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Uncertainties in Biological Responses that Influence Hazard and Risk Approaches to the Regulation of Endocrine Active Substances

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EDITOR'S NOTE:

This is 1 of 5 articles generated from the SETAC Pellston Workshop "Ecotoxicological Hazard and Risk Assessment Approaches for Endocrine-Active Substances (EHRA)" (February 2016, Pensacola, Florida, USA). The primary aim of the workshop was to provide objective advice, based on current scientific understanding, to regulators and policy makers, whether in industry, government, or academia. The goal is to make considered, informed decisions on whether to select an ecotoxicological hazard- or risk-based approach for regulating a given endocrine disrupting substance under evaluation.

ABSTRACT

Endocrine-disrupting substances (EDS) may have certain biological effects including delayed effects, multigenerational effects, and may display nonmonotonic dose–response (NMDR) relationships that require careful consideration when determining environmental hazards. Endocrine disrupting substances can have specific and profound effects when exposure occurs during sensitive windows of the life cycle (development, reproduction). This creates the potential for delayed effects that manifest when exposure has ceased, possibly in a different life stage. This potential underscores the need for testing in appropriate (sensitive) life stages and full life cycle designs. Such tests are available in the Organisation for Economic Co-operation and Development (OECD) tool box and should be used to derive endpoints that can be considered protective of all life stages. Similarly, the potential for effects to be manifest in subsequent generations (multigenerational effects) has also been raised as a potential issue in the derivation of appropriate endpoints for EDS. However, multigenerational studies showing increasing sensitivity of successive generations are uncommon. Indeed this is reflected in the design of new higher tier tests to assess endocrine active substances (EAS) that move to extended one-generation designs and away from multi-generational studies. The occurrence of NMDRs is also considered a limiting factor for reliable risk assessment of EDS. Evidence to date indicates NMDRs are more prevalent in in vitro and mechanistic data, not often translating to adverse apical endpoints that would be used in risk assessment. A series of steps to evaluate NMDRs in the context of endocrine hazard and risk assessment procedures is presented. If careful consideration of delayed, multigenerational effects and NMDRs is made, it is feasible to assess environmental endocrine hazards and derive robust apical endpoints for risk assessment procedures ensuring a high level of environmental protection. *Integr Environ Assess Manag* 2017;13:293–301. © 2016 The Authors. *Integrated Environmental Assessment and Management* published by Wiley Periodicals, Inc. on behalf of Society of Environmental Toxicology & Chemistry (SETAC)

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INTRODUCTION

In recent years, endocrine disruption has become a topic of increasing public and regulatory concern. Consequently, policies and legislation in the major regions have been implemented to regulate endocrine-disrupting substances

(EDS). However, these regulations have become divergent in the approaches proposed. In the United States and Japan, programs such as the Endocrine Disruptor Screening Program (EDSP) (USEPA 1998) and Extend (Ministry of the Environment Japan 2010) are in place to identify endocrine activity. Where necessary, these programs lead to higher tier testing to define concentration–response relationships, perform risk assessment, and conclude on the risk that EDS present to the environment. In Europe, hazard-based cutoff (ineligibility for registration) criteria for EDS are being implemented in some legislations. Most of the cited scientific reasons used to support regulation based solely on hazard center around uncertainties in describing the biological responses of EDS. These are properties, although not exclusive to endocrine-active substances (EAS), which are potentially problematic for the robust derivation of no observed adverse effect levels (NOAELs) required for sound risk assessment. Endocrine-active substances may exhibit delayed or multigenerational effects where an effect is not necessarily manifest at the time of exposure but in a different life stage or generation. These phenomena are discussed and linked to the need for appropriate use of specific ecotoxicological tests from the tool box described by the Organisation for Economic Co-operation and Development (OECD) Conceptual Framework for Testing and Assessment of Endocrine Disrupters (OECD 2012). Similarly, the presence of nonmonotonic dose–response (NMDR) relationships has clouded the toxicological paradigm for EDS. This controversial issue (Rhomberg and Goodman 2012; Vandenberg et al. 2012) continues to be investigated by exhaustive data reviews (National Research Council 2014). Here, we discuss NMDR occurrence and their relation to adversity, and propose a way forward to assess and incorporate NMDRs into test and assessment strategies.

