intra-assay cv: 4.1-9.1	11-KT 100%, T 3.42%, DHT 2.25%
3399	Bieniarz et al. 1986	Carp (C*yprinus carpio*)	serum	E2	-	-	-	-
				11-KT	-	-	-	Low cross-reactivity with T 1.1%

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ref no.	Author/date	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
8006	Venkatesh et al. 1989	goldfish	serum	E2	Recovery 94.5%	Sensitivity: 45 pg	intra-assay cv: 5.6 to 9.0% inter-assay cv: 6.3 to 14.6%, for var. steroids	Cross-reactivity with T 0.02%, 5a-DHT 0.12%, estrone 2.88%, estriol 0.5, other steroids <0.01%
				T	Recovery 96.8%	Sensitivity: 11 pg	intra-assay cv: 5.6 to 9.0% inter-assay cv: 6.3 to 14.6%, for var. steroids	Cross-reactivity with E2 0.19%, Cortisol 0.01%, Androstenedione 1.64%, DHA 3.7%, 5a-DHT 76.86%, 3a-diol 1.1%, 3b-diol 1.1%, other steroids <0.01%
8139	Schulz et al. 1994	African catfish (*Clarias gariepinus*)	testicular tissue	T	-	0.1 ng/ml incubation medium	-	-
				11-KT	-	0.1 ng/ml incubation medium	-	-
			serum	T	-	0.4 ng/ml serum	-	Antisera and cross-reaction rates were previously described (Schulz 1984; 1985; Mayer et al. 1990)
				11-KT	-	0.4 ng/ml serum	-	Antisera and cross-reaction rates were previously described (Schulz 1984; 1985; Mayer et al. 1990)
8980	Larsson et al. 2002	Eelpout (*Zoarces viviparus*)	plasma	E2	-	0.3 ng/ml	-	-
				T	-	0.3 ng/ml	intra-assay cv: 4.2%, inter-assay cv: 6.5%	-
				11-KT	-	0.3 ng/ml	-	Cross-reactivity: 3.0% with T, 1.0% with E2
9602	Schulz 1984	Rainbow trout	serum	E2	-	-	Inter-assay cv: 5.5-10.3%; intra-assay cv: 7.9%	Estriol 2.25%, Estron 3.3%, Androstentrion, T, DHT, 11β-Hydroxytestosterone, 11β-Hydroxyandrostendione, Androstendione, Aldosterone, Cortisone, Cortisol, Progesterone and 17α-Hydroxyprogesterone <0.1%,
				11-KT	-	-	Inter-assay cv: 4.6-9.1%; intra-assay cv: 8.5%	Androstentrion 4.7%, T 3.4%, DHT 2.25%, 11 β-Hydroxytestosterone 0.16%, 11 β-Hydroxyandrostendione,

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ref no.	Author/date	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
								Androstendione, Aldosterone, Cortisone, Cortisol, Progesterone, 17a-Hydroxyprogesterone, Estriol and Estron <0.1%"
10183	Cuisset et al. 1994	Siberian sturgeon (*Acipenser baeri*)	plasma	11-KT	-	-	-	-
11119	Khan et al. 1986	Freshwater silver European eel (*Anguilla L.*)	plasma	T	66.2±1.4	10 pg	Within-assay CV: 10%	Anti-testosterone cross-reacted with 5α-dihydrotestosterone (DHT) and 5β-DHT (45 and 22% respectively) and 11-oxotestosterone (25%).
				11-KT	77.5±1.5	6 pg	Within-assay CV: 19%	Weak cross-reaction with testosterone and 11β-hydroxytestosterone (about 1%) and other 11-oxygenated androgens (less than 0.1%).
11238	Sangalang and Freeman 1977	Brook trout (*Salvenilus fontinalis*)	plasma	T	69.6 to 107%	10 pg	Intra-assay cv: 9.2%; inter-assay cv: 1.3-16.5%	Significant cross-reactivity of T and 11-KT were avoided by chromatography (TLC)
				11-KT	69.6 to 107%	12 pg	Intra-assay cv: 10.5%; inter-assay cv: 5.3-18.3%	Significant cross-reactivity of T and 11-KT were avoided by chromatography (TLC)
		Cod (*Gadus morhua*)	plasma	T	69.6 to 107%	10 pg	Intra-assay cv: 9.2%; inter-assay cv: 1.3-16.5%	Significant cross-reactivity of T and 11-KT were avoided by chromatography (TLC)
				11-KT	69.6 to 107%	12 pg	Intra-assay cv: 10.5%; inter-assay cv: 5.3-18.3%	Significant cross-reactivity of T and 11-KT were avoided by chromatography (TLC)
11718	Dashow et al. 1984	Sea Lamprey (*Petromyzon marinus*)	plasma	E2	-	-	-	-
				estrone	-	-	-	-
				T	-	-	-	-
				DHT	-	-	-	-
13293	Kindler et al. 1989	Bluegill (*Lepomis macrochirus*)	serum	T	-	-	inter-assay cv: 16%; intra-assay cv: 5%	Cross-reactivity <5% with 11KT
				11-KT	-	-	inter-assay cv: 12%; intra-assay cv: 3%	Cross-reactivity <5% with T and <1% with DHT

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ref no.	Author/date	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
15062	Craig et al. 2009	Lake sturgeon (*Acipenser fluvescens*)	plasma	E2		665 pg/ml at 20% binding, 9 pg/ml at 80%	Intra-assay CV: 12%; Inter-assay CV: 3%	Cross-reactivities of antibodies used in these assays with other similar steroids are reportedly <10% according to the vendors (for E2 and T).
				T		299 pg/ml at 20% binding, 6 pg/ml at 80%	Intra-assay CV: 1%; Inter-assay CV: 16%	Cross-reactivities of antibodies used in these assays with other similar steroids are reportedly <10% according to the vendors (for E2 and T).
16266	Schreck et al. 1972	*Salmo gairdneri*	plasma	T	>95%	-	-	-
16381	Kurobe et al. 2016	Delta Smelt (*Hypomesus transpacificus*)	hepatic tissue	E2	-	-	-	-
17351	Prohaska et al. 2013	Atlantic Sharpnose Shark (*Rhizoprionodon terraenovae*)	plasma	E2	71%	-	intra = 7% inter = 8%	-
				T	85%	-	intra = 7% inter = 8%	-
			muscle tissue	E2	59%	-	intra = 10% inter = 10%	-
				T	39%	-	intra = 10% inter = 10%	-
		Spiny Dogfish (*Squalus acanthias*)	plasma	E2	80%	-	intra = 8% inter = 10%	-
				T	87%	-	intra = 10% inter = 11%	-
			muscle tissue	E2	21%	-	intra = 8% inter = 10%	-
				T	23%	-	intra = 10% inter = 11%	-
17737	Rosenblum et al. 1985	Brown bullhead (*Ictalurus nebulosus*)	plasma	E2	-	0.03 ng/ml	intra 3.35%, inter 15.49%	-
				T	-	0.03 ng/ml	intra 5.32% inter 12.68%	-
				11-KT	-	0.05 ng/ml	intra 7.56%, inter 7.65%	-

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ref no.	Author/date	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
		Goldfish (*Carassius auratus*)	serum	E2	-	0.03 ng/ml	intra 3.35%, inter 15.49%	-
				T	-	0.03 ng/ml	intra 5.32% inter 12.68%	-
				11-KT	-	0.05 ng/ml	intra 7.56%, inter 7.65%	-
18023	Venkatesh et al. 1989	*Carassius auratus*	serum	E2	-	45 pg	intra = 5.6-9.0% inter = 6.3-14.6%"	Cross-reactivity with T = 0.19
				T	-	11 pg	intra = 5.6-9.0% inter = 6.3-14.6%	Cross-reactivity with E2 = 0.02
18081	Lamba et al. 1982	Catfish (*Heteropneustes fossilis*)	plasma	E2	Recovery: 78% (63-91)	25 pg	Inter-assay cv: 7.3 % intra-assay cv: 6.8 %	Cross-reactivities: Estradiol taken at 100%; Estrone 1.7 %; Testosterone <0.0002 %; Cortisol <0.0001%
				E1	Recovery: 89% (81-98)	10 pg	Inter-assay cv: 9.0 % intra-assay cv: 6.6 %	Cross-reactivities: Estrone taken as 100%; Estradiol 9.8%
				T	Recovery: 82% (63-97)	5 pg	Inter-assay cv: 4.9 % intra-assay cv: 4.6 %	Cross-reactivities: T as 100%; 5a-Dihydrotestorone 14%; A4-Androstenedione 0.8%; Cortisol <0.0001 %; 5a-Androstanediol 6%; A5-Androstenediol 2.1%
18250	Quérat et al. 1985; Pedernera et al. 1999	Silver eel (*Anguilla L*)	plasma	E2		6.2 pg/tube	Interassay cv: 21%	
				T		10 pg/tube	Interassay cv: 19.1%	
18954	Bykova et al. 2010	Yellow perch (*Perca flavescens*)	plasma	E2	85 – 106%	-	-	-
				T	84 – 106%	-	-	-

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

B.1.3.2. Hypothalamic–pituitary–thyroid axis hormones

The literature search identified 11 studies that used RIA to measure thyroid hormones in fish. Most of the studies identified in the literature search which used RIA to measure HPT relevant hormones were measuring, triiodothyronine (T3) and/or thyroxine (T4), only one study determined Thyroid-stimulating hormone (TSH) by RIA. Details of the identified studies can be found in Table 13: .

The majority of studies measured T3 and/or T4 in plasma or serum samples. One study included other tissues in the analysis of T3 and T4, i.e. embryos and larvae whole tissue samples of *P. promelas* (Crane et al. 2004, reference 305 in Annex B –), and a second looked at both hormones in oocytes of *O. mossambicus* (Weber et al. 1992, reference 3207 in Annex B –). Additional studies determined the T3 content in various somatic tissues of *S. gairdneri* (Fok et al. 1990, reference 11890 in Annex B –) and the T4 levels in whole eggs and fry of *O. keta* (Tagawa and Hirano 1987, ref 13878 in Annex B –).

The earliest studies describe measurement of T3 and T4 in plasma samples. One study describes a procedure developed for measurement of T4 and adapted for T3 in *S. gairdneri* plasma which uses Sephadex columns as stationary phase for the assay (Brown and Eales 1977, reference 4602 in Annex B –). Plasma samples (0.1 ml) were added directly, without prior extraction. The assay sensitivity was 9.5 ng/100 ml for T3 and 12.5 ng/100 ml for T4. Intra-assay CV was 9.3 to 13.5% for T3 and 5.1 to 9.4% for T4; and inter-assay CV was 5.1-15.9% (T3) and 4.9-16.9% (T4).

Another RIA protocol for T4 measurements in *O. kisutch* plasma used 10 µl aliquots of plasma, also without prior extraction (Dickhoff et al. 1978, reference 6755 in Annex B –). One study describing an analytical chemistry method for detection of circulating thyroid hormones measured T3 and T4 in RIA in parallel (Noyes et al. 2014, reference 11800 in Annex B –). They measured circulating T3 and T3 in plasma of four different fish species (*P. promelas*, *F. heteroclitus*, *O. mykiss, O. nerka*). Plasma samples (10 µl) were directly added to the RIA, without prior extraction, approximately following the protocol of Dickhoff et al. (reference 6755 in Annex B –).

One study analysing circulating thyroid hormones measured T3 and T4 in serum of *S. gairdeneri* (Orozco et al. 1992, reference 9122 in Annex B –). The protocol uses trout serum, directly added to the assay without extraction. The authors of the study stress the importance of maintaining serum-homology when measuring thyroid hormones in different animals by RIA, as demonstrated by the differences in the serum species sensitivity by comparing T4 standard curves for bovine, human and trout samples.

A solid-phase RIA was evaluated for measurement of T3 and T4 in trout plasma (Omeljaniuk et al. 1984, reference 10249 in Annex B –). The method employed miniature Sephadex columns and allowed to measure both thyroid hormones simultaneously, thus saving time, sample volume and reagents when measuring T3 and T4 in plasma samples. The method was had consistent recoveries of T3 (87.0 or 86.7%) and T4 (103 or 98%) when added singly or in combination. Inter- and intra-assay CVs were low at < 8% and < 7%, respectively. The same solid-phase RIA protocol was employed with some modifications to measure total circulating plasma T3 and T4 in *S. vitreus* (Picard-Aitken et al. 2007, reference 6160 in Annex B –). Plasma samples were not extracted prior to analysis but added directly to the RIA. Assay sensitivity was 0.27 ng/ml for T3 and 0.2 ng/ml for T4. Intra- and inter assay cv were 10.1% and 12.8% for T3; and 12.2% and 16.6% for T4.

One study investigating the developmental changes of thyroid hormones in *P. promelas* measured T3 and T4 in several tissues, including plasma and whole tissue samples from embryos and larvae (Crane et al. 2004, reference 305 in Annex B –). All tissues were extracted prior to RIA. Plasma (30 µl) was extracted with ethanol, and embryo and larvae samples were extracted with ethanol containing 6-*N*-propyl-2-thiouracil and homogenisation in multiple steps. T3 and T4 were measured by RIA. Extraction efficiency for both thyroid hormones differed depending on matrix (Table 13:). Assay sensitivities for T3 and T4 were 2.04 pg/tube and 8.16 pg/tube. Intra- and inter-assay cv were 9.68% and 11.66% for T3; and 5.33% and 20.37% for T4.

Thyroid hormone measurements in oocytes and serum were performed in samples from *O. mossambicus* (Weber et al. 1992, reference 3207 in Annex B –). The extraction procedures for oocytes included homogenisation in a solution of 6-*N*-propyl-2-thiouracil in methanol and 10 N NaOH followed by

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

repeated extraction with chloroform. Thyroid hormones (T3 and T4) in oocyte extracts were then measured using a modification of a previously described method (Brown and Eales 1977, reference 4602 in Annex B –). The authors also investigated the impact of sample volume on assay outcome and did not find any differences in the T3 or T4 measurements at different sample volumes within the range tested. Intra and inter-assay cv were 6.2% and 9.7% for T3; and 5.8% and 9.0% for T4.

A protocol measuring the levels of T3 in somatic tissue was described for various tissues of *S. gairdneri* (Fok et al. 1990, reference 11890 in Annex B –). Samples from intestine, kidney, liver, stomach, heart, muscle, gill and skin were extracted using a digestion step in phosphate buffer containing 6-*N*-propyl-2-thiouracil and collagenase (muscle and skin) or pronase (for all other tissues). All samples were analysed for T3 by solid phase RIA (Brown and Eales 1977, reference 4602 in Annex B –).

One publication investigated the presence of T4 in eggs of *O. keta* (Tagawa and Hirano 1987, reference 13878 in Annex B –). The study compares two different extraction methods for fish eggs. One used ethanol extraction in combination with homogenisation and the other used homogenisation in methanol followed by methanol:chloroform extraction. The latter successfully removed lipid components, particularly from yolk, which may interfere with the RIA. T4 content of the extract was determined by a modified version of a previously described T4 RIA (Dickhoff et al. 1978, reference 6755 in Annex B –).

Only one of the identified studies determined TSH levels in fish by RIA. Using pituitary extracts from Coho salmon (*Oncorhynchus kisutch*), Moriyama and colleagues isolated salmon TSH and developed an antibody against TSH which was used for RIA and Immunocytochemistry (Moriyama et al. 1997, reference 153 in Annex B –). The RIA method was then applied to 0.1 ml samples of *O. kisutch* pituitary gland extracts, plasma samples and cell culture medium from pituitary incubations. Cross-reactivity with *O. kistuch* GTH I and GTH II, α subunits; GTH I and GTH II, β subunits; growth hormone (GH); prolactin (PRL); somatolactin (SL) was determined in the TSH-RIA and none of the gonadotropins nor the other pituitary hormones showed significant cross-reactivity (< 1.0% displacement). The reported range of the TSH-RIA (n=8 assays over 6-week period) was from 0.4 ± 0.4 ng/tube to 5.1 ± 0.4 ng/tube. Intra- and inter-assay coefficients of variation were 11.2% and 18.1%, respectively.

Overall, the reported sensitivities of RIA measurements for thyroid hormones ranged from 2.04 pg/tube in *P. promelas* samples (plasma, embryo, larvae) (reference 305) to 270 pg/ml in walleye plasma (reference 6160) for T3; and 8.16 pg/tube in *P. promelas* samples (plasma, embryo, larvae) (reference 305) to 200 pg/ml in walleye plasma (reference 6160) for T4; The THS assay had a sensitivity of 400 pg/tube (reference 153).

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 13: Publications retrieved involving analysis of fish samples for thyroid hormones using RIA

Ref no.	Reference	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection /assay range	coefficient of variation (CV)	Specificity
305	Crane et al. 2004	Fathead minnow (*Pimephales promelas*)	Embryo tissue	T3	75.2% (68.0 – 75.3%)	2.04 pg per tube	intra-assay 9.68%, inter-assay 11.66%	Anti-T3 cross-reactivity: 5% with T4; 0.02% with rT3; 3.4% with 3,3′,5′-diiodothyronine. Cross-reaction of 3-monoiodotyrosine and tyrosine negligible.
				T4	69.4% (66.4 - 74.7%)	8.16 pg per tube	intra-assay 5.33%, inter-assay 20.37%	Anti-T4 cross-reactivity: 1.24% with T3; 2.38% with rT3; Cross-reaction of 3-monoiodotyrosine and tyrosine negligible.
			Larvae tissue	T3	63.9% (52.5 – 70.8%)	2.04 pg per tube	intra-assay 9.68%, inter-assay 11.66%	Anti-T3 cross-reactivity: 5% with T4; 0.02% with rT3; 3.4% with 3,3′,5′-diiodothyronine. Cross-reaction of 3-monoiodotyrosine and tyrosine negligible.
				T4	59.5% (50.7-70.0%)	8.16 pg per tube	intra-assay 5.33%, inter-assay 20.37%	Anti-T4 cross-reactivity: 1.24% with T3; 2.38% with rT3; Cross-reaction of 3-monoiodotyrosine and tyrosine negligible.
			Plasma	T3	67.8% (61.9 - 79.0%)	2.04 pg per tube	intra-assay 9.68%, inter-assay 11.66%	Anti-T3 cross-reactivity: 5% with T4; 0.02% with rT3; 3.4% with 3,3′,5′-diiodothyronine. Cross-reaction of 3-monoiodotyrosine and tyrosine negligible.
				T4	69% (60.2 - 86.4%)	8.16 pg per tube	intra-assay 5.33%, inter-assay 20.37%	Anti-T4 cross-reactivity: 1.24% with T3; 2.38% with rT3; Cross-reaction of 3-monoiodotyrosine and tyrosine negligible.
3207	Weber et al. 1992	Tilapia (*Oreochromis mossambicus*)	oocytes	T3	-	-	intra-assay 6.2%; inter-assay 9.7%	-
				T4	-	-	intra-assay 5.8%; inter-assay 9.0%	-
4602	Brown and Eales 1977	Rainbow trout (*Salmo gairdneri*)	Plasma	T3	88.5 to 116%	9.5 ng/100 ml	intraassay CV: 9.3 to 13.5%; interassay CV: 5.1-15.9%	T3 antibody shows less than 1% cross-reaction with T4, 2.8% with tetraiodothyroacetic acid; 20% with triiodothyroacetic acid; 80% with triiodothyropropionic acid.
				T4	98.9 to 120%	12.5 ng/100 ml	intraassay CV: 5.1 to 9.4%;	T4 antibody exhibits less than 1% cross-reaction with T3 and triiodothyroacetic acid; 1.3% with triiodothyropropionic acid; 10% with

