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REVIEW ARTICLE

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A comprehensive review of regulatory test methods for endocrine adverse health effects

M. K. Manibusan[a] and L. W. Touart[b]

[a]Exponent Inc., Washington DC, WA, USA; [b]Equiparent Consulting, Woodbridge, VA, USA

ABSTRACT

Development of new endocrine disruption-relevant test methods has been the subject of intensive research efforts for the past several decades, prompted in part by mandates in the 1996 Food Quality Protection Act (FQPA). While scientific understanding and test methods have advanced, questions remain on whether current scientific methods are capable of adequately addressing the complexities of the endocrine system for regulatory health and ecological risk assessments. The specific objective of this article is to perform a comprehensive, detailed evaluation of the adequacy of current test methods to inform regulatory risk assessments of whether a substance has the potential to perturb endocrine-related pathways resulting in human adverse effects. To that end, approximately 42 existing test guidelines (TGs) were considered in the evaluation of coverage for endocrine-related adverse effects. In addition to evaluations of whether test methods are adequate to capture endocrine-related effects, considerations of further enhancements to current test methods, along with the need to develop novel test methods to address existing test method gaps are described. From this specific evaluation, up to 35 test methods are capable of informing whether a chemical substance perturbs known endocrine related biological pathways. Based on these findings, it can be concluded that current validated test methods are adequate to discern substances that may perturb the endocrine system, resulting in an adverse health effect. Together, these test methods predominantly form the core data requirements of a typical food-use pesticide registration submission. It is recognized, however, that the current state of science is rapidly advancing and there is a need to update current test methods to include added enhancements to ensure continued coverage and public health and environmental protection.

Abbreviations: ACTH: Adrenocorticotropic hormone; AhR: aryl hydrocarbon receptor; AMA: amphibian metamorphosis assay; AOP: adverse outcome pathway; AR: androgen receptor; CLI: Croplife International; DHEA: dehydroepiandrosterone; DNA: deoxyribonucleic acid; DNT: developmental neurotoxicity; EASZY: estrogen active substances zebrafish; ECHA: European Chemical Agency; ECVAM: European Center for the Validation of Alternative Methods; EDC: endocrine disrupting chemical; EDSP: Endocrine Disruptor Screening Program; EFSA: European Food Safety Authority; EOGRT: Extended One Generational Reproduction Test; EPA: US Environmental Protection Agency; ER: estrogen receptor; EU: European Union; FFDCA: Federal Food, Drug and Cosmetic Act; FIFRA: Federal Insecticide, Fungicide, and Rodenticide Act; FOB: functional observational battery; FQPA: Food Quality Protection Act; FSH: follicle stimulating hormone; GHRH: growth hormone-releasing hormone; GnRH: gonadotropin-releasing hormone; GSI: gonado-somatic index; HPA: hypothalamus pituitary adrenal; HPG: hypothalamus pituitary gonadal; HPT: hypothalamus pituitary thyroid; ICCVAM: Interagency Coordinating Committee for the Validation of Alternative Methods; IGF: insulin-like growth factor; LABC: levator ani/bulbocavernosus muscle complex; LAGDA: larval amphibian growth and development assay; LH: luteinizing hormone; MEOGRT: Medaka Extended One Generation Reproduction Test; MIE: molecular initiating event; NIS: sodium-iodide symporter; NTP: National Toxicity Program; OCSPP: EPA Office of Chemical Safety and Pollution Prevention; OECD: Organization for Economic Co-operation and Development; PCB: polychlorinated biphenyls; PCDD: polychlorinated dibenzo-p-dioxin; PCDF: polychlorinated dibenzofurans; PCOS: polycystic ovaries syndrome; PND: postnatal day; PPAR: peroxisome proliferator-activated receptor; PPRE: proliferator-response element; PRL: prolactin; PXR: pregnane X receptor; RAR: retinoic acid receptor; REACH: Registration, Evaluation, Authorization and Restriction of Chemicals; RIA: radio-immuno assay; RXR: retinoid X receptor; SDWA: Safe Drinking Water Act; STTA: stably transfected transcriptional activation; SVHC: substances of very high concern; TDS: testicular dysgenesis syndrome; TG: test guidelines; TPO: thyroid peroxidase; TR: thyroid hormone receptor; TSH: thyroid stimulating hormone; UDS: unscheduled DNA synthesis test; VDR: vitamin D receptor/vitamin D3 receptor; VTG: vitellogenin; WOE: weight of evidence; XETA: xenopus embryonic thyroid signaling assay; α: alpha; β: beta; γ: gamma; δ: delta

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CONTACT Mary Manibusan mmanibusan@exponent.com Exponent Inc., Washington DC, WA, USA

Table of contents

Preface

The scope of this document is centered on determining the adequacy of the existing test methods to detect perturbations of endocrine-related toxicity pathways. To that end, sections addressing the redundancies of measured endpoints across validated test methods, biological pathways, and disease disorders will begin with a brief background of what is currently known about endocrine pathways or diseases/disorders and what types of endpoints are considered diagnostic of the disease or otherwise informative of pathway perturbations. These endpoints will be used collectively to inform whether existing test methods are adequate to measure those same endpoints or those that may be secondary effects.

While this document is broad in its coverage of animal test methods, it is narrowly focused on the state of the current toxicity test methods to explore and probe the known or suspected endocrine-related pathways to define apical endpoints for risk assessment purposes; this document neither covers the human population level effects that are typically reported in human epidemiology studies, nor captures Toxcast/Tox21 *in silico* or high throughput assays that may relate to endocrine bioactivity. The human epidemiology study literature and high throughput assays on endocrine-related health outcomes are databases beyond the scope of this document. It will focus primarily on standard *in vitro* and *in vivo* whole animal test methods to determine whether these test methods adequately address endocrine-relevant adverse effects.

Key terms and definitions

Adaptive effects – Effects that are part of the normal organ/organismal response that reflects compensatory mechanisms. These effects are generally defined as those that are temporary effects or effects that reflect the system's ability to compensate and overcome adverse effects from perturbation of homeostatic mechanisms, while adverse effects are those effects that are irreversible, permanent, and have long-term impacts on the function of the organ and organism (OECD 2016).

Adverse outcome – A specialized type of key event that is generally accepted as being of regulatory significance on the basis of correspondence to an established protection goal or equivalence to an apical endpoint in an accepted regulatory guideline toxicity test (OECD 2016).

Adverse outcome pathway (AOP) – A sequence of key events commencing with initial interactions of a stressor with a biomolecule in a target cell or tissue (i.e. molecular initiating event) progressing through a dependent series of intermediate events and culminating with an adverse outcome (OECD 2016).

Apical endpoints – Results of an *in vivo* assay which describe a response by the organism as a whole (e.g. fecundity or growth) which have possible implications for its biological fitness, rather than a response of the endocrine system alone (including physiological changes dependent on the endocrine system, such as vitellogenin induction). Apical responses may or may not result from endocrine changes (e.g. fecundity may be affected both by some endocrine disrupting chemicals (EDCs) and by some non-EDCs) (OECD 2012).

Endocrine disrupter – An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and, consequently, causes adverse health effects in an intact organism, or its progeny, or (sub)populations (Damstra et al. 2002).

Hormones – Chemical messengers that are released in one tissue and transported via the circulation to reach target cells in other tissues (Martini et al. 1995).

Hormonal activity – These are endpoints in an *in vivo* assay which show whether the endocrine system has been stimulated and often provide information of mechanistic value. In other words, they are not apical endpoints. It is possible in some cases for indicators of hormonal activity to respond to a test chemical while apical endpoints do not respond, while in other cases, both types of endpoints give a response or only apical endpoints respond (OECD 2012).

Key event – A change in biological state that is both measurable and essential to the progression of a defined biological perturbation, leading to a specific adverse outcome (OECD 2016).

Low dose effect – Effects measured at doses below those recommended in validated test methods and/or below the regulatory reference doses (US EPA 2013).

Mode of action – A biologically plausible series of chemical-specific key events starting with the interaction of an agent with a cell, through physiological and tissue or organ changes, resulting in an adverse effect or an adverse outcome (Sonich-Mullin et al. 2001; Dellarco & Fenner-Crisp 2012).

Molecular initiating event (MIE) – A specialized type of key event that represents the initial point of chemical interaction at the molecular level within an organism that results in a perturbation that starts the adverse outcome pathway (OECD 2016).

Risk – The chance of harmful effects to human health or to ecological systems resulting from exposure to an environmental stressor (US EPA 2016).

Weight of evidence (WOE) – A systematic review method that uses a pre-established protocol to comprehensively, objectively, transparently, and consistently identify and evaluate each stream of evidence, including strengths, limitations, and relevance of each study and to integrate evidence as necessary and appropriate based upon strengths, limitations, and relevance of each study and to integrate evidence as necessary and appropriate based upon strengths, limitations, and relevance (Congressional Record Senate 2016).

Executive summary

Testing chemicals for endocrine activity affects stringent regulatory authorities such as the United States Environmental Protection Agency (US EPA), the European Chemical Agency (ECHA), the Japan Ministry of Environment, and other agencies. As a leading example of this regulatory requirement, in 1996 the US Congress directed the EPA, as stated in 1996 with the Food Quality Protection Act (FQPA) (P.L. 104–170), that amended the Federal Food, Drug and Cosmetic Act (FFDCA) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) to identify chemicals with the potential to interact with the endocrine system and, subsequently, to test those that may warrant additional longer-term definitive tests for regulatory determinations. Supplemental to the FFDCA, amendments to the Safe Drinking Water Act (SDWA) (P.L. 93–523), section 1457 specifies

"…the Administrator may provide for testing under the screening program authorized by section 408(p) of such Act, in accordance with the provisions of section 408(p) of such Act, of any other substance that may be found in sources of drinking water if the Administrator determines that a substantial population may be exposed to such substance."

In response to the statutory mandate, the EPA developed its Endocrine Disruptor Screening Program (EDSP) in 1998, which employs a two-tiered screening and testing approach. In partnership with the global regulatory community, the Agency had taken up the charge to scientifically develop and validate test methods, and has issued endocrine test orders to screen and test >50 chemicals for endocrine activity. In 2015, the EPA issued its weight of evidence (WOE) determinations on the screening results for the initial list of 52 chemicals; of those, 32 displayed potential endocrine activity, but 14 were determined to have sufficient information to complete a risk assessment, and only 18 were identified for further testing to better assess potential adverse effects, which may or may not change the current regulatory risk assessments for these chemicals.

EDSP tiered screening and testing approach

1. **Shorter-term screening battery** – to determine whether chemical substances have the potential to interact with the endocrine system.
2. **Longer-term definitive testing** – to test chemical substances to determine the dose response and apical endpoints for risk assessment purposes.

While the EDSP employs a two-tiered screening and testing approach, the Organization for Economic Co-operation and Development (OECD) has developed a tiered conceptual framework. Different from the EDSP tiered screening and testing, the conceptual framework was not developed in response to legislative mandates and should not be interpreted as a regulatory decision-making framework. These five levels are populated with available endocrine-specific and nonspecific assays, depending on the complexity of the test systems.

OECD tiered conceptual framework

1. Existing data and non-test information.
2. *In vitro* assays providing data about selected endocrine mechanisms/pathways, including mammalian and non-mammalian methods.
3. *In vivo* assays providing data about selected endocrine mechanisms/pathways.
4. *In vivo* assays providing data on adverse effects on endocrine-relevant endpoints.
5. *In vivo* assays providing more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the lifecycle of the organism.

To date, there are >40 different ecological and mammalian assays included in OECD's conceptual framework and there are additional assays being developed in other parts of the world for submission to OECD for validation and deliberation by the Endocrine Disrupter Testing and Assessment Advisory Group. Despite the volume and complexity of existing test methods, there is no single assay that is capable of determining endocrine activity or adversity. It is through the collective consideration of multiple assays, along a biological continuum, with the determination of complementarity and necessary redundancies that enhances the overall scientific confidence of the assessment as to whether the chemical elicits an endocrine perturbation that is sufficient to lead to a subsequent adverse health outcome.

Based on the evaluation of whether test methods are adequate in addressing adverse outcomes from disturbances in endocrine-related pathways and diseases, there are approximately 30 different test methods, roughly 75% of the test methods evaluated that are capable of informing whether a chemical substance perturbs one or more of the following endocrine-related biological pathways: steroid synthesis, retinoid, peroxisome proliferator-activated receptor (PPAR), vitamin D, somatotropic, hypothalamus pituitary adrenal (HPA), hypothalamus pituitary thyroid (HPT), hypothalamus pituitary gonadal (HPG)/androgen, and HPG/estrogen. In addition, the evaluation of test methods to inform endocrine-related diseases and disorders identified, approximately 15–34 assays are capable of identifying endpoints informative of endocrine-related human diseases.

In review of the biological pathways related to endocrine perturbation, the number and breadth of relevant assays listed in the OECD Endocrine Conceptual Framework are adequate in the coverage of endpoints, taxonomic groups, sensitive life stages, and routes of exposure. Current test methods have been shown to provide adequate data to ensure that a chemical's ability to perturb the endocrine system to elicit an adverse response would not go undetected.

Conclusions

The currently validated test methods possess sufficient redundancies that allow them to adequately identify substances that have adverse consequences due to a perturbation in the endocrine system. The currently available test methods form the core data requirements of a typical food-use pesticide registration submission.

History and evolution of the endocrine disruption testing program

The human and environmental health concerns for chemicals with potential endocrine activity have a long history. To date, endocrine disruption as a phenomenon has been critically reviewed by several authors, notably by Crisp and colleagues (1998), the National Research Council (NRC 1999), Damstra and coworkers (2002), and Ottinger and vom Saal (2002). A common thread across these reviews is the notion that chemicals that may disrupt the endocrine systems of humans and wildlife by chemicals may also be contaminating their habitats. As late as 1999, Geschwind and others, in reviewing the available literature, concluded that the concern for endocrine-related effects on wildlife is based on a preponderance of circumstantial evidence, which called into question whether the endocrine disruption phenomenon was real or whether it was perceived to be true (Geschwind et al. 1999). Meanwhile, a pandemic of alleged endocrine-related disorders from attention health disorder, autism, diabetes, obesity, childhood cancers, testicular cancer in young men, infertility, male dysgenesis syndrome, hypospadias, low sperm count, loss of semen volume and sperm quality, and increased risk of testicular and prostate cancers had been reported and alleged to be due to endocrine disrupting chemicals (EDCs). All these diseases and disorders have been supposedly increasing in incidence and can allegedly be traced back to some purported prenatal exposure to EDCs (Cottrell & Ozanne 2007; Charbonneau & Koger 2008; Newbold et al. 2008; Sharpe & Skakkeback 2008). To this day, the subject of testing chemicals for potential endocrine disrupting potential have continued to increase in public awareness and contributed to initiation of a number of international endocrine-related research and regulatory activities.

Globally, at the request of member countries and the international industry, the OECD initiated the Special Activity on Endocrine Disrupters Testing and Assessment in 1996 with the objective of providing a set of internationally recognized and harmonized test guidelines (TGs) and assessment strategies for regulatory application. The European Commission also commenced actions to regulate endocrine disrupting substances in 1996. The Community Strategy for Endocrine Disrupters (COM (1999) 706) was adopted in 1999, and was later revised in 2004 (SEC (2004) 1372) and in 2007 (SEC (2007) 1635). In this strategy, short-term action (establishment of a list of priority substances for further evaluation), mid-term action (test method development and research implementation), and long-term action (consideration of methodologies for risk assessment and risk management) have been described for eventual implementation. Also in the European Union's (EU's) 2007 Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), substances "having endocrine disrupting properties" that are also identified from scientific evidence as causing probable serious effects were mentioned as a condition to be authorized as substances of very high concern (SVHC) and regulated accordingly. More recently, the EU has proposed endocrine criteria for establishing chemicals that perturb the endocrine system that involves a WOE approach (EU 2016) that relies on the adverse outcome pathway (AOP) framework as an organizational tool to define an EDC based on evidence supporting an endocrine modality.

Japan similarly responded to emerging concerns with Strategic Programs on Environmental Endocrine Disruptors, a strategic plan more commonly referred to as "SPEED '98", later published in May 1998 [http://www.env.go.jp/en/chemi/ed/speed98/sp98.html]. The specific activities that target the ability to better address endocrine chemicals include: (1) promotion of field investigations into the state of environmental pollution and effects on wildlife, (2) promotion of research and method development, (3) promotion of environmental risk assessment, environmental risk management, and information sharing, and (4) efforts to strengthen international networks. In addition, the Japan Ministry of Environment has hosted the annual International Symposium on Endocrine Disruptors and initiated bilateral joint research projects with the UK, the Republic of Korea, and the USA. Japan's rigorous efforts have continued through enhanced tasks on endocrine disruption (EXTEND 2005) and EXTEND 2010. More recently, Japan's Ministry of Environment has updated this strategic plan in the finalized EXTEND 2016[1], which emphasizes the implementation of hazard and risk assessment in support of future regulatory risk management decisions. The specific assessment framework in this program will subsequently be incorporated into existing regulatory assessment practices, which include setting environmental water quality standards to ensure protection for aquatic life and organisms, implementing a tiered risk assessment approach for industrial chemicals under the Chemical Substances Control Law, and setting standards for registration decisions under the Agricultural Chemicals Regulation Law.

In the USA, two laws were passed by Congress in 1996, the FQPA and amendments to the SDWA. The US EPA was directed to test all pesticide ingredients to determine whether they have estrogenic or other endocrine activity (Federal Register 1998a, 1998b), with the option to include drinking water contaminants that meet the exposure criteria described in the SDWA. Under the FFDCA, amended in 1996, EPA was explicitly directed to develop an endocrine screening program that uses appropriate validated test systems and other scientifically relevant information to determine whether certain substances may have an effect in humans that is similar to effects produced by a naturally occurring estrogen or such other endocrine effects as the EPA may designate (21 U.S.C. 346a(p)). While the language may seem prescriptive, this statutory language provides significant latitude for how the Agency would develop endocrine-specific test methods to implement the EDSP.

In 1998, after external expert consultations and scientific peer reviews, the EPA established the EDSP as a two-tiered screening and testing program to implement the statutory requirements of FFDCA section 408(p) (21 U.S.C. 346a) by considering endocrine bioactivity differently from endocrine adversity. Under Tier 1 EDSP testing, a battery of screening level assays is employed to identify substances that have the potential to interact with the estrogen, androgen, and thyroid hormonal systems. The determination of endocrine biological activity is made based on a WOE approach, taking into account data from the Tier 1 screening level assays and other available scientific information that will inform the decision of whether additional Tier 2 longer-term testing is warranted.

Endocrine Tier 1 screening battery

The EDSP Tier 1 battery was designed to work as a whole with all of the screening assays. The basis for selecting an assay to include in the battery involved two principal aspects: (1) the capacity of an assay to detect estrogen, androgen, and/or thyroid mediated effects by various modes of action, including receptor binding (agonist and antagonist) and transcriptional activation, steroidogenesis, and hypothalamic pituitary gonadal (HPG) or hypothalamic pituitary thyroid (HPT) feedback; and (2) the degree that *in vitro* and *in vivo* assays complemented one another in the battery as summarized. In addition, rodent and amphibian *in vivo* assays were selected for inclusion in the screening battery based on their capacity to detect direct and indirect effects on thyroid function (HPT feedback). Thus, the robustness of the proposed battery is based on the strengths of each individual assay and their complementary nature within the battery to detect effects on the estrogen, androgen, and thyroid hormonal systems.

Application of the Tier 1 validated assays was fully implemented by July 2015 when EPA released its reviews of the Tier 1 screening assay results for the first 52 pesticide chemicals (active and inert ingredients) evaluated by the EDSP almost 17 years after the program's inception in 1998. Among this initial group of chemicals, 32 (62%) were determined to have some level of endocrine system interaction. Of these, 14 were determined to already have sufficient information for EPA to conclude there is no risk attributable to endocrine bioactivity and 18 would require additional testing. Despite these initial conclusions, the Agency has not finalized

the information collection requests for any additional Tier 2 tests. Therefore, final conclusions on whether any of these chemicals are indeed endocrine disruptors and require added regulatory intervention is still several years away. Beyond the List 1 EDSP chemicals, the Agency had finalized the second list of chemicals for Tier 1 screening, drawing largely from the Safe Drinking Water Chemicals on the Candidate Contaminant List (CCL) and the registration review of pesticide-active ingredients under Federal Insecticide Fungicide Rodenticide Act (FIFRA). To date, there has been no additional activities related to the List 2 compounds.

Endocrine Tier 2 definitive test methods

For those chemicals that warrant additional endocrine testing, the Agency may require Tier 2 studies. EDSP Tier 2 test methods include longer-term, more definitive studies in principal taxa that are designed to identify any biologically adverse effects caused by exposure to the substance; these Tier 2 test methods also serve to establish a quantitative relationship between the dose and associated hormonal adverse effect. In such apical organismal tests, the actual modality of an adverse effect may not be definitively obvious. However, within a full chemical risk assessment, the endocrine endpoint is only one of many different health endpoints, with the most sensitive, lower dose endpoint driving the risk assessment. Any endpoint whether endocrine-related or not may serve as the regulatory point of departure if it is protective of all subsequent health effects manifested at higher doses. The determination that the current point of departure is sufficiently protective for all adverse effects was made by the EPA for 14 of the List 1 chemicals that had positive Tier 1 screening results.

Future use of ToxCast/Tox21 high throughput endocrine test assays

To address the slow pace, resource-intensive nature of screening thousands of chemicals, EPA announced a pivot plan on June 18, 2015 (80 FRN 80) for incorporating high throughput (HTP) assays and computational models into the EDSP. These new methods would be used to screen and prioritize chemicals for their potential to interact with the endocrine system (Reif et al. 2010; Rotroff et al. 2014; Browne et al. 2015; Judson et al. 2015; Mansouri et al. 2016). Moving beyond prioritization of chemicals, the EPA has recently proposed to use HTP assays as alternatives for three of the 11 current assays in the EDSP Tier 1 screening battery (estrogen receptor (ER) binding, ER transactivation, uterotrophic). In the near future, EPA plans to develop alternative screening methods for the remaining eight Tier 1 assays using further advancements in HTP assays and computational models. Use of these alternative test methods is expected to quickly accelerate the pace of screening without sacrificing accuracy or quality (Browne et al. 2015).

Are current methods adequate to identify EDCs?

While scientific understanding and test methods have advanced over the past several decades, controversy remains as to whether current scientific methods are capable of addressing the intricacies of a seemingly unpredictable endocrine system. Many critics have noted that because the endocrine system is an interconnected system that operates at molecular levels, with an ability to cross-talk, perturbation at the site of contact may be manifested in multiple endocrine organs distant from the site of initial contact. In addition, changes during sensitive life stages may make the developing organism more vulnerable at discrete periods during organogenesis that may not manifest until later in life, a term described as "*in utero* programming". Transgenerational effects and skipping generations before a phenotypically expressed malformation raise questions on whether any individual or combination of test method(s) is capable of determining effects of different life stages of increased sensitivity. In addition to whether the test methods are capable of identifying endocrine effects across various organs and life stages, some reviewers have questioned whether current test methods are sufficient at capturing non-monotonic dose response curves or low dose effects, defined as doses below those currently employed in validated test methods. Questions on the adequacy of our current test methods and, ultimately, whether regulatory risk assessments are comprehensive enough to ensure human health protection, have been repeatedly raised (Bergman et al. 2013; Diamanti-Kandarakis et al. 2009; Gore et al. 2015) and seldom challenged. Additional comments on the issues around endocrine disruption include:

- Mixture of EDC effects – Individuals and populations are exposed to an ever-changing pattern of chemicals that are released into the environment as a mixture of multiple chemicals. Simultaneous exposures to multiple chemicals with potential endocrine disrupting effects may interact additively, synergistically, or antagonistically (Gore et al. 2015).
- Inadequate Endocrine Test Methods – Available OECD-validated test methods for the identification of endocrine disrupters capture only a segment of the known range of endocrine disrupting effects, mainly focused on estrogenicity, anti-androgenicity, and thyroid disruption. Other aspects of the endocrine system(s) are not considered (Berlayment Declaration on Endocrine Disrupters 2013).
- While internationally harmonized and validated test methods and well-established test systems are available, not all are fully implemented. The current testing and data information requirements for industrial and commercial chemicals under REACH, and those established for plant protection products, are not specifically designed to identify EDCs (Berlaymont Declaration on Endocrine Disrupters 2013; Gore et al. 2015).

Some reviewers have proclaimed that it "is simply not reasonable to assume a chemical is safe until proven otherwise," (Gore et al. 2015) and have invoked the precautionary principle to limit exposures to potential EDCs even before a scientific determination can be made with empirical data, data from animal toxicity testing (EEA, 2012; Gore et al. 2015). While some reviewers have identified some issues with

extrapolations between human and test animals, in 2009 they recognized that endocrine pathways are well conserved across multiple species; and with the recognition that we cannot conduct human testing with environmental contaminants, it is therefore, "reasonable to use animal models for understanding human disease processes, as long as any potential differences are taken into account" (Diamanti-Kandarakis et al. 2009). To that end, the current allegations have implicated EDCs in the etiology of complex human diseases that include precocious puberty, fecundity, adverse pregnancy outcomes, endometriosis and uterine fibroids, testicular dysgenesis syndrome (TDS), breast cancer, prostate cancer, testicular cancer, thyroid cancer, metabolism and development, diabetes mellitus and adrenal disorders, and effects related to immune disorders. As stated by Gore et al. 2015, "determine how much evidence is enough based on rigorous, peer-reviewed science – keeping in mind that absolute proof of harm or proof of safety is not possible."

Objective and evaluation method of this detailed review

The objective of this comprehensive review document is to assess the adequacy of current validated test methods to identify endocrine perturbations through three different analyses that evaluate the available validated test methods for their coverage of:

1. endocrine-related health endpoints,
2. endocrine-related pathways,
3. endocrine-related diseases.

Method of detailed review

The focus of this review is on the adequacy of current validated test methods to inform endocrine-related toxicity pathways and to determine whether there are sufficient levels of protection incorporated into regulatory risk assessments using endocrine-related adverse health outcomes. To that end, this review is focused on validated test methods, as required by section 408(p) of the FFDCA, developed by the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM), as well as the European Center for the Validation of Alternative Methods (ECVAM) and the OECD. The test methods included in this review are described in greater detail in the EPA Office of Chemical Safety and Pollution Prevention (OCSPP) TGs (Supplementary Appendices A and C) and the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters. The period of human and ecological health test methods coverage extends from pre-1998 to 2016.