DELAYED EFFECTS

Delayed effects are a feature of many EAS and they occur when exposure coincides with sensitive windows in the life cycle, yet the effects are seen later on. In such cases, the morphological changes that arise can have severe, lasting consequences even if exposure ceases. Here, we define delayed effects as dose–concentration-dependent effects induced by exposure during critical windows of development or reproduction, which become observable only later on during the life cycle (in another development stage or even in the next generation) after exposure has ceased. This excludes certain effects that may appear later in long term tests as a result of toxicodynamic-toxicokinetic factors.

The case studies developed for the SETAC Pellston Workshop (Matthiessen et al. this issue) provide many examples of delayed effects—particularly in fish species. For example, exposure to potent estrogens such as 17α-ethinylestradiol (EE2) (Örn et al. 2003; Maack and Segner 2004; Nash et al. 2004; Schäfers et al. 2007; Baumann et al. 2014), during the period of sexual differentiation and gonadal development may result in skewed sex ratios, and also, in some instances, lower reproductive outcome in the adult stages. Feminisation caused by exposure to estrogens is partly reversible (Maack and Segner 2004; Schäfers et al. 2007; Baumann et al. 2014), but some EE2 experiments show no recovery of egg production or fertilization rate if exposure was during a critical window (Nash et al. 2004; Xu et al. 2008).

Exposure to nonaromatizable androgens (that cannot be converted to estrogens) during sexual differentiation is known to masculinize fish. Zebrafish (*Danio rerio*) exposed to trenbolone (TRB) during sexual differentiation develop skewed sex ratios and all male populations at 0.01 μg/L and above (Larsen and Baatrup 2010; Morthorst et al. 2010; Boettcher 2011; Baumann et al. 2015) this phenotypic sex change is irreversible (Larsen and Baatrup 2010; Morthorst et al. 2010; Baumann et al. 2015). The TRB-induced reversal to a male phenotype appears more sensitive in zebrafish than in medaka (Örn et al. 2006; Mizukami-Murata et al. 2015). Other endocrine mechanisms can also lead to masculinisation in fish (for a review see Matthiessen and Weltje 2015). Piferrer et al. (1994) found that genetically female Chinook salmon (*Oncorhynchus tshawytscha*) developed into phenotypical males if briefly exposed to the aromatase inhibitor fadrozole during sexual differentiation.

These sex-reversal effects are not delayed per se, but may be considered so, as we are able to measure them only at later life stages (when the gonad is sufficiently developed for histopathological examination). Furthermore, if the change is irreversible, it is likely that a delayed population-level impact may be observed, driven by an inappropriate sex ratio of sexually mature individuals (see Supplemental Data S1 for EE2 and Data S2 for TRB). However, it is important to acknowledge that the typical measure of "sex reversal" is often indirect. Most of the observations cited above are based on phenotypic sex ratios (typically confirmed via gonad histopathology) comparing treated and untreated groups. It is assumed that statistically significant shifts in phenotypic sex ratios are the result of sex reversal (i.e., the proportion of genotypic sex was equal across treatments). Of the commonly used fish test species, currently it is possible to directly measure genotypic sex only in medaka where comparisons of phenotypic versus genotypic sex (via DNA sex probes) can truly be made.

In addition to fish, delayed effects of EDS are not uncommon in the other vertebrate classes. Female Japanese quail (*Coturnix japonica*) exposed in ovo to estrogens like diethylstilbestrol (Kamata et al. 2006) and *o,p'*-DDT (Bryan et al. 1989) lay eggs without shells due to abnormal oviductal differentiation. In red-eared slider turtles (*Trachemys scripta elegans*) that have temperature-dependent sex determination, in ovo exposure to 17β-estradiol (E2) can sex reverse presumptive males (Sheehan et al. 1999). A wide range of delayed adverse effects of EDS have also been reported in mammalian reproduction studies (Gray and Kelce 1996). For example, perinatal estrogen exposure has been shown to alter gender-specific reproductive and nonreproductive

behaviors in rats and mice and to alter reproductive cyclicity in females and shorten their reproductive life span (Gorski 1986).