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ref no.	Reference	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection /assay range	coefficient of variation (CV)	Specificity
							interassay: 4.9-16.9%	tetraiodothyroacetic acid; 25% with tetraiododesaminothyronine
6160	Picard-Aitken et al. 2007	Walleye (*Sander vitreus*)	Plasma	T3	-	0.27 ng/ml	intra-assay: 10.1%; inter-assay: 12.8%	
				T4	-	0.2 ng/ml	intra-assay: 12.2%; inter-assay: 16.6%,	
6755	Dickhoff et al. 1978	Coho Salmon (*Oncorhynchus kisutch*)	Plasma	T4	98%	-	intra-assay: 5%; inter-assay 17%	
9122	Orozco et al. 1992	Rainbow trout (*Salmo gairdneri*)	Serum	T3	-	50 pg (max sensitivity)	inter-assay cv: 8.9%; intra-assay cv: 9.5%	T3 / T4 crossreactivity reported as negligible
				T4	-	50 pg (max sensitivity)	inter-assay cv: 8.3%; intra-assay cv: 6.6%	T3 / T4 crossreactivity reported as negligible
10249	Omeljaniuk et al. 1984	Trout	Plasma	T3	-	-	Intra-assay cv: 7%; inter-assay cv: 6.4%	-
				T4	-	-	Intra-assay cv: 6.6%; inter-assay cv: 7.3%	-
11800	Noyes et al. 2014	Fathead minnow (*Pimephales promelas*)	Plasma	T3	-	-	Intra-assay cv: 6.95%	Cross-reactivity 0.14% with T4, 0.5% with diiodotyrosine and <0.001% with monoiodotyrosine
				T4	-	-	Intra-assay cv: 7.09%	Cross-reactivity <5% with T3, <0.01% with diiodotyrosine and monoiodotyrosine
		Mummichog (*Fundulus heteroclitus*)	Plasma	T3	-	-	Intra-assay cv: 6.95%	Cross-reactivity 0.14% with T4, 0.5% with diiodotyrosine and <0.001% with monoiodotyrosine

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ref no.	Reference	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection /assay range	coefficient of variation (CV)	Specificity
				T4	-	-	Intra-assay cv: 7.09%	Cross-reactivity <5% with T3, <0.01% with diiodotyrosine and monoiodotyrosine
		Rainbow trout (*Oncorhynchus mykiss*)	Plasma	T3	-	-	Intra-assay cv: 6.95%	Cross-reactivity 0.14% with T4, 0.5% with diiodotyrosine and <0.001% with monoiodotyrosine
				T4	-	-	Intra-assay cv: 7.09%	Cross-reactivity <5% with T3, <0.01% with diiodotyrosine and monoiodotyrosine
		Sockeye salmon (*Oncorhynchus nerka*)	Plasma	T3	-	-	Intra-assay cv: 6.95%	Cross-reactivity 0.14% with T4, 0.5% with diiodotyrosine and <0.001% with monoiodotyrosine
				T4	-	-	Intra-assay cv: 7.09%	Cross-reactivity <5% with T3, <0.01% with diiodotyrosine and monoiodotyrosine
11890	Fok et al. 1990	Rainbow trout (*Salmo gairdneri*)	Various somatic tissues	T3	Recovered radioactivity between 55.8 and 83.8%	-	-	-
13878	Tagawa and Hirano 1987	Chum salmon (*Oncorhynchus keta)*	whole eggs and fry	T4	-	-	inter-assay cv: 5.9%; intra-assay cv: 2.9%	-
153	Moriyama et al. 1997	Coho salmon (*Oncorhynchus kisutch*)	Pituitary extract	TSH	-	0.4 ± 0.4 ng/tube to 5.1 ± 0.4 ng/tube	Interassay cv: 18.1% Intraassay cv: 11.2%	*O. kistuch* GTH I and GTH II, α subunits; GTH I and GTH II, β subunits; growth hormone (GH); prolactin (PRL); somatolactin (SL) showed no significant cross-reactivity (< 1.0% displacement).
			Plasma	TSH	-	0.4 ± 0.4 ng/tube to 5.1 ± 0.4 ng/tube	Interassay cv: 18.1% Intraassay cv: 11.2%	

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

B.2. ELISA (and other IA methods) for measuring hormones

As with the RIA, the enzyme-linked immunosorbent assay (ELISA) uses the specificity of the immune system to accurately identify target molecules. Its advantage over the RIA, beyond not employing a radioactive reagent, is that it can directly quantify the target molecule, rather than infer its concentration from the level of bound radiolabelled equivalent. Key to this assay are specially prepared multiwell plates that cause biological molecules to bind to the well bottom. Samples from the target species are incubated in the wells. The plates are then thoroughly washed, leaving only the bound molecules, which will include the target hormones if present. This is followed by the addition of a blocking reagent such as bovine serum albumin to ensure there are no free binding sites in the wells. There are several types of ELISA, with variations in how the remainder of the assay is performed. The simplest is the Direct ELISA. Antibodies raised against the target hormone and modified with an attached enzyme are then added and incubated to allow binding to the target hormone to occur. After another thorough washing to remove any unbound antibody a substrate is added to the wells. The enzyme attached to the antibody will react with the substrate to produce a quantifiable reaction such as the production of a colour, fluorescence or luminescence. While the simplest variant, direct ELISAs have drawbacks, which has led to the development of several other types such as the indirect, sandwich and competitive ELISAs. These have advantages such as lower cost or higher sensitivity in complex samples where many other molecules are present.

The first enzyme immunoassay was developed in 1971 and with ongoing improvements ELISAs continue to be a highly accurate assay for hormone concentration quantification.

B.2.1. Measuring Hormones in Amphibians

B.2.1.1. Hypothalamic–pituitary–gonad axis hormones

The literature search resulted in zero papers describing original and/or detailed methods for the detection of estrogens or androgens in amphibian samples using ELISA or Enzyme immunoassay (EIA) methods.

However, one paper was retrieved which validated a competitive chemiluminescence enzyme immunoassay (ECLIA) for ovarian steroid in *Rhinella arenarum* (Arias Torres et al. 2016, reference 15412 in Annex B –), the method had been previously developed for human samples. Dichloromethane was used to extract steroids from the sample matrices. The LOD for testosterone ECLIA was reported as 0.087 nM with an inter-assay CV of 5-5.4%. Cross reactivity for the T-antibody with other related androgens were as follows: androstenodione <2.5%, DHEA-S (dehydroepiandrosterone sulfate) <0.003%, testosterone propionate <2.46%, 5-α-androsteno-3β,17β-diol <2.11%, 5-α-dihydrotestosterone <0.86%, 11-keto-testosterone <3.22%, 11-β-hydroxytestosterone <18.0%, and nondetectable progesterone.

B.2.1.2. Hypothalamic–pituitary–thyroid axis hormones

The literature search retrieved zero papers describing original and/or detailed methods for the detection thyroid hormones (e.g. T3, T4) in amphibian samples using an ELISA method. One paper was retrieved describing the measurement of Thyroid Simulating Hormone (TSH) in amphibians (Korte et al. 2011, reference 15475, *Xenopus laevis* and *Xenopus tropicalis*). This study developed a Sandwich ELISA to measure intracellular (within pituitaries), secreted (ex vivo pituitary culture), and circulating (serum) amounts of TSH. TSH was extracted from the sample matrices using phosphate/Tween-20 with heating, however how effective this method is unclear as they did not conduct TSH spiked sample extraction recoveries. The LOD of the TSH ELISA was 0.22 ng using 10-20 µL of sample, with inter-assay cv of 5% and intra-assay cv of 5.01% and 5.09% (Korte et al. 2011).

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

B.2.2. Measuring Hormones in Birds

B.2.2.1. Hypothalamic–pituitary–gonad axis hormones

The literature search retrieved six publications which gave sufficient details for measuring estrogens (estrone; E1, 17β-estradiol; E2, estriol E3) and/or androgens (testosterone: T) in avian samples with ELISA or Enzyme immunoassay (EIA) methods. Sample matrices described were varied and included plasma, droppings/faeces, saliva and brain. Sample extraction methods differed depending on matrix and publication.

Four of the six publications included plasma as their sample matrices (Washburn et al. 2007, reference 14030 in Annex B –, Mourning Dove (*Zenaida macroura*), White-eyed Vireo (*Vireo griseus*), Red-eyed Vireo (*Vireo olivaceus*), Indigo Bunting (*Passerina cyanea*); Hahn et al. 2011, reference 752 in Annex B –, Rose-ringed parakeets (*Psittacula krameri*) and Cockatiels (*Nymphicus hollandicus*); Chao et al. 2011, reference 2988 in Annex B –, zebra finch (*Taeniopygia guttata*), and U.S. EPA 2015, reference 13985 in Annex B –, Japanese quail (*Coturnix japonica*)) for sex-steroid measures.

Washburn et al. (2007) diluted avian plasma (12-35 µL) directly in the assay buffer for the testosterone enzyme immunoassay, without solvent extraction (LOD 15 pg/mL, cross-reactivity of testosterone antibody: 1.2% with androstenedione, and 1% for other steroids). Whereas, Hahn et al. (2011) used a diethyl ether extraction of plasma (0.5 mL) for use in the testosterone ELISA (LOD 0.1ng/mL, reported cross-reactivity 0.58% androstedione; <0.1% for all other steroids). Similarly, as with the RIA for sex-steroids, the EPA Test guideline (U.S.EPA 2015) recommends diethyl ether extraction of plasma (100 µL/aliquot) for total T quantification in the recommended ELISA kit (LOD 3.9 pg/mL, cross-reactivity 0.1% with E2). Chao et al. (2011) investigated standard liquid-liquid extraction of plasma and brain samples with diethyl ether, or liquid-liquid extraction followed by SPE. They report the combination of both liquid-liquid extraction followed by SPE provided better recoveries of estrogens from zebra finch brain and plasma samples Chao et al. (2011).

Four of the six publications included analysis of sex-steroids from bird droppings/faeces (Lee et al. 1995, reference 14157 in Annex B –, White-crowned sparrows (*Zonotrichia feucophrys oriantha*); Hahn et al. 2011, reference 752 in Annex B –; Bautista et al. 2013, reference 15304 wild great bustards (*Otis tarda*) and U.S. EPA 2015). In the oldest method, dried droppings were solubilised and diluted directly into the EIA assay buffer (testosterone LOD 1.55 ng/mL, E1 0.31 ng/mL) (Lee et al. 1995). Later methods employ diethyl ether extraction of droppings (Bautista et al. 2013 - 0.1 g, LOD E2 2.9 pg/well, T 4.9 pg/well) and Hahn et al. (2011) also hydrolysed the faeces samples with β-glucoronidase/arylsulfatase before diethyl ether extraction (LOD 0.1 ng/mL). Although the EPA TG recommends diethyl ether extraction of sex-steroids from plasma and egg yolk, it recommends ethanol for extracting sex-steroids from faeces (U.S. EPA 2015).

Hahn et al. (2011) also investigated the feasibility of measuring testosterone in avian (Cockatiel and Rose-ringed parakeet) saliva (without solvent extraction). However, they reported at present saliva does not appear to be an ideal medium for reliable hormone level measurement. See Table 14: for details of above publications.

B.2.2.2. Hypothalamic–pituitary–thyroid axis hormones

The literature search resulted in zero papers describing original and/or detailed methods for the detection of thyroid hormones in avian samples using ELISA or Enzyme immunoassay (EIA) methods.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 14: Publications retrieved involving analysis of bird samples for sex steroid hormones using ELISA

Ref no.	Reference	Species	Sample matrix	Hormone	Extraction efficiency	Limit of Detection	coefficient of variation (CV)	Specificity
14030	Washburn et al. 2007	Zenaida macroura Vireo griseus Vireo olivaceus Passerina cyanea	plasma	T	-	15 pg/mL	-	1.2% with androstenedione, and 1% for other steroids
752	Hahn et al. 2011	Nymphicus hollandicus Psittacula krameri Nymphicus hollandicus	plasma saliva Droppings/faeces	T	-	0.1ng/mL	Intra-assay CV: 4.4% Inter-assay CV: 6.7%	0.58% androstedione; <0.1% for all other steroids
14157	Lee et al. 1995	Zonotrichia feucophrys oriantha	Droppings/ faeces	T	-	1.55 ng/mL	-	-
				E1	-	0.31 ng/mL	-	-
15304	Bautista et al. 2013	Otis tarda	Droppings/ faeces	T	-	4.9 pg/well	Intra- and inter-assay 5.5 % and 8.9 %, respectively.	5-alpha-dyhidrotestosterone 20 %, 4-androstenediol 11.5 %, 5-beta-dihydrotestosterone 5 %, androstenediol 3.5 %, androstenelone 3.2 %, 5-alpha-androstan-3-alpha, 17-beta diol 1 % and <1 % with cortisol, progesterone and estradiol
				E2	-	2.9 pg/well	Intra- and inter-assay 7.4 % and 9.9 %, respectively.	keto-estradiol 6-CMO 100 %, 16 keto-estradiol 16.70 %, 6 keto-estradiol 20.00 %, estriol 8.73 %, estradiol 3-benzoate 3.28 %, estrone 0.50 %, equilin 0.3 % and <1 % with equilenin, progesterone and testosterone

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

B.2.3. Measuring Hormones in Fish

B.2.3.1. Hypothalamic–pituitary–gonad axis hormones

The literature search identified nine studies which describe methods for measurement of estrogens (estrone, E1; 17β-estradiol, E2) and/or androgens (testosterone, T; 11-ketotestosterone, 11-KT) in fish using ELISA. One additional study was identified which used TR-FIA to measure T. A summary of the studies can be found in Table 15: .

Five of the studies measured both, estrogens and androgens by ELISA. The remaining studies looked only at androgens, i.e. testosterone and 11-ketotestosterone. The majority of studies determined sex steroids in plasma or serum samples. Two studies determined E2, T, or 11-KT in tank water (Felix et al. 2013; Friesen et al. 2012, references 1689 and 6129 respectively in Annex B –).

The earliest study using ELISA to analyse sex hormones measured 11-KT in the plasma of Siberian sturgeon (Cuisset et al. 1994, reference 10183 in Annex B –). Their assay included a steroid separation step by HPLC. The assay sensitivity was 7 pg/ml. Intra-assay coefficient of variation (CV) for 11-KT was < 10% and inter-assay CV was 9.5%.

Sampling procedures for serum or plasma were generally similar and used some form of liquid solvent extraction. One study measuring plasma T in *D. labrax* extracted the plasma samples with ice cold methanol (Rodríguez et al. 2000, reference 1598 in Annex B –). Another study extracted plasma from *O. mykiss* with dichloromethane before measuring E2 and T (Nash et al. 2000, reference 3583 in Annex B –). Ether extraction was employed prior to measurement of E1 and T in serum of *P. sieboldi* (Ohta et al. 2001, reference 17968 in Annex B –) or analysis of E2 and T in plasma of *O. kisutch* (Clark et al. 2011, reference 18822 in Annex B –). These studies then mostly followed standard ELISA procedures or used assay kits. More details on assay performance can be found in Table 15: .

One study developed a validation protocol of ELISA kits which are used to measure steroid hormones in fish plasma (Metcalfe et al. 2018, reference 5839 in Annex B –). They tested EIA kits for E2, T and 11-KT to measure plasma samples from three different fish species (anemonefish, *A. melanopus*; Barramundi, *L. calcarifer*; Coral trout, *P. leopardus*). Initially, they measured plasma samples without extraction step, but found that assays were not performing well. Thus, they included a liquid solvent extraction step using ethyl acetate:N-hexane (50:50), which was repeated three times to extract the steroids. Samples were then measured using commercial ELISA kit. In addition, the authors recognised the importance of sample stability during long term storage and the detrimental effect of repeated freeze-thaw cycles, thus snap-freezing aliquoted samples immediately after centrifugation, storage at low temperatures and only thawing required aliquots for assay preparation to preserve sample integrity. The sensitivity of the assays was: LOD: 6.7 pg/ml; LOQ: 23.9 pg/ml for E2; LOD: 3.8 pg/ml; LOQ: 13.4 pg/ml for T; and LOD: 0.7 pg/ml; LOQ: 2.4 pg/ml for 11-KT. More details are listed in Table 15: .

Another publication describes an interlaboratory study to determine the variability in measured concentrations of E2, T and 11-KT (Feswick et al. 2014, reference 6607 in Annex B –). Using plasma from *C. commersoni*, they first performed their own steroid extractions with diethyl ether and measured the three hormones by RIA before sending out plasma samples and extracts to other laboratories for analysis by RIA or ELISA. Whilst they found large inter-laboratory variability, they noted that the intra-laboratory assay variabilities were generally low. To facilitate better reproducibility of data, they stress the need for publication of CV percentages and an agreement of acceptable CV values. Whilst the variability was high in plasma samples and extracts, the authors also point to the need of standardised extraction procedures to guarantee good assay performance and the reporting of extraction efficiencies. They also found that RIA and ELISA produced different results, but that the results were correlated. For testing of endocrine disrupting chemicals, the authors propose that researchers use fold change to describe changes in hormone levels after treatment rather than absolute values when using steroid measurements as endpoint.

Felix et al. (2013) (reference 6129 in Annex B –) discusses the problem with low plasma sample volumes from small fish species such as *D. rerio* (1-5 µl plasma) which often necessitates whole-body hormone

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

measurements, with no possibility of sequential sampling of the same individual. Therefore, they suggested non-invasive methods and presented a study for 11-KT measurements in holding water. Their study measured 11-KT in the plasma and tank water of male and female *D. rerio* (Felix et al. 2013 , reference 6129 in Annex B –). Plasma samples were not extracted, due to the very small volume (1-5 μl), but directly diluted in EIA buffer. Diluted samples were stored at -20°C until analysis. Tank water samples were filtered to remove particulates, and steroids were then extracted using a C18 solid-phase extraction cartridge. Steroids were eluted from thawed columns with 2 x 2ml of ethanol. The eluted steroids were extracted 2x with diethyl ether. The second extraction step was conducted in order to obtain a higher extraction efficiency. The final sample was reconstituted with 1 ml of the buffer solution supplied with the Enzyme Immunoassay (EIA) kit and stored at - 20°C until analysis of 11-KT using a commercial ELISA kit. Intra-assay CV for 11-KT was 4.41% and inter- assay cv was 6.2%.

A second study measured E2 and T in tank water of *P. multicolor victoriae* (Friesen et al. 2012 , reference 1689 in Annex B –). Hormones were extracted from the tank water by solid phase extraction (SPE). Ethyl acetate was used to elute the steroids from the column, as this allowed to measure only the free fraction of steroids from the gills, as proxy for "physiologically active" steroids in the plasma. E2 and T were then measured by commercial ELISA kits. The assay sensitivity was 20 pg/ml for E2 and 6 pg/ml for T. Intra-assay CV was 14% for E2 and 17% for T, inter-assay CV was 20% for E2 and 14% for T.

The literature search also identified one study which reported on the development of an alternative immuno-assay method to measure T in serum of rainbow trout, namely Time Resolved Fluoroimmunoassay (TR-FIA) (Yamada et al. 1997, reference 5476 in Annex B –). Using fluorescence instead of radioisotopes as tracers removes the need of special equipment for performing RIA and using a time resolved-FIA increased sensitivity compared to ELISAs. Serum samples were ether extracted before being subjected to the FIA protocol. The assay was measured with a time resolved fluorometer. The assay sensitivity for T in serum samples was 1.5 pg/ml. Intra-assay and inter-assay CV were 1.62-6.38% and 2.96-8.29%, respectively. Cross-reaction with 11-KT was <0.2%, cross-reaction of 17β-estradiol, progesterone, 17α-hydroxyprogesterone, and cortisol with antiserum was satisfactorily low (<0.01%), except for 5α-dihydrotestosterone (5.8%).

Overall, the reported sensitivities of ELISA measurements for sex steroid hormones were for estrogens;

- E2: 1.6 pg/ml in *O. mykiss* plasma to 24 pg/ml in plasma from three different species (5839),
- E1: 12.5 pg/ml in *P. sieboldi* serum (17968),
- For androgens sensitivity ranged from 0.6 pg/ml in *O. mykiss* plasma (reference 3583) to 13.4 pg/ml in plasma from three different species for T (reference 5839); and 2.4 pg/ml in plasma from three different species for 11-KT (reference 5839) to 7 pg/ml in plasma from *A. baeri* (reference 10183).