Inclusion and exclusion criteria applied:

- Included if test method was validated using ICCVAM, ECVAM, or OECD process between pre-1998 and 2016.
- Included if test method is accepted by a regulatory authority (e.g. EPA, FDA, EU, Japan Ministry of Environment, etc.).
- Included if test method addressed endpoints specific or related to endocrine perturbations, irrespective of whether they address human or ecological health. These would include all OCSPP 890 series that were validated specifically to test chemicals through the EPA/EDSP and those test methods developed through the EPA and Japan Ministry of Environmental Bilateral Agreement.
- Excluded if the test method has not been validated by current method, or OECD performance-based validation process.
- Excluded if the test method does not provide information relevant to any endocrine-related toxicity pathways, diseases, or disorders.
- Excluded if test method used species/strain/taxa not relevant to inform endocrine-related perturbations.

In addition to the evaluation of current test methods, this document also draws on the previously prepared state of the science documents on this topic, inclusive of the European Commission's *State of the Art Assessment of Endocrine Disrupters: Final Report*; *OECD Series on Testing and Assessment No. 178*; the European Food Safety Authority's (EFSA's) *Scientific Opinion on the hazard assessment of endocrine disruptors*, and the *OECD Guidance Document on Standardized Test Guidelines for Evaluating Chemicals for Endocrine Disruption No.150*. These documents form the core foundation of this comprehensive review document, along with more current documents issued on the EDSP on its use of *Weight of Evidence and Advanced High Throughput Assays*. In preparing this report, some more recently published reviews were heavily relied upon to address the current allegations of the extant TGs and validated test methods, particularly the US Endocrine Society Statements "Endocrine-Disrupting Chemicals" for human health endpoints (Diamanti-Kandarakis et al. 2009; Gore et al. 2015).

Endocrine-related health endpoints

This review will focus on the adequacy of current test methods to inform endocrine-related health endpoints for use in regulatory risk assessment. The analyses will include all available test methods used by regulatory authorities and those included in the OECD Conceptual Framework for Test and Assessment of Endocrine Disrupters (OECD 2012).

The OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters provides a tiered framework for organizing *in silico* and other tools as well as validated test methods that are available to evaluate chemicals for potential endocrine disruption, as illustrated in Table 1. The framework, as adopted by the OECD, is not intended to be a regulatory testing strategy, but is intended to provide guidance in prioritizing relevant methods according to the type and level of information needed in a regulatory assessment. Further information regarding the use and interpretation of these test methods are available in Guidance Document No. 150 (OECD 2012).

It is important to recognize that the framework is not all-inclusive and only represents the current status of assays at the time of its adoption (2012). Information and data available in Levels 1 and 2 of the framework are primarily useful for prioritization and informing mechanisms of action, while

Table 1. OECD conceptual framework for testing and assessment of endocrine disrupters.

	Mammalian and non-mammalian toxicology	
Level 1 Existing data and non-test information	• Physical and chemical properties (e.g. MW, reactivity, volatility, biodegradability) • All available (eco)toxicological data from standardized or non-standardized tests • Read across, chemical categories, QSARs, and other *in silico* predictions, and ADME model predictions	
Level 2 *In vitro* assays providing data about selected endocrine mechanism(s)/pathways(s) (mammalian and non-mammalian methods)	• Estrogen or androgen receptor binding affinity • ER transactivation (OECD TG 455 – OECD TG 457) • Androgen or thyroid transactivation (if/when test guidelines become available) • Steroidogenesis *in vitro* (OECD TG 456) • MCF-7 cell proliferation assays (ER antagonist) • Other assays as appropriate	
	Mammalian toxicology	Non-mammalian toxicology
Level 3 *In vivo* assays providing data about selected endocrine mechanism(s)/pathway(s)*	• Uterotrophic assay (OECD TG 440) • Hershberger assay (OECD TG 441)	• Xenopus embryo thyroid signaling assay (when/if TG becomes available) • Amphibian metamorphosis assay (AMA) (OECD TG 231) • Fish reproductive screening assay (OECD TG 229) • Fish screening assay (OECD TG 230) • Androgenized female stickleback screen (Guidance Document 140)
Level 4 *In vivo* assays providing data on adverse effects on endocrine relevant endpoints†	• Repeated dose 28-day study (OECD TG 407) • Repeated dose 90-day study (OECD TG 408) • 1-generation reproduction toxicity study (OECD TG 415) • Male pubertal assay (see GD 150, Chapter C4.3)‡ • Female pubertal assay (see GD 150, Chapter C4.4)‡ • Intact adult male endocrine screening assay see GD 150, Chapter Annex 2.5) • Prenatal developmental toxicity study (OECD TG 414) • Chronic toxicity and carcinogenicity studies (OECD TG 451, 452 and 453) • Reproductive screening test (OECD TG 421 when enhanced) • Combined 28-day/reproductive screening assay (OECD TG 422 when enhanced) • Developmental neurotoxicity (OECD TG 426)	• Fish sexual development test (OECD TG 234) • Fish reproduction partial lifecycle test (when/if TG becomes available) • Larval amphibian growth and development assay (when TG becomes available) • Avian reproduction assay (OECD TG 206) • Mollusk partial lifecycle assays (when TG becomes available)¶ • Chironomid toxicity test (TG 218-219)¶ • Daphnia reproduction test (with male induction) (OECD TG 211)¶ • Earthworm reproduction test (OECD TG 222)¶ • Enchytraeid reproduction test (OECD TG 220)¶ • Sediment water lumbriculus toxicity test using spiked sediment (OECD TG 225)¶ • Predatory mite reproduction test in soil (OECD TG 226)¶ • Collembolan reproduction test in soil (TG OECD 232)¶
Level 5 *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the lifecycle of the organism†	• Extended one-generation reproductive toxicity study (OECD TG 443)§ • 2-generation reproduction toxicity study (OECD TG 416 most recent update)	• FLCTT (Fish lifecycle toxicity test) (when TG becomes available) • Medaka extended one generational reproduction test (MEOGRT) • Avian two-generation reproductive toxicity assay • Mysid lifecycle toxicity test (when TG is available)¶ • Copepod reproduction and development test (when TG is available)¶ • Sediment water chironomid lifecycle toxicity test (OECD TG 233)¶ • Mollusk full lifecycle assays (when TG is available)¶ • Daphnia multigenerational assay (if TG is available)¶

(OECD 2012)

*Some assays may also provide some evidence of adverse effects.

†Effects can be sensitive to more than one mechanism and may be due to non-ED mechanisms.

‡Depending on the guideline/protocol used, the fact that a substance may interact with a hormone system in these assays does not necessarily mean that when the substance is used it will cause adverse effects in humans or ecological systems.

¶At present, the available invertebrate assays solely involve apical endpoints that are able to respond to some endocrine disrupters and some non-endocrine disruptors. Those in Level 4 are partial lifecycle tests, while those in Level 5 are full- or multiple lifecycle tests.

§The extended 1-generation reproductive toxicity study (OECD TG 443) is preferable for detecting endocrine disruption because it provides an evaluation of a number of endocrine-specific endpoints in the juvenile and adult F1, which are not included in the two-generation study (OECD TG 416) adopted in 2001.

Level 3 methods serve in screening for activity *in vivo* and informing possible endocrine mechanisms or toxicological pathways. Level 4 and 5 assays characterize possible adverse effects at sensitive life stages and/or through lifecycle exposures. A more detailed summary of the existing validated test methods is provided in Supplementary Appendix A.

More specific to the analysis of TGs and whether they adequately capture endocrine-related endpoints of concern, Table 2 provides an inventory of the 42 specific test methods considered in the analyses. These test methods have met the previously described inclusion criteria and have been routinely required as part of the pesticide registration dossiers.

The evaluation of the various validated test methods against the endocrine-related endpoints measured included OECD and OCSPP TGs (Supplementary Appendix D). Each TG was reviewed and measured endpoints were inventoried for any directly or indirectly endocrine-related endpoints.

Based on the systematic review of all available test methods, as included in the OECD Conceptual Framework (Table 1) and the OCSPP TGs, and identifying all endocrine-related endpoints measured in each test method, as

Table 2. Test methods available for review.

	Test guideline	OECD	OCSPP
1.	Amphibian metamorphosis assay	231	890.1100
2.	Androgen receptor binding assay	–	890.1150
3.	Aromatase assay	–	890.1200
4.	Estrogen receptor binding	455	890.1250
5.	Estrogen receptor transcriptional activation	455	890.1300
6.	Fish short-term reproduction assay	229	890.1350
7.	Hershberger assay	441	890.1400
8.	Female pubertal assay	–	890.1450
9.	Male pubertal assay	–	890.1500
10.	Steroidogenesis assay	456	890.1550
11.	Uterotrophic assay	440	890.1600
12.	Avian two-generation toxicity test in Japanese quail	–	890.2100
13.	Medaka extended one-generation reproduction test	240	890.2200
14.	Larval amphibian growth and development assay	241	890.2300
15.	Performance-based test guideline for stably transfected transactivation *in vitro* assay to detect ER agonists and antagonists	455	–
16.	Performance-based test guideline for human recombinant estrogen receptor (HRER) *in vitro* assays to detect chemicals with ER binding affinity	493	–
17.	Repeated dose 28-day oral toxicity study in rodents	407	870.3050
18.	Repeated dose 90-day oral toxicity study in rodents	408	870.3100
19.	Repeated dose 90-day oral toxicity study in non-rodents	409	870.3150
20.	Repeated dose 21/28-day dermal toxicity study	410	870.3200
21.	Repeated dose 90-day dermal toxicity study	411	870.3250
22.	Repeated dose 28-day inhalation toxicity study	412	–
23.	Repeated dose 90-day inhalation toxicity study	413	870.3465
24.	One-generation reproduction toxicity study	415	–
25.	Two-generation reproduction toxicity	416	870.3800
26.	Neurotoxicity study in rodents	424	870.6200
27.	Reproduction/developmental screening test	421	870.3550
28.	Combined repeated dose toxicity study with reproduction/developmental screening test	422	870.3650
29.	Prenatal developmental toxicity study	414	870.3700
30.	Developmental neurotoxicity study	426	870.6300
31.	Extended one-generation reproductive toxicity study	443	870.3800
32.	Chronic toxicity study	451	870.4100
33.	Carcinogenicity study	452	870.4200
34.	Combined chronic toxicity and carcinogenicity study	453	870.4300
35.	Developmental thyroid toxicity study (comparative thyroid assay)	–	–
36.	Repeated dose 21-day fish assay	230	–
37.	Fish sexual development test	234	–
38.	Androgen female stickleback screen	148*	–
39.	Fish early life stage toxicity test	210	850.1400
40.	Fish short-term toxicity test on embryo and sac-fry stages	212	–
41.	Fish lifecycle toxicity	–	850.1500
42.	Avian reproduction test	206	850.2300

*Guidance document.

illustrated in Supplementary Appendix C, the level of redundancies in endpoints measured is significant. The analysis emphasizes the comprehensiveness of the two-generation (OECD 416) and extended one-generation (OECD 443) reproduction studies in capturing endocrine-related health endpoints. These longer-term, definitive test methods can inform a multitude of endocrine-specific modalities which comport with the scientific rationale of using these two test methods in the Tier 2 EDSP screening and testing program and Level 5 OECD Conceptual Framework testing. Among the two different test methods, the updated OECD 443 study is the more preferred method because it includes endocrine-sensitive endpoints such as, nipple retention, anogenital distance at birth, and hormonal measurements.

In addition to current validated test methods that have been reviewed, research is underway in developing additional endocrine-related test methods. To that end, OECD has a number of new test methods under development (see Supplementary Appendix B for additional details):

- *Xenopus* Embryonic Thyroid Signaling Assay – Currently undergoing validation and is a potential method for detection of substances that act within the thyroid system.
- Estrogen Active Substances Zebrafish (EASZY) Assay – An *in vivo* mechanism-based assay that uses transgenic zebrafish cyp19a1b-GFP embryos expressing green fluorescent protein under control of the zebrafish *cyp19a1b* promoter.
- HPA (Stress) Axis in Fish – A new test protocol will be designed to identify changes in HPA axis in fish.
- Stably Transfected Transcriptional Activation (STTA) Assay – Assay in the final stages of development and adoption. This assay was designed for the detection of androgenic agonists and antagonist activity of chemicals.
- Performance-Based Test Guideline on Androgen Receptor (AR) Transactivation Assays – A performance-based guideline is being proposed for AR and AR STTA.
- Update of Test Guideline 455 – A proposal to add the Transcriptional ERα CALUX assay to the current OECD 455 TGs.
- Detailed Review Paper on Retinoic Acid Pathway – To evaluate and propose new test methods and

enhancements to existing TGs to specifically address the retinoic acid pathway.
- Reference Chemicals for E-A-S Metabolism – To investigate cell lines and methods for incorporating steroid metabolism in *in vitro* assays.
- Feasibility Study for Minor Enhancements of Test Guideline 414 (Prenatal Developmental Toxicity Study) – Adding additional endocrine-specific endpoints to the OECD TG 414 without impacting the reliability of the test method.

Endocrine-related biological pathways

This review will include the adequacy of current test methods to inform endocrine-related biological pathways. The AOPs addressed in this review include: HPG; steroidogenesis; estrogen; androgen; HPA; HPT; metabolic pathways and somatotropic, PPAR; retinoid, and vitamin D pathways. Recognizing that some AOPs may be better elucidated than others, this review will be an iterative process. As the state of the endocrine science continues to rapidly evolve, AOPs will be better informed.

Adverse disease outcome pathway framework

Toxicity pathways have long been considered and characterized in the analysis of the mode of toxicological action framework, but more recently the expansion of the mode of action analysis which focuses on specific chemicals or chemical classes has taken precedent in being aligned with a broader, chemical-agnostic form of the AOP framework (Ankley et al. 2010). AOPs have been used as a tool to formulate biological pathway linkages from molecular key initiating events, target organ toxicities and organismal impacts and population level toxicities (Figure 1).

This AOP framework will be used as the organizing structure for this review, which will lay out the state of the science of known endocrine-related toxicity pathways and the specific test methods that inform those critical pathways. While each pathway will be described independently of one another, it is important to recognize that cross-talk between different pathways is ubiquitous among endocrine-signaling pathways. Disruption of one endocrine-signaling pathway can have multiple diverse impacts on other AOPs.

For example, in addition to the effects of estrogens on glucocorticoid and growth hormone/insulin growth factor 1-(IGF) signaling, androgen-signaling disruptors can also affect glucocorticoid signaling, thyroid hormone and corticosteroid-signaling disruptors can impact the somatotropic axis and fatty acid-signaling disruptors can impact thyroid hormone signaling. Cross-talk among signaling pathways increases the level of complexity associated with identifying specific chemical-related endocrine effects across multiple assays, from short-term, high dose, screening level assays to apical effects in longer-term tests in whole organisms.

Hypothalamus pituitary gonadal axis

The HPG axis is primarily responsible in vertebrates for regulating sexual development and reproduction. The HPG axis, in general, consists of the hypothalamus and hypophysis (pituitary gland) in the brain, the gonads (testis, ovary), and the liver. Communication between the components of the axis occurs via one or a combination of signals: neuronal secretion of a neurotransmitter or neurohormone to targeted cells; secretion of a hormone by specialized cells within a tissue into extracellular space and transport through the blood to cells in a target tissue; or a paracrine hormone secreted by one cell into extracellular space to diffuse to adjacent cells of a different type. The hypothalamus secretes gonadotropin-

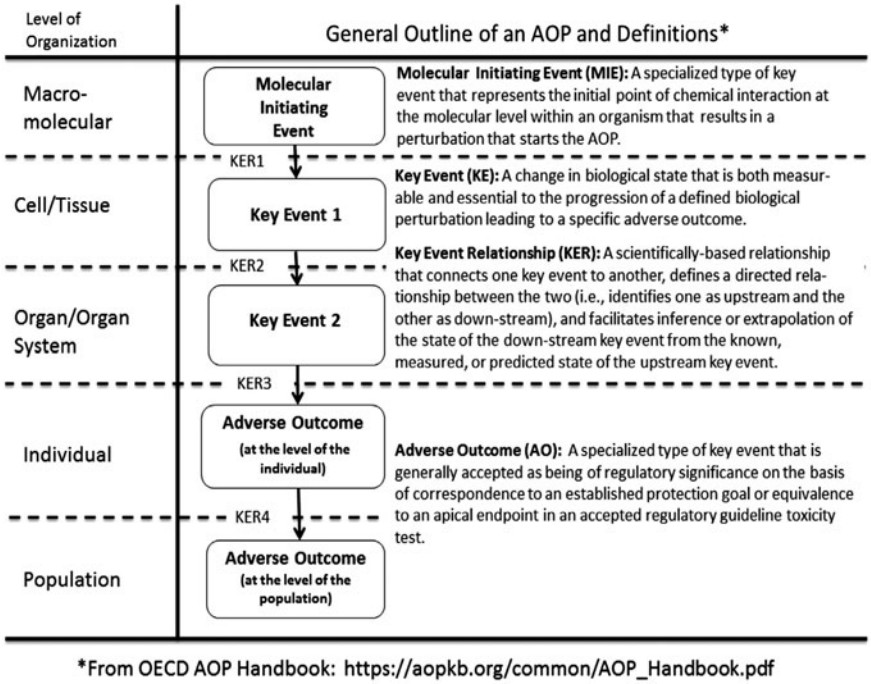

Figure 1. OECD adverse outcome pathway.

releasing hormone (GnRH) that stimulates cells in the pituitary gland to release of the gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which in turn stimulate gonadal development, in particular by induction of sex steroid synthesis. Sex steroids (estrogens and androgens) in the gonads feed back to the hypothalamus and the pituitary, thereby regulating gonadotropin synthesis and release. In addition, non-steroidal feedback regulation of gonadotropins by a FSH-stimulated gonadal peptide, inhibin, contributes to the synchronization of the HPG axis at all stages of the lifecycle.

Key pathways within the HPG axis include the ER signaling pathway, the AR signaling pathway, and the steroid synthesis pathway. This axis and associated pathways function within the broader endocrine system and interact with the other axes (i.e. HPT, HPA, etc.) and existing pathways (i.e. vitamin D, retinoid signaling, etc.). The consequences of disrupting communication or processing at any point within the axis can be significant and reverberate within the axis and across others. Depending on the developmental status of an organism, these detrimental consequences can be categorized as activational or organizational. Activational disturbances are generally associated with later life stages and may occur in reaction to external (environmental) stimuli and are often immediate and mostly recoverable. Organizational responses are essentially permanent changes that occur in early developmental stages and affect differentiation, proper structure, function of tissues, and are latent, that are becoming manifest at subsequent life stages or generations. Therefore, organisms are most susceptible to deleterious perturbations during developmental phases of their lifecycle which may alter phenotypic sex characteristics or during critical activational phases such as normal production/release of gametes in mature adults. Besides their importance for reproduction, the sex steroids are pleiotropic hormones modulating many other physiological functions, such as metabolism, the immune system, the cardiovascular system, and skeletal homeostasis.

Steroid synthesis pathway. Steroid hormones are the principal active agents in tissues within the HPG axis. All vertebrate steroids are synthesized from cholesterol and most cholesterol is synthesized in the liver from acetyl-coenzyme A by glycolysis or fatty acid oxidation. Ovaries and testes within the HPG can also synthesize cholesterol, but generally use the cholesterol derived from the liver and available as a lipoprotein complex in the plasma. Synthesis of the gonadal steroids involves a series of primarily P450 cytochrome enzyme-induced steps which convert the C_{27} cholesterol to C_{21} progestins (gestagens) and corticoids, and then to C_{19} androgens or C_{18} estrogens. Progestins are the obligatory precursors of steroid hormones that are important in the maintenance of a healthy pregnancy and in the initiation or cessation of normal mating behaviors (Norris & Carr 2013). Androgens are considered the "male" or "masculinizing" hormones because they stimulate development of male characteristics. The primary androgen in mammals is testosterone, which is produced in the testis after stimulation by LH from the pituitary. In females, androgens are synthesized in the adrenal cortex and ovaries. Estrogens, "female" hormones, are converted from androgens by the aromatase enzyme. Aromatase synthesis is stimulated by FSH from the pituitary. Although androgens and estrogens are commonly referred to as sex hormones and either "male" or "female", this is misleading. All male vertebrates produce estrogens and progestins and all female vertebrates produce androgens. The sexes do differ in the relative amounts produced of circulating androgens and estrogens due primarily to differences in proportions of steroidogenic enzymes in their gonads. Imbalances in the relative concentrations of these enzymes or interference with their action can lead to an array of endocrine abnormalities.

Interference with the steroid synthesis pathway can disrupt both androgen- and estrogen-regulated processes by limiting availability, altering timing, or perhaps cause an excess of the intended hormone or an unintended one. An AOP for aromatase inhibition based on a small fish model illustrates how such interference directly leads to adverse consequences (Figure 2).

Aromatase inhibition, which prevents conversion of testosterone to estradiol, could also lead to an accumulation of testosterone and increased androgen signaling (agonism). Androgen agonism can greatly affect metabolism and stimulate respiratory metabolism and increase hypertrophy in many tissues (e.g. increased muscle mass), reproductive anomalies, and behavioral problems.

Two *in vitro* assays within the EDSP Tier 1 battery specifically address steroid synthesis: OCSPP 890.1550/OECD TG 456 – Steroidogenesis (Human Cell Line – H295R) and OCSPP 890.1200 – Aromatase (Human Recombinant) Assay. In addition, many of the existing *in vivo* assays can also contribute corroborating information to increase evidence that the steroid synthesis pathway is compromised or to ensure detection of adverse impacts of steroid synthesis disruption (see Table 3 for relevant assays and endpoints).

The number and breadth of relevant assays across endpoints, taxonomic groups, life stages, and routes of exposure as summarized in Table 3 provide comprehensive, redundant, and complementary data to ensure a chemical substance active in an endocrine pathway would be detected and its potential for apical adverse effects characterized. Interpreting results across the broad suite of these assays can be accomplished by examining the results of all the assays together using a WOE approach. The longer-term definitive test methods, including the extended one-generation reproduction test (EOGRT), and the EDSP Tier 2 multigenerational reproduction studies provide data that best inform risk assessment with dose response and apical endpoints for consideration.

Estrogen receptor-signaling pathway. The earliest concern for endocrine disruptors was related to environmental chemicals that mimic estrogen, by binding to the ER, and thereby interfere with the estrogen signaling pathway. This concern led directly to legislation and statutory language [FQPA of 1996] which specifically required chemicals that acted similar to estrogenic compounds to be screened in the interest of protecting human health. Estrogens are important for reproductive function in males and females, including sexual differentiation of the brain and development of secondary female

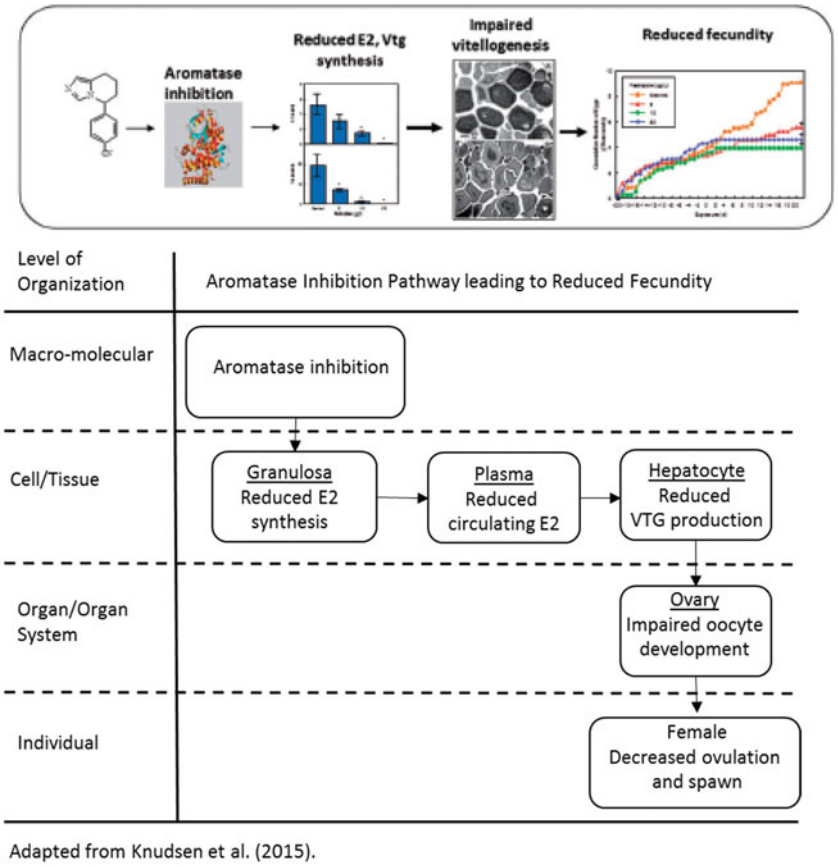

Figure 2. Aromatase inhibition pathway leading to reduced fecundity.

sex characteristics. In addition, estrogens are involved in the structural and functional development of other bodily systems across genders and for maintaining overall homeostasis.

The pleiotropic roles of estrogens both within the HPG axis and across axes to interact and affect other pathways demonstrates the complexity of the endocrine system and the difficulty of assigning a specific activity since estrogens often act in concert with other hormone signals. For instance, it is well known that estrogens along with other hormones play a pivotal role in brain differentiation during early development and that disruption of these processes can result in persistent changes leading to altered timing of puberty and/or behavioral changes (Nelson 1995). Estrogens may also influence the immune system, and there is good evidence for the involvement of sex steroids in the etiology of several inflammatory pathological conditions. In mammals, recent research has focused on potential associations between EDCs and metabolic syndrome. In humans, insulin synthesis by pancreatic β-cells is affected by estradiol, suggesting estrogen agonists could pose a higher risk of type 2 diabetes in exposed individuals. Several estrogenic EDCs have also been reported to impact metabolic pathways and growth.