Delayed effects can also be seen in invertebrate species exposed to some EDS, such as juvenile hormone mimics or ecdysone mimics. Exposure of larval honey bees (*Apis mellifera*) to the juvenile hormone mimic fenoxycarb on day 4 leads to lethal moults (no emergence of viable adults) on day 22 (Aupinel et al. 2007). Similarly, the ecdysone agonist insecticide tebufenozide, which was added on day 4 to a static aquatic test containing first-instar *Chironomus riparius* larvae, led to lethal moults 3 weeks later (Hahn et al. 2001).

Overall for potent EDS acting on the hypothalamo-pituitary-gonadal (HPG) axis, delayed effects in organisms are relatively common (see Supplemental Data S1 for EE2 and Data S2 for TRB). However, these effects are not unique to EDS as they can often be related to exposure during developmental windows similar to delayed effects from teratogens or developmental neurotoxicants. To address this, many extended tests are available in the OECD tool box (Coady et al. this issue). Using these tests ensures that changes induced during critical windows of exposure are followed for long enough that effects are seen, and thresholds for population-relevant apical endpoints can be determined.

MULTIGENERATIONAL EFFECTS

Multigenerational effects can be described as effects seen in the next generation and/or generations and can manifest as an increased sensitivity of a following generation to the test chemical. This may be caused by increased exposure, where the second filial generation (F2) are exposed for longer periods than the first filial generation (F1), or by exposure of F2 over a sensitive window (during which the F1 were not exposed). Multigenerational effects can also result after parental generation (F0) or F1 exposure, where effects are seen in F1/F2 offspring raised without exposure. However, it should be acknowledged that there is also exposure of the F2 as gametes and in utero in the F1 organisms.

To assess the possibility of multigenerational effects of EAS, study designs may include constant exposure to a chemical over more than one generation, or shorter periods in one generation, with targeted exposures to progeny over critical developmental windows or with progeny reared without exposure. Some evidence for such effects is reviewed below.

Some studies have assessed multigenerational effects in fish, where eggs from exposed F0 parents were hatched in clean water. Although, exposure to gametes of the F0 that give rise to the F1 is not excluded. Nash et al. (2004) studied zebrafish eggs (from 0.5 and 4.5 ng/L EE2-exposed F0 parents) and saw no multigenerational effects on F1 or F2 embryos, larvae, or breeding success in the F1. Exposure of rainbow trout (*Oncorhynchus mykiss*) F0 males to 0.8 ng/L EE2 resulted in decreased survival of F1 offspring (Brown et al. 2009). However, the surviving unexposed male F1 trout, when mature (1 and 2 years old), were able to produce normal offspring. F1 threespined stickleback (*Gasterosteus aculeatus*) exposed to high concentrations of sodium perchlorate from fertilization to sexual maturity (1 y) displayed morphological abnormalities at concentrations greater than or equal to 30 mg/L; specifically, impaired formation of calcified plates, fins, and spines (related to perchlorate's actions as a thyroid inhibitor) (Bernhardt et al. 2011). However, surviving F2 raised in clean water for 25 wk were morphologically normal.

If studies have constant exposure over several generations, multigenerational effects cannot be easily separated from early-exposure effects. However, these types of constant-exposure multigeneration studies can assess if there are changes in sensitivity from one generation to the next (see Supplemental Data S1 for EE2 and Data S2 for TRB). A few fish studies show no difference in effects from one generation to the next. For example, F2 zebrafish embryo growth was decreased by EE2 at similar exposure concentrations to F1 growth (Schäfers et al. 2007). Similarly, Boettcher (2011) saw no multigenerational effects of TRB on zebrafish F2 hatching success or survival of eggs over 2 generations.