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 15: Publications retrieved involving analysis of fish samples for sex steroid hormones using ELISA

Ref no.	Author/date	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection /assay range	coefficient of variation (CV)	Specificity
1598	Rodríguez et al. 2000	Sea Bass (*Dicentrarchus labrax*)	Plasma	T		0.009 ng/ml	intra-assay cv: 6.2% inter-assay cv: 9.5%	Testosterone 100.0%, 5α-Dihydrotestosterone (5-DHT) 26.4%, 11β-Hydroxytestosterone (11β-HT) 10.2%, Androstendione (ATD) 5.6%, 11-Ketotestosterone (11-KT) 3.9%, 5α-Androsten-3α, 17β-diol (A-3α-D) 2.4%, 5α-Androsten-3β, 17β-diol (A-3β-D) 1.9%, 11β-Hydroxyandrostendione (11β-HA) 0.3%, 17β-Estradiol (E2) <0.03%
1689	Friesen et al. 2012	African cichlid fish, (*Pseudocrenilabrus multicolor victoriae*)	Tank water	E2		20 pg/ml	intra-assay cv: 14%, inter-assay cv: 20%	
				T		6 pg/ml	intra-assay cv: 17%, inter-assay cv: 14%	
3583	Nash et al. 2000	Rainbow trout (*Oncorhynchus mykiss*)	Plasma	E2		1.6 pg	inter-assay cv: 8.9%; inter- assay cv: <10%	
				T		0.6 pg	inter-assay cv: 7.2%; inter- assay cv: <10%	Antiserum to T cross-reacted with 5α-DHT (46%), 5βdihydrotestosterone (19%), 5α-androstane-3α,17β-diol (3.7%), 11β-hydroxytestosterone (3.3%), 5α-androstane-3β,17βdiol (2.7%), 5β-androstane-3α,17β-diol (2.5%), 11-ketotestosterone (0.85%), estradiol (0.54%), 4-androstenedione (0.47%), 4-androstenetrione (0.31%) and 17,20βP (0.18%)
5839	Metcalfe et al. 2018	Red and black anemonefish (*Amphiprion melanopus*)	Plasma	E2	Spike recovery: 113.9±10.2%	LOD: 6.7 pg/ml; LOQ: 23.9 pg/ml	Inter-plate cv: 9.5; Intra-plate cv: 5.3, 13.7	spiked samples showed good specificity
		Barramundi (*Lates calcarifer*)	Plasma	E2	Spike recovery: 102.6±18.5%	LOD: 6.7 pg/ml; LOQ: 23.9 pg/ml	Inter-plate cv: 7.3 Intra-plate cv: 7.3, 7.9	spiked samples showed good specificity

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ref no.	Author/date	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection /assay range	coefficient of variation (CV)	Specificity
				T	Spike recovery: 110.2±8.7%	LOD: 3.8 pg/ml; LOQ: 13.4 pg/ml	Inter-plate cv: 6.5 Intra-plate cv: 4.8; 7.7	spiked samples showed good specificity
				11-KT	Spike recovery: 108.4±3.7%	LOD: 0.7 pg/ml; LOQ: 2.4 pg/ml	Inter-plate cv: 5.4 Intra-plate cv: 3.9; 7.7	spiked samples showed good specificity
		Common coral trout (*Plectropomus leopardus*)	Plasma	E2	Spike recovery: 94.6±4.6%	LOD: 6.7 pg/ml; LOQ: 23.9 pg/ml	Intra-plate cv: 13.8	spiked samples showed good specificity
				T	Spike recovery: 102.2±14.1%	LOD: 3.8 pg/ml; LOQ: 13.4 pg/ml	Intra-plate cv: 10.1	spiked samples showed good specificity
				11-KT	Spike recovery: 93.1±5.2%	LOD: 0.7 pg/ml; LOQ: 2.4 pg/ml	Intra-plate cv: 13.1	spiked samples showed good specificity
6129	Felix et al. 2013	Zebrafish (*Danio rerio*)	plasma	11-KT	-	-	intra-assay cv: 4.41%, inter- assay cv: 6.2%	-
			tank water	11-KT	-	-	intra-assay cv: 4.41%, inter- assay cv: 6.2%	-
6607[(a)]	Feswick et al. 2014	white sucker (*Catostomus commersoni*)	plasma	E	-	-	-	
				T	-	-	-	
				11-KT	-	-	-	
10183	Cuisset et al. 1994	Siberian sturgeon (*Acipenser baeri*)	plasma	11-KT		7 pg/ml	Intra-assay cv: < 10%; Inter-assay cv: 9.5%	11-KT 100%, T 6.8%, 11 β-Hydroxytestosterone 6.2%, 5β-Androstane 3β,17β-diol 0.33%, 5α-DHT 3.5%, 3α-Hydroxy-5β androstane-11, 17-dione 0.7%, Adrenosterone 1.2%; Rest: 4-Androstene 3,17-dione, 11 β-Hydroxy-4-androstene-3,17-dione, Cortisone, Cortisol, I l-Ketoprogesterone, I l β, 17α-Dihydroxy-4-pregnene-3,20 dione all < 0.01%"
17968	Ohta et al. 2001	Bambooleaf wrasse	serum	E1	-	Sensitivity: 12.5 pg/ml	Intra-assay cv: 4.8%; inter-assay cv: 11.0%	cross reactivities for E1: E2, 5%; estriol, 1.2%; estrone-3-sulfate, 0.4%; estrone-3-

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Ref no.	Author/date	Species	Matrix	Hormone	Extraction efficiency	Limit of Detection /assay range	coefficient of variation (CV)	Specificity
		(*Pseudolabrus sieboldi*)						glucuronide, 0.15%; 2-methoxyestrone, 0.05%; P5, 0%; progesterone, 0%; cortisol, 0%; cortisone, 0%; AD 0%; T, 0%; DHEA, 0%; 5α-dihydrotestosterone, 0%
				T	-	Sensitivity: 9.5 pg/ml	Intra-assay cv: 11.3%; inter-assay cv: 16.6%	cross reactivities for T: 5α-dihydrotestosterone, 7.3%; AD, 2.1%; androsterone, 0.28%; 5-androstene-3β,17β-diol, 0.15%; 5α-androstane-3α,17β-diol, 0.10%; 5β-androstane-3α,17β-diol, 0.09%; cortisol, 0.02%; corticosterone, 0.01%; progesterone, 0.01%; P5, <0.01%; 17-hydroxypregnenolone (17-P5), <0.01%; aldosterone, <0.01%; DHEA, <0.01%; E2, <0.01%.
18822	Clark et al. 2011	Coho salmon (*Oncorhynchus kisutch*)	plasma	E2	-	-	-	-
				T	-	-	-	-
5476[b] TR- IA	Yamada et al. 1997	Rainbow trout	Serum	T		1.5 pg/ml	Intra-assay cv: 1.62-6.38%; Inter-assay cv: 2.96-8.29%	Cross-reaction with 11KT <0.2%. Cross-reaction of estradiol-17b, progesterone, 17a-hydroxyprogesterone, and cortisol with antiserum was satisfactorily low (<0.01%), except for with 5a-dihydrotestosterone (5.8%)

(a): Ringtest, comparing performance of different labs and therefore included;

(b): TR-FIA study, not ELISA

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

B.2.3.2. Hypothalamic–pituitary–thyroid axis hormones

The literature search retrieved only one study where thyroid hormones were measured by ELISA in fish.

The study measured T3 and T4 by ELISA in plasma from juvenile fish from two different species, Sea Bass (*Dicentrarchus labrax L.*) and Sea Bream (*Sparus aurata L.*) (CerdaReverter et al. 1996, reference 9522 in Annex B –). *D. labrax* plasma samples were pooled before analysis, whereas *S. aurata* samples were tested individually. The method used a minimum of 100 µl plasma or diluted plasma, which was extracted with methanol (3 extraction steps), before being evaporated and resuspended in EIA buffer. T3 and T4 were determined using standard ELISA procedures. The portion of the standard curves ranging to 15–85% of binding corresponds to concentrations from 1.25 to 0.02 ng/ml (62.5–1 pg/well) and 6.25 to 0.2 ng/ml (312.5–10 pg/well) for T3 and T4. The authors concluded that the assay had a comparable degree of sensitivity to RIAs for T3 and T4.

Forward citation searches

By investigating some of the citations for the above publications we can see how well these methods have been adopted by the research community. The method for improving sex-steroid extraction from plasma for RIA detailed in Newman et al. (2008) has been used (cited) by researchers working primarily with bird tissues (e.g. Taves et al. 2010, Shah et al. 2011, Fokidis et al. 2013; Dickens et al. 2014, Heimovics et al. 2016, Prior et al. 2017, Merrill et al. 2019), although it has also been employed with lizard (Zena et al. 2019), snakes (Rensel et al. 2015), fish (Lorenzi et al. 2012) and mouse (Overk et al. 2013) samples. There was also some cross-over with many of the papers which cited the SPE extraction methods of Newman et al. also citing Charlier et al. (2010) SPE method for extracting steroids from tissues (brain) for RIA or Chao et al. (2011) liquid-liquid plus SPE method for plasma and tissues extraction for ELISA.

B.3. Chemical analysis for measuring hormones

Rapid recent developments in analytical chemistry instruments have enabled the detection and quantification of ever lower concentrations of compounds of interest. The more common methods of instrumental chemical analysis employ chromatography, more specifically liquid or gas chromatography. Accordingly, when screening studies for eligibility, manual methods such as thin layer or column chromatography that have now been superseded by instruments were not considered further unless they reported particularly salient information about sampling procedures or sample preparation.

The principles of chromatographic separation rely on differences in affinity of different compounds in a sample between two phases; a stationary phase that remains fixed in place while the mobile phase carries the components of the mixture through the medium being used. The stationary phase acts as a constraint on many of the components in a mixture, slowing them down to move slower than the mobile phase. The rapidity of the transport of the various components in the mobile phase depends on their interactions with the mobile and/or stationary phases. Some compounds move faster than others, thus facilitating the separation of the components within that mixture.

Gas chromatography (GC)

Gas chromatography refers to analytical separation techniques used to analyse **volatile** substances in a gas phase; the components of a sample are dissolved in a solvent and vaporised in order to separate the analytes. The mobile phase is a chemically inert gas that serves only to carry the molecules of the analyte through the **heated column** and as such GC is one of the sole forms of chromatography where the mobile phase does not interact with the analyte. The stationary phase is either a solid adsorbant or a liquid on an inert support. The latter gas-liquid chromatography is the method most commonly used to separate organic compounds. For non-volatile and/or thermolabile compounds, such as hormones, an additional step in sample preparation termed **derivatisation** is necessary to modify the chemical structure of the compounds such that they can be analysed by the technique.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

GC columns are coupled with a detector. In theory, any property of the analytes that is different from the carrier gas can be used as a detection method. In modern instruments, the sensitivities of the detectors are in the range of 10^{-8} to 10^{-15} g of solute per second.

High Performance Liquid Chromatography (HPLC)

HPLC is an analytical technique used for the separation of compounds **soluble in a particular solvent**, developed as some of the shortcomings of standard liquid chromatography were overcome. Columns can be packed with solids such as silica or alumina or a liquid stationary phase bonded to a solid support, usually silica or alumina. The mobile phase, or solvent, is typically a mixture of polar and non-polar liquids whose composition depends on the composition of the sample. In **normal phase** HPLC, the stationary phase is more polar than the mobile phase whereas if the stationary phase is less polar than the mobile phase, the separation is **reverse phase**. Normal phase columns are generally packed with alumina or silica whilst alkyl, aliphatic or phenyl bonded phases are used for reverse phase separation. When the composition of the mobile phase is maintained constant throughout the HPLC separation, the separation is referred to as an **isocratic elution**. It may be necessary to vary the ratio of polar to non-polar compounds in the mobile phase during analysis in order to elute all of the compounds in the sample in a reasonable amount of time, while still maintaining peak resolution, particularly if the analytes within a sample exhibit a wide range of polarities. This is referred to as **gradient** chromatography.

The HPLC detector must record the presence of various components of the sample, but not detect the solvent. Most medium to large molecules absorb UV radiation and UV absorption detector were commonly applied to HPLC.

Mass spectrometry (MS)

Mass spectrometers are popular detectors coupled with both GC and HPLC. They can create charged particles (ionisation) from minute quantities of samples. Magnetic and/or electric fields may (or may not) then be applied to these charged ions and depending on their trajectory, a mass analyser can determine the mass/charge ratios of these ions and thereby provide information about the molecular weight of the compound and its chemical structure. There are many types of mass spectrometers with a variety of ionization techniques (e.g. electrospray (ESI) and atmospheric pressure chemical ionization (APCI)), ionization modes (e.g. negative ion (NI) and positive ion (PI)) and monitoring modes (e.g. selected ion monitoring (SIM)) which allow a wide range of analyses.

It is possible that several analytes could potentially have the same m/z ratio as the analyte of interest. Therefore, in order to increase both specificity and sensitivity, modern instruments often couple two mass spectrometers (MS-MS), able to analyse first a precursor ion and a further fragment ion to add selectivity to the analysis. This technology has allowed the development of very specific and sensitive analytical methods.

GC-MS refers to gas chromatography coupled with mass spectrometry. LC-MS and LC-MS/MS refer to HPLC coupled to one or two mass spectrometers, respectively.

B.3.1. Measuring Hormones in Amphibians

B.3.1.1. Hypothalamic–pituitary–gonad axis hormones

The literature search retrieved two references describing an analytical chemistry method to measure steroid estrogens in amphibians. Mensah-Nyagan et al. (1996) coupled HPLC separation with GC/MS characterisation to detect testosterone in brain samples of the Marsh frog (reference 3309 in Annex B –). Because the method described was designed to uncover mechanistic information about brain steroidogenesis and interested in detecting rather than quantifying testosterone, it will not be discussed further.

Chang et al. (2010) however did quantify the estrogens (E1, 17β-E2, 17α-E2) in African clawed frogs (*Xenopus laevis*), (see reference 1407 in Annex B –). This liquid chromatography–electrospray tandem mass spectrometry (LC–ESI-MS/MS) was developed specifically to enable the simultaneous

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

quantification of not only steroid estrogens but also a variety of phenolic compounds, including halogenated contaminants.

It was applied to 0.3-1ml samples of frog plasma. The method employed a derivatisation step with to increase sensitivity of the method for steroid estrogens and further allow simultaneous determination of up to 30 phenolic compounds. Additionally, the method required both a liquid-liquid extraction (prior to derivatisation) and silica gel chromatography purification steps to reduce matrix effects in the sample extract and remove excess derivatising reagents. The authors report accuracies between 95% and 101%.

According to the reported linear calibration range, the method is applicable for concentrations between 0.5–1000 ng/mL. The LOQs were 4, 3 and 2 pg/g for E1, 17β-E2 and 17α-E2, respectively. With regards to precision, the intrabatch and interbatch CV reported were all ≤ 12%.

This LC-ESI-MS/MS method was also validated in a bird species. A citation search (65 citations on Web of Science) revealed that the method has been applied in fish to measure 17β-E2 in transgenic male Japanese medaka exposed to dichlorodiphenyltrichloroethane (DDT) and its metabolites (Sun et al. 2016) and to measure both estrogens and androgens in female rainbow trouts exposed to selenomethionine (Wiseman et al. 2011). The method has also been applied to the clinical setting, to environmental samples and *in vitro* in the H295R steroidogenesis assay.

B.3.1.2. Hypothalamic–pituitary–thyroid axis hormones

The literature search retrieved three references reporting on analytical chemistry methods employed to measure thyroid hormones in amphibians, all based on liquid chromatography. Simon et al. (2002) measured iodide, the active hormones T4 and T3, as well as (inactive) reverse T3 (rT3), and the synthetic precursors of TH, monoiodotyrosine (MIT), and diiodotyrosine (DIT) in whole body homogenates of tadpoles of African Clawed frogs (*Xenopus laevis*) using liquid chromatography inductively coupled plasma mass spectroscopy (LC-ICP-MS) (reference 8692 in Annex B –). Luna et al. (2013) used ultrahigh-performance liquid chromatography and isotope dilution tandem mass spectrometry (UPLC-ID-MS-MS) to quantify total T4 in a small volume (10 µL) of plasma also from tadpoles and adult *Xenopus laevis* (reference 3463 in Annex B –). Hansen et al. (2016) validated a LC-MS/MS method to quantify absolute and total concentrations of eleven thyroid hormones and associated metabolites in both 50 µL plasma of adult *Xenopus* or 50 µL serum of tadpoles (American bullfrog, *Rana (Lithobates) catesbeiana*) (reference 8142 in Annex B –).

The method by Simon et al. (2002) differed from the other two both with regards to the sampling matrix, and therefore sample preparation, and the instrumentation (use of the LC-ICP-MS as opposed to the more selective LC-MS/MS methods). According to the method as reported, after homogenisation in buffer and centrifugation, the pooled samples were simply digested with pronase E to destroy thyroid-hormone binding to carrier proteins before LC-ICP-MS analysis. The performance of the method (accuracy, precision, sensitivity) that had been applied in both tadpoles and zebrafish is not reported or discussed by the authors. A citation search (24 citations on Web of Science) did not discover any further application of the described method in amphibians or fish.

The LC-MS/MS methods described by Luna et al. (2013) and Hansen et al. (2016) do not only differ in their scope. The method described by Luna et al. (2013) allowed quantification of T4 in smaller plasma sample volumes, that as a result did not need to be pooled even when handling tadpoles. By contrast, Hansen et al. (2016) were able to avoid pooling tadpole serum samples by focusing on a larger species, the American Bullfrog. A further important difference between the two methods is related to the preparation of the samples, more specifically, the step necessary to release the bound thyroid hormones from plasma proteins; to denature proteins, Luna et al. (2013) employed acid hydrolysis prior to SPE whereas on the strength of their prior stability investigation, Hansen et al. (2016) opted for strong basic conditions (1 % ammonia hydroxide) in combination with the chaotropic agent urea and heat.

As detailed in Table 16: , while the method by Hansen et al. (2016) requires higher sample volumes, overall it was shown to perform better in terms of its absolute recoveries, sensitivity and precision in

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

addition to allow the quantification of a wide range of relevant thyroid hormones and related compounds.

Table 16: Comparison of LC-MS/MS methods to measure T4 in the plasma or serum of amphibians

Reference	Sample volume	Sample preparatio n	Linear range	Recovery	Accuracy	LOD	LOQ	RSD
Luna et al. 2013 (3463 in Annex B –)	10 µL	Acid hydrolysis followed by SPE	2-100 ng/mL	6-42%	88-129%	0.20 ng/ml	0.5 ng/ml	Intraday 5% Interday 10%
Hansen et al. 2016 (8142 in Annex B –)	50 µL	Urea and heat followed by SPE	0.5-/20 pmol/mL	89.40%	100-159%	70 pg/ml	0.27 pmol/ml	Intraday 2.36% Interday 3.94%

Citation analyses revealed that Luna et al. (2013) had been cited 3 times (Web of Science), including by Hansen et al. (2016), and that their sample preparation method has also been applied *in vitro* in tadpole thyroid gland explant culture experiment as well as *in vivo* in tadpoles (Hornung et al. 2015). A citation search (cited 20 times according to Web of Science), revealed that the method has been cited by authors developing their own methods in non-mammalian species (e.g. egg yolk, zebrafish embryos or sea lampreys, included in the corresponding sections of this report) as well as in the clinical and mammalian settings. To date, only its application in the white whale has been reported in the literature (Hansen et al. 2016).

B.3.2. Measuring Hormones in Birds

B.3.2.1. Hypothalamic–pituitary–gonad axis hormones

Androgens

Androgens have been measured in variety of avian species and from different sampling matrices including plasma or serum from blood, brains, egg yolk but also non-invasive sample matrices such as feathers. Notably, no chemical analytical method has been described for fecal samples in the retrieved literature. Of the ten studies identified by our systematic search of the literature as related to the measurement of androgens in birds, seven will be discussed in more detail in this section. Culbert (1973) describes extraction and purification on a florisil column for steroid hormones from avian plasma (reference 5287 in Annex B –), we would expect such sample preparations steps to have been further optimised since 1973 and the performance of the method is not discussed further. Similarly, Jallageas and Attal (1968) describe early efforts with gas chromatography and we would expect the performance of modern instruments and more recent methods to have been optimised since 1968 (reference 17615 in Annex B –). Fokidis et al. (2019) (reference 7869 in Annex B –) actually applied the method developed by Prior et al. (2016) (reference 8141 in Annex B –) and only the latter is discussed in more detail in this section.