The ER-mediated reproductive impairment AOP describes the linkage between the event that initiates the pathway, binding to the ER, and key events made at successively higher and more complex levels of biological organization. The pathway progresses from the molecular initiating event (MIE) through cell and tissue level gene transcription and translation, continuing through organ effects to an adverse outcome observed at the individual and/or population level. In lower vertebrates, such as fish and amphibians, most studies on endocrine disruption are related to perturbations of male reproductive physiology due to exposure to estrogenic EDCs, resulting in the feminization phenomena such as intersex gonads (e.g. occurrence of testicular oocytes) or shifts in sex ratio. In some cases, genotypic males are being phenotypically expressed as female. With a plausible pathway to an adverse outcome described, a rationale is provided for using chemical interaction at the MIE as a basis for prioritizing chemicals for further screening using *in vivo* assays that incorporate endpoints at higher biological levels of organization to confirm or better characterize an adverse outcome.

Interference with ER signaling within the HPG can disrupt developmental-, growth-, and reproduction-regulated processes by limiting availability, altering timing, or perhaps cause an excess of the intended hormone or an unintended one. An illustration of the AOP for ER agonism leading to an increased risk of endometrial cancer demonstrates how such interference directly leads to adverse consequences is provided in Figure 3.

The ER-signaling pathway is well covered with several assays capable of detecting specific estrogenic bioactivity. The EDSP Tier 1 battery includes five primary assays which have estrogen pathway specific endpoints and many other established assays, *in vitro* and *in vivo*, can also contribute corroborating information to increase evidence that the

Table 3. Test methods and endocrine pathway relevant endpoints.

		Relevant endocrine pathway								
Test method	Endpoints	S	E	A	T	H	M	D	P	R
OCSPP 890.1100/OECD TG 231 – Amphibian metamorphosis (frog)	• Developmental stage progression				✓					
	• Asynchronous development				✓					
	• Hind limb length				✓					
	• Snout-vent length				✓					
	• Thyroid gland histopathology				✓					
	• Enlarged larynx, forelimb (agonism)			✓						
	• Body weight		✓	✓		✓	✓	✓	✓	✓
	• Delayed developmental stage						✓			
OCSPP 890.1150 – Androgen receptor binding (rat prostate)	• Displacement of ligand from receptor (binding cannot distinguish between agonism or antagonism)			✓						
OCSPP 890.1200 – Aromatase (human recombinant) assay	• Inhibition of aromatase (CYP19) enzyme activity	✓								
OCSPP 890.1250 – Estrogen receptor binding	• Displacement of ligand from receptor (binding cannot distinguish between agonism or antagonism)		✓							
OCSPP 890.1300 – Estrogen receptor transcriptional activation (human cell line HeLa-9903)	• Activation of reporter gene linked to ER (agonism)		✓							
	• Inhibition of activation of reporter gene linked to ER (antagonism)		✓							
OCSPP 890.1350/OECD TG 229 – Fish short-term reproduction	• Vitellogenin induction in males (agonism)		✓							
	• Change in male 2° sex characteristics	✓	✓	✓						
	• Gonad histopathology (e.g. Leydig cell hyperplasia)	✓	✓	✓						
	• Vitellogenin depression in females (antagonism, assuming no systemic toxicity)	✓	✓							
	• Changes in estradiol and testosterone	✓	✓	✓						
	• Fecundity depression		✓	✓		✓	✓	✓	✓	✓
	• Abnormal behavior		✓	✓		✓	✓	✓	✓	✓
OCSPP 890.1400/OECD TG 441 – Hershberger (rat)	• Increase in weight of ventral prostate, seminal vesicles, LABC, Cowper's glands, glans penis (positive agonist outcome if 2 or more tissues are increased)			✓						
	• Reduction of androgen stimulated weights of ventral prostate, seminal vesicles, LABC, Cowper's glands, glans penis (positive antagonist outcome if 2 or more tissues are decreased)			✓						
	• Changes in serum hormones (optional)			✓						
OCSPP 890.1450 – Female pubertal (rat)	• Decreased female estradiol	✓	✓							
	• Weight change of ovaries	✓	✓	✓						
	• Histopathology in uterus & ovaries	✓	✓	✓						
	• Age change at first estrus		✓	✓						
	• Changes in estrus cyclicity	✓	✓	✓						
	• Age change at vaginal opening	✓	✓	✓						
	• Weight change of uterus	✓	✓	✓						
	• Increased thyroid weight				✓					
	• Possible liver weight increase (in combination with other thyroid-related endpoints)				✓					
	• Histopathology in thyroid (follicular cell height increase & colloid area decrease)				✓					
	• Serum T4 decreased, TSH increased				✓					
	• Changes in weight of pituitary and/or adrenals					✓	✓	✓	✓	✓
OCSPP 890.1500 – Male pubertal (rat)	• Age at preputial separation	✓	✓	✓						
	• Weight of seminal vesicles (+ coagulating glands), ventral prostate, dorsolateral prostate, LABC, epididymides	✓	✓	✓						
	• Histopathologic changes in testes, epididymides	✓	✓	✓						
	• Serum hormone measures	✓	✓	✓						
	• Testis weight	✓	✓	✓						
	• Increased thyroid weight				✓					
	• Possible liver weight increase (in combination with other thyroid-related endpoints)				✓					
	• Histopathology in thyroid (follicular cell height increase & colloid area decrease)				✓					
	• Changes in weight of pituitary and/or adrenals					✓	✓	✓	✓	✓
OCSPP 890.1550/OECD TG 456 – Steroidogenesis (human cell line – H295R)	• Interference with steroidogenesis/inhibition and induction of estradiol and testosterone synthesis	✓								

(*continued*)

Table 3. Continued

Test method	Endpoints	Relevant endocrine pathway								
		S	E	A	T	H	M	D	P	R
OCSPP 890.1600/OECD TG 440 – Uterotrophic (rat)	• Uterine weight (wet and blotted) increase [aromatizable androgens can increase uterine weight in both immature and ovariectomized female rats]		✓	✓						
	• Keratinization and cornification of vagina		✓							
	• Proliferation of endometrial epithelium		✓							
	• Changes in uterine histopathology		✓							
	• Reduction of estrogen stimulated uterine weight		✓							
	• Endpoints in the immature option where the HPG axis is intact may respond to other HP signaling modes of action (e.g. related to growth releasing hormone (GHRH) release)					✓	✓	✓	✓	✓
OCSPP 890.2100 – Avian two-generation toxicity test in the Japanese quail	• Changes in estradiol and testosterone	✓	✓							
	• Gonad histopathology (e.g. Leydig cell hyperplasia)	✓	✓	✓						
	• VTG depression in females (assuming no systemic toxicity)	✓	✓							
	• 1st appearance of foam		✓	✓						
	• Time to first egg laid		✓	✓		✓	✓	✓	✓	✓
	• Sexual behavior		✓	✓		✓	✓	✓	✓	✓
	• Egg production	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Eggshell thickness, strength, integrity		✓	✓	✓	✓	✓	✓	✓	✓
	• Embryo viability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Hatching success	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Body weight		✓	✓	✓	✓	✓	✓	✓	✓
	• Gross pathology		✓	✓	✓	✓	✓	✓	✓	✓
	• Weight of organs		✓	✓						
	• Testicular spermatic counts		✓	✓						
	• Induction of male 2° sex characteristics in females (agonism)			✓						
	• Depression of male 2° sex characteristics (antagonism)		✓							
	• Reduced fertility	✓	✓	✓	✓	✓				
	• Male-biased phenotypic sex ratio			✓						
	• Vitellogenin induction in males		✓							
	• Serum hormone measures	✓	✓	✓	✓					
	• Thyroid weight				✓					
	• Histopathologic changes in thyroid (follicular cell height increase & colloid area decrease)				✓					
	• Behavior		✓	✓		✓				
	• Gross morphology		✓	✓	✓	✓	✓	✓	✓	✓
	• Gonado-somatic index		✓	✓		✓	✓	✓	✓	✓
	• Multiple organ histopathology		✓	✓		✓	✓	✓	✓	✓
OCSPP 890.2200/OECD TG 240 – Medaka extended one-generation reproduction test	• Time to maturity (time to first spawn)	✓	✓	✓	✓	✓				
	• Vitellogenin depression in females (assuming no systemic toxicity)	✓	✓							
	• Depression of male 2° sex characteristics			✓						
	• Induction of male 2° sex characteristics in females	✓		✓						
	• Gonad histopathology (e.g. Leydig cell hyperplasia)	✓	✓	✓						
	• Reduced fecundity	✓	✓	✓	✓	✓				
	• Reduced fertility	✓	✓	✓	✓	✓				
	• Histopathologic changes in follicle cells (follicular cell height increase & colloid area decrease)				✓					
	• Male-biased phenotypic sex ratio			✓						
	• Female-biased phenotypic sex ratio		✓							
	• Vitellogenin induction in males		✓							
	• Altered levels of estradiol and/or testosterone	✓	✓	✓						
	• Hatching success		✓	✓	✓	✓				
	• Weight		✓	✓	✓	✓	✓	✓	✓	✓
	• Length		✓	✓	✓	✓	✓	✓	✓	✓
	• Altered behavior		✓	✓	✓	✓	✓	✓	✓	✓
	• Gross morphology		✓	✓	✓	✓	✓	✓	✓	✓
	• Gonado-somatic index		✓	✓		✓	✓	✓	✓	✓
	• Multiple organ histopathology		✓	✓		✓	✓	✓	✓	✓
OCSPP 890.2300/OECD TG 241 – Larval amphibian growth and development assay)	• Asynchronous development				✓					
	• Developmental stage progression				✓					
	• Hind limb length				✓					
	• Snout-vent length				✓					
	• Thyroid gland histopathology				✓					
	• Gonad histopathology		✓	✓		✓				

(*continued*)

Table 3. Continued

Test method	Endpoints	Relevant endocrine pathway								
		S	E	A	T	H	M	D	P	R
	• Male-biased phenotypic sex ratio			✓						
	• Female-biased phenotypic sex ratio		✓							
	• Vitellogenin increase in males and females		✓			✓				
	• Vitellogenin depression in males and females	✓	✓							
	• Time to metamorphosis (NF stage 62)		✓	✓	✓	✓				
	• Nuptial pad development		✓	✓						
	• Abnormal behavior					✓	✓	✓	✓	✓
	• Body weight		✓	✓	✓	✓	✓	✓	✓	✓
	• Gross pathology				✓	✓	✓	✓	✓	✓
OECD TG 455 – Performance-based test guideline for stably transfected transactivation *in vitro* assays to detect estrogen receptor agonists and antagonists	• Activation of reporter gene linked to ER (agonism)		✓							
OECD TG 493 – Performance-based test guideline for human recombinant estrogen receptor (hrER) *in vitro* assays to detect chemicals with ER binding affinity	• Inhibition of activation of reporter gene linked to ER (antagonism)		✓							
	• Displacement of ligand from receptor (binding cannot distinguish between agonism or antagonism)		✓							
OCSPP 870.3050/OECD TG 407 – Repeated dose 28-day oral toxicity study in rodents	• Histopathology changes in testes, epididymides, prostate + seminal vesicles with coagulating glands	✓	✓	✓						
	• Histopathology changes in ovary, uterus/cervix, vagina	✓	✓	✓						
OCSPP 870.3100/OECD TG 408 – 90-day oral toxicity in rodents	• Changes in weight of testes, epididymides, prostate + seminal vesicles with coagulating glands	✓	✓	✓						
OCSPP 870.3150/OECD TG 409 – 90-day oral toxicity in nonrodents	• Histopathology in mammary glands (females)	✓	✓	✓						
	• Weight change of uterus and ovary	✓	✓							
OCSPP 870.3200/OECD TG 410 – 21/28-day dermal toxicity	• Body weight			✓		✓	✓	✓	✓	✓
	• Serum hormone measures	✓	✓	✓	✓					
	• Possible liver weight increase (in combination with other thyroid-related endpoints)				✓					
OCSPP 870.3250/OECD TG 411 – 90-day dermal toxicity	• Histopathologic changes in thyroid (follicular cell height increase & colloid area decrease)				✓					
OECD TG 412 – subacute inhalation toxicity: 28-day study	• Increased thyroid weight				✓					
	• Histopathology in adrenal					✓	✓	✓	✓	✓
	• Changes in adrenal weight					✓	✓	✓	✓	✓
OCSPP 870.3465/OECD TG 413 – 90-day inhalation toxicity	• Histopathology in pituitary and mammary glands					✓	✓	✓	✓	✓
	• Tumor types		✓	✓	✓	✓	✓	✓	✓	✓
	• Clinical biochemistry		✓	✓	✓	✓	✓	✓	✓	✓
OECD TG 415 – One-generation reproduction toxicity study	• Change in AGD in male and/or female pups	✓	✓	✓						
	• Changes in estrus cyclicity (P, F1)	✓	✓	✓						
	• Age at vaginal opening (F1)	✓	✓	✓						
	• Age at preputial separation (F1)	✓	✓	✓						
	• Change in weights of: (P, F1) uterus, ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands)	✓	✓	✓						
	• Histopathology in uterus, ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands)	✓	✓	✓						
	• Serum hormone measures	✓	✓	✓	✓					
	• Time to mating		✓	✓	✓	✓	✓	✓	✓	✓
	• Male fertility		✓	✓	✓	✓	✓	✓	✓	✓
	• Female fertility			✓	✓	✓	✓	✓	✓	✓
	• Gestation length		✓	✓	✓		✓	✓	✓	✓
	• Dystocia		✓	✓	✓	✓	✓	✓	✓	✓
	• Placental weight		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of implantations, corpora lutea		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of live births and pre-and post-implantation loss		✓	✓	✓	✓	✓	✓	✓	✓
	• Litter size		✓	✓	✓					
	• Sex ratio (F1)		✓	✓	✓					
	• Litter/pup weight		✓	✓	✓					
	• Pup survival index		✓	✓	✓					
	• Abnormalities in pup development (F1)		✓	✓	✓					
	• Changes in sperm parameters: sperm numbers, sperm motility, sperm morphology (P)	✓	✓	✓	✓					
	• Clinical biochemistry					✓	✓	✓	✓	✓
	• Changes in adrenal weight					✓	✓	✓	✓	✓

(*continued*)

Table 3. Continued

Test method	Endpoints	Relevant endocrine pathway								
		S	E	A	T	H	M	D	P	R
	• Histopathology in adrenal					✓	✓	✓	✓	✓
	• Possible liver weight increase (in combination with other thyroid-related endpoints)				✓					
	• Histopathologic changes in thyroid (follicular cell height increase & colloid area decrease)				✓					
OSCP 870.3800/OECD TG 416 – Two-generation reproduction toxicity	• Changes in AGD in male pups and/or female pups	✓	✓	✓						
	• Changes in estrus cyclicity (P, F1)	✓	✓	✓						
	• Changes in age at vaginal opening (F1)	✓	✓	✓						
	• Changes in age at preputial separation (F1)	✓	✓	✓						
	• Changes in weights of: (P, F1) uterus, ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands)	✓	✓	✓						
	• Histopathology in uterus, ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands)	✓	✓	✓						
	• Changes in sperm parameters: sperm numbers, sperm motility, sperm morphology (P, F1)	✓	✓	✓						
	• Time to mating		✓	✓	✓	✓	✓	✓	✓	✓
	• Male fertility		✓	✓	✓	✓	✓	✓	✓	✓
	• Female fertility		✓	✓	✓	✓	✓	✓	✓	✓
	• Gestation length		✓	✓	✓	✓	✓	✓	✓	✓
	• Dystocia		✓	✓	✓	✓	✓	✓	✓	✓
	• Placental weight		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of implantations, corpora lutea		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of live births and pre-and post-implantation loss		✓	✓	✓	✓	✓	✓	✓	✓
	• Litter size		✓	✓	✓					
	• Sex ratio (F1, F2)		✓	✓	✓					
	• Litter/pup weight		✓	✓	✓					
	• Pup survival index		✓	✓	✓					
	• Abnormalities in pup development (F1, F2)		✓	✓	✓					
	• Serum hormone measures	✓	✓	✓	✓					
	• Clinical biochemistry					✓	✓	✓	✓	✓
	• Changes in adrenal weight					✓	✓	✓	✓	✓
	• Histopathology in adrenal					✓	✓	✓	✓	✓
	• Possible liver weight increase (in combination with other thyroid-related endpoints)				✓					
	• Histopathologic changes in thyroid (follicular cell height increase & colloid area decrease)				✓					
	• Increased thyroid weight				✓					
OCSPP 870.3550/OECD TG 421 – Reproduction/developmental toxicity screening test	• Histopathology changes in ovary, uterus/cervix, vagina, and female mammary gland	✓	✓	✓						
	• Weights of: (P, F1) uterus, ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands)	✓	✓	✓						
	• Histopathology changes in testes, epididymides, prostate, seminal vesicles(+coagulating glands)	✓	✓	✓						
	• Changes in vaginal smears		✓	✓						
	• Histopathology in mammary glands (males)	✓	✓	✓						
	• Uterus and ovary weight	✓	✓	✓						
	• Litter size		✓	✓						
	• Changes in adrenal weight					✓	✓	✓	✓	✓
	• Histopathology in adrenals					✓	✓	✓	✓	✓
	• Gestation length					✓	✓	✓	✓	✓
	• Changes in fertility, reproduction or fetal development					✓	✓	✓	✓	✓
	• Dystocia		✓	✓		✓	✓	✓	✓	✓
	• Placental weight		✓	✓		✓	✓	✓	✓	✓
	• Number of implantations, corpora lutea		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of live births and pre- and post-implantation loss		✓	✓	✓	✓	✓	✓	✓	✓
	• Body weight			✓		✓	✓	✓	✓	✓
	• Food consumption					✓	✓	✓	✓	✓
	• Clinical biochemistry					✓	✓	✓	✓	✓
	• Developmental abnormalities			✓	✓					
	• Serum hormone measures	✓	✓	✓	✓					
	• Possible liver weight increase (in combination with other thyroid-related endpoints)				✓					
	• Histopathologic changes in thyroid (follicular cell height increase & colloid area decrease)				✓					
	• Increased thyroid weight				✓					

(*continued*)

Table 3. Continued

Test method	Endpoints	Relevant endocrine pathway								
		S	E	A	T	H	M	D	P	R
OCSPP 870.3650/OECD TG 422 – Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test	• Histopathology changes in ovary, uterus/cervix, vagina and female mammary gland	✓	✓	✓						
	• Weights of: (P, F1) uterus, ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands)	✓	✓	✓						
	• Histopathology changes in testes, epididymides, prostate, seminal vesicles (+coagulating glands)	✓	✓	✓						
	• Changes in vaginal smears		✓	✓						
	• Histopathology in mammary glands (males)	✓	✓	✓						
	• Uterus and ovary weight	✓	✓	✓						
	• Litter size		✓	✓	✓					
	• Changes in adrenal weight					✓	✓	✓	✓	✓
	• Histopathology in adrenals					✓	✓	✓	✓	✓
	• Gestation length		✓	✓	✓	✓	✓	✓	✓	✓
	• Changes in fertility, reproduction or fetal development		✓	✓	✓	✓	✓	✓	✓	✓
	• Dystocia		✓	✓	✓	✓	✓	✓	✓	✓
	• Placental weight		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of implantations, corpora lutea		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of live births and pre- and post-implantation loss		✓	✓	✓	✓	✓	✓	✓	✓
	• Body weight			✓		✓	✓	✓	✓	✓
	• Food consumption					✓		✓		
	• Clinical biochemistry					✓	✓	✓	✓	✓
	• Developmental abnormalities		✓	✓	✓					
	• Serum hormone measures	✓	✓	✓	✓					
	• Possible liver weight increase (in combination with other thyroid-related endpoints)				✓					
	• Histopathologic changes in thyroid (follicular cell height increase & colloid				✓					
	• Increased thyroid weight				✓					
OCSPP 870.3700/OECD TG 414 – Prenatal developmental toxicity study	• Pregnancy rate	✓	✓	✓	✓	✓	✓			
	• Total number corpora lutea		✓	✓	✓	✓	✓	✓	✓	✓
	• Total number of implantations		✓	✓	✓	✓	✓	✓	✓	✓
	• Total number of litters	✓	✓	✓	✓	✓	✓			
	• Fetal mortality, structural abnormalities, or altered growth	✓	✓	✓	✓	✓	✓			
	• Assessment of maternal effects					✓	✓			
	• Serum hormone measures	✓	✓	✓	✓					
	• Visceral and skeletal evaluation					✓	✓	✓		
	• Clinical biochemistry					✓	✓	✓		
OCSPP 870.6300/OECD TG 426 – Developmental neurotoxicity study	• Gross neurologic and behavioral abnormalities, including the assessment of physical development, behavioral ontogeny, motor activity, motor and sensory function, and learning and memory	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Clinical observations and bodyweight	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Brain weight (post fixation), brain weight (unfixed)	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Neuropathology (immersion or perfusion fixation)	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Sexual maturation	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Other developmental landmark	✓	✓	✓	✓	✓	✓	✓	✓	✓
OECD TG 443 – Extended one-generation reproductive toxicity study	• Changes in AGD in male pups and female pups	✓	✓	✓						
	• Changes in estrus cyclicity (P, F1)	✓	✓	✓						
	• Changes in age at vaginal opening (F1)	✓	✓	✓						
	• Changes in age at preputial separation (F1)	✓	✓	✓						
	• Weights of: (P, F1) uterus, ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands)	✓	✓	✓						
	• Histopathology in uterus, ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands), and mammary glands	✓	✓	✓						
	• Changes in sperm parameters: sperm numbers, sperm motility, sperm morphology (P, F1)	✓	✓	✓						
	• Time to mating	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Male fertility	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Female fertility		✓	✓	✓	✓	✓	✓	✓	✓
	• Gestation length		✓	✓	✓	✓	✓	✓	✓	✓
	• Dystocia		✓	✓	✓	✓	✓	✓	✓	✓
	• Placental weight		✓	✓	✓	✓	✓	✓	✓	✓

(*continued*)

Table 3. Continued

Test method	Endpoints	Relevant endocrine pathway								
		S	E	A	T	H	M	D	P	R
	• Number of ovarian follicles		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of implantations, corpora lutea		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of live births and pre-and post-implantation loss		✓	✓	✓	✓	✓	✓	✓	✓
	• Litter size		✓	✓	✓					
	• Sex ratio (F1, F2)		✓	✓	✓					
	• Litter/pup weight		✓	✓	✓					
	• Pup survival index		✓	✓	✓					
	• Abnormalities in pup development (F1, F2)	✓	✓	✓	✓					
	• Genital abnormalities	✓	✓	✓						
	• Nipple retention		✓	✓						
	• Changes in apical endpoints from the developmental, neuro-, and immunotoxicity cohorts	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Serum hormone measures	✓	✓	✓	✓					
	• Possible liver weight increase (in combination with other thyroid-related endpoints)				✓					
	• Histopathologic changes in thyroid (follicular cell height increase & colloid area decrease)				✓					
	• Increased thyroid weight				✓					
	• Change in adrenal weight					✓	✓	✓	✓	✓
	• Histopathology in adrenal					✓	✓	✓	✓	✓
	• Clinical biochemistry					✓	✓	✓	✓	✓
OCSPP 870.4100/OECD 451 – Chronic toxicity	• Histopathology changes in testes, epididymides, prostate + seminal vesicles with coagulating glands	✓	✓	✓						
OCSPP 870.4200/OECD 452 – Carcinogenicity	• Histopathology changes in ovary, uterus/cervix, vagina	✓	✓	✓						
OCSPP 870.4300/OECD 453 – Combined chronic toxicity/ carcinogenicity	• Uterus and ovary weight	✓	✓	✓						
	• Changes in weight of testes, epididymides, prostate + seminal vesicles with coagulating glands	✓	✓	✓						
	• Histopathology in mammary glands (males)	✓	✓	✓						
	• Body weight			✓		✓	✓	✓	✓	✓
	• Serum hormone measures	✓	✓	✓	✓					
	• Possible liver weight increase (in combination with other thyroid-related endpoints)				✓					
	• Histopathologic changes in thyroid (follicular cell height increase & colloid area decrease)				✓					
	• Increased thyroid weight				✓					
	• Histopathology in adrenal					✓	✓	✓	✓	✓
	• Histopathology in pituitary and mammary glands					✓	✓	✓	✓	✓
	• Changes in adrenal weight					✓	✓	✓	✓	✓
	• Tumor types		✓	✓	✓	✓	✓	✓	✓	✓
	• Clinical biochemistry		✓	✓	✓	✓	✓	✓	✓	✓
Developmental thyroid toxicity study (comparative thyroid assay)	• Serum T3, T4 and TSH				✓					
	• Thyroid weight and histopathology				✓					
	• Colloid depletion				✓					
	• Follicular cell size and cell height				✓					
	• Follicular cell hyperplasia				✓					
	• Fetal sex and body weight				✓					
	• Gross external anomalies/lesions	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Any neurological signs observed	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Gross necropsy of organs (optional)	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Uterine weight (optional)	✓	✓							
	• Number of corpora lutea (optional)		✓	✓	✓	✓	✓	✓	✓	✓
	• Number of implantations (optional)		✓	✓	✓	✓	✓	✓	✓	✓
OECD TG 230 – 21-day fish assay	• Induction of male 2° sex characteristics (agonism)			✓						
	• Vitellogenin induction in males (agonism)		✓							
	• Depression of male 2° sex characteristics	✓	✓	✓						
	• Vitellogenin depression in females (assuming no systemic toxicity)	✓	✓							
	• Abnormal behavior		✓	✓	✓	✓		✓	✓	✓
OECD TG 234 – Fish sexual development test	• Biased phenotypic sex ratio	✓	✓	✓						
	• Vitellogenin depression in females	✓	✓							
	• Abnormal behavior			✓		✓				
	• Length and weight	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Embryo viability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Hatchability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Morphological abnormalities				✓		✓	✓	✓	✓
OECD GD 148 – Androgenized female	• Spiggin induction (agonism)			✓						
	• Spiggin depression (antagonism)			✓						

(*continued*)

Table 3. Continued

Test method	Endpoints	Relevant endocrine pathway								
		S	E	A	T	H	M	D	P	R
stickleback screen										
OCSPP 850.1400/OECD TG 210 – Fish early-life stage toxicity test	• Embryo viability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Hatchability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Length & weight	✓	✓	✓	✓	✓	✓	✓	✓	✓
OECD TG 212 – Fish short-term toxicity test on embryo and sac-fry stages	• Embryo viability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Hatchability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Length & weight	✓	✓	✓	✓	✓	✓	✓	✓	✓
OCSPP 850.1500 – Fish life-cycle toxicity	• Embryo viability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Hatchability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Length & weight	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Fecundity	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Fertility	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Appearance (2° sex characteristics)	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Altered behavior		✓	✓	✓	✓		✓	✓	✓
OCSPP 850.2300/OECD TG 206 – Avian reproduction test	• Egg production	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Cracked eggs		✓	✓	✓	✓	✓	✓	✓	✓
	• Eggshell thickness		✓	✓	✓	✓	✓	✓	✓	✓
	• Embryo viability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Hatchability	✓	✓	✓	✓	✓	✓	✓	✓	✓
	• Body weight		✓	✓	✓	✓	✓	✓	✓	✓
	• Gross pathology		✓	✓	✓	✓	✓	✓	✓	✓

S: steroidogenesis; E: estrogen pathway; A: androgen pathway; T: thyroid pathway; H: HPA axis pathway; M: somatotropic (metabolic) pathway; D: vitamin D pathway; P: PPAR pathway; R: retinoid pathway

ER-signaling pathway is disturbed and to ensure detection of adverse impacts within the estrogen-signaling pathway (see Table 3 for relevant assays and endpoints).