However, some fish multigenerational studies show increasing effects and lower NOECs from subsequent generations. F1 swim-up success of Chinese rare minnow (*Gobiocypris rarus*) exposed to EE2 was negatively affected at 0.91 ng/L, whereas parental F0 survival at swim-up (exposed from egg stage) was not affected until 13.6 ng/L (Zha et al. 2008). Fathead minnow (*Pimephales promelas*) F1 larval length and weight were decreased at 0.16 ng/L EE2, whereas F0 length and weight were decreased at a higher concentrations (12 ng/L) as they were exposed from the fertilized egg stage only (Länge et al. 2001). There is also some evidence for increased effects of TRB over several generations of exposure in fish. Cripe et al. (2010) conducted a 3-generation flow-through study exposing sheepshead minnow (*Cyprinodon variegatus*) to TRB. Reproduction was significantly reduced at levels of 0.87, 0.027, and 0.027 μg TRB/L in the F0, F1, and F2 generations, respectively. The differences in sensitivity may be related to exposure duration and timing, where F0 were exposed from the adult stage onward, whereas F1 and F2 were exposed in ovo and throughout their lives (Cripe et al. 2010).

These studies provide evidence of some effects occurring at lower concentrations in subsequent generations of fish in comparison to earlier generations. However, in most cases the differences in sensitivity may be caused by exposure of the 2nd and 3rd generations for longer periods than the parental generation or during sensitive juvenile stages (see Case Studies in Supplemental Data S1 for EE2 and Data S2 for TRB). These results suggest the uptake of some EAS into the egg during its development in the exposed female parent fish may be important. Whereby, the egg's exposure during its maturation inside the female increases the sensitivity of hatched larvae once the egg is laid and fertilized.

In mammals, there are a multitude of effects in males and females seen only in the F1 (offspring) and not in the P0

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(parent) generation including delayed effects, effects on reproductive life span, etc. (Gray et al. 1994, 1997; Bigsby et al. 1999; Vidaeff and Sever 2005; Hotchkiss et al. 2007). A variety of protocols are in use to assess multigenerational effects in rats and mice including multigeneration, one-generation, and enhanced one-generation protocols (examples are the ECHA enhanced one-generation protocols and the OECD Extended One-Generation Reproductive Toxicity Study). In addition, the NIEHS NTP uses a different protocol, they identified as a modified-one-generation test (NIEHS 2015) (https://ntp.niehs.nih.gov/testing/types/mog/index.html).

A review by Schwindt (2015) finds some evidence of EAS-related multigenerational effects in aquatic species, where exposure of the parent can result in changes in offspring. However, he also concludes that there appear to be no clear demonstrations of heritable transgenerational effects in aquatic species exposed to EAS. Rorije et al. (2011) found that for 49 of 50 of compounds tested in rat multigenerational tests, the additional information provided by the second generation did not influence the chemical classification and labeling decisions under the European Chemical Agency guidance (Rorije et al. 2011).

Overall, for EDS, there is little evidence for multigenerational effects beyond the second generation. Slight decreases in LOECs are often due to increased exposure from maternal transfer, in addition to the continued exposure of the F2 organism. Standardized extended one-generation tests are now available to assess these potential multigenerational effects of EAS so information can be obtained relating to whether multigenerational effects occur. These characteristics and considerations apply equally to substances with other specific or nonspecific modes of action as they are not unique to EDS.

NONMONOTONIC DOSE RESPONSES

Nonmonotonic dose–responses (NMDRs) are frequently observed in toxicological experiments. Nonmonotonic dose–responses may originate from various causes (reviewed in Lagarde et al. 2015) such as: nutritional value of the test compound for the test organism (e.g., essential metals with a deficiency, optimum, and toxicity part of the curve); poor control performance (suboptimal control values, optimal performance at low concentrations, toxicity at higher concentrations); hormesis phenomena such as overcompensation or some kind of stimulation (e.g., enzyme induction by a test compound at low concentrations followed by toxicity at higher concentrations); metabolism of the test compound (whereby the metabolite induces a different type of effect than the parent compound); and chance findings (emphasizing the need for repeatability and an appropriate number of replicates and not too widely spaced dose groups) etc. Often, NMDRs can be explained by 2 distinct mechanisms or effect types operating simultaneously with 1 mechanism dominating the low concentration response and the other the high concentration response, the latter frequently is associated with overt toxicity.