Endogenous male and female hormones are potent growth promoters whose use in farming, including poultry farming, is prohibited in the European Union (Union, The European Parliament and the Council of the European 2008). Enforcement has required the use of validated method to ensure compliance to measure hormone levels in meat or muscle tissue but also the serum of farmed animals as described by McDonald et al. (2010) (reference 10281 in Annex B –). The LC-MS/MS method is able to detect both testosterone and epitestosterone, requires one SPE preparation step and is validated in accordance with Commission Decision 2002/657/EC and ISO/IEC 17025:2005 in poultry in turkey serum. It required a 5ml sample volume which would be a limited factor if investigating smaller bird species. Method performance is detailed in Table 17: . The citation search (4 citations according to Google Scholar) did

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

not reveal any evidence that the method has been applied in a non-mammalian ecotoxicology testing context.

Koren et al. (2012b) (reference 12969 in Annex B –) and Prior et al. (2016) (reference 8141 in Annex B –) describe LC-MS/MS method developed to measure hormones in much smaller volumes of avian plasma. Koren et al. (2012b) validated sample preparation for simultaneous quantitation of not only testosterone and dehydroepiandrosterone (DHEA) but also E2, corticoids and progestagens in five avian species (European starlings, zebra finches, black-browed albatross, house sparrows and song sparrows) using 100 µL of serum or plasma. The method does not require protein precipitation, hexane is used to remove lipids during the washing step. The SPE step was automated allowing high-throughput. The citation search (36 citations in Scopus) revealed that the method has been applied in ecology to investigate wild birds (Crossin et al. 2013) and in aquaculture (Guzmán et al. 2015). Prior et al. (2016) adapted a method used in the clinical setting to avian plasma to examine a total of eight androgens and progestins. The method requires a liquid-extraction step and a derivatisation step but allowed the analysis of smaller samples (~ 30 µL). Reported details of the performance of the assay can be found in Table 17: . The method has been cited 4 times and applied by the same research group in birds (Fokidis et al. 2019; Prior et al. 2017).

Because obtaining blood samples from wildlife is challenging, and hormones levels can change within a few minutes and is affected by the stress of capture, non-invasive sampling methods have been developed with e.g. faeces and feathers. This systematic evidence map uncovered two LC-MS/MS methods to measure androgens in feathers. Koren et al. (2012a) (reference 2229 in Annex B –) determined testosterone and corticoids in the feathers of wild house sparrows, whereas Bílková et al. (2019) (reference 7209 in Annex B –) recently optimised an analytical method that also simultaneously determines testosterone and cortisone from feather samples collected from the flanks of seven different species of passerine birds. A significant difference between the two methods is an additional derivatisation step in the latter that aims to address some reliability issues with cortisone measurements, whilst SPE could be automated in the former. As can be seen in Table 17: , the sensitivity of the method described by Bílková et al. (2019) compares favourably with that of the Koren et al. (2012a) method.

Mi et al. (2014) (reference 18009 in Annex B –) describes a method to measure simultaneously a total of 26 steroids, including testosterone and five other androgens in eggs from hen, quail, duck and pigeon. They also cite two other methods to measure steroid hormones levels in eggs, also developed for food safety purposes; one using GC-MS (Hartmann et al. 1998), the other using LC-MS/MS was the first to implement $ZNCl_2$ precipitation of the high levels of phospholipids found in eggs prior to SPE (Wang et al. 2010). Mi et al. (2014) usefully compare the limits of detection of analytical methods available at the time of writing with the range of concentrations of steroids, including testosterone, with the naturally occurring ranges reported for hen eggs. It becomes evident that minima for androgens and estrogens overlap with LOQs and it is unclear whether these methods could be usefully applied in an ecotoxicology testing context. A citation search (12 citations in Scopus) did not discover any evidence that such methods designed for food safety purposes were sensitive enough to be applied to avian toxicology investigations.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 17: Comparison of LC-MS/MS methods to measure testosterone in avian samples

Reference (number in Annex B –)	Matrix	Sample amount	Sample preparation	Recovery	Accuracy	LOD	LOQ	Precision
Koren et al. 2012a (2229)	feathers	21–84 mg	liquid extraction & SPE				0.1 ng/ml	
Bílková et al. 2019 (7209)	feathers	25 mg	Solvent extraction, SPE clean-up. Derivatisation	61-81%		1.0 pg/ml	3.3 pg/ml	RSD: 3-12%
Mi et al. 2014 (18009)	Egg yolk	2g	Solvent extraction, SPE			0.015 ng/g	0.050 ng/g	3.4-4.8%
Prior et al. 2016 (8141)	plasma	~ 30 µl	Solvent extraction, derivatisation	> 80%		0.01 - 0.02 ng/mL		
Koren et al. 2012b (12969)	plasma	100 µl	SPE	T: 87%			0.1 ng/ml	Intraday CV: 5.70% Interday CV: 7.18%
McDonald et al. 2010 (10281)	serum	5 ml	SPE		Trueness: -1.4%	CCα: 0.04 µg/l	CCβ: 0.06 µg/l	Repeatability within lab: 13%

Oestrogens

By contrast with androgens, the systematic literature search retrieved several methods developed to measure estrogens in bird **faeces**, but none in feather samples. All three analytical chemistry methods described here were developed in China and the motivation for measuring hormone levels in livestock manure appears to be related to both the existence of concentrated animal feeding operation (CAFOs) and the risk that estrogens could be discharged into the aquatic environment via surface runoff from both land application or composting. Fu et al. (2013) (reference 9676 in Annex B –) and Lu et al. (2014) (reference 16886 in Annex B –) describe similar HPLC method coupled with fluorescence detection that appeared to perform comparably on the basis of available information (Table 18:). The rationale given for selecting fluorescence detection rather than mass spectrometry was related to the complexity of faecal matter as a sampling matrix and the susceptibility of the MS detector to matrix effects. Fluorescence is a sensitive detection method that can be used for hormone analysis to enable sufficiently low detection limit, and a more practical level it requires less stringent clean-up rendering high-throughput possible. Zhang et al. (2014) (reference 15305 in Annex B –) describe a LC-MS/MS analytical method to quantify free estrogens (E1, 17β-E2, E3) and conjugated estrogens (estrone-3-sulfate sodium salt (E1-3S), 17β-estradiol-3- sulfate sodium salt (E2-3S), estrone-3-glucuronide sodium salt (E1-3G), and 17β-estradiol-3-glucuronide sodium salt, (E2-3G)). The method does require a 5-step sample preparation and clean-up but does achieve 10-fold lower sensitivity (Table 18:).

Methods to measure estrogens in plasma or serum invariably apply LC-MS/MS analytical methods. The method applied by Chang et al. (2010) (reference 1407 in Annex B –) in the plasma of African clawed frogs and described in detail in section B.3.1.1 was also applied in double-crested cormorants. There were reported species difference with regards to methodological performance (Table 18:). The LC-MS/MS methods developed by Koren et al. (2012b) (reference 12969 in Annex B –) to determine multiple steroids in avian plasma and by McDonald et al. (2010) (reference 10281 in Annex B –) to determine

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

hormones in livestock animal serum have been discussed in detail with respects to androgens in this section. Their performance for E2 is given in Table 18: . They present the distinct advantage of allowing simultaneous quantification of both estrogens and androgens.

Wang et al. (2011) (reference 6327 in Annex B –) constructed pseudo molecularly imprinted polymers (PMIPs) which they used as dispersive solid-phase extraction (DSPE) materials as the sample preparation step before HPLC analysis with UV detection. They applied their method to muscle/tissue samples including chicken meat. The systematic literature search retrieved an earlier HPLC-UV method to measure estrogens in meat samples whose performance has not been considered in detail in this report (Medina and Sherman 1986). It is unclear whether such technological advances in separation are commercially and economically available at this present time.

Finally, two methods were concerned with estrogens in egg samples, including the method of Mi et al. (2014) (reference 18009 in Annex B –) already discussed in the previous section on androgens (Table 18:). The said LC-MS/MS method, in addition to testosterone and five other androgens, can simultaneously measure E1, E2 and E3 in eggs from hen, quail, duck and pigeon. Its performance is detailed in Table 18: . Additionally, Azzouz and Ballesteros (2015) (reference 1688 in Annex B –) have described a less sensitive GC-MS that required an additional derivatisation step designed to measure E1 and E2 in addition to 20 pharmaceuticals in egg samples.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 18: Comparison of analytical chemistry methods to measure E2 in avian samples

Reference (number in Annex B –)	Sampling matrix	Sample amount	Sample preparation	Method	Linear range	Recovery	Sensitivity	Precision
Fu et al 2013 (9676)	faeces	-	Ultrasonic extraction & SPE	HPLC	1. 00 ~1000 µg/L	86%	LOD: 2.13 µg/kg	RSD: 2.2-5.7%
Lu et al 2014 (16886)	faeces	5g	Ultrasonic extraction & SPE	HPLC		73 ± 15%	LOD: 4 µg/L	
Zhang et al 2014 (15305)	faeces	1g	liquid–liquid extraction, Florisil cleanup, aqueous alkali extraction, hydrophilic–lipophilic balance (HLB) enrichment, NH2 cartridge clean-up	LC-MS/MS		67±2.4%	LOD: 0.2 µg/kg	repeatability (RSD): 2-20%
Wang et al 2011 (6327)	Muscle/ tissue	5g	used pseudo molecularly imprinted polymers (PMIPs) as dispersive solid-phase extraction (DSPE) materials	EE2-PMIPs–DSPE coupled with HPLC–UV	0.1–1.0µmol L^{-1}	90.3-95.2%	LOD: 5.4 µg/L	RSD: 2.5-5.7%
Azzouz et al 2015 (1688)	Egg	2g homogenised egg	Solvent extraction, SPE, derivatisation	GC-MS	10-10,000 ng/kg	96-99%	LOD: 2.6 ng/kg; LOQ: 8.0 ng/kg	Intraday CV: 5.5%; interday CV: 6.1%
Mi et al 2014 (18009)	Egg	2g	Solvent extraction, SPE	LC-MS/MS	0.5–500 ng/mL	85.6-87.9%	LOD: 0.075 ng/g; LOQ: 0.250 ng/g	0.3-0.5%
Chang et al 2010 (1407)	plasma	0.3–1 mL	extraction, derivatisation with dansyl chloride, silica gel chromatography	LC-MS/MS	0.5–1000 ng/mL	99-101%	3 pg/g	Intrabatch CV: 5.3-9.8%; Interbatch: 6.6-12%
Koren et al. 2012b (12969)	plasma	100 ul	SPE	LC-MS/MS		94%	LOQ: 0.1 ng/ml	intraday CV: 7.89%; interday CV: 4.49%
McDonald et al 2010 (10281)	serum	5 ml	SPE	LC-MS/MS	0–2.0 µg/L	Trueness: 0.6%	CCα: 0.02 µg/l; CCβ: 0.04 µg/l	Repeatability within lab: 16%

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Steroidogenesis

(Liere et al. 2019, reference 3788 in Annex B –) adapt a GC-MS/MS method developed by the same group to measure steroids in rat brain to quail brains. They measured a wide range of estrogens, androgens and their precursors or conjugated forms including 13 sex steroids in order to investigate brain steroidogenesis in both brains and plasma. Important points to note include the fact that birds were rapidly killed by decapitation without anaesthesia to avoid potential changes in steroid concentrations. They reported detection limits of 0.002 ng/mL for both E2 and T in plasma, which compares favourably to LC-MS/MS methods (Table 17: , Table 18:) but the method required a larger sample volume (1mL). The LODs for telencephalons and pre-optic area were 0.005 ng/g and 0.05 ng/g for both T and E2, respectively.

B.3.2.2. Hypothalamic–pituitary–thyroid axis hormones

The systematic evidence map retrieved only two references that were related to chemical methods to measure thyroid hormones in avian samples. (Wang et al. 2015) (reference 7269 in Annex B –) present a LC-MS/MS method suitable to determine T4, T3 and rT3 in 5g of chicken meat and chicken egg samples. They report detection limits below 0.04 ng/g. It has only cited once (CrossRef) and there is no evidence that the method would be suitable for application in the ecotoxicology setting as opposed to food safety.

By contrast, the study of Ruuskanen et al. (2018) (reference 11791 in Annex B –) presents an extremely sensitive nanoflow LC-MS/MS method specifically suited for toxicological research in small animals and small sample volumes. They applied their method in 5-400mg egg yolk samples from five both domestic and wild bird species. They report limits of detection in the attomolar range (10^{-18} mol).

B.3.3. Measuring Hormones in fish

B.3.3.1. Hypothalamic–pituitary–gonad axis hormones

Androgens

Most methods to measure androgens in fish retrieved by our literature search have been developed to measure hormones in the plasma. In the 1980s and 1990s, both HPLC (Huang et al. 1983; Khan et al. 1997) (references 16495 and 10755 in Annex B –, respectively) and GC-MS (van Dam et al. 1989) (reference 6384 in Annex B –) were already in use. They did, however, require 5-8ml of plasma. Validated test guidelines tend to use small fish such as the fathead minnow, from which usually a maximum of 20 µL plasma can be obtained from an individual fish.

For both analytical methods, LODs in the low ng/mL can now be achieved with as little as 10 µL of plasma (Table 19:). Focusing first on GC methods, the method developed by Budzinski et al. (2006) (reference 179 in Annex B –) was able to measure five androgens, five progestins and two estrogens in fish plasma cans also applied in fish bile. This specific sampling matrix however requires an additional preparation step; enzymatic hydrolysis deconjugation of the conjugated hormones before the first SPE step. A very similar method was applied by the same research group in the turbot exposed to EE2 and gonadal tissue in addition to plasma and bile were analysed (Labadie and Budzinski 2006) (reference 5959 in Annex B –). This latter study demonstrates the usefulness of the assay in the ecotoxicological testing setting, exposure to EE2 in this case representing a positive control for an estrogenic mode-of-action. Noaksson et al. (2004) (reference 1880 in Annex B –) applied a method originally developed for the measurement of hormones in meat samples (Hartmann and Steinhart 1997) to measure androstenedione and testosterone in adult female Perch. This research group later expanded the scope of the androgens (and estrogens) that could be measured with the method to include eight androgens and they further optimised the method and sample preparation in order to achieve LODs in the pg/g range (Widell et al. 2011) (reference 9025 in Annex B –). Their method still requires relatively large samples. Whilst Margiotta-Casaluci et al. (2013) (reference 5987 in Annex B –) describe a less sensitive GC-MS/MS method to measure androgens, their method is able to handle smaller sample volumes of 200 µl. Margiotta-Casaluci et al. (2013) has been cited 20 times (Scopus) in the context of fish

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

ecotoxicology. However, citing work makes specific reference to the scientific findings of the study rather than adopt the analytical method to measure androgens. When citing papers have measured androgen levels, they did so using ELISA kits or LC-MS/MS. Widell et al. (2011) has been cited once and the focus of the citing work was corticosteroids.

Yang et al. (2006b) (reference 1566 in Annex B –) developed a manual solid-phase microextraction (SPME) with on-fibre derivatisation procedure as sample preparation step for GC-MS that they applied to the measurement of T and DHEA in the serum of female carps. SPME is an extraction technique using a fused-silica fibre coated outside with an appropriate stationary phase that has a number of advantages in terms of simplicity, cost and solvent-free. This research group went on to develop a fully automated method amenable to high-throughput that achieved better accuracy and precision (LOQ = 0.413 ng/ml for T) (Yang et al. 2006a). Both works have been cited 116 (Scopus) and 38 times, respectively, and the technology is evidently attracting much interest.

With regards to liquid chromatography, LC-MS/MS methods achieve similar LODs compared with GC-MS or GC-MS/MS. Although Zhang et al. (2009) (reference 986 in Annex B –) and Hala et al. (2011) (reference 6556 in Annex B –) employed different sample preparation methods (although both included a derivatisation step) and ionisation techniques for MS detection, the performances of both approaches is broadly similar (Table 19:). More recently, researchers have developed very sensitive LC-MS/MS methods with streamlined sample preparation in the Sea lamprey (Wang et al. 2016) (reference 3228 in Annex B –) and Stingrays (Lyons and Wynne-Edwards 2019) (reference 77 in Annex B –). The method by Wang et al. (2016) was also applied to tissue samples (rope tissue and testes) to simultaneous determine 16 steroids (including 8 androgens), whilst the method developed by Lyons and Wynne-Edwards (2019) was applied to uterine fluid to determine 13 steroids (including 4 androgens). Blasco et al. (2009) (reference 15744 in Annex B –) achieved similarly low LOQ (0.2 ng/ml for T) with a LC-MS method to simultaneous quantify testosterone, 11-ketotestosterone and 11-β hydroxyandrostenedione in Goldfish serum, although the volume of sample necessary is unclear.

Analytical and adapted sample preparation methods for the quantitative analysis of androgens in fish have also been described in sampling matrices other than those already described. In particular, methods have been developed to enable the enforcement of food safety requirements and residues of natural and synthetic hormones in animal products intended for human consumption, including fish meat. Wang et al. (2012) (reference 5352 in Annex B –) report efforts related to sample preparation method with the aim of increasing sample throughput and reducing the consumption of the organic solvent. They describe a dynamic microwave-assisted extraction coupled with salting-out liquid-liquid extraction as pre-treatment to LC-MS/MS analysis that achieved sensitivities in the low ng/g tissue range.

Zebrafish (*Danio rerio*), for a number of reasons including its relatively short lifecycle, is a prominent animal model in both human toxicology and ecotoxicology. However, its small size can make the measurement of certain endpoints challenging, including the volume of plasma available for the measurement of circulating hormones. For these reasons, methods developed for whole body homogenates are of particular interest. Yang et al. (2015) (reference 3467 in Annex B –) developed a method based on ultrahigh performance LC-MS/MS that allowed the simultaneous determination of 26 EDCs (including five estrogens, eight androgens, and two thyroid hormones) in fish and water was developed and applied to whole body homogenates of zebrafish. The method performed adequately in term of accuracy and precision and achieved limits of detection in the ng/g range. The interpretation of the biological significance of any changes in measured levels of hormones in whole body homogenate may however be complicated by the fact that such measurements do not allow the localisation of hormones either in specific organs or the circulation.

Estrogens

Chemical analysis of steroid estrogens in fish plasma is typically done simultaneously with determination of androgens. Most methods described in the previous section on androgens also measure E1 and E2 and sometimes E3. The performance of these method for estrogens is detailed in Table 20:). The only method that specifically targeted estrogens is a relatively recently published LC-MS/MS method that

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

uses a dansyl derivatisation step and achieves sensitivities in the pg/ml range (Bussy et al. 2017a) (reference 2261 in Annex B –).

The situation is similar for the analysis of serum samples. The manual and automated SPME methods with on-fibre derivatisation coupled with GC-MS described in the previous section on androgens are also able to determine estrogens simultaneously (Yang et al. 2006b; Yang et al. 2006a) (reference 1566 in Annex B –). Our search did however retrieve a reference describing a novel electrochemical sensor for E2 from molecularly imprinted polymeric microspheres and multi-walled carbon nanotubes grafted with gold nanoparticles (Futra et al. 2016) (reference 18041 in Annex B –). This ultrasensitive sensor was developed for the aquaculture industry to measure E2 in fish sera in order to determine the sex of the animals. It required very low sample volumes (9µl) and boasted extremely sensitivity ($2.5*10^{-16}$ M) but little other information about the method performance was reported, e.g. in terms of specificity, accuracy and precision.