Androgen receptor-signaling pathway. Androgens are compounds that stimulate the development of male characteristics. In males, the primary source for circulating androgens is the testis, while the pituitary gland is where LH stimulates the synthesis and release of androgens into the blood. For the AR-signaling pathway, specific assays for detecting compounds that perturb this signaling pathway include those that identify sexual differentiation and development of secondary sex characteristics in the male organism, as well as for a wide variety of male and female reproductive and non-reproductive functions. Sex steroids, in particular, testosterone and its derivatives, are anabolic hormones that are known to induce muscle growth in mammals, as well as other vertebrates. Accordingly, interference by an EDC with androgen signaling can have effects on metabolism and growth in exposed organisms. While estrogens are better known as female hormones that impact female characteristics, estrogens are also synthesized in males and have critical roles in male reproduction and development, just as androgens do for females. Anti-androgens can induce responses that manifest similarly to estrogenic effects, such as attenuating expression of male secondary sexual characteristics, or impaired spermatogenesis and reduced sperm numbers. Furthermore, the induction of intersex has been reported in male fish, as well as in amphibians exposed to model anti-androgens, suggesting a possible shift in the estrogen/androgen ratio.

Interference with AR signaling within the HPG can disrupt developmental-, growth-, and reproduction-regulated processes by limiting availability, altering timing, or perhaps cause an excess of the intended hormone or an unintended one. Figure 4 illustrates an AOP for AR antagonism leading to impaired reproduction and how such interference directly leads to adverse consequences.

Currently, four screening level assays within the EDSP Tier 1 screening battery and the extended one-generation study are collectively capable of detecting whether a chemical interacts and perturbs the androgen hormonal pathway. Together, these assays are expected to detect chemicals with androgenic and anti-androgenic activities; these assays include: (1) AR binding, (2) Hershberger, (3) pubertal male, (4) fish short-term reproduction assays, and (5) extended one-generation reproduction study. Of the five assays, the one *in vitro* assay provides specific mechanistic information at the receptor level, while the four *in vivo* assays provide evidence of the impacts on the reproductive system at the whole organism level, with complete metabolic and compensatory mechanisms.

The AR-signaling pathway is well covered with several assays capable of detecting androgenic and anti-androgenic bioactivity. The EDSP Tier 1 battery includes four primary assays, which have androgen pathway-specific endpoints, and many other established assays, *in vitro* and *in vivo*, can also contribute corroborating information to increase evidence that the AR-signaling pathway is disturbed and to ensure detection of adverse impacts within the androgen-signaling pathway (see Table 3 for relevant assays and endpoints).

Hypothalamus pituitary thyroid axis

Thyroid hormones are essential for normal physiological functions, including neurodevelopment, growth, and cellular metabolism. Exposure to a wide range of structurally diverse environmental chemicals, including polychlorinated biphenyls (PCBs), dioxins (tetrachlorodibenzo-p-dioxin, TCDD),

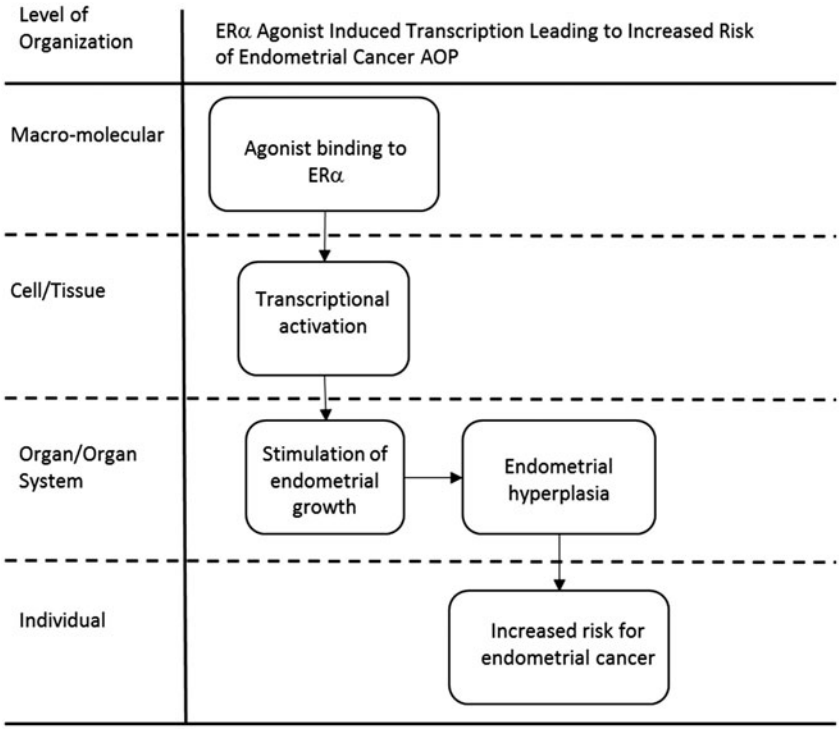

Figure 3. ERα agonist induced transcription leading to increased risk of endometrial cancer AOP (Becker et al. 2015).

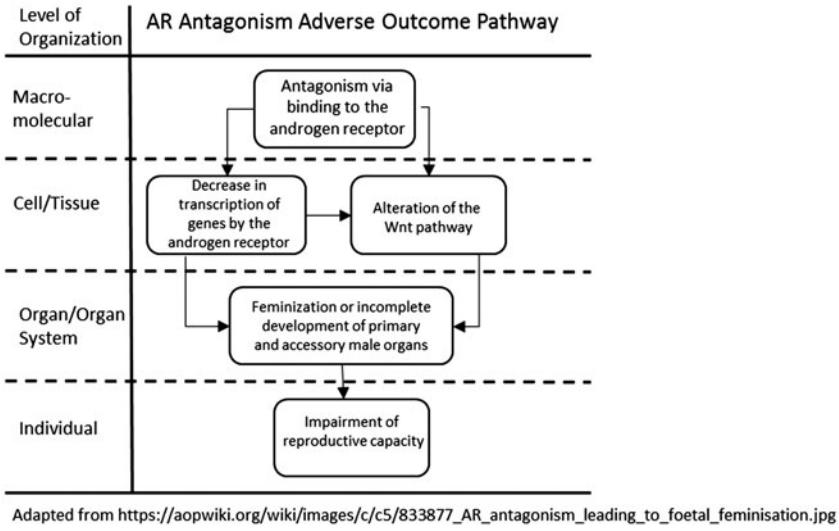

Figure 4. AR antagonism AOP.

polychlorinated dibenzofurans (PCDFs), bisphenol A (4,4′ isopropylidenediphenol or BPA), polybrominated diphenyl ethers (commonly known as flame retardants), phthalates, perchlorate, halogenated pesticides, and others, such as parabens, are known to disrupt thyroid axis signaling, homeostasis, and function (Bruckner-Davis 1998; Crofton 2008; Jugan et al. 2010). The thyroid system is highly complex, and thyroid hormone homeostasis involves a complex network of homeostatic regulatory interactions (Crofton 2008). Zoeller et al. (2007) and Murk et al. (2013) provide a detailed review and general background information on the HPT axis and summarize several *in vitro* and *in vivo* assays that could address several points within the thyroid endocrine system that may be disrupted by these toxicants across vertebrate taxa (LeBlanc et al. 2012).

The generalized vertebrate HPT axis, including the liver and peripheral tissues and the thyroid follicular cell, can be affected at several sites, including: (1) inhibition of iodide uptake at the sodium-iodide symporter (NIS), (2) inhibition of the iodinating activity of thyroid peroxidase (TPO), (3) increased elimination via upregulation of deiodination and conjugation reactions, (4) competitive displacement of TH from carrier proteins in the blood, such as transthyretin, and (5) altered local deiodination at the tissue level.

The complexity of HPT regulation poses some perplexing interpretational problems. Some tissues can regulate their own sensitivity to thyroid hormone by changes in the expression of various enzymes and transporters (Kampf-Lassin & Prendergast 2013). Thyroid disrupting chemicals can also interfere with thyroid hormone action in a complex manner

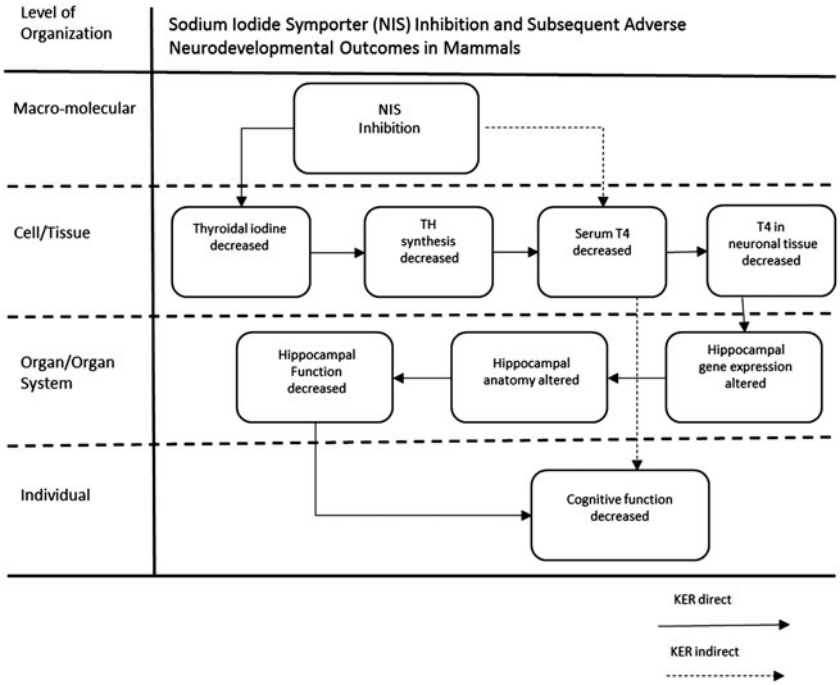

Figure 5. NIS inhibition and subsequent adverse neurodevelopmental outcomes in mammals.

in the thyroid gland, the hypothalamus, or pituitary, or in thyroid hormone-regulated tissues and cells (Zoeller 2010).

Interference with the HPT pathway can disrupt developmental-, growth-, and reproduction-regulated processes by limiting availability, altering timing, or perhaps cause an excess of the intended hormone or an unintended one. Figure 5 illustrates an AOP for iodide uptake inhibition at the NIS, leading to cognitive dysfunction and illustrating how such interference directly leads to adverse consequences.

Thyroid hormones are essential for normal development and maintenance of physiological functions in vertebrates. Delivery of thyroid hormones to tissues and cells is highly regulated throughout life and is governed by complex physiological processes involving the HPT axis, including peripheral organs and tissues. Environmental factors, such as the presence of specific toxicants, can perturb this system at various points of regulation, inducing a variety of responses that can be detected with thyroid-related endpoints in the *in vivo* assays. Assays for detecting chemicals that affect the HPT axis and for identifying environmental compounds that have the potential to alter the hormonal regulation of reproductive function involving the estrogen and androgen hormonal pathways include assays in the EDSP Tier 1 screening battery, which also provides relevant information about the potential of a chemical to interfere with thyroid function.

The HPT axis is well screened with several assays capable of detecting thyroid axis bioactivity. The EDSP Tier 1 battery includes three assays which have HPT axis-specific endpoints, and many other established assays, *in vitro* and *in vivo*, can also contribute corroborating information to increase evidence that the HPT-signaling pathway is disturbed and to ensure detection of adverse impacts within the thyroid-signaling pathway (see Table 3 for relevant assays and endpoints).

Hypothalamus pituitary adrenal axis

The HPA axis governs stress responses, maintenance of lipid and glucose homeostasis, brain function, osmotic, and integrity of the immune response. Symptoms of HPA dysfunction include obesity, metabolic syndrome, diabetes mellitus, immunodeficiency, and improper stress response. The adrenal cortex is under the strict control of the hypothalamic pituitary axis, linking putative effects of EDCs in the brain to changes in glucocorticoid secretion and action. There is also an important interaction between the adrenal and the immune system: glucocorticoids are potent immune suppressors. It has been proposed that EDC exposure during the development of the autoimmune system could cause type 1 diabetes in children (Chester-Jones & Phillips 1986).

The HPA axis of vertebrates is primarily a regulator of metabolism and modulates the immune system, growth, and reproduction in vertebrates. Many aspects of early development, as well as the timing of important events, such as puberty and reproductive organ development, are regulated by glucocorticoids from the adrenal cortical tissue in all vertebrate groups. The HPA axis responds to a great variety of stressors and is pivotal in homeostasis within the entire endocrine system. Additionally, the HPA axis affects cardiovascular functions, ionic regulation, and memory. Because of the role of the HPA axis in metabolism, virtually all body tissues are affected by the actions of HPA axis hormones.

There are no reports dealing with effects of EDCs on the adrenal medulla, but many studies indicate unwanted actions on the HPA axis. There is a great deal of literature on the effects of EDCs and other xenobiotics on adrenal and glucocorticoid receptor function in experimental animals; many different factors interfere with glucocorticoid homeostasis. For example, Dichlorodiphenyltrichloroethane (DDT) metabolites are known inhibitors of adrenal function, acting by direct

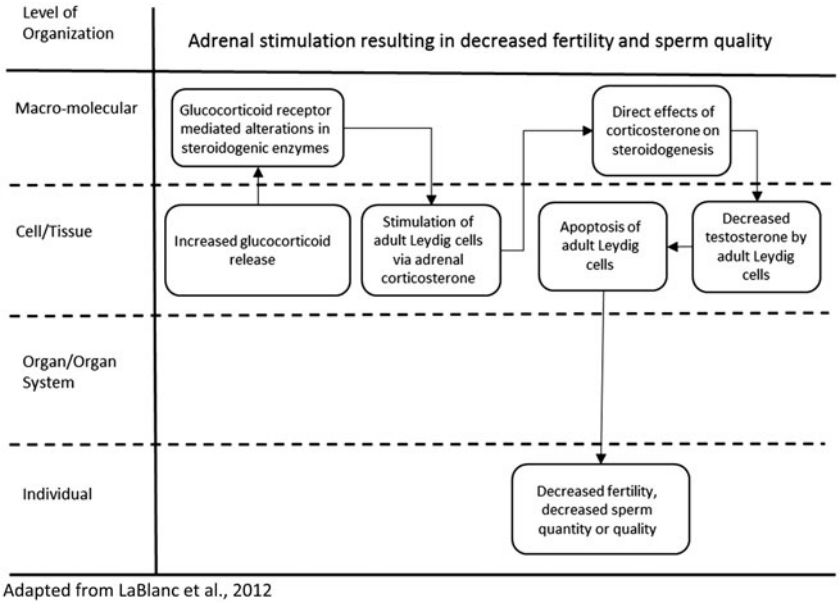

Figure 6. An AOP for adrenal stimulation resulting in decreased fertility and sperm quality.

cytotoxicity to adrenocortical cells. Emerging data in experimental animals indicate potent modulatory action of many EDCs on global steroidogenesis, including effects on adrenocortical cells. With the exception of glucocorticoids, however, immune cells are non-typical targets of traditional endocrine hormones, and the concept of endocrine disruption is not well defined for this biological system (Greenberg & Wingfield 1987). Numerous substances affect leucocytes, and it is difficult to distinguish toxic and immune activating suppressive effects from those that may count as endocrine disruption.

Interference with the HPA axis can disrupt developmental-, growth-, and reproduction-regulated processes by limiting availability, altering timing, or perhaps cause an excess of the intended hormone or an unintended one. Figure 6 illustrates what is known of an AOP for adrenal stimulation resulting in decreased fertility and sperm quality and illustrates how such interference directly leads to adverse consequences. Stress may result in excess release of glucocorticoids from the adrenal gland which will affect steroidogenic enzymes and steroidogenesis in Leydig cells resulting in reduced testosterone production and Leydig cell death and ultimately impact spermatogenesis and sperm production.

Although there are no assays in the EDSP Tier 1 battery specific to the HPA axis, many established assays can contribute corroborating information to increase evidence that the HPA axis may be disturbed and to ensure detection of adverse impacts resulting from HPA axis disruption (see Table 3 for relevant assays and endpoints).

Somatotropic axis

This pathway is responsible for the release of growth hormone and insulin-like growth factors. These hormones regulate a variety of functions related mainly to growth, maturation, and metabolism (Figure 7) (LeBlanc et al. 2012). The somatotropic axis consists of the signaling cascade that originates at the hypothalamus with the secretion of growth hormone-releasing hormone (GHRH) and consists of

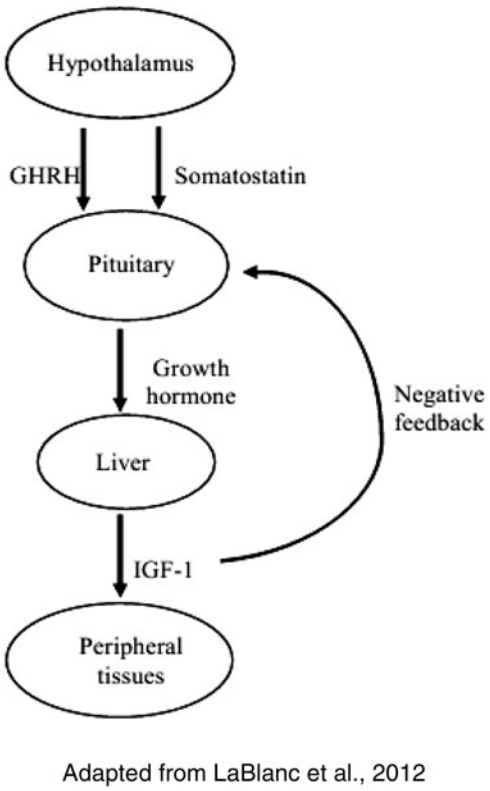

Figure 7. The somatotropic axis.

neuroendocrine signaling of growth hormone release by the hypothalamic hormones GHRH and somatostatin. In the liver, growth hormone regulates enzymes involved in steroid metabolism and in the production of IGF-1 and IGF-2. IGF-1 is the primary cell-signaling form of IGF. IGF-1 is largely responsible for the growth-promoting activities associated with the somatotropic axis, exerting multiple effects at various tissues relating to growth. Along this interconnected axis, other hormones like insulin, leptin, glucocorticoids (from the HPA), or thyroid hormones (from HPT) are involved in this mechanism

by modulating growth hormones and/or IGF-I synthesis and availability. GHRH and somatostatin are released in a coordinated fashion, resulting in a patterned release of growth hormone from the pituitary gland (Renaville et al. 2002).

Interference with normal functioning of the somatotropic axis can result in several adverse physiological conditions. Suppression of the axis can lead to increased body fat, an abnormal lipid profile, impaired cardiac function, reduced muscle mass, atherosclerosis, insulin resistance, and immunodeficiency. Excitation on the other hand can increase body size, increase risk of heart disease, interfere with thyroid function, cause hypertension, menstrual disturbances, and upset salt/water balance.

The somatotropic axis is influenced by cross-talk with other endocrine pathways. Estrogenic chemicals have been shown to have a suppressive effect on the somatotropic axis. The suppressive effect of estrogens on the axis may be mediated by the down regulation of the hepatic growth hormone receptor (GHR), preventing the induction of hepatic IGF-1 production by growth hormone (Krattenmacher et al. 1994; Shved et al. 2008). Thyroid hormones, on the other hand, may stimulate the somatotropic axis through its induction of pituitary growth hormone synthesis or through direct action on hepatic IGF-1 synthesis. Corticosteroids from the HPA axis can suppress somatotropic axis signaling in fish and mammals. This effect is accompanied by no change in pituitary or plasma content of growth hormone with a decrease in hepatic IGF-1 gene expression. These observations suggest that corticosteroids desensitize the liver to growth hormone (i.e. suppress expression of the GHR) or directly suppress IGF-1 gene expression.

Figure 8 illustrates an AOP for somatotropic axis modulation resulting in decreased growth and illustrates how such interference directly leads to adverse consequences.

Although there are no assays in the EDSP Tier 1 battery specific to the somatotropic axis, many established assays can contribute corroborating information to increase evidence that the somatotropic axis may be disturbed and to ensure detection of adverse impacts to development, growth, and/or reproduction resulting from somatotropic axis disruption (see Table 3 for relevant assays and endpoints).

Vitamin D pathway

Vitamin D is a steroid hormone and, similar to other steroids, the biological effects of vitamin D are initiated through its hormone receptor, vitamin D receptor (VDR). Characteristically, vitamin D is necessary for normal bone development and remodeling but also functions in many other areas. Elucidation of the changes in calciotropic hormones occurring during pregnancy and lactation has revealed a critical role for vitamin D. During pregnancy, the changes in vitamin D metabolism occur as the increase in the maternal plasma levels of 1,25-$(OH)_2$ D due to a putative placental synthesis of the hormone, but this does not seem to impact maternal vitamin D levels (Bikle 2010). Because transfer of vitamin D from mother to fetus is important for establishing the newborn's growth rate, the goal of ensuring adequate vitamin D levels is critically important. In lactating women there appears to be no direct role for vitamin D because increased calcium needs are regulated by parathyroid-related peptide, and recent studies have failed to show any change in vitamin D metabolites during lactation (Danks et al. 2011; Licata & Lerma 2012). Levels of VDR and vitamin D have shown an inverse relationship with the incidence of multiple cancers, including breast, colon, and prostate cancers (Figure 9). Additional research is needed to further explore this pathway as it is unclear whether and how a change in vitamin D and/or VDR levels functions to perturb the endocrine hormonal system.

Interference with the vitamin D pathway may disrupt developmental-, growth-, and reproduction-regulated processes by limiting availability, altering timing, or perhaps cause an excess of the hormone or its action in a plethora of different tissues. An AOP for VDR binding resulting in neurodevelopmental abnormalities or reduced skeletal growth illustrates how such interference may directly lead to adverse consequences (Figure 10).

Although there are no assays in the EDSP Tier 1 battery specific to the vitamin D pathway, many established assays can contribute corroborating information to increase evidence that the vitamin D pathway may be disturbed and to ensure detection of adverse impacts resulting from vitamin D pathway disruption (see Table 3 for relevant assays and endpoints).

PPAR pathway

Concerns have been published on whether endocrine active chemicals play a role in the increased incidence of neurodevelopmental, metabolic, and other adverse health outcomes. The molecular mechanisms involved in metabolic syndrome disorders such as diabetes mellitus, obesity, and certain cardiovascular diseases are multifaceted and are still largely unknown, but alteration of gene expression after binding to the aryl hydrocarbon receptor (AhR), PPARα, PPARγ, and ERs appear to play some role (LeBlanc et al. 2012). Although regulatory efforts have emphasized estrogen, androgen, and thyroid receptors, nuclear hormone receptors beyond these include progesterone receptors (PRs), glucocorticoid receptors, PPARs, and other nuclear hormone superfamily members, as well as membrane steroid hormone receptors. PPARs are ligand-activated transcription factors belonging to the nuclear receptor family. PPAR receptor expression has been shown in adrenocorticotropic hormone (ACTH)-secreting cells in both normal and adenomal pituitary, as well as in normal and tumor adrenal cortex, suggesting a possible HPA or stress-induced interaction which may also trigger activation.

Peroxisome proliferators regulate gene expression through a PPAR/retinoid X receptor (RXR) heterodimeric complex binding to a peroxisome proliferator-response element (PPRE). However, there is the possibility of several variations on this pathway:

1. The peroxisome proliferator may interact with the PPAR that preexists as a deoxyribonucleic acid (DNA) complex with associated corepressor and in association with a coactivator, resulting in the classical mechanism.

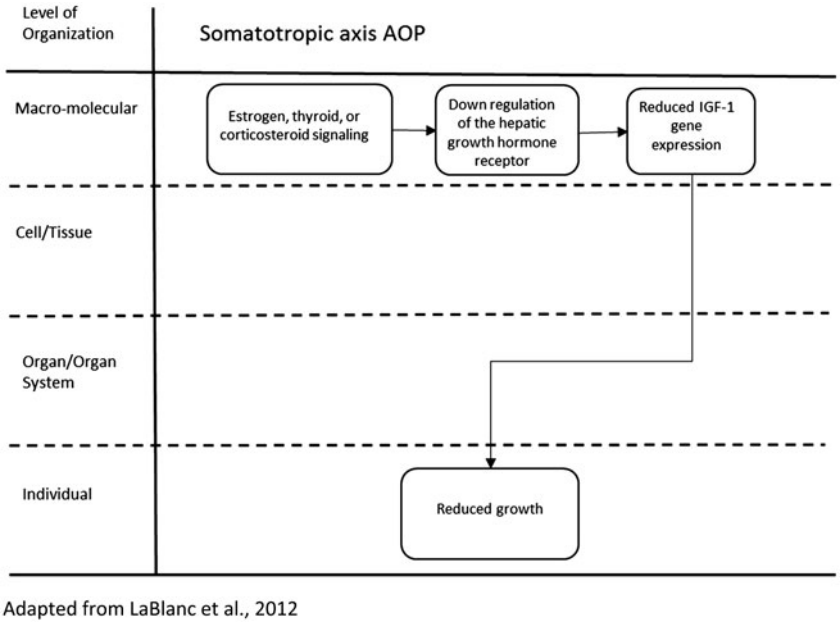

Figure 8. AOP for somatotropic axis modulation leading to decreased growth.