Nonmonotonic dose–responses are not specific to EAS (Calabrese 2008; EFSA 2013; Beausoleil et al. 2016), but are found across all types of chemicals in all kinds of test systems including in vitro and in vivo tests. Nevertheless, there is concern that, particularly for EDS, the occurrence of NMDRs might confound the setting of robust NOAELs, thus compromising the risk assessment. Clearly, for risk assessment, only the in vivo apical adverse effect NMDRs would be of interest as in general only adverse effects are used in risk assessment.

Although most scientists agree that NMDR phenomena can occur (Ankley and Villeneuve 2015), many contend that the NMDRs demonstrated to date would not lead to unidentified hazards or risks for human health and the environment (van der Woude et al. 2005). Arguments include statistical insignificance of some NMDR data, erroneous combinations of different endpoints as a single response, mischaracterization of oscillatory biochemical and cellular phenomena that counteract rather than produce adverse effects, lack of reproducibility, and the inability to explain more numerous, well-established monotonic relationships (Witorsch 2002; Kamrin 2007; Rhomberg and Goodman 2012; Fussell et al. 2015). Regardless of this debate, it is clear that current (eco)-toxicological test methods identify NMDRs regularly, and therefore it is prudent to develop clear criteria for identifying NMDRs (Lagarde et al. 2015; Beausoleil et al. 2016) and for assessing dose–response phenomena in regulatory decision making (Borgert et al. 2015).

Evaluation of NMDRs in the context of endocrine hazard and risk assessment

Based on the considerations presented above, a scheme is proposed to evaluate NMDRs for both nonapical (mechanistic) and apical (adverse) endpoints. A flowchart for NMDRs in the context of endocrine hazard and risk assessment procedures is presented; one for in vitro mechanistic and/or in vivo biomarker endpoints and one for adverse apical endpoints (Figures 1A and 1B, respectively). The flowcharts consider aspects of reproducibility and biological plausibility and, for the apical endpoints, if a threshold (i.e., NOAEL) can reliably be determined.

If a NMDR is observed for a mechanistic endpoint, the flowchart (Figure 1A) can be used to identify whether further investigation of potential downstream effects on apical endpoints is warranted. For apparent mechanistic NMDRs that have no plausible biological explanation and are not reproducible, lower weight is placed on these findings and testing should proceed without special consideration. Mechanistic NMDRs that are reproducible, even if not well understood, may need to be considered in in vivo experiments to detect apical endpoints. There are many examples of EAS that produce NMDRs in mechanistic but not apical endpoints, and, therefore, have no impact on the risk assessment (e.g., propiconazole [Goetz et al. 2009; Skolness et al. 2013], perchlorate [Li et al. 2011; Petersen et al. 2015], TRB [Ankley et al. 2003; Ekman et al. 2011], and vinclozolin [Monosson et al. 1999]).