Another important difference between the chemical analysis of estrogens and androgens in fish is the number of methods that have focused on sampling matrices other than plasma or serum. Whereas the systematic evidence mapping exercise discovered one or at most two further methods that had considered androgens in bile or whole-body homogenates, there were 5 and 4 additional methods for estrogens in these matrices, respectively. Some details about methods to determine estrogens in fish bile, fish tissue or whole-body homogenates are given in Table 21: . For fish bile samples, the species in which such investigations have been carried out is noteworthy as this is a matrix favoured in wildlife species for eco-epidemiological investigations and there are doubts over the feasibility of using this sampling matrix in smaller species as typically favoured in ecotoxicological investigations. Similar concerns arise in relation to methods measuring estrogens in tissue samples. Such methods have been designed to respond more specifically to the needs of the food industry, as previously mentioned. An interesting aspect nonetheless of many of these published methods is their focus on efficient and when possible fully automated sample preparation techniques.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 19: Comparison of the performance of recent analytical chemistry methods to measure T in fish plasma

Reference (number in Annex B –)	Androgen	Species	Sample amount	Sample preparation	Method	Recovery	Sensitivity	Precision
Noaksson et al. 2004 (1880)	T	Perch (*Perca fluviatilis*)		Protein precipitation, lipid reduction by sequential extraction with n-hexane plus SPE, NH2-phase clean-up. Derivatisation, Liquid-liquid extraction	HRGC/HRMS		LOD: 0.28 ng/g	intraassay CV < 10%
Budzinski et al. 2006 (179)	T, A, KT, DHA, DHT	Rainbow trout and European flounder	1g	C_{18} SPE extraction, NH_2 SPE purification, derivatisation	GC-MS	101±3%	LOD: 0.1 ng/g	Intraassay CV <10%
Labadie and Budzinski 2006 (5959)	T, KT, DHT, DHA, A, DHEA	Turbot (*Psetta maxima*)	1g	Extraction, fractionation, purification and conjugate cleavage, derivatisation	GC-MS	121 ± 1 %	LOD: 0.1 ng/g	
Widell et al. 2011 (9025)	T, KT, A, DHT, OHT, DHEA, OHA, Adiol	Perch (*Perca fluviatilis*)	0.5–1 mL	Protein precipitation, lipid reduction by sequential extraction with n-hexane plus SPE, NH2-phase clean-up. Derivatisation	HRGC/HRMS	101±2%	LOD: 0.002 ng/g; LOQ: 0.01 ng/g	Intraassay CV: 2%
Margiotta-Casaluci et al. 2013 (5987)	T, DHT	Fathead minnow (*Pimephales promelas*)	200 µL	Enzymatic hydrolysis, solvent extraction, SPE, liquid-liquid extraction, SPE	GC-MS/MS		LOD: 0.05 ng/mL; LOQ: 0.10 ng/mL	
Khan et al. 1997 (10755)	T, KT, A, DHT, OHT, OHA & 3 more	Arctic Charr (*Salvelinus alpinus*)	5ml	Urea and heat, SPE, deconjugation, SPE	HPLC	> 94%		
Zhang et al. 2009 (986)	T, KT	Fathead minnow (*Pimephales promelas*)	10 µL	Derivatisation with dansyl chloride	LC/APPI-MS/MS	89.2-98.7%	LOQ: 1 ng/mL	Intraassay RSD: 2.4-11.4%; Interassay RSD: 2.6-8.6%
Hala et al. 2011 (6556)	KT	Fathead minnow (*Pimephales promelas*)	20-25µL	Liquid-liquid extraction, derivatisation	LC-ESI+/MS/MS	89-129%	LOD: 1.25 ng/ml	12-49%

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Reference (number in Annex B –)	Androgen	Species	Sample amount	Sample preparation	Method	Recovery	Sensitivity	Precision
Wang et al. 2016 (3228)	T, KT, DHT, DHEA, A & 3 more	Sea lamprey (*Petromyzon marinus*)	200 µL	SPE	UPLC-MS/MS	102%	LOD: 0.06 ng/mL; LOQ: 0.2 ng/mL	intra 2.5%, inter 3.2%
Lyons & Wynne-Edwards 2019 (77)	T, KT, A	Stingrays	50 µL	Protein precipitation	LC-MS/MS		LOQ: 0.05 ng/ml	

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 20: Comparison of the performance of recent analytical chemistry methods to measure E2 in fish plasma

Reference (number in Annex B –)	Estrogen	Species	Sample amount	Sample preparation	Method	Linear range	Recovery	Sensitivity	Precision
Labadie & Budzinski, 2006 (5959)	E1, E2, E3	Turbot (*Psetta maxima*)	1g	Extraction, fractionation, purification and conjugate cleavage, derivatisation	GC-MS		92±6%	LOD: 0.1 ng/g	
Budzinski et al 2006 (179)	E1, E2	Rainbow trout (*Onchorhyncus mykiss*) and European flounder (*Platichthys flesus*)	1g	C_{18} SPE extraction, NH_2 SPE purification, derivatisation	GC-MS		68-104%	LOD: 0.1 ng/g	Intraassay CV <10%
Noaksson et al 2005 (1880)	E1, E2	Perch (*Perca fluviatilis*)		Protein precipitation, lipid reduction by sequential extraction with n-hexane plus SPE, NH2-phase clean-up. Derivatisation, Liquid-liquid extraction	HRGC/HRMS			LOD: 0.1 ng/g	
Widell et al, 2011 (9025)	E1, E2	Perch (*Perca fluviatilis*)	0.5–1 mL	Protein precipitation, lipid reduction by sequential extraction with n-hexane plus SPE, NH2-phase clean-up. Derivatisation	HRGC/HRMS		96±18%	LOD: 0.002 ng/g; LOQ: 0.01 ng/g	Intraassay CV: 2%
Zhang et al 2009 (986)	E2	Fathead minnow (*Pimephales promelas*)	10 uL	Derivatisation with dansyl chloride	LC/APPI-MS/MS	1.0–250 ng/ml	80.4-115.4%	LOQ: 1 ng/mL	Intraassay RSD: 3.1-9.4%; Interassay RSD: 2.6-10.8%
Wang et al 2016 (3228)	E2	Sea lamprey (*Petromyzon marinus*)	200 uL	SPE	UPLC-MS/MS	0.05–10 ng/mL	102%	LOD: 0.01 ng/mL; LOQ: 0.04 ng/mL	Intraassay CV: 2.5%; Interassay: 2.6%
Bussy et al 2017 (2261)	E1, E2, E3	Lake trout (*Salvelinus namaycush*) and Sea lamprey (*Petromyzon marinus*)	200µL	LLE and protein precipitation	UPLC-MS/MS	5–5000 pg/ml	68.9 ± 11.7%	LOQ: 0.5 pg/mL	Intraday CV: 5.1-8.6%; Interday CV: 5.7-13.1%

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Lyons & Wynne-Edwards 2019 (77)	E1, E2, E3	Stingrays	50 uL	Protein precipitation	LC-MS/MS			LOQ: 0.05 ng/ml	

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 21: Methods to determine estrogens in fish bile, tissue or whole-body homogenate

Reference number (Annex B –)	Reference	Species	Sampling amount	Analytical method	Sensitivity
Bile					
155	Gibson et al. 2005	Rainbow trout and Roach		GC-MS	
5959	Labadie and Budzinski 2006	Turbot	100mg	GC-MS	
179	Budzinski et al. 2006	Rainbow trout and European flounder	0.1g	GC-MS	LOD: 1.6-14 ng/g
639	Vallejo et al. 2010	Grey mullets	100 µl	GC-MS	LOD: 5 µg/g
4250	da Silva, Denis A M et al. 2013	English sole	50-100 µl	LC-MS/MS	LOQ: 6.3 ng/ml
2989	Ros et al. 2015	Thicklip grey mullet	100 µl	GC-MS	LOD: 0.4 ng/ml
Tissue					
6474	Dong et al. 2009	Not specified	1g	HPLC	LOD: 0.02 µg/ml
5033	Hu et al. 2010	Not specified	5g	HPLC	LOD: 2.39 µg/l
5352	Wang et al. 2012	Frozen fish samples (cod, hairtail, whitebait, yellow croaker, and mackerel)	3g	LC-MS/MS	LOD: 0.12 ng/g
16006	LU et al. 2013	Tilapia	4g	GC-MS	LOD: 1µg/kg
1370	Lan et al. 2014	Not specified	2g	HPLC	LOD: 0.4-1.7 ng/g
14511	Cohen et al. 2017	American eel	0.5g	LC-MS/MS	LOD: 72 pg/g
Whole-body homogenate					
13033	Navarro et al. 2010	Zebrafish	1g	GC-MS	LOD: 1.5 ng
17314	Al-Ansari et al. 2011	Goldfish	2g	GC-MS	LOD: 0.8 pg (on column)
15482	Arukwe et al. 2013	Atlantic salmon (larvae)		GC-MS	LOD: 0.6-0.8 ng/g
3467	Yang et al. 2015	Zebrafish	5g	LC-MS/MS	LOD: 5.17 ng/g

B.3.3.2. Hypothalamic–pituitary–thyroid axis hormones

Far fewer methods to measure thyroid hormones in fish samples have been published than methods to measure sex steroids. It may be related to the fact that analytical chemistry methods to quantify thyroid hormones have been restricted to liquid chromatography, or that fish was considered a poor model for thyroid disruption until relatively recently. The LC-MS or LC-MS/MS methods identified by our search are summarised in Table 22:). We have already encountered and discussed many of these methods in previous sections; the method reported by Simon et al. (2002) has also been validated in amphibians (section B.3.1.2); the method described by Yang et al. (2015) allows the measurement of not only thyroid hormones from the whole body homogenates of zebrafish, but simultaneously also that of androgens and estrogens (section B.3.3.1); and the method of Wang et al. (2015) is applicable to other types of food of animal origin such as chicken meat and eggs (section B.3.2.2).

Perhaps, the most significant finding of this literature mapping in the context of developing guidance for the measurement of hormones in certain taxa with regards to the EDC criteria is related to the method described by Bussy et al. (2017b) (reference 3833 in Annex B –). This very sensitive method which allows the quantification of T3, T4 and rT3 in relatively small samples, from various matrices in the Sea lamprey (plasma, liver, gill and kidneys), has been fully validated according to FDA guidance.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 22: Methods to measure thyroid hormones in fish

Number in Annex B –	Reference	Hormones	Species	Matrix	Sample amount	Sample preparation	Method	Sensitivity (LOD)	Precision
8692	Simon et al. 2002	T3, T4	Zebrafish	Whole body homogenate	-	Digestion with pronase E, separation with HPLC	LC-ICP-MS		
11800	Noyes et al. 2014	T3, T4	Rainbow trout, fathead minnow, mummichog, sockeye salmon, coho salmon	Plasma	50µL	Antioxidant, SPE extraction	LC-ESI/MS/MS	T4: 0.42 ng/g; T3: 0.24 ng/g	<13%
3467	Yang et al. 2015	T3, T4	Zebrafish	Whole body homogenate	5g	QuEChERS	LC-MS/MS	T4: 0.41 ng/g; T3: 0.14 ng/g	<11%
7269	Wang et al. 2015	T3, T4, rT3	Cod, salmon	Tissue	5g	SPE	LC-MS/MS	<0.04 ng/g	RSD: 1.4-20%
3833	Bussy et al. 2017b	T3, T4, rT3	Sea lamprey	Plasma, liver, gill and kidney	100µL	Enzymatic digestion followed by protein precipitation	LC-MS/MS	<1pg/ml	<20%
843	Chen et al. 2018	T3, T4, rT3, 3,5-T2, 3,3'-T2	Zebrafish embryos or larvae	Whole	150 larvae	Digestion with pronase, solvent extraction, SPE clean-up, derivatisation	LC-MS/MS	0.2-0.24 ng/g	0.5-12.4%

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Appendix C – Results of the extensive review of the literature on pathology of endocrine organs in birds

C.1. Thyroid

Six of the articles retrieved in the search specifically focused on thyroid disruption. Four were from one group; (Pandey and Mohanty 2015) (reference **3** in Annex C –), (Mohanty et al. 2017) reference **2** in Annex C –), (Pandey and Mohanty 2017) (reference **11** in Annex C –) and (Pandey et al. 2017) (reference **29** in Annex C –) investigated the effects of mancozeb (MCZ) and imidacloprid (IMI) and their mixture in red avadavat. Adult males (n-8/group) were fed organically grown ground wheat grain coated in olive oil (dose vehicle) at 0 (control), 0.25% LD50 MCZ, 0.25% LD50 IMI, 0.25% equimixture MCS & IMI, 0.5% LD50 MCS & IMI for 30 days during preparatory period before annual breeding phase. Endpoints included pathology and histopathology of the thyroid (Pandey and Mohanty 2015, reference **3** in Annex C –, and Pandey and Mohanty 2017, reference **11** in Annex C –) pathology and histopathology of the testis (Mohanty et al. 2017, reference **2** in Annex C –), and immunohistochemistry of the testis (Pandey et al. 2017, reference **29** in Annex C –). The pathology measures in these publications relate to weight and volume of the thyroid or testis. The histopathology measured the height of testicular capsule, width of intertubular space, diameters of seminiferous tubules, density of Leydig cells and Sertoli cells and density of apoptotic and necrotic cells in interstitium and seminiferous tubules in the testis and the number of follicles containing colloids, volume of colloids, epithelial cell height, nucleus size and nucleus-to-cytoplasm ratio in epithelial and stromal cells in the thyroid. In addition to the pathology and histopathology, hormone (luteinising hormone, LH, follicle stimulating hormone, FSH, prolactin, PRL, testosterone, T, estradiol, E2, thyroid stimulating hormone, TSH, triiodothyronine, T3 and thyroxine, T4) measurements were made on the birds plasma or serum with commercial ELISA kits. Although the kits were not designed for this test species LH, FSH, PRL, T and E2 were validated for this bird by adsorption test using avian antigens which were provided by National Hormone and Pituitary Program (NHPP), USA as well as parallelism assays (Pandey et al. 2017, reference **29** in Annex C –). Mohanty et al. (2017) (reference **2** in Annex C –) also sought to validate the use of human ELISA kits in red avadavat by immunohistochemistry, staining pituitary hormone producing cell types of red avadavat against human antibodies (anti-hTSH, anti-h LH, anti-hPRL). However, the use of not target species TSH ELISA was not specifically validated.

The fifth article specifically investigating thyroid disruption, was by Jacobsen et al. (2017) (reference **8** in Annex C –) who investigated the developmental histopathological consequences of exposure to flame retardants (Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP; CAS No 13674-87-8) and Dechlorane Plus (DP; CAS No 13560-89-9)) in ovo. Japanese quail eggs were injected with 500 ng/µl of DP (n=9) or TDCIPP (n=8), while the mixture group contained 500 ng/µl of DP and 500 ng/µl of TDCIPP (n=10), a control group was not injected at all (n=9). Thyroid and liver tissues were investigated in the 14-day-old chicks. For the thyroid, proliferation of follicular cells and size and number of thyroid follicles was measured. For the liver, lymphoid cell aggregates (granulomas and infiltrates), necrosis, lipid vacuoles, and sinusoidal dilatation were recorded (Jacobsen et al. 2017, reference **8** in Annex C –).

Fowles et al. (1997) (reference **22** in Annex C –), investigated the effects of Aroclor 1254 on the thyroid gland of mallard ducks (*Anas platyrhynchos*). Adult male mallards (n=6/treatment) were exposed to 0, 4, 20, 100, 250, and 500 mg/kg Aroclor 1254 by gavage twice per week for 5 weeks, at the end of the experiment spleen, liver, and thyroid glands were weighed and examined for histopathology. A dose-dependent increase in liver weight was observed with PCB treatment, thyroid weight also increased dose-dependently. Plasma T3 concentrations were measured and reduced dose-dependently with Aroclor 1254 treatment. However, Aroclor 1254 did not induce significant histopathological lesions in the thyroid, with the exception of increased vacuolization and a slightly enlarged thyroidal colloid in the 500 mg PCB/kg treatment (Fowles et al. 1997, reference **22** in Annex C –).

Another article (Harr et al. 2017) (reference **17** in Annex C –) focused on the effects of crude oil exposure on internal organs of a diving bird, these included thyroid specific endpoints, however the study is not specifically suggesting endocrine mode of action as mortality and gross pathology is likely

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

related to overt toxicity of the crude oil. This study used wild caught adult double-crested cormorants (*Phalacrocorax auritus*). Harr et al. (2017) (reference **17** in Annex C –) exposed adult double-crested cormorants via their diet or dermally to artificially weathered MC252 oil (Diet: control n = 8, 7 male, 1 female, 5 ml oil/kg bw/day n = 9, 6 male, 3 female, and 10 ml oil/kg bw/day n = 9, 7 male, 2 female. Dermal: 13ml of oil (exposed n=11) or water (control n=11) was applied to the birds every three days through Day 15 of the trial (on Days 0, 3, 6, 9, 12, and 15). A large number of the orally exposed bird died or had to be euthanised before the end of the 21 day experiment for humane reasons, this included all 9 high dose animals. Absolute and relative kidney and liver weights increased in oil exposed bird, whereas there was no significant change in brain, heart or spleen weight. Histopathology was conducted on a large range of organs and tissues including: pancreas, thyroid gland, kidney, brain, heart, small intestine, colon, spleen and lungs. In the thyroid gland, follicular hyperplasia and colloid atrophy were recorded, follicular hyperplasia was seen in all treatments (including controls) however it was more frequent in dermally exposed birds (Harr et al. 2017) (reference **17** in Annex C –).

C.2. Reproductive organs

One article was retrieved that focused on anti-androgenic effects. Quinn et al. (2008) (reference **24** in Annex C –) investigated the effects of dichlorodiphenyldichloroethylene (DDE) on Japanese quail. One hundred eggs per treatment were randomly assigned to either sesame oil (vehicle control), 20 (low) or 40 µg/egg (high) DDE. At 18 weeks old birds were sampled (10 per sex). For females, ovary weights relative to body mass and the number of mature yellow follicles were measured. For males, combined testes weights were expressed as relative to body weight. One testis per male was used for histopathology to assess the relative area occupied by spermatozoa within a seminiferous tubule, and spermatogenic stages were checked to verify normal spermatozoan maturation. The left epididymis was used to investigate sperm motility. Prior to sampling, at 10 weeks of age, the pairs of Japanese quail were also assessed for their reproductive behaviour and egg production (Quinn et al., 2008).

Henry et al. (2012) (reference **12** in Annex C –) investigated possible effects of exposure to the androgen 17β trenbolone (17β-TB) in Japanese quail via dietary exposure. At four week old birds were paired (12 per treatment 0, 5 & 20 mg 17B-TB per kg feed), their reproductive function (e.g. onset of sexual maturation, no. of eggs laid, percentage eggs fertilised), testosterone levels, gross pathology (Male; weights and area of the cloacal gland and total gonadal weights were recorded. Female; total number of yellow yolk follicles, mean weights for yellow yolk follicles/ovary were recorded from a subset of the females) and histopathology (testes only) were assessed. There were no differences in body and testes weight, testes histology, plasma testosterone concentrations, or size and weight of the foam glands in males. Although there were no effects on ovary weight, females in the 20 mg group produced fewer yellow yolk follicles, laid less eggs and had lower percentage of fertile eggs (Henry et al. 2012, reference **12** in Annex C –).

Quinn et al. (2007) (reference **26** in Annex C –) investigated the effect of trenbolone acetate on reproductive development and function in Japanese quail. Quail embryos were exposed to sesame oil control or 0.05, 0.5, 5, or 50 µg trenbolone at embryonic day 4. At four weeks, 10 males and 10 females per treatment level were separated for onset of puberty assessment, copulatory behaviour, and reproductive assessments. Birds were sampled at 18 weeks of age. Relative testes and ovary weight were recorded. No significant differences in relative testes or ovary weight were found, and no effect on ovarian follicle counts were observed. Testis seminiferous tubule size was marginally reduced at the highest dose, whereas the size of the proctodeal gland was significantly reduce at the highest two doses (Quinn et al. 2007, reference **26** in Annex C –).

Tokumoto et al. (2013) (reference **14** in Annex C –) investigated effects of clothianidin (CTD) exposure on the reproductive system of male Japanese quail. Twenty-six, 13 weeks old males were assigned to four groups: Control (CTD 0 mg/kg), CTD0.02 (CTD 0.02 mg/kg), CTD1 (CTD 1 mg/kg), CTD50 (CTD 50 mg/kg) and orally administered water or CTD dissolved in water daily for a period of 30 days. There were no significant differences in relative weights of testis and spleen between the groups, however in the highest dose liver weight was increased. An increase of vacuolization in the seminiferous epithelia and the number of germ cells having fragmented DNA in seminiferous epithelia increased in a dose-

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

dependent manner. A decrease of the number of germ cells were also detected in a dose-dependent manner (Tokumoto et al. 2013, reference **14** in Annex C –).