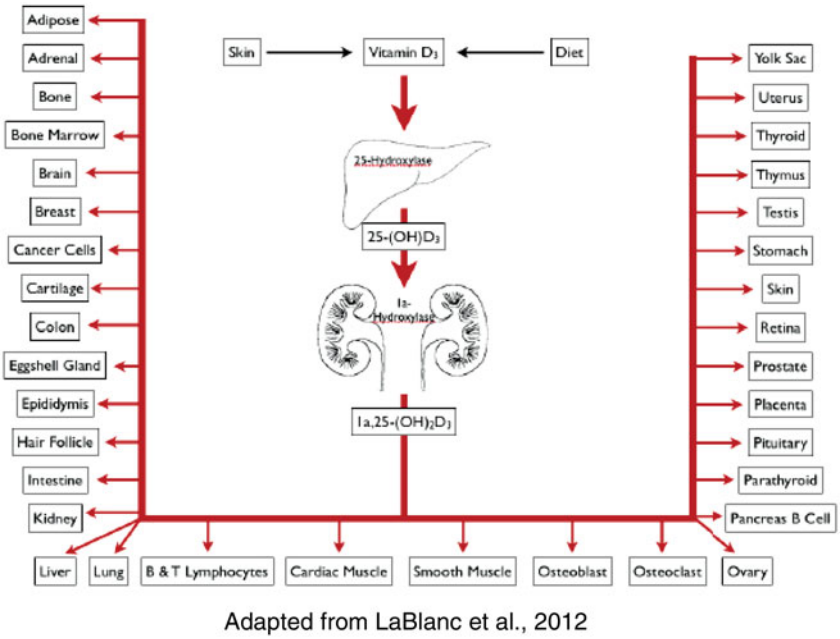

Figure 9. Vitamin D synthesis and sites of action.

2. The peroxisome proliferator may interact with the PPAR as a soluble member of the nucleus. The binding of ligand results in pregnane X receptor (PXR) heterodimerization, DNA binding, and coactivator recruitment.
3. PPAR exists in the cytosol, perhaps complexed to heat shock protein 90 and/or other chaperone protein. Binding of peroxisome proliferator causes a conformational change promoting translocation into the nucleus.
4. PPAR may be capable of forming DNA binding heterodimers with several nuclear receptors, including the thyroid hormone receptor (TR). The binding site for this non-RXR heterodimer may not be the classic DR-1 motif found in the PPRE.
5. PPAR may participate in the regulation of gene expression without binding to DNA (Corton et al. 2014; Klaunig et al. 2003). By association with transcription factors such as c-jun or p65, PPAR diminishes the ability of AP1 or NFκB to bind to their cognate DNA sequences, respectively. Most importantly, growth factor signaling may have a more pronounced effect on PPAR via post-translational modification.

Any interference with the PPAR-signaling pathway can disrupt steroid synthesis, and androgen- and estrogen-regulated processes by limiting the availability, altering the timing, and perhaps cause an excess of the intended hormone or an unintended one. PPAR receptor expression has been shown in ACTH-secreting cells in both normal and adenomal pituitary as well as in normal and tumor adrenal cortex. Figures 11 and 12 provide examples of AOPs for PPAR that illustrate

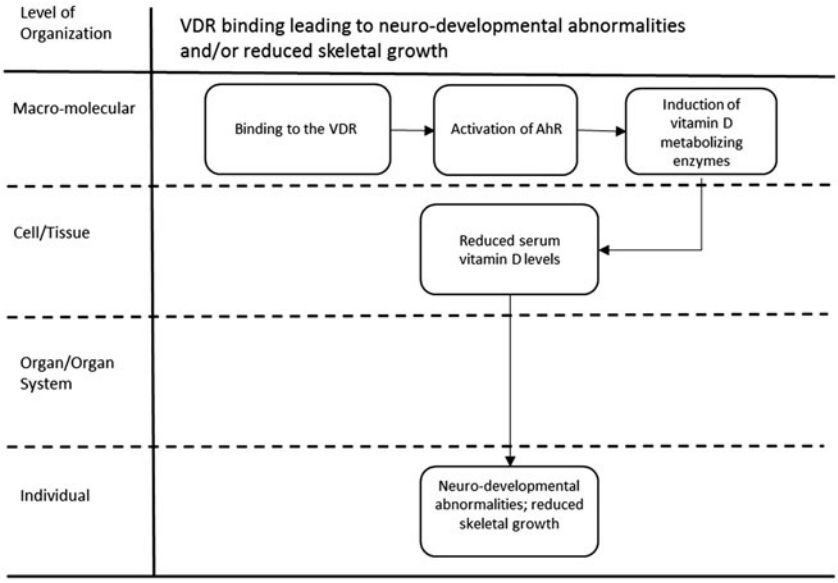

Figure 10. VDR binding leading to neurodevelopmental abnormalities and reduced skeletal growth.

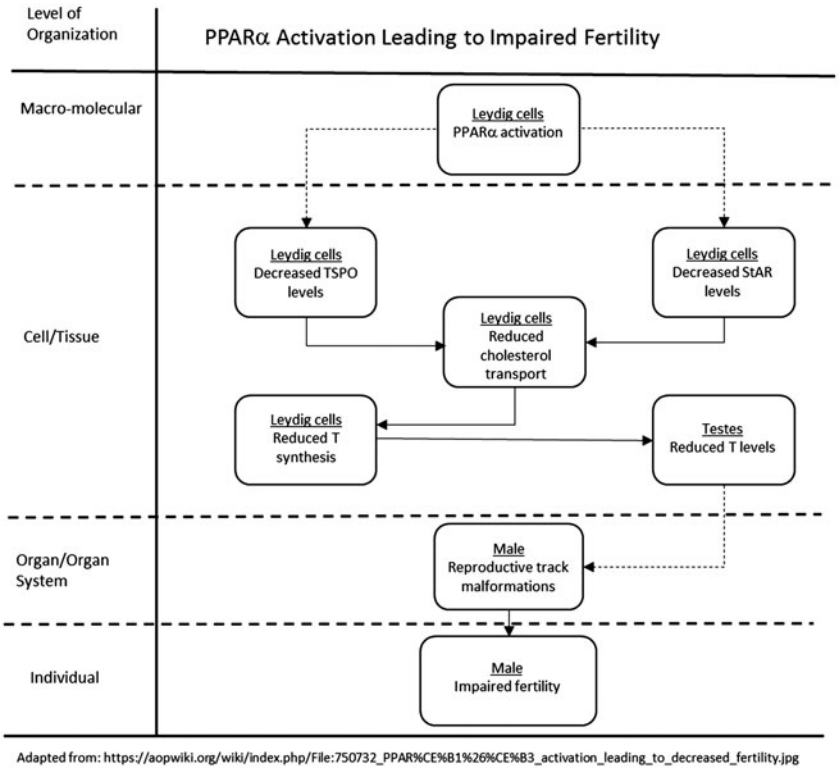

Figure 11. PPAR activation leading to impaired fertility.

how such interference directly leads to adverse consequences.

Although there are no assays specific to the PPARα/PPARγ pathways included among the validated test methods, many established assays can contribute corroborating information to increase evidence that the PPARα/PPARγ pathways may be disturbed and to ensure detection of adverse impacts resulting from PPARα/PPARγ pathways disruption (see Table 3 for relevant assays and endpoints).

Retinoid-signaling pathway

Vitamin A (retinol) is a fat-soluble vitamin that is derived from dietary sources of both animal and plant origins. Retinol is metabolized to biologically active retinoid through oxidative reactions catalyzed by alcohol and retinol dehydrogenases. Retinoid signaling in the body is further regulated by the level of retinol and retinoic acid binding to binding proteins and the level of metabolic inactivation, which is largely driven by members of the CYP26 family of cytochrome P450 enzymes (Lefebvre et al.

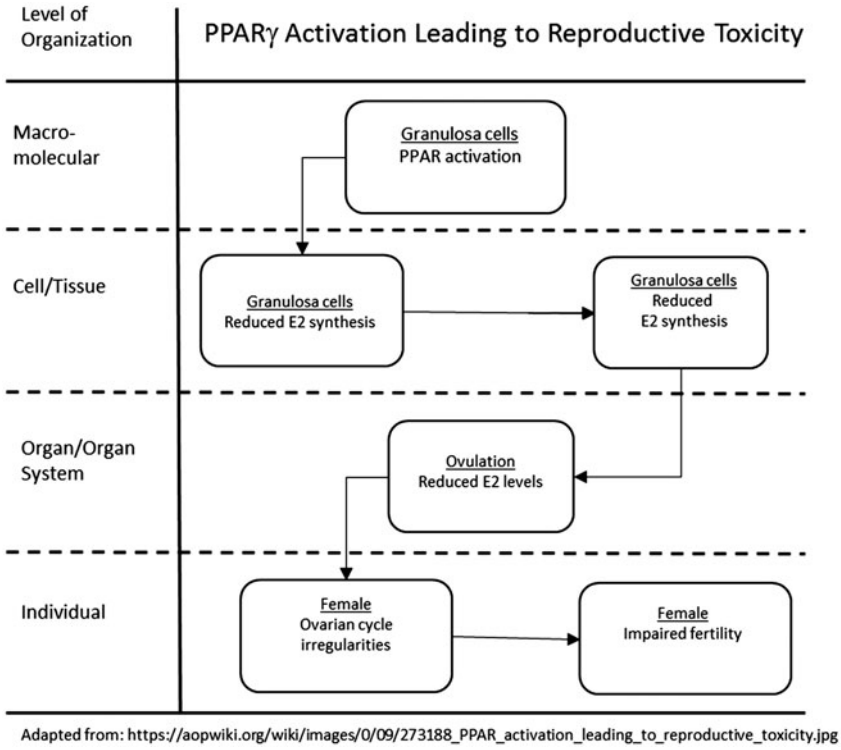

Figure 12. PPARγ activation leading to reproductive toxicity.

2010). The retinoid compounds serve as signaling molecules that regulate pleiotropic activities relating to development and differentiation in vertebrates. This hormonal regulatory activity is mediated through association of the retinoid with the retinoic acid receptor (RAR) and the RXR in vertebrates. Excess or suboptimal levels of retinoid during development result in developmental abnormalities (Damstra et al. 2002).

There are many reports of associations among environmental pollutants, altered retinoid levels in exposed wildlife, and physiological responses consistent with altered retinoid signaling. Retinoid signaling has been shown to be disrupted by various, diverse xenobiotics both *in vitro* and *in vivo*. Mechanisms include reductions in endogenous retinoid reserves, retinoid receptor activation by agonists, and receptor inactivation by antagonists. The physiological consequences of activation of RXR by tributyltin have been well described as related to disruptions in lipid homeostasis. In rodent models, tributyltin has been shown to cause differentiation of multipotent stromal stem cells into adipocytes. AhR ligands, such as some polychlorinated dibenzo-p-dioxins (PCDDs), PCDFs, and PCBs, have the ability to disrupt retinoid signaling by depleting endogenous retinoid reserves. However, the precise mechanism of action resulting in loss of retinoids is not fully understood.

Interference with the retinoid-signaling pathway can disrupt various activities related to development and differentiation in vertebrates. Figure 13 provides what is currently known of an AOP for retinoid signal disturbance resulting in excess lipid accumulation and obesity.

Although there are no assays specific to the retinoid-signaling pathway, many established assays can contribute corroborating information to increase evidence that the retinoid-signaling pathway may be disturbed, and to ensure detection of adverse impacts resulting from retinoid-signaling pathway disruption (see Table 3 for relevant assays and endpoints).

Recommendations on endocrine-related pathway test method enhancements

While current test methods are judged to be sufficiently adequate for the identification of endocrine mediated adverse outcomes for risk assessment purposes, there are recommended enhancements to current TGs to better determine the specific endocrine modality. The recommendations provided in this section include an update and summary to those provided in the 2012 OECD *Detailed Review Paper on the State of the Science on Novel in Vitro and in Vivo Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors, No. 178* (LeBlanc et al. 2012).

Hypothalamus pituitary gonadal axis

The HPG axis, estrogen- and androgen-signaling pathways, as well as steroidogenesis, are currently major components of endocrine disruption TGs. Although apical effects of HPG axis disruption are well considered, current TGs may not include specific biomarker endpoints that can unambiguously confirm all modalities *in vivo*. As the development and advancement of AOPs and delineation of more key events and their relationships, the ability to interpret existing test methods and identify useful enhancements and new methods will improve.

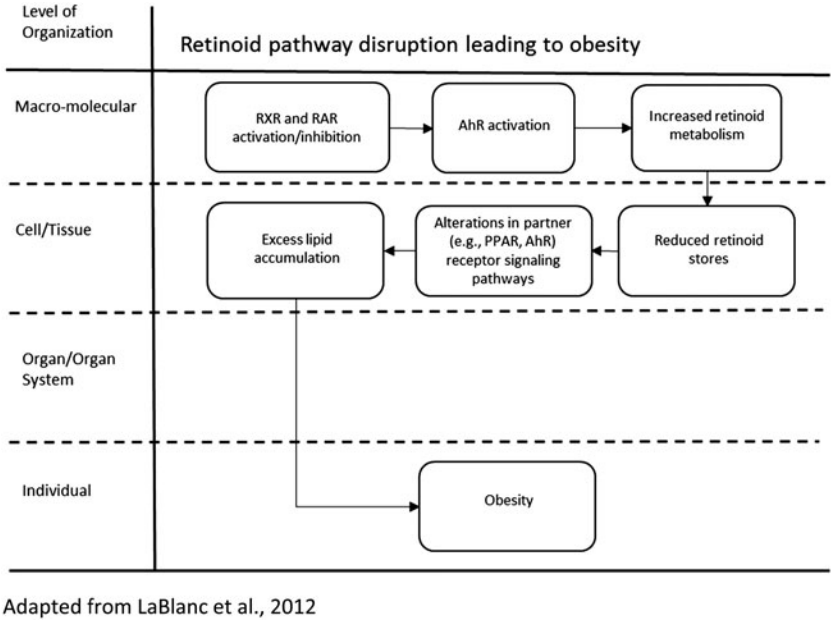

Figure 13. Retinoid disruption AOP leading to obesity.

An example of these types of enhancements is illustrated with the validation of OECD 443. The extended one-generation reproductive toxicity study is the most comprehensive of the available tests for assessing HPG- and HPT-specific disturbances and general adverse effects from other pathways, especially if there is a need to consider effects associated with the second generation. Similarly, tests such as the two-generation reproduction study could be enhanced with the measurement of endpoints found in the extended one-generation reproductive toxicity study, and others found in the developmental neurotoxicity (DNT) test and the chronic/cancer bioassays.

Additional observations on behavior could be beneficial in corroborating androgen-signaling perturbations.

1. A new endpoint may be added to the Medaka Extended One Generation Reproduction Test (MEOGRT) for assessing GnRH neuron development in the brain could be informative, but there are some logistical and cost disadvantages for the limited value added.
2. More expanded behavioral assessments are recommended for addition to any of the *in vivo* methods for indications of androgen-signaling amplification.
3. Enhancements and new methods are also suggested for assessing gestagen (e.g. progesterone) signaling in the HPG, which are not directly considered in any of the established TGs, include:
    a. A new screening assay for PR membrane binding and or PR transactivation.
    b. A new endpoint to the fish short-term reproduction assay and/or MEOGRT to assess oocytes and sperm quality or a new *ex vivo* method for assessing oocytes and sperm derived from these fish assays.

Although current TGs do not include specific biomarker endpoints that can unambiguously confirm all modalities *in vivo,* specific modalities *in vitro* and apical effects of HPG axis disruption *in vivo* are well considered .

Hypothalamus pituitary thyroid axis

The HPT axis is well covered for apical effects with several existing *in vivo* methods; however, established *in vitro* assays for specific mode of action confirmation are lacking. Additional assays could be considered that would strengthen the linkage between initiating events and adverse apical effects along the AOP.

1. Thyroid transactivation reporter assays and cell proliferation assays are available that would definitively evaluate the ability of xenobiotics to bind the TR and function as an agonist or antagonist.
2. TPO inhibition assay and the iodide uptake assay both could provide information on thyroid hormone-signaling disruption in a screening format.
3. Assessment of HPT-regulated gene expression could be incorporated into existing *in vivo* assays designed as a linkage between molecular events and apical outcomes under the same experimental design.
4. The xenopus embryonic thyroid-signaling assay (XETA) TG is advancing in its validation effort and is expected to provide another useful and rapid assessment tool for assessing potential HPT axis disruption.

Although current TGs do not include specific *in vitro* assays or specific biomarker endpoints that can unambiguously confirm all modalities *in vivo*, apical effects of HPT axis disruption are generally well considered.

Hypothalamus pituitary adrenal axis

The HPA axis contributes to many physiological processes, including maintenance of lipid and glucose homeostasis, brain function, osmotic balance, and integrity of the immune response and stress response. Symptoms of dysfunction include obesity, metabolic syndrome, diabetes mellitus,

immunodeficiency, and improper stress response; however, little precedence exists for the use of endpoints related to these processes in assessing *in vivo* HPA disruption. Several *in vitro* assays exist that could be used to evaluate disruption of corticosteroid signaling including:

1. A glucocorticoid reporter assay and an adrenal steroid synthesis assay.
2. *In vivo* TGs could be enhanced with addition of ACTH release and stress response-relevant endpoints.

A couple *in vitro* assays that have been considered and recommended for further development and validation to provide more specific indications of HPA axis disruption include GHR transactivation assay and adrenal steroid synthesis assay (or addition to the existing steroidogenesis assay). A few additional endpoints could be added to existing clinical biochemistry to include measures beyond cholesterol such as glucocorticoids and corticotropin (ACTH).

Although current TGs do not consider the HPA axis directly, apical effects of HPA axis disruption are generally well considered among the currently available validated test methods. Despite the lack of HPA axis specific assays, the probability of not detecting HPA axis-related endpoints is relatively low.

Somatotropic axis

The somatotropic axis should be better exploited in assessing endocrine disruption because several endocrine-signaling pathways converge on this pathway. That is, disruption of androgen, estrogen, corticosteroid, and thyroid signaling could be detected by measuring alterations in a key somatotropic axis biomarker such as circulating IGF-1 levels.

1. While not diagnostic of a specific mode of action, changes in IGF-1 levels could be added to intact organism assays to determine the occurrence of endocrine disruption in general, or could be applied to longer-term, animal exposures to detect overt endocrine disruption during these exposures.
2. Alterations in the somatotropic axis also can be detected by more closely examining weight and length of fetal rodents and growth rates in other vertebrates like fishes and birds.
3. The addition of TH receptors and GHR transactivation assays would also help inform somatotropic axis involvement.
4. Addition of hepatic GHR messenger ribonucleic acid (mRNA) levels in fish/mammals *in vivo* assays and analyses of serum IGF-1 in clinical biochemistry in the rodent tests or hepatic IGF-1 mRNA levels in the fish/mammal *in vivo* assays would increase the diagnostic ability of existing TGs for somatotropic axis activity. Note: IGF-1 can be influenced by a variety of exogenous factors and the endpoint has not been extensively used to assess endocrine disruption (EFSA 2013).
5. It should be noted that current measurements of fetal birth weight and length in rodent tests and in the growth measures in fish assays are informative to this pathway.

Although current TGs do not consider the somatotropic axis directly, apical effects of somatotropic axis disruption are generally well considered.

Vitamin D-signaling pathway

Despite the important role vitamin D plays in the development and maintenance of various systems, including bone, immune, cardiac, and neurological overall wellbeing, few methods exist that can directly assess the impact of chemical exposure on this signaling pathway. Studies typically have evaluated chemical effects on some apical endpoint (e.g. bone development), which may or may not be related to effects on vitamin D signaling. Additional research is needed to identify biomarker endpoints that can serve to diagnose activity related to disruption in vitamin D signaling.

1. Two *in vitro* assays that would inform possible activity in the vitamin D-signaling pathway would be: (1) a VDR transactivation assay and (2) an AhR transactivation assay.
2. Some endpoints that could aid in interpreting vitamin D influence to apical endpoints, such as growth and reproduction, would be measuring vitamin D hydroxylase and ethoxyresorufin-O-deethylase (EROD) activity.
3. Serum radio-immuno assay (RIA) or enzyme linked immunosorbent assay for vitamin D levels could be added to clinical biochemistry measures.
4. Current measurements of brain size in rodent offspring and bone length in juveniles would be informative of the possible activities in this pathway.

Although current TGs do not consider the vitamin D-signaling pathway directly, apical effects of vitamin D pathway disruption are generally well considered.

Retinoid-signaling pathway

The retinoid signaling pathway is important in regulating various facets of reproduction, development, and lipid homeostasis through its heterodimerization with other nuclear receptors. Among its heterodimer partners are PPAR, TR, vitamin D_3 receptor (VDR), and the RAR. The RXR has been shown to be highly susceptible to activation by some xenobiotics, such as tributyltin, resulting in alterations in lipid homeostasis and intersex conditions in some invertebrates. RXR is expressed in almost all vertebrate species thus far examined. Transactivation reporter assays are commercially available for RXR and RAR. In addition, AhR agonists have the ability to deplete retinoid levels, thus disrupting this signaling pathway. Aryl hydrocarbon receptor (AhR) reporter assays also are commercially available and should be included in the conceptual framework. Adipocyte differentiation assays, as

described for PPAR, are informative with regards to RXR since RXR agonists can activate the RXR:PPAR complex, resulting in alterations in adipocyte differentiation and lipid accumulation. Serum retinoid levels can be informative in whole-animal exposures since AhR ligands can deplete retinoid levels and disrupt normal retinoid signaling. Additionally, lipid accumulation and serum retinoid level measures could be added to certain *in vivo* methods. The addition of EROD activity measures and CYP1A mRNA or protein quantification to the clinical biochemistry measures in *in vivo* assays would be informative. Finally, careful attention to weight gain measures by assessing adipose tissue mass, lipid accumulation, and retinoid levels would enhance the diagnostic ability of these endpoints.

Although current TGs do not consider the retinoid-signaling pathway directly, apical effects of retinoid pathway disruption are generally well considered and as previously noted, an OECD Detailed Review Paper is underway to evaluate and potentially propose new test methods and enhancements to existing TGs to address this specific pathway.

PPAR-signaling pathway

The PPAR pathway, which is typically activated by fatty acids, is clearly involved in lipid and glucose homeostasis, inflammation, and aspects of development. The AOP involving PPARγ is reasonably well established, with activation of the receptor leading to adipocyte differentiation, lipid accumulation, and weight gain. The AOP involving PPARα mediated rodent liver tumors is well established (Corton et al. 2014), but the potential impact on the endocrine system is less well defined, and little is known of the AOP involving PPARβ/δ. Assays that could be used to assess disruption of normal signaling have been well developed although none have been formally adopted. Screening assays are available for the rapid assessment of PPAR signal disruptors, as are apical endpoints that could be incorporated into current *in vivo* animal assays. Among *in vitro* screening assays, prioritization should be given to PPAR transactivation reporter assays and adipocyte differentiation assays. Prioritization also should be given to incorporating peroxisome proliferation and lipid accumulation measures into certain *in vivo* assays.

Although current TGs do not consider the PPAR-signaling pathway directly, apical effects of PPAR pathway disruption are generally well considered.

Conclusions on the adequacy of test methods for endocrine biological pathways

Existing test methods (e.g. EPA and OECD TGs) are fully capable of detecting and characterizing potential apical adverse effects whether from endocrine or non-endocrine etiologies. However, individual TGs have limitations such that a fully comprehensive assessment of potential endocrine disruption requires a near full complement of available tests. Several test methods can provide diagnostic confirmation of specific endocrine activities, but usually this requires a WOE evaluation of multiple studies to ensure that other contributing or confounding factors are considered. Recommendations for additional test methods are largely to address earlier, more diagnostic precursor key events in the AOP, and while they are informative, the absence of these assays does not limit the ability of current test methods to provide regulatory endpoints that are public health and environmental protective against adverse endocrine-related health outcomes, as demonstrated in this review of biological pathways.

Endocrine-related diseases and disorders

This review evaluates whether and to what extent the current test methods can identify effects associated with human endocrine-related disease outcomes. The human diseases addressed in this section include human female reproductive health; human male reproductive health; hormonal cancers; metabolic syndromes, including adrenal disorders; vitamin D pathways; DNT; and immune disorders. Note: some disease pathways are better understood than others. As recognized at the outset, this review is subject to change based on the rapidly evolving state of endocrine science.

Female reproductive health effects

Evidence suggests that EDCs have been demonstrated to cause a number of reproductive health outcomes, including abnormal puberty, irregular cyclicity, reduced fertility, infertility, polycystic ovarian syndrome, endometriosis, uterine fibroids, preterm birth, and adverse birth outcomes (Gore et al. 2015). The sections below will provide an evaluation of how adequate current test methods are in the evaluation of endocrine-related diseases and disorders.

Precocious puberty

Precocious puberty has been associated with endocrine perturbation. The normal onset of puberty involves the secretion of high-amplitude pulses of GnRH by the hypothalamus. These high amplitude pulses of GnRH result in pulsatile increases in the pituitary gonadotropin-LH and FSH. Increased LH levels stimulate production of sex steroids by testicular Leydig cells or ovarian granulosa cells. Pubertal levels of androgen or estrogens cause the physical changes of puberty, including penile enlargement and sexual hair in boys and breast development in girls. These levels also mediate the pubertal growth spurt. Increased FSH levels cause enlargement of the gonads in both sexes and eventually promote follicular maturation in girls and spermatogenesis in boys (Kaplowitz & Kemp 2015).

In humans, precocious puberty is the appearance of physical and hormonal signs of pubertal development earlier than normal. Precocious puberty for girls typically occurs before the age of eight and for boys, onset of puberty before the age of nine. Precocious puberty refers to the conditions in which increased production of sex steroids is gonadotropin-independent. Currently, the GnRH stimulation test is the

standard method used to verify the activation of the HPG axis, but other biochemical and clinical characteristics include pubertal stage, height, height standard deviation score, weight, weight standard deviation score, body mass index, body mass index standard deviation score, yearly growth rate, basal LH, basal FSH, basal E2, IGF-1, IGF-1 standard deviation score, IGF binding protein-3 and standard deviation score (Kaplowitz & Kemp 2015; Suh et al. 2013).

Current animal test methods. Current animal test methods are capable of informing assessors of potential for precocious puberty. Chemical influence on sexual development is assessed in offspring as anogenital distance in male and female rodents and nipple retention in male rodents. Chemical influence on pubertal timing is assessed as the age of vaginal opening in female rodents and preputial separation in male rodents, and anogenital distance in both male and female rodents. Vaginal opening is a physiological manifestation of the estrogenic rise that accompanies the onset of puberty and the first sign of gonadotropin-dependent ovarian activity (Rasier et al. 2006). The combination of age and body weight at vaginal opening and preputial separation are well recorded in the guideline required two-generation reproduction study (OECD TG 416; OCSPP 870.3800) in F1 exposed *in utero*, as well as validated TGs such as the female pubertal study (OCSPP 890.1450), the extended one-generation reproduction study (OECD 443) and the DNT study (OECD TG 426; OSCPP 870.6300). Age at first estrus is also used to assess the completion of the pubertal development in females. Other estrous cyclicity information evaluated in the female pubertal study includes the length of the cycle, the percent of animals cycling, and the percent of animals cycling regularly. Information on estrous cyclicity is also included in the reproduction studies, and the phase of the estrous cycle at study termination can be recorded in repeated dose guideline toxicity studies of the appropriate exposure duration. Hormonal measurements can be optionally included in the majority of the guideline-validated toxicity studies and most often include measurements of testosterone levels in male pubertal studies, although LH, FSH, estradiol, progesterone, and prolactin (PRL), as well as other androgens such as dehydroepiandrosterone (DHEA). Serum T levels are useful to determine if the test substance induces liver metabolism of testosterone, lowering serum levels. Without the T data, such an effect might appear to be via an anti-androgenic mechanism. Both LH and FSH levels are important. LH levels provide information about the ability of an anti-androgen to reduce organ weights, affect hypothalamic-pituitary function, which in long-term studies can induce testicular tumors, and FSH is an important hormone for spermatogenesis.

Female organ weights and histopathological findings are evaluated in many validated TG studies. In the repeated dose studies, female organ weights include the uterus and ovaries and histopathological evaluations include the uterus, ovaries, and the female mammary gland. In the female pubertal study and reproduction studies, histopathology includes the vagina, the uterus with cervix, and the ovaries. Ovarian histology is conducted and includes an evaluation of follicular development (including presence/absence of tertiary/antral follicles, presence/absence of corpora lutea, changes in corpus luteum development, and changes in number of both primary and atretic follicles) in addition to any abnormalities/lesions, such as ovarian atrophy. Uterine histology in these studies includes evaluation of uterine hyper- or hypotrophy as characterized by changes in uterine horn diameter and myometrial, stromal, or endometrial gland development.

Male organ weights and histopathological findings are evaluated throughout the guideline validated toxicological studies. Male reproductive organs are routinely evaluated in the repeated dose studies, including testes, epididymides, and seminal vesicles and prostate glands. The Male Pubertal Study and reproductive studies evaluate the organ weight and histopathology of male reproductive organs including the seminal vesicle plus coagulating glands (with and without fluid), ventral prostate, dorsolateral prostate, levator ani/bulbocavernosus muscle complex (LABC), epididymides and testes, while the Hershberger Assay (OECD 441, OCSPP 890.1400) includes organ weight and histopathological evaluation of androgen-sensitive male tissues including the seminal vesicles, ventral prostate, LABC, Cowper's glands, and glans penis. In the male pubertal and reproduction studies, testicular histopathological examination includes evaluation of retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen. Histopathological evaluation of the epididymis includes the caput, corpus, and cauda and lesions such as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium. Sperm evaluations are included in the repeated dose or reproductive toxicity studies and are included in the male pubertal studies. These studies evaluate the total cauda epididymal sperm number, percent progressively motile sperm, percent morphologically normal sperm, and percent of sperm with identified abnormality and are optionally included the repeated dose toxicity studies.

In addition to the male and female reproductive organs, repeated dose studies as well as pubertal studies, reproduction studies, and other higher tiered studies such as the DNT studies, also evaluate organ weight and histopathology of the adrenal gland and pituitary. Effects on the thymus may be considered secondary to adrenal effects and contribute to the WOE evaluation. Organ weight and histopathological analysis of the thymus is also included in the majority of the repeated dose and higher tiered toxicology studies.

Current ecological test methods. Due to the conservation of endocrine pathways across species, current ecological test methods in fish, frogs, and birds are capable of informing assessors of endocrine activities indicative of a potential for precocious puberty in mammals. Conversely, the effects on this pathway in the mammalian studies also support the potential for estrogen pathway interactions for wildlife (Ankley & Gray 2013). Ecological studies in fish, frogs, and birds include the measurement of vitellogenin (VTG), a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active females of all oviparous species. The production of VTG is controlled by interaction of

estrogens with the ER. Significantly, males maintain the capacity to produce VTG in response to stimulation with ER agonists; as such, induction of VTG in males and immature females has been successfully exploited as a biomarker specific for estrogenic compounds in a variety of fish species (OECD 2004). Evaluation of secondary sexual characteristics, ratio of gonad weight to body weight (gonadosomatic index, GSI), sex-linked behavior, and time to sexual maturity are recorded in ecological receptors as well as histopathology of male and female reproductive organs (Avian Two-Generation Study (OCSPP 890.2100)). Hormone measurements are optional measurements in ecological studies. Secondary sex characteristics in fish are hormonally controlled, making them viable endpoints for the evaluation of endocrine disruption. All of the species considered have some secondary sex characteristics, such as females having distinct genital papilla. The male fathead minnow has distinct breeding tubercles on the snout and dorsally located fat pad. The male zebrafish and medaka have larger or longer anal fins than the females, and the male medaka dorsal fin has a cleft. The mature male fathead minnow and medaka have distinct coloration. The GSI is also frequently reported as a general measure of gonad maturation and spawning readiness and is based on the broad assumption that proportionally larger gonads indicate greater development (OECD 2004). In the Avian Reproduction Study (OECD 2016) and the Avian Two-Generation Study, eggshell parameters including the total egg production per hen, the number of eggs cracked, the number of eggs broken, the number of abnormal eggs (plus description of abnormalities), the number of eggs set, the egg fertility (ED 8), the embryo viability (viability and embryonic deaths; ED 15), the eggshell thickness (nm), the eggshell strength (Newtons), the number of eggs that hatch, and the number of 14-day-old survivors per hen are all recorded. In addition, in the Avian Two-Generation Toxicity Test (OCSPP 890.2100) histopathology of the parent and offspring reproductive organs (ovary, oviduct, shell gland, cloacal glands, bursa in females and testis, cloacal glands, bursa epididymis/vas in males) as well as adrenal glands are performed and time to sexual maturity and secondary sexual characteristics are recorded. These same ecological methods are equally informative to other hormonally related disease pathways.

Enhancements and additional studies. Effects on estrogenic and androgenic pathways are well investigated in both the standard mammalian and ecological guideline studies. While screening and testing for endocrine disrupters routinely include critical endpoints related to pubertal development, there may be a consideration of other important parameters such as breast development and adrenal competence to detect peripheral precocious or delayed puberty (Kortenkamp et al. 2012).

Despite logistical complications, adding hormonal measurements in repeated toxicity studies would enhance the studies. It should be noted that the variability within animals, depending on the time of the day or phase of the menstrual cycle, the variability between animals, and a consistent methodology for measuring hormones in various species and studies, would complicate the evaluation of hormonal measurements.

Fecundity

Fecundity is defined as the biological capacity for conception to occur, which is somewhat different from fertility that refers to the ability to deliver a live-born infant. Fecundity is a necessary but insufficient component of fertility (Buck Louis et al. 2006). In general, female fecundity captures a wide spectrum of endpoints related to the ability to conceive including hormonal profile, menstruation, early pregnancy loss, ovarian reserve and failure, and reproductive senescence or menopause. Recognizing that there are many risk factors associated with decreased fecundity, female biological factors such as age and menstrual cycle length have been among the more important predictors of fecundity than other lifestyle factors. Shorter cycles were less likely to be followed by conception, but shorter and longer cycles were associated with spontaneous abortion (Small et al. 2006). Important to note that a majority of spontaneous abortions are due to chromosomal abnormalities; and cytogenetic studies indicated that anomalies occur in 21% to 50% of first term spontaneous abortion and aneuploidy is among the most common identified chromosomal abnormality in 10% of the clinically recognized pregnancies (Hunt et al. 2016).

Menstrual cycle is an important factor in addressing female human fecundity. During each menstrual cycle, the growths of primordial follicles into primary follicles are stimulated to occur. The primary follicles evolve into secondary and tertiary follicles through a process that involves proliferation and differentiation into cumulus and granulosa cells. In humans, follicle growth takes 85 days compared to two weeks in mice. Ovarian steroidogenesis promotes follicle growth and differentiation via direct intraovarian action and endocrine feedback to the hypothalamus and pituitary. Estradiol and progesterone are the active hormones that mediate early follicular development and later maturation of healthy follicles, ovulation, and luteal development.

Pre-ovulatory surge of gonadotropins induces a cascade of events that culminate in ovulation. The gonadotropin surge that precedes ovulation is characterized by an increase in LH that stimulates the terminal differentiation of granulosa cells that switch from estradiol production to the production of both estradiol and progesterone (luteinization).

In summary, the parameters that can affect fecundity include measurements of the spacing and length of the menstrual cycle, and blood levels of LH, FSH, estradiol, and progesterone levels. Evaluation of the ovary and time to pregnancy would also be informative.

Current animal test methods. There are a number of studies that provide information on effects that may contribute to the evaluation of female fecundity including endpoints related to the ability to conceive, including hormonal profile, menstruation, early pregnancy loss, ovarian reserve and failure, and reproductive senescence or menopause. Studies assessing reproductive capabilities are required in

toxicological studies and include the two-generation reproductive toxicity study or the extended one-generation reproductive toxicity study. These studies, as well as the one-generation reproductive study and the reproductive screening studies expose the animals to the test material before mating to determine effects on reproductive ability throughout mating, gestation, parturition, lactation, and weaning. The multiple generation reproduction studies include a repeated process with the offspring of the parental animals. The complex design of the reproduction studies allows for evaluation of a chemical's ability to interfere with mating, copulation, fertilization, conception, maintenance of pregnancy, and the ability to deliver the offspring.

Estrous cycle information from the reproduction studies and female pubertal studies include the age at first estrus, the length of the cycle, the percent of animals cycling, and the percent of animals cycling regularly. Estrous cyclicity information in repeated dose animal studies record the phase of the estrous cycle at study termination and can be used to evaluate the phase of the estrous cycle and could detect reproductive senescence. As stated in the previous sections, the reproduction studies, female pubertal and repeated dose toxicity studies of various durations and routes of exposure adequately evaluate parameters relative to reproductive toxicity including organ weights of female reproductive organs (uterus and ovaries), adrenal gland and pituitary gland and histopathology of reproductive organs (uterus, ovaries, cervix, vagina, and oviducts), mammary gland, adrenal gland and pituitary gland. In the female pubertal study and reproduction studies, ovarian histology is conducted and includes an evaluation of follicular development (including presence/absence of tertiary/antral follicles, presence/absence of corpora lutea, changes in corpus luteum development, and changes in number of both primary and atretic follicles) in addition to any abnormalities/lesions, such as ovarian atrophy. Uterine histology in these studies includes evaluation of uterine hyper- or hypotrophy as characterized by changes in uterine horn diameter and myometrial, stromal, or endometrial gland development.

The uterotrophic study also mainly evaluates effects on uterine weight in ovariectomized female rats, and is specifically designed to detect estrogenic effects of compounds. The uterotrophic study can also include uterine histopathology. In addition, all of the repeated dose studies can include hormonal analysis. Hormonal measurements can be optionally included in the majority of the guideline validated toxicity studies and most often include measurements of LH, FSH, estradiol, progesterone, and PRL, which would enhance the overall ability of these studies to discern endocrine-specific effects from exposure. Supplemental to the repeated dose studies, the prenatal developmental toxicity studies, conducted in two species - rats and rabbits - evaluate the effect of exposure during critical periods of gestation on maternal toxicity and the ability to maintain pregnancy.

Current ecological test methods. Current ecological test methods are capable of identifying potential effects on fecundity in fish, frogs, and birds. The estrogen pathway is generally considered well conserved among vertebrate species; the effect on this pathway in the mammalian studies also supports the potential for estrogen pathway interactions for wildlife (Ankley & Gray 2013). Fecundity is evaluated in fish and birds through the evaluation of the number of eggs produced and cumulative eggs laid, number of embryos, and fertilization success.

Enhancements and additional studies. Effects on estrogenic and androgenic pathways are well investigated in both the standard guideline studies in mammalian and ecological receptors. Although consistent hormonal measurements would enhance the studies, the variability within animals, depending on the time of the day or stage of menstrual cycle, the variability between animals, and a consistent methodology for measuring hormones in various species and studies, do not allow for easy evaluation of hormonal measurements.

Polycystic ovaries syndrome

Polycystic ovaries syndrome (PCOS) is a heterogeneous condition that is defined by the association of clinical and or biochemical evidence of androgen excess with chronic anovulation. It is often associated with obesity and the metabolic syndrome and secondary PCOS may occur in association with disorders characterized by adrenal androgen excess. The confirmed presence of polycystic ovaries is necessary for the development of the syndrome, but it is important to recognize that not all women with polycystic ovaries have PCOS (Yildiz & Assis 2007). Overall, 6% to 8% of reproductive aged women are diagnosed with PCOS, making this disorder one of the more prevalent endocrine abnormalities.

The specific etiology of PCOS remains unclear, but researchers have suggested that genetically determined hypersecretion of androgens by the ovary during puberty or during fetal development is involved, leading to disruption of the HPA axis to such an extent that excessive levels of LH are secreted. Progesterone and estrogen levels are reduced and gonadotropin secretion is increased. The elevation of gonadotropins stimulates excessive production of androgenic steroids like DHEA and androstenedione that cannot be aromatized to estrogens in the absence of granulosa cells. Androgens are reportedly responsible for the masculinization that is associated with PCOS. However, animal studies show that exposure of the fetus to excess androgens in the maternal circulation results in many of the features of PCOS as the animal reaches adolescence. Thus, the role of exogenous factors leading to excess androgen action during the development/imprinting of the HPA cannot be excluded. Typical diagnosis of PCOS includes the following observations:

- **Irregular periods** – This is the most common characteristic. Examples include menstrual intervals longer than 35 days, fewer than eight menstrual cycles a year, failure to menstruate for four months or longer, and prolonged periods that may be scant or heavy.

- **Excess androgen** – Elevated levels of androgens may result in physical signs, such as excess facial and body hair, adult acne or severe adolescent acne, and male pattern baldness.
- **Polycystic ovaries** – Polycystic ovaries become enlarged and contain numerous small fluid-filled sacs surrounding the eggs.

Current animal test methods. Current animal toxicology test methods such as the longer-term, extended one-generation study or multi-generation studies require histopathology of reproductive organs and the number of primordial and small growing follicles should be enumerated. The use of rodents in guidelines studies offer advantages in relatively short lifecycle spans and a homogenous genetic background. Excess androgen in rodents suppresses the ability of the hypothalamus and pituitary to generate the LH surge necessary to induce ovulation in response to rising estradiol levels; the ovary in those cases appear to be functionally unaffected. Rodent models can detect compounds acting via androgenic mode of action. In animal test methods, measurements of irregular estrous cyclicity, elevated male hormones, and increased ovary weight may be indications of this disease. Additional hormonal measurements include LH, FSH, total and free testosterone, DHEA sulfate, PRL, androstenedione, progesterone[2].

Adverse pregnancy outcomes

Adverse pregnancy outcomes include spontaneous abortion, ectopic pregnancies, fetal death, stillbirth, preterm delivery, low birth weight, sex ratio, and certain congenital defects. It is important to note that while these endpoints are those focused on female reproductive health, there are studies that indicate some effects associated with male reproductive health (Silbergeld & Patrick 2005). Many of these adverse effects are associated with dysfunction of the uterus and also impact other tissues such as the oviduct, cervix, and upper vagina. Effects on these reproductive organs have been demonstrated to lead to infertility, pregnancy loss, or fetal compromise and abnormal development. They all share a similar mechanism of action based on oxidative damage resulting in the disruption of endocrine processes which prepare the uterus for pregnancy during the menstrual cycle.

Although most adverse pregnancy outcomes appear to be the result of oxidative stress, this can also be related to disorders of implantation. Without the relevant steroidal hormones for proper implantation, adverse pregnancy outcomes will follow. It is important to be reminded that not all adverse pregnancy outcomes are due to EDCs. In fact, the 2002 *Global Assessment of the State of the Science of Endocrine Disruptors* (Damstra et al. 2002) document concluded there are substantial gaps in our current knowledge about whether exposure to environmental chemicals has any impact on adverse pregnancy outcomes such as spontaneous abortion rates.

Current animal test methods. To address adverse pregnancy outcomes that may be due to endocrine chemical perturbations, a number of TG studies have been validated to detect endocrine activity and uterine endpoints. For example, the OECD 440 validated uterotrophic assay uses the hypertrophic response of the rodent uterus to estrogenic stimulus to detect EDCs that perturb the estrogenic mode of action pathway. It is a relatively simple assay that measures the change in uterine weight. Beyond the organ level studies, longer-term studies, like those mentioned in OECD conceptual framework Level 5, such as the extended one-generation reproduction toxicity study and multi-generation reproduction study include a multitude of endpoints that are related to fertility and pregnancy outcomes such as a record of the duration of gestation, signs of dystocia or abnormal and difficult labor, the number, sex and weight of pups, the number of live and still births, and any additional signs of gross anomalies. After the parent generation females have been sacrificed, they are examined for the presence and number of implantation sites.

Enhancements and additions. Effects on estrogenic and androgenic pathways are well investigated in both the standard guideline studies in mammalian and ecological receptors. Although consistent hormonal measurements would enhance the studies, the variability within animals, depending on the time of the day or phase of the menstrual cycle, the variability between animals, and a consistent methodology for measuring hormones in various species and studies, do not allow for easy evaluation of hormonal measurements.

Endometriosis and uterine fibroids

Endometriosis. Endometriosis is defined by the presence of endometrial glands and stroma outside the uterine cavity. The uterine endometrium is an endocrine-sensitive tissue whether it be eutopic or ectopic. Clinically, the disorder presents with irregular bleedings, abdominal pain, and infertility. The disease is largely estrogen dependent. Endometriotic lesions can occur adjacent to the eutopic endometrium, within the myometrium or fallopian tubes, ovaries, Douglas pouch, uterine ligaments, vagina, vulva, or perineum or within the pelvic cavity, septum rectovaginal, intestine, and ureter (Tariverdian et al. 2007). The proliferation of endometriotic lesions appear to be dependent on estrogens and it has been hypothesized that estrogenic compounds from the environment could play a role in other forms of pathogenesis (Mclachlan et al. 2006). Experimental studies and treatment protocols in human medicine clearly show that the course of endometriosis can be modified by estrogens. Both ERα and ERβ are overexpressed in endometriotic tissue; ERβ and ERα at 142-fold and 9-fold higher, respectively, compared to normal endometrium. Aromatase is also known to have a role in endometriotic growth as it is found in the endometriotic stroma where it can convert androgens to E1 which is then converted to E2 by 17β-HSD type 1 (Tariverdian et al. 2007). Prostaglandin E2 is a potent inducer of aromatase activity in endometriotic cells.

The etiology of this disorder appears to be due to a combination of genetic susceptibility, altered immune and hormonal response, and environmental factors, but the specific

etiological mechanisms involved in disease onset remain elusive.

Uterine fibroids. Uterine fibroids are benign tumors derived from the myometrium; leiomyomas are known to respond to endogenous levels of estrogen and progesterone and this response has been characterized at different levels of biological organization. There are significantly higher concentrations of both ERα and ERβ in leiomyomas compared to normal myometrium and levels of both isoforms of mRNA fluctuate in a similar manner during menstrual cycle. Levels of estradiol in fibroids are also elevated compared to normal myometrium. Increased levels of aromatase and low levels of arylsulfatase are thought to contribute to the accumulation of estradiol, leading to hyper-responsiveness to estrogen, up regulation of ERs, PRs, and proliferation (Parker 2007).

Current animal test methods. Animal test methods that may be informative in identifying endometriosis and uterine fibroids include assays that focus on the estrogenic pathway, including ER binding, ER transactivation assays, AR binding, steroidogenesis and aromatase assay. The *in vivo* studies focusing on the uterus include uterotrophic, female pubertal and repeat short and longer-term dose studies provided added refinement to our collective understanding of how chemicals may perturb the endocrine pathway and elicit endometriosis.

Enhancements and additions. Effects on estrogenic and androgenic pathways are well investigated in both the standard guideline studies in mammalian and ecological receptors. Although consistent hormonal measurements would enhance the studies, the variability within animals depending on the time of the day or phase of the menstrual cycle, the variability between animals, and a consistent methodology for measuring hormones in various species and studies, do not allow for easy evaluation of hormonal measurements.

Male reproductive health effects

Testicular dysgenesis syndrome

Male reproductive health problems such as poor semen quality, testicular cancer, hypospadias, and cryptorchidism are captured under TDS because they often occur together and there is evidence (Walsh et al. 2009) to their common origin in fetal development (Skakkebaek et al. 2001), but not all cases of cryptorchidism, hypospadias or poor semen quality develop as part of TDS. Not all TDS are due to endocrine perturbations. There are certain mutations that predispose to cryptorchidism and hypospadias without being related to diminished androgen action. For example, poor semen quality may result from exposure to a toxic drug or occupational exposure and therefore not a sign of a preceding developmental disorder. But when cryptorchidism, hypospadias and/or testicular cancer are found in association with poor semen quality, the underlying problem is hypothesized to be a TDS caused by androgen insufficiency during the early development of the male reproductive organs, impacting the proper functioning of Sertoli cells and Leydig cells where androgen synthesis takes place.

Cryptorchidism

Cryptorchidism is the most common congenital malformation in male babies at birth. Normal testicular descent in two phases: (1) transabdominal phase and (2) transinguinal phase. The second phase is androgen dependent and disruption of this phase appears to be the most common cause of cryptorchidism at birth. The initial burst of androgen synthesis occurs in the first 3 to 5 months after birth reverses many instances of cryptorchidism diagnosed at birth. The earlier transabdominal phase of descent is triggered by insulin-like factor 3, a peptide hormone and when disrupted can lead to complete failure of testicular descent.

Hypospadias

Androgen action during fetal life is crucially important to ensuring the proper location of the urethral opening at the tip of the glans penis. If androgen is diminished, the urethra opens on the underside of the glans penis.

Reduced semen quality

Semen quality is determined by sperm counts, sperm motility, sperm concentration, ejaculation volume, and other parameters. It can be affected by a number of different factors such as abstinence, ethnicity, infectious disease, season, and clothing.

Current animal test methods: TDS (cryptorchidism and hypospadias)

Animal studies have shown that effects mimicking those of TDS can be induced following *in utero* exposure of rodent models to chemicals that interfere with androgen synthesis and/or action (e.g. Bay et al. 2006). For example, the multigeneration and extended one-generation reproduction study incorporate the relevant endpoints for the detection of chemicals that might interfere with male sexual development. Specific measurements include testosterone levels, anogenital distance and sperm motility and morphology. *In vitro* assays sensitive to AR antagonists and to inhibition of steroidogenesis are available as screening levels assay that could potentially inform *in vivo* effects. In addition, the Hershberger assay detects *in vivo* AR antagonist activity.

Genotoxicity tests are also available to evaluate the possible mutation to spermatogenic cells or chromosome aberrations. Studies such as analysis in spermatogonia, Unscheduled DNA Synthesis Test (UDS) in testicular cells, Mammalian Spermatogonial Chromosome Aberration test (OECD 483), and Spermatid Micronucleus Assay are a few of the specific genotoxicity studies that could be conducted.

Enhancements/additional studies – testicular dysgenesis syndrome

Androgen effects are well covered in current toxicological testing along with evaluation of sperm parameters and male reproductive organs.

Hormonal cancers health effects

Breast cancer

Human breast cancer is one of the most common disease in females, with an estimated 1.4 million new cases diagnosed every year (IARC/GLOBOCAN 2010). A proposed role for human exposure to EDCs in the causation of breast cancer follows on from the association of steroidal estrogens with breast cancer. The discovery of estrogenic properties of many chemical pollutants found in the environment, food, and consumer products has led to the suggestion that the significant rise in breast cancer cases in industrialized countries is potentially due to chemical exposure (Davis et al. 1993).

While the precise mechanism has not been defined, research indicates the breast tissue is uniquely vulnerable to cancer-causing influences during two specific periods of duct structure growth: (1) during development in the womb, when the breast tissue is laid down (Soto et al. 1998) and (2) during puberty when the breast experiences the first significant growth phase of the ductal system.

Current animal test methods. A plethora of experimental systems exist to study the potential for breast cancer formation from exposure to chemical substances. Genotoxicity assays are available to determine DNA reactivity and ability to initiate a mutagenic mode of action to potentially elicit tumor formation; important to note that direct interaction with DNA may not always lead to a linear extrapolation, but exhibit a threshold dose response. Additional assays are available to probe estrogen specific pathway, which include ER binding, ER transactivation, Uterotrophic and Pubertal assays that form the Tier 1 EDSP assays and OECD conceptual framework. In addition, the prenatal developmental and multigenerational and extended one-generation reproduction studies provide unique insights on the critical time windows of exposure, ranging from organogenesis, development, to longer-term, transgenerational effects with specific evaluations of the mammary gland in both males and females. The chronic cancer bioassays provide empirical evidence of mammary tumor formation histopathologically over a lifetime of exposure, covering adulthood and aging. The carcinogenicity bioassays, conducted often in both rats and mice, have the statistical power to identify carcinogenic potential with a rich understanding of historical controls. More specifically, the rat is considered to be a better model than the mouse for the purpose of identifying potential human breast carcinogens because mice are generally more resistant to developing chemically induced mammary gland tumors (Mollard et al. 2011), however the high background tumor rates in some rat species can complicate the detection and evaluation of mammary tumors. Repeated dose studies in multiple species allow for identification of precursor changes (hypotrophic or hypertrophic) in the mammary tissue or changes in other organ systems supporting an estrogenic mode of action. As with other non-routine endpoints in repeated dose toxicity studies, any gross pathological change in mammary tissue (males or females) are subject to further histopathological analysis and if effects on mammary tissue (male and female) are expected, then histopathology of the mammary tissue may be included in the study design. The animal cancer bioassay is both adequate to test chemicals with a genotoxic mode of action and chemicals with a hormonally mediated mode of action.