Another article (Singh et al. 2013) (reference **20** in Annex C –) primarily focused on male reproductive endpoints after exposure to an antibiotic. Twenty-two male Japanese quail were treated with norfloxacin at 20 mg/kg body weight with an additional 14 males as un-dosed as controls. Birds were sampled for serum testosterone, pathology, histopathology and molecular assessments (Androgen receptor expression) at 7 days (n= 4) and 14 day (n= 4) of exposure from the norfloxacin treatment, 4 birds were also sampled from the control treatment at 14 days for these endpoints. The remaining birds from both treatments were investigated for sperm parameters (semen collection and sperm counts). A second experiment (14 days) investigated effects on reproductive performance (10 pairs, control or norfloxacin), only the males were given the norfloxacin treatment. The article reported effects of exposure on gene expression, sperm concentration, cloacal gland area, and serum testosterone as well as histopathological changes to spermatogenesis and secretary tissue level in the cloacal gland. These effects were mirrored in the reproductive performance assay. (Singh et al. 2013, reference **20** in Annex C –).

Shibuya et al. (2005) (reference **6** in Annex C –) employed OECD TG 206, but with additional endpoints. Sixteen pairs of 10-week-old Japanese quail were fed a low phytoestrogen diet containing E2 at 0 (control), 10, 100, and 1000 ppm for 6 weeks, and parent quails (F0), eggs and offspring (F1) were examined. F1 chicks were maintained up to 14 days of age. Gross pathology and histopathology were conducted on male and female F0 birds and offspring (F1). In F1 birds, testis, ovary, oviduct, liver, spleen, and cloacal gland were weighed and the organ-to-body weight ratios were calculated based on the body weight. Grossly, atrophy of the testis, ductus deferens, and the cloacal gland, swelling of the liver, and discoloration of the kidneys were observed in male birds exposed to the higher concentrations of E2. In female birds, atrophy and degenerate ova of the ovary and discoloration of the kidneys were observed in the higher concentrations of E2. Histopathologically, atrophy and degeneration of the seminiferous tubules and a marked decrease in spermatogenesis in the testis, atrophy of the ductus deferens and decreased number of the sperm in the ductus deferens. There was atrophy of the cloacal gland with glandular epithelial cells possessing few secretory granules in their cytoplasm and decreased secretory activity. Disruption to the spleen, kidney and liver were also recorded in the higher doses (Shibuya et al. 2005, **6** in Annex C –). In addition to the gross and histopathological endpoints, the viability of embryos from F0 breeding pairs, and the female yolk protein, vitellogenin, and E2 were also measured in males and females (F0).

Yamashita et al. (2011) (reference **1** in Annex C –) also investigated the effects of E2 on Japanese quail. Sixteen pairs of 10-week-old quails were fed a low phytoestrogen diet containing E2 at 0 (control), 0.3, 3, and 30 ppm for 6 weeks, and parent quails, eggs and offspring were examined and F1 chicks were maintained up to 14 days or 10 weeks of age. In males, at necropsy, atrophy of the testis, ductus deferens and cloacal gland, swelling and yellowish discoloration of the liver and kidneys, and discoloration of the heart were recorded at significantly high incidences. Relative cloacal gland weight had reduced and relative liver weight had increased in the high dose F0 males. In F0 females, at the highest dose, atrophy and degenerate ova of the ovary and swelling and discolouration of the kidney were observed. In F0 females the relative cloacal gland weight had also reduced at the higher dose (Yamashita et al. 2011, reference 1 in Annex C –). Histopathological changes in the gonads were also observed at significant levels at the highest dose. For example, ovary atrophy with degeneration and decreased numbers of oocytes and ova in females and degeneration of the seminiferous tubules with decreased spermatogenesis in the testis of males. As with Shibuya et al. (2005) (reference **6** in Annex C –) vitellogenin, and E2 were also measured in the quail, with significant increases at higher doses. Histological changes were also observed in reproductive organs of F1 14-day old chicks.

Razia et al. (2006) (reference **10** in Annex C –) investigated histopathological effects of E2 and NP on developing Japanese quail endocrine organs. Eggs were injected on day 3 of incubation with 0 (control) 1, 10 100 ng/g E2 and 1, 10, 100 ug/g NP in 20ul peanut oil. Embryos were dissected on day 15 of incubation, thyroid and testis were assessed histologically. For the testis, the presence of oocytes developing in the otherwise male gonad were the main endpoint reported. In the thyroid the height of

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

simple cuboidal epithelial cells and area of thyroid follicles were measured (Razia et al., 2006) (reference **10** in Annex C –).

Another article (Yang et al. 2012) (reference **18** in Annex C –) focused primarily on female specific endpoints relating to cadmium exposure. Ninety 50-day-old hyline white chicken hens were randomly divided into three groups (30 hens per group): a control group was fed with basal diet, a low dose group was fed with basal diet containing 140 mg/kg CdCl2 and a high dose group was fed with basal diet containing 210 mg/kg CdCl2. Ten hens were selected from each group and were killed, respectively, at the 20th, 40th and 60th days after Cd exposure. The ovary was assessed via histopathology, ovarian lesions with severe necrosis and degeneration of ovarian follicle and interstitial cell were reported. In addition, the ovary was assessed via electron microscopy ovary (TEM), an apoptosis assay (TUNEL) of the ovary was conducted, a range of oxidative stress measures were taken, and oestradiol and progesterone were also measured in the bird's serum (via RIA). The number of apoptotic cells in the ovary was increased in the Cd treatment and the ovary had severe damage to it from Cd exposure. Oestradiol and progestogen levels fell significantly in Cd treated hens. However, the authors concluded that oxidative damage of hens' ovary tissue may underlie the reproductive toxicity induced by Cd (Yang et al. 2012, reference **18** in Annex C –).

Swartz (1984) (reference **25** in Annex C –) assessed 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane (DDT) on gonadal development in the chicken (*Gallus gallus domesticus*) embryos. Fertile white Leghorn eggs were exposed via intravitelline injections to dosages of 5.0, 10.0, or 20 mg DDT in olive oil prior to incubation. Control embryos received only the olive oil vehicle. The eggs were incubated until day 5 or 12. Histopathology was conducted on the gonads of both male and female chicks. At 5 days post-exposure the number of primordial germ cells aggregating in the gonadal area was not impacted by DDT exposure. However, in the 12-day exposed embryos, effects were seen in both male and female gonads. Male DDT-exposed embryos consisted of mostly stroma with fewer seminiferous cords than controls. In DDT-exposed females ovaries contained a larger number of distended medullary cords as well as a difference in the distribution of these cords when compared to controls (Swartz 1984, reference **25** in Annex C –).

Cheng et al. (2017) (reference **13** in Annex C –) Japanese quail were treated for 18 weeks to either control, 1 µg L, 10 µg L, or 100 µg L nonylphenol (NP) via their drink water. Each treatment consisted of three replicate cages each with two females and one male. Quail were sampled for histological endpoints prior to exposure, directly after exposure and at the end of the experiment. It seems at each time point one cage per treatment (n=3: F=2, M=1) was sampled. Cheng et al. (Cheng et al. 2017, reference **13** in Annex C –) report that NP, at all exposure levels, reduced spermatogenesis in seminiferous tubules and visible shedding of spermatogenic cells in to seminiferous tubule cavities was observed. However, no quantification or frequency of these effects are given. No histopathological effects on the ovary were found by Cheng et al. (2017) (reference **13** in Annex C –). The same exposure regime and number of animals was used separately for reproductive assessment.

In a second article, Cheng et al. (2019) (reference **9** in Annex C –) also investigated the reproductive and histopathological effects of NP on Japanese quail via dietary exposure. 14-day old quails were given 2 weeks acclimation before being exposed to NP via their diet 0 (control), 10 mg/Kg, 20 mg/Kg or 50 mg/Kg NP. The gonads of the quails from three cages (n=3: F=2, M=1) per treatment were assessed using histopathology, these tissue samples were collection at week 2 (before exposure), week 18 (directly after exposure), and at week 23 (end of the feeding experiment/with depuration). It seems at each time point only one cage per treatment (n=3: F=2, M=1) was sampled. After NP exposure, Cheng et al. (2019) report a reduction in spermatogenesis compared to pre exposure and control, and that sperm cell were shedding into the lumen. Fertility and hatchability were reduced in NP exposed breeding groups. No histopathological effects on the ovary were found (Cheng et al. 2019, reference **9** in Annex C –). The same exposure regime and number of animals was used separately for reproductive assessment.

Ahmed et al. (2015) (reference **16** in Annex C –) investigated the effect of P-Nitrophenol (PNP) on male Japanese quails. Twenty-eight days old quails were exposed orally to four different treatments control (0), 0.01, 0.1, 1 mg/kg PNP for two and a half months. Birds were sampled throughout the study (n=5

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

per treatment on 0.1, 1, 1.5, 2, 2.5 months). Testes were weighed separately and the cloacal gland area were measured. Testes were the focus of the histopathology. In addition, a range of hormones were measured via RIA (testosterone, corticosterone and LH). PNP treatment induced testicular atrophy, and significant differences were observed in testis weight at 2 months in the mid and high PNP treatment, but not at the later date. A decrease in spermatids and spermatozoa populations was found in the highest dose compared to the control, and testicular degeneration score increased with dose. Ahmed et al. (Ahmed et al. 2015, reference **16** in Annex C –) also reported PNP exposure increased cloacal gland atrophy.

Oshima et al. (2012) (reference **21** in Annex C –) investigated the feminising effects of Bisphenol A (BPA) or NP on Japanese quail embryos. Eggs were injected with 0 (control), 200, 2,000, 20,000, and 200,000 ng NP or 0 (control), 20, 200, 2,000, and 20,000 ng BPA. At incubation day 16, all embryos were sampled, gonads were assessed for gross pathology and histopathology. Three female birds from the BPA treatments (1 each at 20, 2000 and 20,000 ng BPA) had a small right-hand ovary (which female birds do not normally have) which had a normal ovarian appearance under histological investigation. The occurrence of ovotestis in males was significantly increased in NP and BPA treatments compared to controls (although no does dependent effect was seen), however ovotesis were only detected in the left testis of embryos (Oshima et al. 2012, reference **21** in Annex C –).

Aire (2005) (reference **23** in Annex C –) investigated the pathological and histopathological effects of carbendazim (fungicide) on the testis of Japanese quail. Twenty adult male quail were exposed to 400 mg/kg carbendazim via oral gavage, five bird were given oil base only as controls. Five carbendazim exposed birds were sampled at 5 h, 3, 8 and 13 days post-exposure. Three birds per treatment were used for histopathology and two for electron microscopy. Aire 2005 Aire (2005) (reference **23** in Annex C –) report that relative testis weight reduced at 3, 8 and 13 days post exposure. Histopathology indicated marked changes to the structure of the gonad which included deformed spermatids, atrophic and multinucleated cells Aire (Aire 2005, reference **23** in Annex C –).

Grote et al. (2008) (reference **27** in Annex C –) investigated effects of epoxiconazole fungicide on avian fertility and reproduction, using the Japanese quail. Adult quail were exposed for three weeks at dietary levels of 10, 50, and 500 ppm epoxiconazole. Six males were assessed per treatment group for gonad histopathology. Histopathology revealed degeneration of seminiferous tubuli, disruption to spermatogenesis, marked reduction of mature spermatids, spermatocytes and spermatogonia, and as well as sloughing of spermatocytes into the lumen. However, no impact was observed on testis weight, hormone levels, fertility, and reproductive outcome (Grote et al. 2008, reference **27** in Annex C –).

Niemann et al. (2010) (reference **28** in Annex C –) reviewed articles to assess if histopathology could be used to assess endocrine toxicity. Chemicals in the review included vinclozolin, epoxiconazole, methyl testosterone and Fentin hydroxide. Niemann et al. (2010) (reference **28** in Annex C –) concluded that histological examination of the testis and counting of spermatids might be suitable and sensitive methods to elucidate the mechanism if a male-mediated decline in reproductive success is suspected.

C.3. Test species selection

The US EPA Avian Two-generation Toxicity Test (EPA OCSPP 890.2100 (US EPA, 2015)) is validated for the Japanese quail (*Coturnix japonica*), and the OECD Avian Reproduction Test (TG 206 (OECD, 1984)) recommends the use of Japanese quail, bobwhite quail (*Colinus virginianus*) and/or mallard duck (*Anas platyrhynchos*). These recommended test species are generally reflected in literature, with the majority (17) of the articles included in this review employing Japanese quail as their test model. The remaining eight articles used the domestic chicken (*Gallus gallus domesticus*, 2 articles), mallard duck (1 article), an Asian finch the red avadavat (*Amandava amandava*, 4 articles) or double-crested cormorant (*Phalacrocorax auritus*, 1 article). Some of the more unusual test species relate to specific exposure scenarios, i.e. the double-creased cormorant was used to test crude oil toxicity. All four articles employing red avadavat originated from the same research group investigating effects of pesticides on seed eating birds.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

C.4. Age/life stage and sex

OECD TG 206 (OECD, 1984) was designed primarily to investigate toxicity, reproductive output, egg shell thickness and early chick survival. This is largely due to the types of organochlorine pesticides historically used at the time of TG 206's development Although adult (F0) and 14-day old chicks (F1) are included, effects on F0 adults are investigated in more detail than the F1 chicks (i.e. chicks limited to mortality, food consumption and total weight). Proposals have previously been made for an OECD two-generation study (to include endpoints to specifically analyse possible endocrine disrupting effects in birds), however, there is no avian Level 5 test under the current OECD Test Guideline framework. Here we include information on the US EPA Avian Two-generation Toxicity Test (EPA OCSPP 890.2100, (US EPA, 2015)) guidance document for reference and comparative purposes only – the US EPA two-generation test is not a requirement in European chemicals regulation. The US EPA Avian Two-generation Toxicity Test (EPA OCSPP 890.2100) guidance document stipulates that F0 and F1 adults, F1 and F2 embryos and 14 days-old chicks or adult F2 birds should be assessed for gross pathology and histopathology. In all life stages both males and females should be assessed (EPA OCSPP 890.2100 (US EPA, 2015)).

From the literature pathology and histopathology have been investigated in terms of possible endocrine disruptive effects in multiple life stages. Reports in adults were identified in 21 articles, 8 articles investigated pathology/histopathology in both adult males and females (Yamashita et al. 2011, reference **1** in Annex C –; Shibuya et al. 2005, reference **6** in Annex C –; Cheng et al. 2019, reference **9** in Annex C –; Henry et al. 2012, reference **12** in Annex C –; Cheng et al. 2017, reference **13** in Annex C –; Harr et al. 2017, reference **17** in Annex C –; Quinn et al. 2008, reference **24** in Annex C –; Quinn et al. 2007, reference **26** in Annex C –). However, another 11 articles focused only on adult males (Mohanty et al. 2017, reference **2** in Annex C –; Pandey and Mohanty 2015, reference **3** in Annex C –; Pandey and Mohanty 2017, reference **11** in Annex C –; Tokumoto et al. 2013, reference **14** in Annex C –; Ahmed et al. 2015, reference **16** in Annex C –; Singh et al. 2013, reference **20** in Annex C –; Fowles et al. 1997, reference **22** in Annex C –; Aire 2005, reference **23** in Annex C –; Grote et al. 2008, reference **27** in Annex C –; Niemann et al. 2010, reference **28** in Annex C –; Pandey et al. 2017, reference **29** in Annex C –) and one article focused only on adult females (Yang et al. 2012, reference **18** in Annex C –). Only two articles reported on pathology and histopathology in juveniles (chicks) and adults of both sexes (Yamashita et al. 2011, reference **1** in Annex C –; Shibuya et al. 2005, reference **6** in Annex C –). Three articles investigated reproductive pathology and/or histopathology in embryos (Razia et al. 2006, reference **10** in Annex C –; Oshima et al. 2012, reference **21** in Annex C –, and Swartz 1984, reference **25** in Annex C –) and one article reported in chicks only (Jacobsen et al. 2017, reference **8** in Annex C –).

C.5. Animal care

OECD TG 206 (OECD) provides guidance on the age and housing conditions to be used during the test (Table 23:). TG 206 also states that adult birds must be kept at 22 ± 5 °C and 50-70% humidity. For incubation, hatchlings and young birds the environmental conditions are also given and are listed in Table 24: .

Table 23: Recommended age and conditions for adult birds (OECD TG 206)

Species	Age at beginning of test	Age range within a test	Minimum floor area of pen per pair[(a)]
Mallard duck	9-12 months	± 2 weeks	1 m^2
Bobwhite quail	20-24 weeks	± 1 week	0.25 m^2
Japanese quail	[(b)]	± ½ week	0.15 m^2

a, if larger groups are used the floor space should be increased in proportion

b, it is recommended that Japanese quail are proven breeders before use in the test, so as to reduce variability with this species

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Table 24: Recommended conditions for eggs and young birds (OECD TG 206)

	Temperature (°C)	Relative humidity (%)	Turning
Mallard duck			
Storage	14-16	60-85	Optional
Incubation	37.5	60-75	Yes
Hatching	37.5	75-85	No
Young first week	32-35	60-85	-
Young second week	28-32	60-85	-
Bobwhite quail			
Storage	15-16	55-75	Optional
Incubation	37.5	50-65	Yes
Hatching	37.5	70-75	No
Young first week	35-38	50-75	-
Young second week	30-32	50-75	-
Japanese quail			
Storage	15-16	55-75	Optional
Incubation	37.5	50-70	Yes
Hatching	37.5	70-75	No
Young first week	35-38	50-75	-
Young second week	30-32	50-75	-

The US EPA Avian Two-generation Toxicity Test (US EPA, 2015) guidance document also outlines husbandry conditions for Japanese quail temperature, humidity and minimum floor area are given in Table 25: . The EPA guidelines suggest pens for both adults and chicks should preferably be of stainless or galvanized steel or other inert materials. If the floor of the pens is wire mesh, the wire mesh should be of a size sufficient to prevent foot injury (trauma or pododermatitis) but large enough to allow excreta to drop through. Pens should have a ventilation of 8 to 15 air changes per hour. Birds should be exposed to light intensity of at least 10 lux, measured at the level of the feeder. Chicks should be reared in thermostatically controlled rearing pens or cages free of drafts and radiators (e.g., ceramics) are recommended to maintain adequate temperatures (Table 25:). Adult bird should be housed in pairs (one male/one female) and chicks should be identified individually or by pen of origin, and may be housed together, in groups of approximately equal number, preferably by treatment group (U.S. EPA 2015).

Table 25: Housing Conditions from US EPA Avian Two-generation Toxicity Test (EPA OCSPP 890.2100)

Age (week)	Temperature (°C)	Relative humidity (%)	Minimum floor area (cm^2/bird)
1	35-38	40-80	50
2	30-35	40-80	75
3-4	23-27	40-80	100
>4	16-27	40-80	625

Note: The acceptability of housing conditions of adults will be evaluated on the basis of results of reproductive performance.

EU legislation (Directive 2010/63/EU) also outline minimum condition for birds used in research and regulatory testing in Europe.

C.6. Anaesthesia / Euthanasia

OECD Avian Reproduction TG 206 (OECD, 1984) provides no specific guidance on anaesthesia or euthanasia methods. The US EPA Avian Two-generation Toxicity Test (U.S. EPA, 2015) states that birds should be humanely euthanized, also but provides no specific guidance. In the EU, euthanasia methods

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

should adhere to Directive 2010/63/EU. Under specific conditions, anaesthetic overdose, decapitation and cervical dislocation are all recognised methods of euthanasia under Directive 2010/63/EU.