Animal models that are responsive to mammary carcinogenesis by estrogenic compounds are available. In addition, assays that monitor the activation of ER signaling with subsequent transactivation and organ level effects are available and in combination with longer-term and transgenerational *in vivo* test methods, can adequately address chemicals with the potential to elicit mammary tumors.

Enhancements and additional studies. Effects on estrogenic pathways are well investigated in both the standard guideline studies in mammalian and ecological receptors. The rodent (rat and mouse) carcinogenicity studies provide a good model for detection of these tumors, however due to the mouse being relatively resistant to mammary gland tumors and some rat strains having high spontaneous mammary gland tumor rates, there are some difficulties with the ability of these studies to predict breast cancer in humans (Russo & Russo 1996; Mollard et al. 2011). As an enhancement to the cancer bioassay for the detection of mammary tumors, the whole mount procedure was discussed among OECD experts but it was decided to be too difficult to implement based on the high degree of uncertainty with respect to the interpretation. A protocol for evaluating whole mounts of mammary glands was judged to need additional development and interlaboratory validation to demonstrate consistency among different laboratories (Osborne et al. 2014). Regulatory experts concurred that the whole mount procedure was not ready for incorporation into the OECD 452 and 453 TGs at that time.

In addition, while some information suggests that *in utero* exposure has been associated with delayed mammary gland tumor formation, the overall WOE has not been consistent and until there is confirmatory data, development of additional test methods would be deemed premature; there is no currently available TG toxicity model for evaluating exposure from conception through lifetime, but as described later in this document, there are combinations of various test methods that could inform sensitive life stage effects. Taken together, the current test methods are considered adequate in the identification and protection of effects associated with development of mammary tumors.

Prostate cancer

The developing prostate gland is sensitive to estrogen exposure. Prostate morphogenesis occurs in fetal life during the second and third trimester and it is typically complete at the time of birth. Although estrogens together with androgens play critical roles in normal prostate development, several lines of evidence support that estrogen exposure during morphogenesis can profoundly alter the development trajectory of the gland, leading to increased susceptibility of initiating cell proliferation, hyperplasia and cancer later in life.

Evidence of the developmental vulnerability of the prostate gland to strong exposures comes from experimental studies with rodents where the details of the key events that

underlie this process have been published (Harkonen & Makela 2004; Huang et al. 2004; Prins et al. 2007). Rats exposed to estradiol during prostate morphogenesis (perinatal life in rats) showed disorganized prostate epithelia. With aging, these lesions develop into epithelial piling, neoplasia and carcinoma. These experiments show that estrogen exposure during morphogenesis induces disruptions of normal epithelial cell differentiation that persist into later life.

The AR is also a key steroid receptor in epithelial and stromal cells of the developing rodent prostate. Androgens that reach the prostate induce stromal cells to produce paracrine factors important for gland development and differentiation. During the process of differentiation, AR levels increase and the expression of ERβ is induced in epithelial cells. In summary, there is a switch from a prostate characterized by androgen signaling to one regulated predominately by estrogens, progesterone and retinoid. Together, this leads to disruptions in the finely coordinated expression of critical genes involved in prostate gland development and differentiation.

Current animal test methods. Chronic exposure to a combination of testosterone and estradiol induces prostate cancers in rodents. Scientific evidence suggests that chronic administration of testosterone in combination with estradiol can lead to prostate dysplasia in rodents. With the example of bisphenol A, evidence indicates that exposure during the prostate gland developmental time window is also critical (Nelles et al. 2011). In addition, prostatic inflammation and hyperplasia in current rodent reproductive, repeated dose and cancer bioassay toxicity models are relevant for human diseases.

Enhancements and additional studies. Available rodent carcinogenicity models have exhibited low prostate tumors rates; prostate cancer is the most common cancer in human males. Genetically engineered mouse models (PTEN and Myc transgenic mice models) have been developed to study factors related to the pathogenesis of experimental prostate cancer and it may be helpful to search for rodent strains that are more sensitive to development of prostate cancer. Additionally, there are not validated study protocols that begin with *in utero* exposure or early post-natal exposure during prostate duct development and continue for a chronic duration study, but there are opportunities to optimize current test methods to inform each of those life stages. More extensive histopathology of the prostate was also recommended, as chemically induced preneoplastic changes may have value for assessing human carcinogenic potential.

Testicular cancer

Inadequate fetal exposure to androgens is hypothesized to be a cause of testicular cancer. Concerns have been raised concerning the potential for estrogenic and anti-androgenic chemicals to exacerbate cancer of the testis and other disorders attributed to disruption of fetal androgen action. Although some studies have proposed a role from environmental chemical exposure, the mechanism of action has not yet been elucidated. The etiology of human testicular tumors is poorly defined. With the possible exception of prenatal estrogen exposure, no specific chemical exposures have been associated with testicular cancer risk in men. Prenatal as well as postnatal estrogen treatments induce testicular tumors in some mouse strains, but not in other mouse strains or in rats. It is noted, however, that Cryptorchidism is a consistent risk factor for testicular cancer in men. Prenatal estrogen exposure also causes cryptorchidic testes in mice and possibly in rats.

Current animal test methods. Studies in rabbits have demonstrated that exposure to certain endocrine active compounds appear to lead to the formation of germ cell atypia, a lesion that has similarities to neoplastic cells in humans (Veeramachaneni et al. 2007; Veeramachaneni 2008). However, it is not known whether this lesion will develop into germ cell tumors in the rabbit. While germ cell tumors are rare in rodents, National Toxicity Program's (NTP's) long-term carcinogenicity rodent bioassays have reported chemicals that can cause testes tumors. In addition, spontaneous Leydig cell adenomas have been reported to be common in F344/N rat strain and this high background incidence of Leydig cell adenomas is thought to make the F344/N rat unsuitable for the detection of chemical-induced tumors of the testes. In contrast, however, the B6C3F1/N mouse shows a fairly low spontaneous Leydig cell tumor incidence. The 129.MOLF-Chr19 mouse strain, which is predisposed to developing male germ cell tumors, is a possible model for germ cell tumors in men (Thayer & Foster 2007). Chronic exposure to estrogenic compounds in mice can lead to testicular tumor formation.

Testicular descent occurs in two phases controlled by Leydig cell-derived hormones insulin-like peptide 3 (INSL3) and testosterone. Disorders in fetal androgen production/action or suppression of Insl3 are mechanisms causing cryptorchidism in rodents (Virtanen & Adamsson 2012). Additionally, cryptorchidism and low sperm count are not associated with testicular cancer in rodents, but would still act as a marker for human testicular cancer.

Enhancements and additional studies. Germ cell tumors are rare in rodents, but possible models like the 129 MOLF-Chr19 mouse strain can be explored for use (Youngren et al. 2003). Initiating studies with *in utero* exposure or early post-natal exposure during the critical windows for development of potential testicular dysgenesis and continuing for a chronic duration study is not a guideline toxicity model, but as mentioned above using precursors for early prediction of germ cell tumors such as cryptorchidism and low sperm counts might serve as good early predictors.

Thyroid cancer

Perturbation of thyroid and pituitary hormone levels are known factors of thyroid cancer, but whether these changes cause or are the direct consequence of thyroid carcinogenicity is not clear. There is no current evidence for a direct oncogenic role of thyroid stimulating hormone (TSH) in

human thyroid carcinogenesis but recent findings suggest that TSH may play a role in the progression of thyroid carcinomas. A number of studies have suggested that elevated serum levels of TSH are associated with a subsequent diagnosis of thyroid cancer (Boelaert et al. 2006; Jonklaas et al. 2008; Polyzos et al. 2008). Iodine deficiency causes a reduction in the level of circulating thyroid hormones associated with a consequent rise in serum TSH concentrations, and chronic iodine deficiency is a well-established risk factor for the development of follicular thyroid carcinoma (Feldt-Rasmussen 2001; Nagataki & Nystrom 2002). Therapy with suppressive doses of thyroxine (T4) has long been known to positively affect outcomes in differentiated thyroid cancer (Mazzaferri & Jhiang 1994) and more recently, prospective studies have indicated reductions in thyroid cancer-related deaths and relapse with TSH suppression therapy (Hovens et al. 2007).

In addition to TSH, activation of ERs appears to play a role in human thyroid carcinogenesis. A study demonstrated the presence of ERα and ERβ in thyroid cells derived from human goiter nodules and in a human thyroid carcinoma cell line HTC-TSHR (Manole et al. 2001). There was no difference between the expression levels of ERα in males and females, but there was a significant increase in expression levels in response to estradiol. Stimulation of benign and malignant thyroid cell with estradiol resulted in an increased proliferation rate and an enhanced expression of cyclin D1 protein, which plays a role in the regulation of G1/S transition in the mitotic cell cycle (Manole et al. 2001). The rapid effects of ER signaling, such as the activation of phosphatidylinositol-3-OH kinase signaling cascade, are becoming increasingly recognized as a common feature of thyroid follicular neoplasms (Yeager et al. 2008).

Current animal test methods. The EPA has considered thyroid follicular tumors formed in the rodent model and has issued science policy guidance on how it will consider the relevance of rodent thyroid tumors when assessing the human carcinogenic potential of chemicals (US EPA 1998).

"Quantitatively, if humans develop cancer through thyroid-pituitary disruption, it appears that humans are less sensitive to the carcinogenic effects than are rodents. Rodents show significant increases in cancer with thyroid pituitary disruption; humans show little, if any" (US EPA 1998).

The Agency has determined that the chemicals producing thyroid follicular cell tumors formed via disruption of thyroid-pituitary function are not likely to be carcinogenic to humans because of significant quantitative differences in anticipated levels of exposure. Rodents do not have the thyroxine binding globulin that humans have and therefore have a heightened sensitivity to perturbations of thyroid hormone levels. The rodent bioassay therefore is considered conservative for predicting carcinogenicity in the human population. In addition to the cancer bioassay, there are existing endocrine-specific assays that measure T_3 and TSH levels such as the pubertal and amphibian metamorphosis assay (AMA) in frogs, recognizing that the endocrine HPT pathway is conserved across species.

Therefore, if a chemical is not genotoxic, current animal models and subsequent mechanistic studies conducted to elucidate the mechanism of carcinogenesis are considered to be conservative estimates for humans. In addition, the toxicological database with repeated dose studies allows for precursor effects of epithelial cell (follicular cell) hypertrophy and hyperplasia, colloid changes to be evaluated through different durations of studies, in different species and through difference routes of exposure. Thyroid hormone levels can be optionally measured in repeated dose and reproductive toxicity studies.

Ecological test methods. There are multiple studies that measure effects in the thyroid gland, including the avian two-generation study, AMA and larval amphibian growth and development assay (LAGDA) assay; all of these test methods requires the evaluation of thyroid histopathology with the option to include thyroid hormone levels. In addition, the AMA and LAGDA studies also provide morphometric analysis to evaluate the influence of possible thyroid hormone level disruptions on growth and development.

Enhancements and additional studies. The current animal models provide useful information on the possibilities of cancer in humans, and the understanding of physiological differences allow for potential determinations of whether some thyroid specific carcinogenic effects are relevant in humans.

Developmental neurotoxicity

While there are many alleged mechanisms in support of a role for endocrine disruptors in DNT, or impact on the neuroendocrine system, most of the data appears to be focused on perturbations of thyroid hormones during development. Thyroid hormone function is considered essential for normal brain development and plays a crucial role in cerebellar development (Ahmed et al. 2008; Koibuchi 2008; Howdeshell 2002). Thyroid hormones have effects on neuronal proliferation, migration, synaptogenesis and myelinations (Howdeshell 2002; Darras 2008). It is important to recognize that while impacts on thyroid hormone may have a profound impact on the developing nervous systems, there are multiple factors, chemical and non-chemical stressors, that contribute toward the neurological disease outcome pathways. The wide range of neurological endpoints defining DNT include IQ deficits, motor impairment, memory loss, learning and memory, neuropsychological deficits, cognitive function, attention deficit disorder, cerebral palsy and autism (Damstra et al. 2002). While not comprehensive, some characteristics associated with these neurological disease outcomes are listed below.

Autism – characterized by impaired social interaction and limited communication, repetitive behaviors and some intellectual impairment.

Attention deficit disorder – characterized by lack of attention, impulsivity, and hyperactivity.

Cerebral palsy – characterized as a disorder of movement or posture, resulting from static abnormality in the brain with the condition acquired early in life (Blair & Watson 2006).

Neural tube defects – characterized as malformation of the brain, skull and spinal column that occurs early during prenatal development.

Specific endocrine mechanisms of DNT include interference with neuroendocrine (hypothalamus and pituitary) function, a key to reproductive and sexually dimorphic behavior and interference with circulating hormones including thyroid hormones and estrogens and androgens which control neurodevelopment. The interactions between brain development and thyroid hormone have been studied extensively. The fetal mechanisms involved in neurodevelopmental disorders have been reviewed and include the endocrine disruption of cell programs (Connor et al. 2008), developmental trajectories, synaptic plasticity and maturation of oligodendrocytes.

Current test methods. While some guideline studies evaluate neurotoxicity in adult animals, there are two studies designed specifically to evaluate neurotoxicity in offspring – the DNT study and the extended one-generation reproduction study. These studies assess autonomic function including ranking of the degree of lacrimation and salivation, presence or absence of piloerection and exophthalmos, ranking or count of urination and defecation, including polyuria and diarrhea, pupillary function such as constriction of the pupil in response to light or a measure of pupil size, and the degree of palpebral closure (e.g. ptosis in maternal animals and offspring). These studies report the description, incidence, and severity of any convulsions, tremors, or abnormal movements, posture and gait abnormalities, unusual or abnormal behaviors, excessive or repeated actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, deposits around the eyes, nose and mouth, and any other observations. Offspring are monitored for motor activity on postnatal days (PNDs) 13, 17, 21 and 60, subject to an auditory startle test around weaning and day 60, and learning and memory tests around day 60. Neuropathological examinations are conducted on PND 11 and at the termination of the study (after PND 60). In the extended one-generation study, offspring are subjected to auditory startle (PND 24), functional observational battery (FOB) and motor activity (between PND 63 and 75), and neuropathology assessments. The FOB includes a thorough description of the subject's appearance, behavior and functional integrity. This is assessed through observations in the home cage, after removal to a standard arena for observation (open field) where the animal is moving freely, and through manipulative tests (similar to those listed above for the DNT study).

Additional neurotoxicity (behavioral and neuropathology) is assessed in adults in acute neurotoxicity studies, repeated dose neurotoxicity studies and FOB assessments included in at least one repeated dose toxicity study. Clinical signs are monitored in every toxicological study and brain weights and histopathology of the brain (including sections of medulla/pons, cerebellum and cerebrum), peripheral nerves, spinal cord and eye are included in the repeated dose toxicity studies.

If a chemical affects cholinesterase activity, measurement of cholinesterase activity in plasma and erythrocytes and brain are added to the toxicology studies as a marker of systemic toxicity. A comparative cholinesterase study is available, which assesses the level of cholinesterase inhibition in offspring compared to maternal animals.

There are OECD TGs that are informative of a chemical's ability to induce potential neurological effects and these TGs include OECD TG 418 Delayed Neurotoxicity of Organophosphorus Substances Following Acute Exposure, OECD TG 419 Delayed Neurotoxicity of Organophosphorus Substances: 28-day Repeated Dose Study, OECD TG 424 Neurotoxicity Study in Rodents and OECD TG 426 Developmental Neurotoxicity Study.

While these four TGs are informative, the DNT test study protocol (OECD TG 426) has received the most recognition, with scientific critiques and reviews on the extensive resources required to conduct the study, as well as its complexity and limited ability to discern DNT effects; the study protocol is a validated OECD study design that has demonstrated sufficient power to elucidate developmental neurotoxic effects from exposure to chemicals that elicit neurotoxic outcomes.

In addition to these studies, the comparative developmental thyroid toxicity study. Female and male pubertal in rats, and the AMAs are capable of identifying effects on the developing organism. They are also capable of addressing endocrine disrupting effects that covers both human and ecological species.

Enhancements and additional studies. The current toxicological studies cover neurotoxicity and the potential for DNT. Neural tube toxicants could be determined through existing studies, if the mechanism of toxicity was determined to be similar between human and animals. However, while clinical effects in offspring and adults could be similar to those reported in autism, attention deficit disorder, and cerebral palsy, without a mechanistic understanding of those diseases and their etiologies, it is unclear what parameters in animal studies would directly correspond with human neurodevelopmental outcomes, but to the extent that the animal models provide sufficient conservatism when the most sensitive end point is selected among the effects at the lowest dose tested, this provides added assurances that human neurodevelopmental health outcomes will often times be associated with higher levels of exposure than the regulated dose from animal studies, based on margin of exposure type of calculations.

Obesity and diabetes

Obesity is one of the greatest public health challenges of the 21st century. Its prevalence has tripled in many countries of the World Health Organization European Region since the 1980s, and the numbers of those affected continue to rise at an alarming rate. In addition to causing various physical disabilities and psychological problems, excess weight drastically increases a person's risk of developing a number of non-communicable diseases, including cardiovascular diseases, cancer, and diabetes (WHO 2015).

While the prevalence of this disease is clear, this is a multifactorial disease that includes factors such as: genetic

predispositions, high caloric, high fat dietary intakes, and lack of exercise, sedentary lifestyles, and other environmental factors. Specific EDCs that impact the estrogenic, androgenic, glucocorticoid and peroxisomal pathways may play some role in contributing to the multifactorial metabolic disease outcome.

Estrogen receptor(s) – The role of ERs in the control of energy and glucose homeostasis has been reviewed and studies in ERα and ERβ knock out mice have shown that these receptors participate in many of the processes that govern energy homeostasis, food intake, energy expenditure, insulin sensitivity in liver and muscle, growth of adipocytes, deposition of adipose tissue, and pancreatic function (Ropero et al. 2008). Membrane forms of ERs play crucial roles in the function of the islet of Langerhans and the hypothalamus which contribute to the control of energy balance and glucose homeostasis.

Androgen receptor(s) – Prenatal androgen exposure produced a metabolic syndrome in adult female rats that include increases in body weight, parametrial and subcutaneous fat, serum insulin, cholesterol, triglycerides (Demissie et al. 2008). Prenatal testosterone exposure and dihydrotestosterone (DHT) increase body fat in adult male rats (Lazic et al. 2010). Testosterone but not DHT, increased serum glucose levels and the authors further notes that this may indicate a mechanism that involves aromatase activity (converting testosterone to estrogen) rather than a direct androgenic action.

Glucocorticoid receptor(s) – Glucocorticoids are a part of the HPA axis and have critical roles in metabolism, development, immunosuppression and anti-inflammatory reactions. The glucocorticoid receptor signaling is important in the overall control of adipocyte differentiation, along with activation of the peroxisome PPAR γ (Sargis et al. 2010).

Peroxisome proliferation (nuclear receptors) – The PPARγ is a nuclear receptor that is a major regulator of adipogenesis; it promotes adipocyte differentiation, lipogenesis and metabolic homeostasis through activation of genes involved in energy balance. Disruption in receptor activation would result in adipocyte differentiation and predisposition to obesity through an increased sensitivity to a high caloric dietary intake (Grun & Blumberg 2006).

Despite the evidence that the endocrine system is involved in the control of metabolism through specific endocrine glands such as the pancreas, adipose tissue, adrenal and thyroid gland, clear associations with obesity and diabetes have not yet been established. The intersection between obesity and type 2 diabetes will be explored at the upcoming National Academy of Medicine (NAM 2016).

Current animal test methods. Available OECD TGs include many of the diagnostic measurements that are informative to metabolic syndromes including: body weight changes, food efficiency, clinical chemistry parameters such as changes in serum cholesterol, triglyceride, and glucose levels (urinary and serum). Organs of direct relevance for metabolic disorders include measurements in the repeated dose studies of the pancreas, adrenals, and thyroid glands. Additionally, gross pathological evaluation will identify gross changes in visceral fat accumulation and histopathological evaluation will identify fatty accumulation in the liver. On the *in vitro* assays, the EDSP Tier 1 screening levels assays include the estrogen and androgen pathways, inclusive of the HPG axis. The aromatase assay will also capture any aromatization process that requires conversion of testosterone to estradiol for subsequent impact on metabolic syndrome-related organs. The EDSP Tier I Uterotrophic and Hershberger assays will capture *in vivo* effects mediated through the estrogen or AR pathways, respectively. The EDSP Tier I Male and Female Pubertal studies include thyroid organ weight, histopathology and optional thyroid hormone measurement.

Current ecological receptor test methods. In birds, fatty liver is a consequence of obesity and multi-yolked and large eggs are also indications of obesity. The egg parameters are evaluated in both the avian reproduction study and the avian two-generation study, while liver histopathology is included in the avian two–generation study. Body weight and food consumption are included in the ecological receptor studies.

Enhancements and additional studies. Parameters specific to diabetes include serum and urinary glucose and histopathology evaluations of the pancreas. Obesity would be reflected in body weight, body weight gain, food efficiency, food consumption and other secondary effects. If a pathway for a metabolic syndrome effect was identified, then more specific mechanistic studies could be conducted.

Adrenal disorders: adrenocortical hyperplasia

Along with a suite of metabolic disturbances, including high blood sugar levels (hyperglycemia), redistribution of body fat and an increased protein catabolism, gross enlargement of the adrenal cortex is frequently observed in this disease. The reversed situation resulting from adrenal hypotrophy and hypo-secretion of adrenal steroid hormones may result in Addison disease, a potentially fatal condition depending on the extent of tissue destruction in the adrenal cortex.

Addison's disease is characterized by a reduction in cortisol resulting in hypersecretion of ACTH. Hyperpigmentation occurs in most cases due to increased levels of ACTH and its ability to bind to α-MSH receptors on melanocytes. This disease is typically associated with hypoglycemic due to lack of cortisol and reduced capacity for gluconeogenesis. Absence of aldosterone causes muscle weakness, water loss, hypotension, and salt craving. Addison's disease is seen with a bilateral atrophy of the adrenal gland and congenital deficiencies in steroidogenic enzymes.

Congenital adrenal hyperplasia, however, is a genetic defect in one or more genes coding for steroidogenic enzymes involved in corticosteroid synthesis. This can cause reduced glucocorticoid synthesis and excessive ACTH secretion due to the absence of normal feedback. Hypersecretion of ACTH causes hypertrophy and hyperplasia of the adrenal cortex in the zona fasciculata and zona reticularis with an increase in adrenal androgen production caused by the elevated levels of ACTH.

Current animal test methods. The repeated dose toxicities studies routinely evaluate effects on the adrenal gland including organ weight and histopathology. The release of glucocorticoids from the adrenal cortex is triggered by the hypothalamus and the pituitary gland and kidneys trigger the release of mineral corticoids from the adrenal cortex. The thymus and pituitary organ weights and histopathology are also routinely analyzed in repeated dose toxicity studies. Histopathologic evaluation of the hypothalamus is limited to the neurotoxicity studies, including the acute and sub chronic neurotoxicity studies, which evaluate effects in the adult and the DNT and extended one-generation reproduction study, which evaluate neurotoxicity in offspring. The adrenal cortex also releases male and female sex hormones, so evaluation of the male and female reproduction systems is also applicable. The reproduction studies also evaluate the effect of the test material *in utero*, throughout weaning and development capable of evaluating untoward adrenal effects on various organ systems and the pubertal studies evaluate effects in juvenile animals.

Applicable clinical chemistry endpoints routinely measured in toxicological studies include glucose, triglycerides, cholesterol, potassium, and sodium, which would include effects of energy (glucose), fat distribution, and potassium and sodium secondary to aldosterone on the kidneys. Major changes in electrolytes such as potassium and sodium can sometimes be detected in clinical effects such as tremors and inactivity. Effects on body weight, food consumption as well as food conversion/food efficiency calculations can indicate the relationship between food intake and body weight.

Currently, if a specific effect on the adrenal gland via a specific hormone is indicated, hormonal measurements can be included in routine toxicological studies or special mechanistic studies. Additionally, considered together with the results from estrogen and androgen binding assays, the estrogen transcription assay and the Uterotrophic and Hershberger studies, the collective releases male and female sex hormones from the adrenal cortex effects associated with adrenocortical hyperplasia may be determined.

Current ecological receptor studies. Histopathology of the kidneys is included in avian two-generation reproduction study, the medaka extended one-generation reproduction fish study and the larval growth and development frog study. The adrenal glands are evaluated by histopathology in the avian two-generation reproduction Study. Effects on male and female reproductive organs, food consumption and body weight, would be considered adrenal-sensitive endpoints currently available other ecological receptor studies. Eggshell parameters may also predict changes in adrenal hormones, for example in birds administered corticosterone, elevated eggshell thickness was reported without altering weight and strength, suggesting possible changes in shell structure.

Enhancements and additional studies. The adrenal glands are evaluated in multiple species (dogs, mice, and rats) upon multiple routes of entry, exposure durations, and life stages. As such, the adrenal changes or related effects are well covered in existing toxicological studies.

It is recognized that measurements of cortisol (hydrocortisol), corticosterone, aldosterone, ACTH levels are not routinely conducted in animal or ecological receptor studies. As a study protocol enhancement, measurement of hormones that directly affect the adrenal gland or are released from the adrenal gland could be incorporated in to routine toxicological studies.

Human immune-related disorders

The immune system is a functionally rather than anatomically defined organ, spread throughout the body with a major role of defending the host against infections. It is composed of antigen specific lymphocytes and their products, constituting the acquired and adaptive component of the host defense, and an innate nonspecific part that constitutes the first line of defense. The latter also includes cells that are unable to recognize antigens directly but are nevertheless indispensable to securing a fortunate outcome in the battle against invading microorganisms.

Current animal test methods. The available current animal toxicology studies include hematological evaluation of immune parameters, as well as gross and histopathological evaluations of immune-related organs. Hematological parameters include red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential, such as prothrombin time or activated partial thromboplastin time. Changes in a specific white cell type can be detected, as well as changes indicative of more wide spread hematological effects. Routine histopathology evaluations are completed for the following organs: two different sets of lymph nodes (one in close proximately to the route of entry and one distal), bone marrow, adrenal gland, thymus and spleen.

In addition, the immunotoxicity TGs, the study protocol specifically requires the evaluation of immune system suppression, which might occur as a result of repeated exposure to a test chemical. The immunotoxicity study evaluates the functional responsiveness of major components of the immune system and includes functional tests used to determine the response to antigen administration and/or the effect on antibody-producing cells from the spleen, immunoglobulin quantification, natural killer cell activity, and enumeration of splenic or peripheral blood total B cells, total T cells, and T cell subpopulations. Spleen and thymus weights are also included in this study.

The Extended-One Generation Study specifically evaluates immunotoxicity in offspring exposed *in utero*. The developmental immunotoxicity cohort evaluates T-cell dependent antibody response assay (the primary IGM antibody response to a T-cell dependent antigen, such as sheep red blood cells or keyhole limpet hemocyanin). General hematological parameters are also evaluated in parental animals in this study and include hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte

count, platelet count and blood clotting time/potential. Parental animals and offspring have adrenal gland, spleen and thymus organ weights recorded and histopathological evaluations. Weights of lymph nodes associate with and distal from the route of entry are recorded in offspring and splenic lymphocyte subpopulation analysis conducted (CD4 + and CD8 + T lymphocytes, B lymphocytes, and natural killer cells).

Current ecological receptor endpoints. At this time, there are no specific studies applicable to the evaluation of immune-response in ecological receptors.

Enhancements and additional studies. Specific functional immune tests and evaluations like those incorporated into the Immunotoxicity study could be included more routinely in repeated dose toxicological studies.

Vitamin D pathway-related disease and disorders

The vitamin D endocrine system is complex, involving the skin, liver, and kidney for synthesis of the vitamin D metabolites and, primarily, the intestine and bone for biologic expression. Numerous factors and disorders affecting the skin, gastrointestinal tract, and kidney will adversely affect vitamin D metabolism.[3] Vitamin D is well known as a hormone involved in mineral metabolism and bone growth. Its most dramatic effect is to facilitate intestinal absorption of calcium, although it also stimulates absorption of phosphate and magnesium ions. In the absence of vitamin D, dietary calcium is not absorbed at all efficiently. Vitamin D stimulates the expression of a number of proteins involved in transporting calcium from the lumen of the intestine, across the epithelial cells and into blood. As a transcriptional regulator of bone matrix proteins, it induces the expression of osteocalcin and suppresses synthesis of type I collagen. In cell cultures, vitamin D stimulates differentiation of osteoclasts.

Current animal test methods. While vitamin D levels and precursors are not measured in routine guideline toxicity studies, other clinical chemistry endpoints affected by vitamin D deficiencies are routinely included. For example, calcium and phosphorus are often included in the clinical chemistry parameters evaluated and magnesium would be added to the list of clinical chemistry parameters if the mode of action indicates a possible effect on magnesium.

Furthermore, adverse effects on the liver and kidney that may influence vitamin D synthesis are included in almost every repeated dose toxicity and reproductive toxicity study. The parathyroid, a major inducer of 1-alpha-hydroxylase[4], is routinely evaluated by histopathology in routine toxicological studies. Gross pathological observations in body structure and evaluations of body weight in the animal toxicity studies would also indicate an effect of bone growth or strength. Any adverse clinical findings related to limping or gait would be reported in toxicological studies, and those animals evaluated closely for fractures in routine toxicological studies.

The developmental toxicity studies evaluate skeletal variations and malformations in the offspring of rats and rabbits and the reproduction and developmental toxicity studies would be able to evaluate gross effects in offspring.

Importantly, if an adverse effect(s) was detected in bone, calcium and/or phosphorus level in a toxicological study (28-day, 90-day, developmental toxicity study, or chronic duration study) additional endpoints or mechanistic studies would be conducted to elucidate the mode of action, threshold of effect, and relevance to humans.

Current ecological receptor studies. Studies in fish, frogs and birds all include measurements of physiological growth, which would detect alterations in bone and cartilage. Effects on delays or defects in growth will be detected in the fish and frog studies. In addition to forming new bone, calcium is needed for the formation of eggshells, therefore the bird studies and eggshell analysis would be indicators of changes in calcium levels.

Enhancements and additional studies. Although certain enzymes are routinely measured in repeated dose toxicity studies, it would be possible to evaluate the enzymes responsible for the conversion of vitamin D to the active form including the P450 mitochondrial enzyme 25-hydroxylase (CYP2R1), which hydroxylates cholecalciferol to 25-hydroxycholecalciferol in the liver and 1-alpha-hydroxylase, which converts 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol by the addition of one –OH group, the biologically active form in the kidney. These enzymes could be added to routine repeated dose toxicity studies, such as the 28-day and 90-day subchronic toxicity studies.

In addition to calcium and phosphorus, routinely measured in toxicological studies, serum analysis of magnesium as well as the different forms of vitamin D could be added to routine toxicological studies. Additional biochemical markers of bone metabolism (bone-specific alkaline phosphatase (ALP), serum osteocalcin, serum C-telopeptide and urinary N-telopeptide) could also be included.[5]

Genotoxicity studies to evaluate genetic defects in the VDR due to a number of different mutations have been identified in humans that lead to hereditary vitamin D resistance could be added. Also investigations into activation and regulation of transcription, as vitamin D is identified as a transcriptional regulator of bone matrix proteins and of protein expression involved in calcium absorption[6].

While, histopathological evaluation of the bone marrow is routine in toxicological studies, histopathological analysis of the bone, including osteoclasts and osteoblasts could be included in studies where an effect of vitamin D regulation was expected. Evaluation of bone mineral content and density could also be included.

Summary and conclusions

The endocrine system is a complex, interconnected system that enables regulation of growth, development and

reproduction. Responsiveness of the system to environmental and internal stimuli provides the means for a wide suite of toxicity studies and their associated sensitive health endpoints to reflect multiple perturbations of endocrine-related pathways.

This review evaluates the adequacy of current toxicity test methods to inform the means by which chemicals can perturb known endocrine-related biological pathways that can subsequently result in diseases and disorders. In this review, specific test methods and measured endpoints were evaluated to determine the extent to which endocrine-related adverse effects can be detected and quantified. To address any existing test method gaps, identification of any needed research and/or enhancements to current test methods have also been provided.

Available test methods

Many existing test methods are required for registration of pesticide chemicals (Supplementary Appendix C); these test methods include studies of different species, durations and routes of exposure, with multiple doses including a dose with clear toxicity. 42 extant test methods are available to inform endocrine activity and effects. Of these test methods, 25% are *in vitro* and 75% are *in vivo* assays. While some assays may be diagnostic of a specific endocrine modality, typically a specific combination of assays (e.g. EDSP Tier 1 battery of assays) provides for a more rigorous evaluation of potential interaction with particular endocrine-related biological pathways. The extant test methods are also capable of characterizing potential apical adverse effects whether from endocrine or non-endocrine etiologies.

Although it may be difficult to confirm a specific endocrine causality from existing methods, any adverse apical effect to critical life processes should be sufficiently quantified. Confounding factors such as hormesis or stress-induced HPA axis crosstalk would not mask an adverse consequence. Overall, based on this analysis, the likelihood that adverse effects of an EDC would go undetected when evaluated through existing regulatory test methods is remote.

This conclusion is based on the current evaluation and those of previous regulatory agencies. In 2013, EFSA published its "scientific opinion on the hazard assessment of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment" (EFSA 2013) and concluded similarly that "… a reasonably complete suite of standard assay for endocrine activity and for endocrine hazard identification and/or characterization is available for EATS modalities relevant for mammals and fish…" Furthermore, EFSA states that "… there must be a reasonable evidence-based for a biologically plausible causal relationship between induced endocrine activity and the adverse effect(s) seen in an intact organism, or its progeny, or (sub)population. Evidence for this relationship should be obtained from the OECD conceptual framework or from other investigations and assessed on a weight-of-evidence basis" (EFSA 2013). Since the EFSA 2013 documentation, there have been a number of significant, endocrine-specific, longer-term definitive test methods that have been validated through OECD and EDSP (US EPA 2014). This current analysis updates all previous test method assessments for endocrine effects, incorporates all available validated test methods, and applies the AOP framework for endocrine-related biological pathways.

Endocrine-related biological pathways

Highlighting the interconnected endocrine system, disruption within one endocrine pathway will have effects through homeostatic checks and balances with likely, pleiotropic effects across and within other endocrine-related biological pathways. Attention should be paid to the pattern of responses to guide the weight(s) afforded to these various responding endpoints and assays and to the amount of corroboration observed across studies. Among the suite of *in vitro* and *in vivo* assays, however, it is the longer-term, more definitive test methods that can inform a multitude of endocrine modalities; these include the EOGRT (OECD TG 443), and the EDSP Tier 2 Multigenerational Reproduction Toxicity studies (OECD TG 416). The OECD 443 study protocol is the preferred endocrine test method to the OECD 416 because of the comprehensive coverage of endpoints in the juvenile and F1 life stages and because it captures endpoints sensitive to endocrine disruption, including areola/nipple retention, anogenital distance at birth, measurement of thyroid hormones T_3, T_4 and TSH levels (OECD 2012). Together, these studies provide the most comprehensive data that would best inform risk assessment with dose response and apical endpoints for multiple endocrine-related biological pathways (Supplementary Appendix D).

In review of the biological pathways related to endocrine perturbation, the number and breadth of relevant assays across endpoints, taxonomic groups, sensitive life stages, and routes of exposure provide adequate data to ensure a chemical substance that is active in any of the endocrine-related biological pathways would be detected (Figure 14). Recommendations of test methods enhancements, as summarized in Table 4, are provided only to supplement the understanding of the apical endpoints in the context of earlier precursor key events that are more specific and diagnostic of endocrine perturbations (Figure 14).

Endocrine-related diseases and disorders

In the evaluation of current test methods, it was determined that 15 to 34 assays can inform endocrine-related diseases and disorders. While current test methods are adequate in capturing downstream adverse apical events, some additional research or test methods enhancements may be warranted to address more upstream, diagnostic earlier precursor key events. Table 5 provides a summary of the recommended test methods enhancements.

In the evaluation of test methods for endocrine-related diseases (Figure 15), two core recommendations were provided to supplement the existing assays: (1) adding hormonal

Table 4. Summary of recommended test methods enhancements to address endocrine-related biological pathways.

Endocrine-related biological pathways	Test method enhancement	Added measurement(s)
Hypothalamus pituitary gonadal axis	Repeated dose mammalian studies	Additional observations on behavior could be beneficial in corroborating androgen signaling perturbations Added hormonal measurements (e.g. progesterone, etc.)
	Fish short-term reproductive assay and/or Medaka extended one-generation reproduction toxicity test	New endpoint for assessing GnRH neuron development in brain but some logistical disadvantages with minimal added value Assess oocytes and sperm quality or a new *ex vivo* method for assessing oocytes and sperm derived from these fish assays.
Hypothalamus pituitary thyroid axis	Early precursor key events upstream of adverse outcomes	*In vitro* assays to address diagnostic, early precursor key events (e.g. thyroid transactivation reporter assays, cell proliferation assays, TPO inhibition assay, HPT regulated gene expression assays)
Hypothalamus pituitary adrenal axis	Enhancements to existing repeated dose mammalian studies	Addition of stress response relevant endpoints, glucocorticoids and corticotropin (ACTH)
	New assays for consideration of earlier key events in AOP	GhR transactivation assay (*in vitro*)
	Enhancement to the existing steroidogenesis assay (OCSPP 890.1550)	Adrenal steroid synthesis assay (*in vitro*)
Somatotropic axis	Enhancements to existing repeated dose studies	Add measurements of IGF-1 serum levels or IGF-1 mRNA levels
Vitamin D signaling pathway	Vitamin D transactivation assay and AhR transactivation assay	Research on identifying biomarker endpoints that can be diagnostic of vitamin D disruption
	Enhancements to existing repeated dose studies	Vitamin D hydroxylase and EROD activity
Retinoid signaling pathway	Enhancements to existing repeated dose studies	Serum retinoid levels, EROD induction activity, CYP1A1 mRNA or protein quantification in *in vivo* assays
Peroxisome proliferator-activated receptor signaling pathway	PPAR transactivation reporter assays and gene microarray library as early precursor key events to supplement apical endpoints	Peroxisome proliferation measurements at different time courses and lipid accumulation measurements may be added to repeat dose toxicity studies

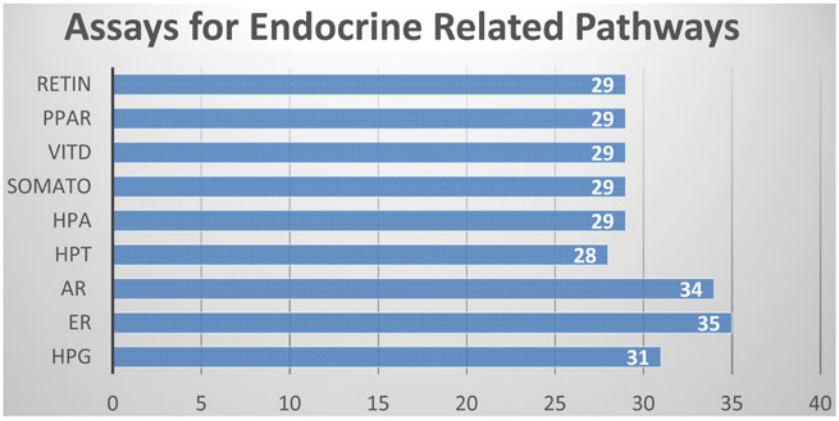

Figure 14. Assays for endocrine-related pathways.

measurements to existing repeated dose studies and (2) an *in utero* to end of life study to capture effects that manifest later in life. Of the various disease and pathways evaluated, the HPA and adrenal disorders and diseases have been identified as an area that may warrant additional test method enhancements. While the adverse effects from HPA perturbation may be captured in current test methods, diagnostic measurements such as cortisol (hydrocortisol), corticosterone, aldosterone, ACTH levels are not routinely conducted in animal and ecological studies. Measurements of hormones that directly affect the adrenal gland or are released from the adrenal gland could be readily incorporated into routine toxicological studies.

For example, adding hormonal measurements in the 28/90 day studies are routinely done on a proactive basis and the current TGs allow for these to be considered; these enhancements increase the ability to identify endocrine diagnostic effects through a WOE approach.

The recommendations of conducting the *in utero* to lifetime study protocol, however, may be more complex and less practical to implement; with longer-term studies, there are more opportunities to introduce laboratory errors and technical difficulties while trying to maintain a study for that duration. The number of animals with multiple reproductive cycles, and measurements, would increase the likelihood of problems in the execution of the study.

Table 5. Summary of recommended test methods enhancements to address endocrine activity.

Endocrine-related disease/disorder	Test method enhancement	Added measurement(s)
Male and female reproductive health: precocious puberty Female reproductive health: fecundity Female reproductive health: polycystic ovaries syndrome Female reproductive health: adverse pregnancy outcome Female reproductive health: endometriosis and uterine fibroids	Repeated dose toxicity studies (e.g. 28/90-day study)	Hormonal measurements (e.g. LH, FSH, testosterone, estradiol, progesterone, prolactin, DHEA)
Male reproductive health: testicular dysgenesis syndrome	No enhancements needed at this time	No enhancements needed at this time
Hormonal cancers: breast cancer	Adapt existing assays and diagnostic techniques	A study to address *in utero* to end of life duration of exposure, or from conception through lifetime. Genetically engineered mouse models (GEMMS) or transfected organism models that have been developed to study factors related to the pathogenesis of experimental prostate, ovarian, and other cancer subtypes and using more sensitive rodent strains for endocrine-related cancer (NAS, 2016)
Hormonal cancers: prostate cancer	Adapt existing assays and diagnostic techniques	
Hormonal cancers: testicular		
Hormonal cancers: thyroid	*In vitro* assays to establish earlier key events	TR reporter assays, TPO assay, and HTP thyroid specific assays (NIS)
Neurodevelopment disorders	Additional research to elucidate the mode of action and key events confirming the primary role of endocrine activity in the manifestations of neurodevelopmental, obesity and diabetes multifactorial health outcomes.	
Metabolic syndrome – obesity and diabetes		
Human adrenal disorders: adrenocortical hyperplasia	Repeated dose toxicity studies (e.g. 28/90-day study)	Measurements of cortisol (hydrocortisol), corticosterone, aldosterone, adrenocorticotropic hormone levels
Immune-related disorders: inflammatory, immune cancer, childhood respiratory disease	Repeated dose toxicity studies (e.g. 28/90-day study)	Functional immune tests and evaluations included in the immunotoxicity study
Vitamin D-related disorders	Repeated dose toxicity studies (e.g. 28/90-day study)	25-hydroxylase measurements, vitamin D and magnesium, biochemical markers of bone metabolism (bone specific ALP, serum osteocalcin, serum C-telopeptide and urinary N-telopeptide)

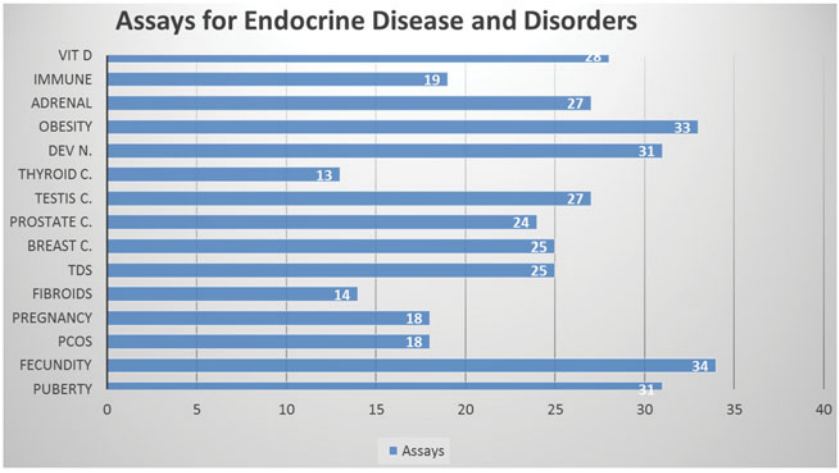

Figure 15. Assays for endocrine diseases and disorders.

Alternatively, the potential of early life exposure to increase the susceptibility of the organism to adverse effects seen later in life, could be addressed by integrating results from a variety of available studies; For example, the integration of new Tox21 methods, new genomic technologies, and HTP cellular and molecular approaches, 2) prenatal developmental study (OCSPP 870.3700), 3) female and male pubertal assays (OCSPP 890.1450; 890.1500), 4) extended one-generation (OCSPP 870.3800; OECD 443 or multigenerational reproduction studies (OCSPP 870.3800; OECD 416) and 5) the chronic/cancer bioassays (OCSPP 870.4200 and 870.4300 OECD 452 and 453) would capture the sensitive

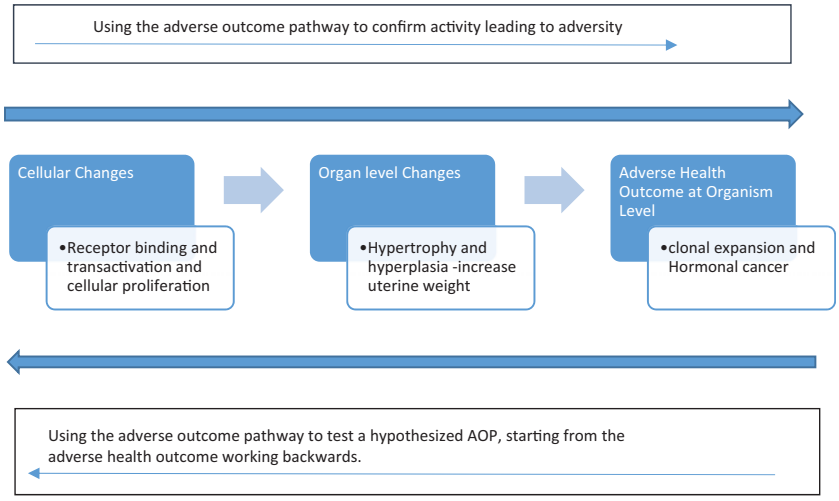

Figure 16. Applications of the adverse outcome pathway framework.

life stages intended to be addressed by the *in utero* to life time study protocol.

Evidence of germinal genotoxicity and heritable mutations (Mouse Heritable Translocation Assay) (OECD 485) could be evaluated with the results from the multiple generation reproduction, extended one-generation reproduction studies and the longer-term chronic cancer bioassays, noting, however, that available methods for detecting human germline mutations are not currently considered adequate for risk assessments.

Future research may include the considerations of advancing genetically engineered mouse models that model the morphological and biological changes of a particular histologic subtype of endocrine-related cancers (e.g. prostate, ovarian, testicular, etc.), which are considered useful for the study of tumor biology (NAS 2016). While research is needed to better determine endocrine modalities, integrating existing studies results would adequately provide relevant information on the sensitivities of each life stage and determine whether there is increased susceptibility in the young. The existing toxicity studies adequately capture transgenerational exposures and effects, with lifetime exposures addressed by the chronic/cancer bioassay (OECD 452, 453).

Endocrine disease pathways are interconnected. This is demonstrated by Hunt et al. (2016), who noted that "a higher risk of autoimmune disorders and cancer have been suggested for women with endometriosis and PCOS, a higher risk of gestational diabetes and metabolic or cardiovascular disease among women with PCOS, and a greater risk of cancer among infertile women in comparison with unaffected women." Mechanistic studies that could inform earlier, precursor events and additional parameters added to existing higher tiered studies such as the additional hormonal measurements and enzymatic analyses, could be included to better elucidate the etiology of the endocrine-related adverse responses. As the state of the knowledge and science advances to better understand the endocrine-related AOPs, it will be important to update the current test methods to include additional parameters to increase the power of these test methods to identify endocrine-specific pathways.

Future perspectives

Endocrine-specific test methods are rapidly evolving. As additional test methods are developed that better inform endocrine-related AOPs, expansion of the current knowledgebase will better inform the diagnosis of those adverse apical endpoints caused by endocrine disruption. Downstream adverse health effects, as demonstrated though this evaluation, are adequately captured by current test methods, but research is needed to identify earlier precursor key events that would be more specific and diagnostic of endocrine-related pathways (Figure 16).

Further understanding and insights into the mechanism of actions of EDCs would facilitate the design of a HTP battery of tests for early indications and screening of chemicals for endocrine disrupting effects (EEA 2012). Understanding the endocrine-related adverse health outcomes would allow multidirectional (up and down the AOP) research to elucidate the key events that may be used to prioritize further testing and/or determine whether higher tiered studies are needed to more fully explore the potential for endocrine activity. Scientific techniques that can better elucidate perturbation early in the AOP or disease state are becoming available; e.g. endocrine-specific HTP assays, new transcriptonomics, proteomics, genomics and metabolomics information, and Tox21 methodologies. Achieving improved understanding of pathways will provide the means to realize the practical outcome of new assays that better elucidate whether chemicals perturb key events early in the disease progression pathway or diagnose causes of observed adverse outcomes (Figure 16).

Notes

1. http://www.env.go.jp/press/102328.html.
2. http://www.obgyn.net/infertility/hormone-levels-and-pcos#sthash.LZ9vQse5.dpuf
3. http://www.ncbi.nlm.nih.gov/pubmed/6367121
4. http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/other-endo/vitamind.html
5. http://www.fda.gov/downloads/Drugs/NewsEvents/UCM399622.pdf
6. http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/other-endo/vitamind.html

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Declaration of interest

Mary Manibusan is a former employee of the US EPA and is currently a Senior Managing Scientist at Exponent. Dr. Leslie Touart is also a retired, former employee of the EPA and is currently an independent consultant for Equiparent Consulting. Exponent is an independent consulting firm with a broad range of expertise, while Equiparent Consulting is a small, privately owned, independent consulting firm that provides expert assessments on ecotoxicology data. Exponent has provided engineering, scientific, environmental, and health consulting services to corporations, insurance carriers, government agencies, law firms, and individuals. Exponent utilizes the best available scientific data to support its technical work products. For this specific project, CropLife International (CLI) sponsored the evaluation completed by Exponent and Equiparent Consulting.

CLI is an association based in Brussels, Belgium that promotes agricultural technologies such as pesticides and plant biotechnology. CLI sponsored this independent expert review, but had no role in the review, writing, or development of the manuscript. CLI received the draft/final document and had an opportunity to review the document, but decided that the journal's peer review process would be sufficiently thorough and any comments and/or changes from CLI would be redundant to the journal's peer review process. CLI did not provide any comments or suggested changes prior to or during the journal submission process.

As indicated, the authors have both been previously employed by the EPA and each worked directly in the EDSP and managed the test method validation process for endocrine methods, including those used in the screening and testing program. Both Mary Manibusan and Dr. Leslie Touart left the Agency in 2015. Previously, Ms. Manibusan worked at the EPA for 18 years as a chemical risk assessor, regulatory manager, and chief of the toxicology and epidemiology branch in the Office of Pesticide Programs; more recently, she had served as director of the EDSP from 2012 through 2015 and had led the Federal Insecticide Fungicide Rodenticide Act (FIFRA) Scientific Advisory Panel peer review of the validation for the EDSP Tier II methods and the WOE assessments of the Tier 1 screening battery of assays. Dr. Touart worked at the EPA for 37 years as an ecological toxicologist and served as senior science advisor for the EDSP before his retirement from government service. He participated in the evolution of the program from 1998 through 2015. He was also directly involved in the test method validation efforts for the EDSP and Organization for Economic Cooperation and Development (OECD) programs. Dr. Touart led the bilateral agreement with the Japan Ministry of Environment and the development and validation of the Tier II endocrine test methods. Neither Ms. Manibusan nor Dr. Touart has testified (written or oral) before government agencies in the content of the document or been involved in any litigation related to the content of the document. Views expressed are solely those of the authors and may not necessarily represent those of CLI.

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Supplementary material

Supplemental material for this article is available online here.

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