The American Veterinary Medical Association Guidelines for the Euthanasia of Animals (Leary et al. 2020) reviews appropriate methods for euthanasia of animals. The most acceptable method for avian species were reported as intravenous injection of barbiturates. However, injectable agents can precipitate in tissues and can induce artefacts at necropsy and on histopathologic examination. Exposure to high concentrations of inhaled anaesthetics (e.g., halothane, isoflurane, sevoflurane, with or without N_2O) were also considered acceptable, with conditions, for euthanasia for birds (Leary et al. 2020). Birds exposed to high concentrations of inhaled anaesthetic gases lose consciousness rapidly. Euthanasia by exposure to gas anaesthetics also induces minimal tissue damage and results in the least amount of tissue artefact for necropsy (Leary et al. 2020). Based on information currently available, the AVMA also considers decapitation to be acceptable, with conditions, for euthanasia of small (< 200 g) birds. An adult female Japanese quail can weight up to 160 g (U.S. EPA, 2015). Decapitation is preferred over cervical dislocation in birds, which should only be performed by well-trained personnel who are regularly monitored to ensure proficiency (Leary et al. 2020).

From the literature search, decapitation was the most widely reported means of euthanasia (10 out of 25 articles) (Mohanty et al. 2017, reference **2** in Annex C –; Pandey and Mohanty 2015, reference **3** in Annex C –; Jacobsen et al. 2017, reference **8** in Annex C –; Pandey and Mohanty 2017, reference **11** in Annex C –; Henry et al. 2012, reference **12** in Annex C –; Tokumoto et al. 2013, reference **14** in Annex C –; Ahmed et al. 2015, reference **16** in Annex C –; Yang et al. 2012, reference **18** in Annex C –; Grote et al. 2008, reference **27** in Annex C –; Pandey et al. 2017, reference **29** in Annex C –). Two articles used ether inhalation as the means of euthanasia (Yamashita et al. 2011, reference **1** in Annex C –; Shibuya et al. 2005, reference **6** in Annex C –), one article used an intraperitoneal injection of barbiturate (Sagatal) (Aire 2005, reference **23** in Annex C –) and one article use CO_2 inhalation (Fowles et al. 1997, reference **22** in Annex C –). However, nine articles did not specifically report the method of euthanasia (Cheng et al. 2019, reference **9** in Annex C –; Razia et al. 2006, reference **10** in Annex C –; Cheng et al. 2017, reference **13** in Annex C –; Harr et al. 2017, reference **17** in Annex C –; Singh et al. 2013, reference **20** in Annex C –; Quinn et al. 2008, reference **24** in Annex C –; Quinn et al. 2007, reference **26** in Annex C –; Oshima et al. 2012, reference **21** in Annex C –; and Swartz 1984, reference **25** in Annex C –).

C.7. Tissue choice, sample collection and storage

The OECD TG 206 recommends gross pathology should be conducted on “all adult birds”, however it does not stipulate which tissues and endpoints should be investigated (OECD, 1984). The US EPA Avian Two-generation Toxicity Test (US EPA, 2015) specifies that all adult birds (F0 and F1) should undergo necropsy and gross examination. Appendix 5 of the EPA guidance document (US EPA, 2015) provides detailed guidance and a photographic atlas to aid dissection and sampling.

In the EPA guideline, in addition to the gross pathology, from the F0 and F1 birds, eight males and eight females from each treatment and control group should be collected and processed for histopathology. The EPA guidance document stipulates that the following organs are excised and preserved for histopathological examination: kidneys, liver, adrenal glands, reproductive organs and associated structures (cloacal gland and bursa), and right thyroid gland. For the 14-day old chicks (F2), eight males and eight females from each treatment should terminated, undergo necropsy and gross examination, and be processed for histopathology. Embryos are also assessed, two from each mating pair are randomly selected and tissues are preserved for histopathology. For chicks and embryos, the same tissues are included for analysis as in the adults, however only the kidneys, liver and adrenal glands are excised for fixation, the reproductive organs and associated structures (cloacal gland and bursa) should be fixed *in situ*. Davidson's fixative (or a suitable alternative) is recommenced to fix and preserve the

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

tissues for histopathology, samples should be fixed for approximately 24 hours, then rinsed in 70% ethanol and transferred to 10% neutral buffered formalin (NBF) or ethanol for storage (U.S. EPA, 2015).

Four of the articles in the search identified OECD TG206 in their methods (Yamashita et al. 2011, reference **1** in Annex C –; Shibuya et al. 2005, reference **6** in Annex C –; Cheng et al. 2019, reference **9** in Annex C –; and Cheng et al. 2017, reference **13** in Annex C –). None of the articles, identified in the literature search, referred to the EPA Avian Two-generation Toxicity Test guidelines. However, across the articles all life-stages and sexes were documented, although the majority of assessments in the literature were conduction on F0 individuals (generally exposed either as adults orally, or in ovo via injection).

Within the literature search, the testis was by far the most investigated endocrine tissue (17 studies, Table 26:). Both the weight of the testis and histopathological endpoints (e.g. seminiferous tubule structure, spermatogenic cell population) were regularly applied to assess endocrine toxicity. In males, the area, and in a few cases the histopathology, of the cloacal gland were also assessed, especially in studies that investigated reproductive output or success.

In terms of gross female pathology, it is important to note that unlike many vertebrates, adult female birds generally only have one ovary (i.e. not paired). In the female chick embryo, only one gonad and accessory structures fully develop, with the result that the adult hen has only one ovary and a single oviduct – both on the left side (Guioli et al. 2014). Therefore, the presence of a right-hand ovary could be considered a sign of disruption (Oshima et al. 2012, reference **21** in Annex C –). Generally, the weight of the ovary, the number of mature yellow follicles and, in some cases, ovarian histopathology was also evaluated in the extracted articles (Table 26:). However, in many cases the metrics by which the ovarian histopathology was grades was not clear.

Within the literature search, the thyroid gland was investigated less frequently than reproductive tissues (Table 26:). Histopathology and weight, and in some cases volume, was employed to gaged disruption. Proliferation of thyroid follicular cells, follicles containing colloids, volume of colloids and epithelial cell height were frequently measured to assess possible disruption.

Table 26: Summary of tissues and methods of assessment employed in the extracted articles

Reference (number in Annex C)	**Major endocrine tissues assessed for gross and histopathology**			
	Thyroid	**Ovary**	**Testis**	**Cloacal gland**
Quinn et al. 2008 (24)	-	Weight Mature yellow follicles	Weight Histopathology	-
Henry et al. 2012 (12)	-	Weight Mature yellow follicles	Weight Histopathology	cloacal gland area
Quinn et al. 2007 (26)	-	Weight Mature yellow follicles	Weight Histopathology	cloacal gland area
Tokumoto et al. 2013 (14)	-	-	Weight Histopathology	-
Singh et al. 2013 (20)	-	-	Weight Histopathology	cloacal gland area Histopathology
Shibuya et al. 2005 (6)	-	Weight Histopathology	Weight Histopathology	cloacal gland area Histopathology
Yamashita et al. 2011 (1)	-	Weight Histopathology	Weight Histopathology	cloacal gland area Histopathology
Razia et al. 2006 (10)	Histopathology	-	Histopathology	-
Yang et al. 2012 (18)	**-**	Histopathology	-	-
Swartz, 1984 (25)	**-**	Histopathology	Histopathology	-

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Cheng et al. 2017 (13)	-	Histopathology	Histopathology	-
Cheng et al. 2019 (9)	-	Histopathology	Histopathology	-
Ahmed et al. 2015 (16)	-	-	Weight Histopathology	cloacal gland area
Oshima et al. 2012 (21)	-	Weight Histopathology	Weight Histopathology	-
Aire, 2005 (23)	-	-	Weight Histopathology	-
Grote et al. 2008 (27)	-	-	Weight Histopathology	-
Pandey & Mohanty, 2015 (3)	Weight/Volume Histopathology	-	-	-
Mohanty et al. 2017 (2)	-	-	Weight and volume Histopathology	-
Pandey & Mohanty, 2017 (11)	Weight/Volume Histopathology	-	-	-
Pandey et al. 2017 (29)	-	-	Immunohistochemistry	-
Jacobsen et al. 2017 (8)	Histopathology	-	-	-
Fowles et al. 1997 (22)	Weight Histopathology	-	-	-
Harr et al. 2017 (17)	Histopathology	-	-	-

From the literature search, the majority of articles (10) used Bouin's as a histological fixative (Aire 2005; Mohanty et al. 2017; Niemann et al. 2010; Pandey & Mohanty 2015; Pandey et al. 2017; Quinn et al. 2007, 2008; Razia et al. 2006; Singh et al. 2013; Swartz 1984), NBF was employed in six studies (Jacobsen et al. 2017; Niemann et al. 2010; Oshima et al. 2012; Shibuya et al. 2005; Yamashita et al. 2011; Yang et al. 2012). Niemann et al. (2010) (reference **28)** had some studies with tissues fixed in NBF and others with Bouin's. Paraformaldehyde was used by four articles (Ahmed et al. 2015; Cheng et al. 2017, 2019; Tokumoto et al. 2013), the histological fixative was not reported in three articles (Harr et al. 2017; Henry et al. 2012; Pandey & Mohanty 2017).

C.8. Analysis and Quantitation – Pathology

The OECD TG 206 recommends gross pathology should be conducted on "all adult birds" and the US EPA Avian Two-generation Toxicity Test (U.S. EPA, 2015) specifies that all adult birds (F0 and F1) should undergo necropsy and gross examination. However, how these gross measures are used in a weight of evidence approach to determine endocrine disrupting activity is not specified in either guidance document. A very recent article, assessing historical data (Eurofins avian laboratory) reported a range of gross pathology endpoints in control birds (Temple et al. 2020). Temple et al. (2020) reported that a number of gross pathologies which may be considered to be driven by endocrine disruption (e.g. small testes, inactive ovaries) are also frequently observed in control birds. These 'reproductive pathologies' were often correlated to husbandry and/or breeding status/cycle. For example, Temple et al. (2020) report that in their control data inactive ovaries was correlated with reduced weight gain in birds. The authors concluded that gross pathology observations were useful when used as corroborating information alongside other measures, such as reproductive output. However, gross pathology should not heavily biase a weight-of-evidence assessments for endocrine disrupting effects (Temple et al. 2020). Temple et al. (2020) also support the use of historical gross pathology observations in control birds, to provide baseline data to evaluate potential treatment-related effects.

C.9. Analysis and Quantitation – Histopathology

US EPA Avian Two-generation Toxicity Test (U.S. EPA, 2015) specifies that F1 birds that have been exposed *in ovo* and throughout life should be examined for systemic toxicity prior to investigating endocrine endpoints (in F0, F1 and F2 birds). It recommends examining the histopathology of the liver, kidney, adrenal, thyroid, reproductive tissues for signs of systemic toxicity in the control and highest

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

treatment first. If no signs of overt general toxicity are observed among F1 birds in the high treatment group, histopathological samples from F0, F1, and F2 birds (adults, chicks and embryos, as collected) can be limited to reproductive tissues and thyroid glands. However, if signs of overt toxicity are observed in the high treatment group, the potential of overt toxicity mimicking or masking endocrine related effects cannot be ruled out, and all tissues should be examined in the next highest concentration (adult F1) until indications of overt toxicity are not observed (U.S. EPA, 2015). Appendix 5 in the U.S. EPA Avian Two-generation Toxicity Test (U.S. EPA, 2015) guidance document provides detailed descriptive and photographic information on how to prepare tissues for histopathological analysis and also multiple figures describing the hematoxylin and eosin stained slides of each tissue. The EPA guidance document recommends that pathologists reading histology slides should be experienced in reading toxicological pathology studies and be familiar with normal avian thyroid and gonad histology, physiology, and general responses of these organs to toxicological insult (U.S. EPA, 2015). It is recommended that initially slides (control and highest concentration) are read un-blinded to aid the identification of subtle differences between exposed and unexposed animals (i.e., to avoid false-negative results). After this initial familiarisation with the pathology, all potential treatment-related findings should be re-evaluated by the pathologist in a blinded manner (i.e. without knowledge of treatment), in order to prevent the reporting of false-positive results. Appendix 5 of the U.S. EPA guidance document also provides details of how pathologies should be reported (i.e. severity grading scores, presences/absence of conditions, etc.) and guidance on how to determine treatment related effects using weight-of-evidence (WOE) approaches. It highlights that to determine endocrine-related effects additional ancillary data, results of other assays, and the published literature, including mechanistic studies should also be considered (U.S. EPA, 2015).

From the literature search, none of the articles provided the high level of detail and rationale for histopathological assessment as outlined in the EPA guidance (U.S. EPA, 2015). None of the articles reported if final histopathological analysis was conducted on blinded slides. The majority of articles only reported which endpoint were considered different between treatments and control e.g. 'reduced spermatogenesis' but without specifying how these were qualified or quantified (e.g. scoring system, image analysis). In addition, most of the articles do not consider general toxicity in their assessment and how this may impact the interpretation of findings.
However, some articles did provide criteria in the methods for how histomorphic variations were characterised and measured. For example, Ahmed et al. (2015) (reference **16** in Annex C –) referred to previous publications' criteria to evaluate histopathological changes of testes, included how many seminiferous tubules per animal were assessed and provided a severity scoring system. Razia et al. (2006) (reference **10** in Annex C –) cited an earlier publication with regard to identifying testicular oocytes (intersex) in birds, and used image software to measure the follicle area of the thyroid and the height of the epithelial cells. Razia et al. (2006) (reference **10** in Annex C –) also reported how many sections of tissue were analysed, how many follicles or cells were used for the measurements, and that two individuals were involved in the observations. Oshima et al. (2012) (reference **21** in Annex C –) reported how image analysis was used to quantify the area of ovotestis was either ovary or testis. Harr et al. (2017) (reference **17** in Annex C –) provides a detailed description of the types of histological lesions found in each tissue analysed, they also provide details of the lesion distribution (e.g. diffuse, focal, multifocal), the qualitative (mild, moderate, severe) and quantitative (number score) lesion grade and the number of animals affected (Table 26: , Harr et al. 2017, reference **17** in Annex C –).

C.10. Statistical analysis

The OECD TG 206 (OECD, 1984) states that test groups should be compared to the control by a statistical procedure designated in the study plan, and that any generally acceptable statistical method, such as ANOVA, or other applicable method given in Finney 1978 (Grimm 1979) may be used.

US EPA Avian Two-generation Toxicity Test (US EPA, 2015) and more recent OECD test guidelines which incorporate pathology and histopathology endpoints (e.g. OECD TG 241; LAGDA (OECD, 2015)) specify that histopathology severity scores can be analysed using Rao Scott Cochran-Armitage by slices (RSCABS, (Green, Springer, Saulnier, & Swintek, 2014)). This approach accounts for both severity and

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

frequency of specific pathologies. Other valid statistical approach can be used, if appropriately explained and justified (US EPA, 2015)..

None of the articles used the RSCABS method recommended by the U.S. EPA. Within the extracted literature, ANOVA (one-way, or two-way) or Kruskal-Wallis (for non-parametric data) followed by post-hoc test (e.g. Dunnetts, Least Significant Difference, Tukey's) were frequently employed to test for significance. Fishers exact test (Chi squared) was also used to analyse incidence or frequency data. Histopathological data, which is often in the form of severity 'scores', can often be miss-analysed as quantitative data e.g. a low score could be given a 1 and a high score 4 and then these 'numbers' could be collected and analysed in a t-test or ANOVA. Fishers exact test is acceptable for analysing frequency of abnormality, however it doesn't take into account the severity of the condition. The RSCABS (Green et al. 2014) method was specifically designed to overcome some of these issues when assessing statistical significance in histopathology data.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Appendix D – Results of online survey of ecotoxicology laboratories

Appendix D can be found in the online version of this output ('Supporting information' section)

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Appendix E – Summaries of follow-up telephone interviews

E.1. Telephone interview 1

We're a contract research organisation that carries out ecotoxicity testing, chemical properties and environmental safety studies. The only species we work on with endocrine disruptors are fish, specifically medaka, zebrafish and fathead minnow. They would probably be the only species that we offer testing in the future. There are no plans to include amphibians or rodent or any other mammals in our testing portfolio.

In relation of hormone measurements, the only thing we can do at the moment is vitellogenin with commercially available ELISA kits. We are not a molecular biology lab and it's likely similar for other ecotox labs that we have had little experience with these methods so our preferred method will always be the one that is easiest to handle. At the moment, there is little demand from clients. We have done some method establishment work performing OECD 229 tests, including measuring vitellogenin in medaka with an ELISA kit and it worked well. From our experience with vitellogenin, a major challenge for us has been that we need to extract and handle proteins and avoiding their accidental degradation. That is something few of our staff have previous experience of.

In terms of interpretation of the results, it will be important to consider the full picture, that is not only the hormone measurements alone but in the context of other endpoints such as secondary sex characteristics and fecundity. This is also something that we will need to gain experience of. With our method development for the OECD 229 guideline including vitellogenin measurements, we used substances we knew would have a clear effect. It may be more difficult to interpret results with substances that exert more subtle endocrine disrupting effects. A difficulty we encountered measuring vitellogenin with medaka was the sample size, specifically the weight of individual livers. The guidance in that respect was unclear and made it appear easier than it was, for instance in terms of the sizes of test tubes for protein extraction. So an important point to keep in mind when developing guidance is for it to be appropriate for different species and corresponding likely sampling volumes. Depending on fish species and sampling matrix, it will not always be possible to follow standard procedures and therefore there is a need for training and method development before such measurements are implemented.

In terms of anaesthesia or euthanasia before hormone measurements, it likely to be easier in fish compared to rodents because the fish are killed. So it's overdose anaesthesia. We typically use tricaine and benzocaine. The guideline recommends tricaine because it's better for the tissue for histopathology. For husbandry, an issue we have encountered is the sample size recommended in test guidelines, mainly OECD 229 and 240. There is an adaption period of 1 or 2 weeks to evaluate if the fish get along before we find out if egg production is sufficient. This will be very important as it will also have an influence on hormone production and it will be crucial to monitor this adaptation period beforehand. It can take us up to three weeks and replacement of some animals to find the best social groupings. This is particularly true for the TG240 the Medaka Extended One Generation Reproductive Toxicity Study where fish have to be set up in pairs in a stage where animal replacement is no longer possible. If the animals do not reproduce well with each other, the validity of the entire test is at risk easily.

E.2. Telephone interview 2

Particular challenges with including steroid measurements in fish assays for regulatory use will be to account for diurnal variation in hormone levels, stress levels, the diet and even origin of the fish. We have standard operating procedures for husbandry, handling, enrichment and diet for the species we work with based on many years of experience. As a result, we get very high survival rate (over 98%) and would doubt any data derived from fish with reported colony survival rates below 75%. We also record origin of all individual fish and always know which fish have been sampled at what time. The number of fish in OECD test guidelines can be an issue (too low) as the tests have been validated for other endpoints. We typically use larger sample sizes than those recommended in guideline studies to study effects on steroid levels, as they are inherently more variable that OECD Test endpoints.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Another important issue is related to the sampling volume; all validated species are small and as such provide a very small amount of blood which can provide measurements typically for one steroid, sometimes two.

Another issue I see is the almost exclusive use of zebrafish and in particular the fish sexual development test. Beyond small sampling volume, there are all sort of peculiar issues with the zebrafish such as the lack of genetic sex and the rather peculiar type of sexual differentiation they display (first they all become females and then some develop as males).

Furthermore, to gain a full picture of endocrine disruption in fish, it's important to include steroids other than oestrogens and androgens and in particular testosterone; we must also include 11-ketotestosterone and other steroids such as progestogens.

We developed and validated the tank water assay. This method provides many advantages such as avoiding anaesthetising the fish that would cause stress and interfere with hormone measurements as a result. It is also possible to carry out individual fish measurements. The fish are trained to spend some time alone in the sampling tank to again avoid inducing stress during sampling. This method also allows sampling in smaller species where blood sample volume may be a challenge to carry out desired hormonal analyses. Furthermore, it allows the determination of up to 8 steroids simultaneously.

For plasma samples, we favour RIA assays. Based on our experience, we have actually found them to be more reliable (in terms of both sensitivity and specificity) than ELISA kits.

In summary, measuring hormones in fish requires a lot of hard work and experience but it can be done.

E.3. Telephone interview 3

We work with fish and amphibians. We typically only carry out measurement required in guideline studies. We could measure additional endpoints if it were required by the client but in our experience so far, there has been no demand for hormonal measurements. In frogs we measure apical endpoints and some tissues in some cases and in fish we measure vitellogenin. ELISA is the preferred method for VTG measurements.

Mammals are tested in our facility but I am not personally involved. However, there would potentially be in house capacity for hormonal measurements (both steroids and thyroid) as we have both in vitro and in vivo facilities. If a study sponsor did request hormone analysis, it would not be too challenging for us because we are already familiar with sampling techniques for matrices such as plasma or mucus and we have enough experience within the organisation to perform hormone analysis.

Because we are a contract lab, we work under GLP and so all new procedures need to be internally validated in house before we can offer a specific service.

A challenge we could anticipate in fish, depending on the test species, is the sampling volume and being able to obtain sufficient amount of plasma. We often work with fathead minnow and this is quite a large fish so we collect the blood from the tail. For zebrafish, which are much smaller, you have to perform a cardiac puncture to obtain the blood sample and this is more challenging procedure.

As a large organisation, we have a lot of in-house knowledge and capacity between our different sites. We are the only site that carries out testing in fish and frogs but could draw on the experience of our colleagues working with mammalian species. Furthermore, measuring hormones in fish and/or frog would offer useful additional information for people working on hazard or risk assessment of substances.

E.4. Telephone interview 4

We are a company that primarily carries out short acute test in microalgae and vertebrate species including fish. We have never carried out hormonal measurements in fish. We are not really equipped for flow-through experimental set-ups so we do not currently cover endocrine disruption test guidelines and I do not expect us to have the capacity to offer this kind of analysis in the near future. I do not know of many labs that offer endocrine work but get the impression that interest in has increased a lot

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

in the past year and many labs are considering increasing their capabilities in endocrine work. Currently, there are only a handful in Europe.

If we need to, we take blood samples and send them for analysis to another facility. We do have some existing standard operating procedures for taking blood samples. Generally, the fish are anaesthetised using buffered MS222 and depending on the size of the fish, we follow the guidelines of our supplier to minimise the stress. We do not yet have standard procedures regarding the timing of the sampling. Blood samples are stored in the fridge for 24hours before spinning. After the serum has been collected, we store in the freezer at -20oC although some clients prefer us to store samples in the fridge to avoid dry hard shipment. If it were a requirement to source a GLP compliant lab for analysis, this may act as a barrier for us being able to offer this type of analysis. It is easier to find contractors that follow ISO and/or are GMP compliant. It would also make the study more complicated on our side because then there are different levels of quality assurance and we would need to appoint a principal investigator.

If hormonal measurements were required as part of the testing that we carry out we would need some guidance on standardisation of methods particularly in relation to the conditions for the testing, anaesthesia protocols, steps to minimise stress, time of day, storage and transport of samples and recommended method of hormonal analysis.

E.5. Telephone interview 5

We carry out whole organism bioassays for the oil sector, driven by the OSPAR regulations, essentially for the registration of chemicals that are used offshore. We use specific standard protocols but the assessment of endocrine disrupting properties is not part of the requirements. Skeletonema is the relevant ISO standard for the algal test, then the OECD protocols for *Acartia tonsa* for invertebrates with marine species of fish, the sheepshead minnow tends to be the standard test species. The testing we are involved in is related to the OCNS, Offshore Chemical Notification Scheme. It is a prerequisite that the chemicals that are used offshore are REACh-registered but we do not routinely carry out testing for REACh registration; our portfolio does include freshwater tests. Typically, the chemicals we test will already have undergone the REACh registration process.

We have also been involved in risk-based assessment for the oil sector organised by the Department for Business, Energy and Industrial Strategy (BEIS), the regulator. There is a key requirement to assess the potential impacts on the environment of produced water that is discharged offshore. We do so using both actual data and modelling. We have not done any in situ field studies as it's not currently required for the assessment. However, in situ field testing is stipulated by some other countries. By extension, it seems logical that any impacts in terms of organisms in the field are likely to be related to sublethal effects. Although we're not directly involved in field evaluation, endocrine effects is something that the industrial sector should be aware of. Any impacts of the chemicals used by operators and companies should be taken into account. As a company, we are also looking to extend our portfolio in terms of the tests we offer and endocrine disruption testing is something that we identified as a probable future need. Any guidance on standardisation, standardised protocols or international harmonisation would therefore be of high interest. The International Organisation for Oil and Gas Producers (IOGP) are currently working on their own guidance document (which we are commissioned to draft) to support risk-based approaches to environmental impact assessments. There are disparities internationally in terms of what is practised routinely and there is tremendous scope for global harmonisation, and issues such as EDCs are important in terms of all aspects related to the environment. We are interested in extending our portfolio. In the past, we have extended it from marine testing to freshwater.

Because we are situated in a remote island, there are challenges in terms of geography and logistics. However, in terms of the calibre of our employees and our set-up, we do not envisage any specific problems. We have certain constraints in terms of our facilities. For example, we have limited analytical chemical capability in terms of instrumentation. In terms of toxicological testing and prerequisite requirements that samples must be tested with a specific time frame, because of where we are located, there may be challenges in terms of getting samples to the lab. They would need to be shipped or transported by helicopter. The staff themselves do not live on the island and commute by ferry every morning and this can on rare occasion be impacted by weather conditions. We do not however see any

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

specific challenges in terms of the implementation of the testing. This set of specific circumstances is something that we are used to overcome. We have for example implemented video facilities to monitor the fish behaviour. We are one of the few labs in our sector to have facilities for culturing and testing in-house and we hold a Home Office license, so we are aware of restrictions and ethical considerations in terms of necessary testing and reducing the stress of the animals.

We're a GLP lab and work towards being a centre of excellence. When developing new test methods, it is a prerequisite that we conduct our own in-house validation. In fact, we regularly participate in laboratory trials and are involved in the development of new standards. Our laboratory staff is skilled in whole organism bio-assays, so the preparation of samples may be a little outside our current capabilities but it's not something beyond our means.

With regards to the whole sector and infrastructure, there are very few labs that can implement any new regulatory requirements and considering the existing infrastructure is extremely important.

E.6. Telephone interview 6

We have many years' experience working in the context of pesticide and biocide regulations. In Europe, the required avian tests are acute toxicity in one species and the reproductive toxicity, usually in a quail species. Since we are developing our product for global users, we also have to keep in mind what the other legislations require and therefore, for most of our products, we have more data than the EU requires. For example, for the US, we have to submit two acute oral toxicity tests with different species, usually quail and passerine bird (canary or zebrafinch). This depends on the length of the study and the birds we can source. With passerine birds, it can be difficult to get the quality and the number of birds needed for this test. Then we have a 5-day dietary study that is currently required by the US but also, for example, China, Brazil, also done in two species, quail and duck. For the US and other legislations like China, we have to do the reproduction study, according to OECD TG206, which is quite old one, also done in quail and duck. We usually test in the bobwhite quail although TG206 also accepts the Japanese quail (but this species is not accepted by the US). Bobwhite quail studies are only offered at the moment in the US. There are several labs in Europe that offer the TG206 study but not in the bobwhite quail. If we were to carry out a study in the Japanese quail in Europe, we would have to repeat the study in the bobwhite quail in the US. For this reason, we conduct a bobwhite quail and duck test in the US. Then there are some special testing requirements from certain countries like India, Brazil, China that require acute studies with the finished product.

Current avian test guideline date from 1984, so they are definitely not state-of-the-art studies and there are some fundamental flaws in their design without even considering their sensitivity to detect endocrine disrupters. It's not a successful study from the perspective of investigating effects of chronic exposure on reproduction. There have been various initiatives over the years to refine it, which is partly why the USEPA has a different study design. But this OECD test guideline has definitely not been successfully validated for the detection of endocrine disrupters. It is one of the worse designs in ecotoxicology. We have so many spurious, chance findings. We measure a lot of different parameters, we calculate ratios. When we find a statistically significant difference, not dose-response related, it is difficult to interpret within the wider context of effects detected. There often seem to be no biological plausibility behind it. But it is statistically significant and therefore significant for regulatory authorities. For every parameter we may add, we also increase the likelihood of spurious findings. In our experience, every second study has to be repeated because we cannot interpret the results. We were involved in efforts with the German UBA in 1991-92 to revise the guideline but they were blocked by the USEPA for almost 10 years. Some of these frustrations regarding this assay are reflected in the OECD guidance document 150 as well as in the ED guidance. It's just not designed to be sensitive to these endpoints. It may be sensitive to but not diagnostic of those endpoints.

In terms of hormone measurements, there's no kind of chemistry done, there's negligible sampling unless for kinetics reasons. These are not routine endpoints. Our understanding is that taking blood samples may even create problems. For example, for the USEPA, if you take blood samples, you are disturbing the birds and may decrease sensitivity if you decrease the performance of control birds. Therefore it is difficult to add parameters or change anything to this guideline. A pair, male and female

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

birds, are kept in a cage for weeks and expected to produce nearly an egg a day, and the female gets exhausted. This is a very forced situation compared to what would happen in the wild. There is also a question about the best timing of any blood sampling, because every day the female is doing the same thing.

It's important when interpreting results to consider the context and the protection goal. In recent times, it's not clear that pesticides have contributed to endocrine-mediated effects in bird populations. The case study that would come to mind would be DDT but that doesn't represent the pesticide profiling of today. It is part of the history for why these regulations are in place. Regulations in that respect are working and products like DDT would not be authorised onto the market today. Considering the current guidance document p107 Table 17 showing the main investigated parameters in avian studies that fall in levels 4 and 5 of the OECD conceptual framework, none of these endpoints are diagnostic. In comparison with mammalian assays, we do not have the mechanistic understanding in birds. It's difficult to ascertain what the findings really mean. We have a study which runs for at least 20 weeks. We expose the birds for an 8-week period on a short daylight regime. Then we increase the day length and after 2 to 4 weeks they start laying eggs and we collect the eggs for 10 weeks. The birds start laying eggs after 10-12 weeks exposure and we don't know how long it takes for the effects to kick in. For pesticides, we generally don't have a 12 weeks exposure period in the field. These test guidelines are from 1984, a time where organochlorines were in use. These compounds are stable and lipophilic and it takes some time for the birds to get the relevant body burdens. So the test was appropriate for this class of compounds, but nowadays we have a completely different type of chemistry. Compounds that bioaccumulate would not be authorised. We have also got rid of the compounds that exert acute toxicity such as organophosphate and carbamates. The purpose of this TG206 is to predict population level impacts, it was never expected to provide sophisticated mechanistic information. A completely different study design would be needed if we really wanted to elucidate, prove or disprove an endocrine mechanism. A separate investigation would be required to find something meaningful. If we did have liver or thyroid findings in adults, it would need to be correlated with an effect in the egg, the progeny, to be considered a population level impact. A histopathology finding in the adult does not necessarily constitute evidence of an adverse effect on the population. That's an important distinction for the ecotox assays. It gets a little complicated when we use ecotox models such as fish and frogs embryos to predict endocrine activity to be extrapolated to predict human sensitivity.

The test guideline creates a completely artificial scenario where female lay almost one egg a day whereas in the wild they would lay 10 eggs, 2 clutches. Then it is difficult to leverage the results to understand what would happen in the field. Additionally, we are trying to understand the mechanism through which adverse effects arise. If we add parameters like histopathology, we are likely to find something, or not, but we lack an understanding of what is normal in these birds. After 22 weeks exposure or 22 weeks in a cage for the controls, the birds are really exhausted. So it's not clear how to interpret a change in the histopathology of the thyroid for example. We are lacking a baseline. When comparing with the academic literature on avian histopathology, it is worth keeping in mind that the exposure duration is usually quite different. On a global scale, the pathology of these birds is so rarely examined. There is less than a handful of pathologists that can interpret those studies. There's a huge knowledge gap. Further, compared to the mammalian assay, a hormonal measurement in the laying hen would not be informative about what happens for the egg, this is a physically separate compartment. There is no ongoing exchange between the parents and the offspring during incubation. So even if the maternal thyroid levels were changed, it's really not clear what impact that would have on the development of the separate egg. This is major difference with the mammalian in utero system. In terms of the stress the animals are under in this test system, it is normalised by the control birds. Further in terms of husbandry, parameters like the type of cages the birds are kept in can make a difference. There are some cages with a metal wall that looks like a mirror and some of the male birds would fight their own reflection.

This assay can be so difficult to implement only two labs that carry out this type of assays have survived in the US, down from maybe 10 in the 1980s. There are two main reasons for this; first there is no great demand for these studies, and you need to be able to maintain quality over years. Previously, the main pesticide manufacturers conducted their avian studies in house. This is no longer the case, if you

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

develop one new compound a year, that equivalent to a couple of studies that year. This is not sufficient to justify retaining the expertise and maintaining facilities. Contract labs do maybe five or six studies a year. Some of these studies will not meet the validity criteria. The validity criteria are quite low and few but if you observe a significant effect in the lowest dose but no effects are seen in the mid and high dose, the USEPA would conclude that you do not have a NOEC and the study would not be acceptable. The endpoints measured can be highly variable, it can depend on the supplier or on the time of the year during which the study is conducted. If the birds are already in breeding conditions, they need to be brought ack to non-breeding conditions, and that does have an impact on the quality of the study. There are a lot of difficulties stemming from the surrounding infrastructure that is not as standardised or normalised as for the toxicology of rodents. The test populations are more heterogenous than rodent strains and there is more inherent variability in avian studies. There are usually 16 or 18 pairs per dose group and it's not uncommon for a pair not to lay any eggs and this pair is lost to the study. There were propositions to start with proven breeders but this was not implemented. Additionally if a pair lays just one or two eggs in a treatment group, it's impossible to ascertain whether this was treatment related or not.

There is quite a bit of literature from the USEPA EDSP as they had huge discussions about the avian study.

E.7. Telephone interview 7

I am responsible for ED evaluation in fish and amphibians. In our organisation, we do not have the capacity or facilities to measure hormones in-house so we rely on the services of contract research organisations (CROs). CROs do have the expertise and equipment to measure hormones in fish tests and potentially amphibians but they do not carry out these types of measurements on a regular basis. Hormone measurements in current test guidelines for fish are at the moment optional and are not conducted in a systematic way. In my experience CROs do not offer these measurements in their initial quotation but will carry them out if they are specifically requested by their customers. The current position of the industry is that they are interested in any endpoint that could be useful for ED evaluation. So hormone measurements are one option, but they are also considering swim bladder inflation in the fish early life stage test as an additional endpoint for thyroid disruption. This endpoint was investigated by the USEPA and is mentioned on a recurring basis by EFSA in their discussions. Currently, there are still questions on the specificity of the swim bladder inflation toward thyroid disruptors It's important to consider the appropriate endpoints to investigate endocrine activity reliably and industry is considering different options and supporting the development of methods.

There may be opportunities to measure hormones in current guideline studies but what is currently not well defined and would be useful to get some guidance on is the optimal timing for taking samples as well as sample sizes. A difficulty is that there is no large database for historical controls available. Making a parallel with vitellogenin measurements, which are fairly comparable, the variability within the controls is fairly high. It would be quite important to have similar information on hormone levels in historical controls in fish.

The selection of test species is an important parameter also. Some species are more difficult to handle due to their small size or their sensitivity to manipulation. Due to the potential of manipulation to induce stress, which might have important consequences to identify endocrine mechanisms, we are currently investigating non-invasive sampling methods for vitellogenin. We are interested in non-invasive methods but we are not sure if we can do the same for hormones. The fathead minnow is probably the species most commonly used for endocrine disruption evaluations, but the zebrafish is also used on a regular basis. The medaka is used more and more frequently. In such species, we already have some background knowledge so they should be prioritised. Both the medaka and zebrafish are small species, but the zebrafish may be a particularly problematic species with respect to e.g., sex determination.

In terms of interpretation of results, I anticipate the same difficulties interpreting of changes in thyroid hormone levels in mammalian models, for which we do have a lot of background knowledge, in fish models for which we have less information. So there is a case for building our knowledge base of the normal variations in thyroid hormones in those species before we can correctly interpret data on the

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

effect of chemicals on thyroid hormone levels. Important potential confounding factors include stress and food, but also the development of microbes, including pathogens, in the test medium. We do not yet have a good understanding of the influence of these factors on thyroid hormones. Steroid hormones may be less sensitive to external factors. We have a better understanding of thyroid hormones in amphibians as a specific model for investigating thyroid disruption. We probably need to gain the same level of knowledge for fish, with the added difficulties that several different species are used and this may change from one species to another. This links back to the selection of the most appropriate test species for investigating thyroid hormones.

For amphibians, the Larval Amphibian Growth and Development Assay (LAGDA) is a level 4 assay in the OECD Conceptual Framework and will not inform population-relevant endpoints. It includes endpoints related to growth and development but these may not be the most relevant endpoints in terms of population level effects. This test was not validated with specific thyroid disrupting substances and it does not offer much more information than the Amphibian Metamorphosis Assay (AMA). In terms of evaluation of endocrine disruption, the LAGDA is not really suitable. It's also a test that uses many animals, so there are also issues in terms of reducing the use of animals in testing. If you extend the AMA, you get nearly the same information as the LAGDA and some adaptation to the AMA test guideline may be more appropriate. Including some time-to-stage measurements is something that was already considered in the literature.

The situation is different for fish because we have different types of tests, the fish short-term reproduction assay (FSTRA), the fish sexual development test, the fish full lifecycle test (FFLCT) and extended one-generation reproduction test. Depending on the question, one may select a shorter assay like FSTRA or a longer assay like FFLCT. We probably need very good guidance on how to use the context of those tests to implement hormone measurements, e.g. about the timing of hormone measurements in FFLCT. This may be very complex in longer term assays compared to shorter tests such as FSTRA for which there may only be a couple of time points suitable for sampling. There is a need to balance the effort depending on the type of test being conducted.

In terms of the interpretation of results, hormonal measurements should be considered in the context of other endocrine sensitive endpoints and will be indicative of an endocrine mode-of-action rather than an adverse effect. For example, we frequently see effects on vitellogenin without any effects on reproduction. Similarly, for swim bladder inflation, the USEPA published 3 papers that concluded that swim bladder inflation is not necessarily very sensitive nor highly specific for thyroid disruption. We are currently collaborating with universities to clarify the sensitivity and specificity of swim bladder inflation and vitellogenin. Vitellogenin is sought to be associated with reproduction and this is only partially true as vitellogenin also has non-reproductive roles. It's also involved in immunological response and oxidative stress. There could be confounding factors related to the fish environment rather than exposure to a chemical that affect vitellogenin, such as the development of bacteria in the aquaria in which the fish were exposed. Hormonal measurements are part of the weight-of-evidence assessment but cannot support a firm conclusion in isolation. When they are associated with other endpoints, as part of an AOP, then one can draw more robust conclusions.

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Annex A – Protocol development for the systematic evidence map of methods to measure hormones related to estrogen, androgen and thyroid pathways in fish, birds and amphibians

Annex A can be found in the online version of this output ('Supporting information' section) and includes the following documents:

1. Pilot searches
2. Data extraction template
3. Draft protocol for open review
4. Draft Protocol open review feedback form
5. Comments on draft protocol
6. Responses to comments

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Annex B – Data extraction for the systematic evidence map of methods to measure hormones related to estrogen, androgen and thyroid pathways in fish, birds and amphibians

Annex B can be found in the online version of this output ('Supporting information' section) and includes the following documents:

- Data extraction file_hormones

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Annex C – Data extraction for the extensive review of the literature on pathology in birds

Annex C can be found in the online version of this output ('Supporting information' section) and includes the following documents:

- Data extraction file_bird pathology

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).

Annex D – Supplementary files related to the online questionnaire

Annex D can be found in the online version of this output ('Supporting information' section) and includes the following documents:

- Participant Information Sheet
- Interview Consent form
- Online questionnaire

The present document has been produced and adopted by the bodies identified above as author(s). This task has been carried out exclusively by the author(s) in the context of a contract between the European Food Safety Authority and the author(s), awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the author(s).