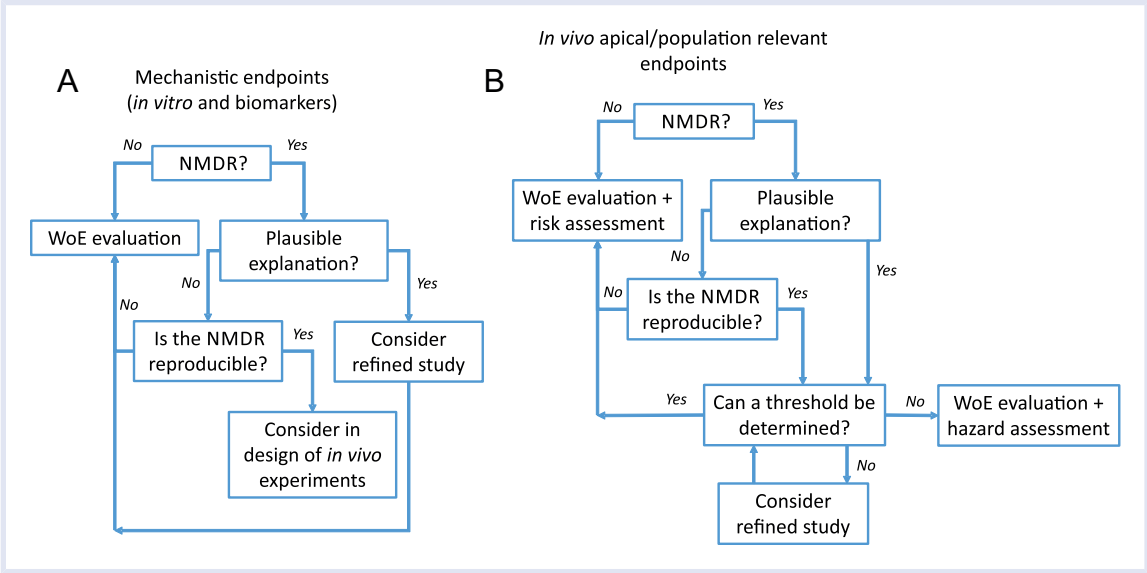

Figure 1. (A) Proposed flowchart scheme for evaluation of NMDRs for mechanistic endpoints (in vitro and biomarkers). (B) Proposed flowchart scheme for evaluation of NMDRs for in vivo apical and/or population-relevant endpoints.

The second flowchart (Figure 1B) starts with the observation of an apparent NMDR in an apical endpoint and is used to establish whether a threshold can be determined. Qualitative criteria for identifying NMDRs have been discussed by Calabrese (2008) and Lagarde et al. (2015). For apparent apical adverse NMDRs that have no plausible biological explanation and are not reproducible, a weight-of-evidence analysis may continue without special consideration. If the existence of an NMDR is supported by mechanistic evidence, the study design may be refined to further characterize the response and to establish whether a threshold can be determined. An example of a nonmonotonic apical response to an EAS is the masculinization of genetically female Chinook salmon exposed as juveniles to 17α-methyltestosterone (MT). With increasing concentration, a higher proportion of fish developed testes, culminating in 100% masculinisation at 400 μg/L; however, higher concentrations of MT resulted in decreased proportion of fish with testes and the development of ovaries in up to 11.8% of fish (Piferrer et al. 1993). The underlying mechanism for these effects is now understood to be the biological conversion of MT to 17α-methylestradiol via the aromatase enzyme (Hornung et al. 2004), which also explains the observation of NMDR in vitellogenin production of juvenile zebrafish exposed to MT (Örn et al. 2003). Another example is the observation that fish exposed to low doses of EE2 produce more eggs than control groups, and fish exposed to higher concentrations produce fewer eggs than control groups (Nash et al. 2004; Pawlowski et al. 2004; Parrott and Blunt 2005). It is plausible that increased egg production at low concentrations of EE2 is mediated via stimulation of vitellogenin production in females (Kime et al. 1999), but it is unclear what mechanism and/or mechanisms may account for decreased egg production at higher concentrations of EE2 or whether it is related to male reproductive pathology. As is the case with many NMDRs, this could be explained by the overlay of 2 distinct mechanisms operating simultaneously, with one dominating at the low and the other at the high concentration. In such situations, refined studies should be considered to characterize each mechanism's dose response separately and to establish whether a threshold can be determined. Further studies may also be needed to determine if and under which circumstances NMDRs such as increased egg production are adverse effects that should be considered for risk assessment.

In summary, for nonreproducible NMDRs and plausible and/or reproducible NMDRs in apical endpoints for which a threshold can be determined, risk assessment is the proposed approach. However, if a threshold for an apical endpoint cannot be determined, hazard assessment may be the preferred option.

THRESHOLDS

The discussion around thresholds and the existence thereof has similarity with those around hypotheses: they cannot be proven, only disproven. Nevertheless, when a hypothesis holds despite numerous attempts to disprove it, it becomes part of accepted scientific theory. "Proving" no effect in an experiment (e.g., determining a NOEL) is based on a statistical comparison (with usually 5% uncertainty) of the performance in the treatment groups with that in the control, and thus it does not necessarily imply zero effect. The NOEL is a selected test concentration whereas the mathematical description of the dose–response curve is often asymptotic (i.e., no zero response) and merely illustrates the difference between theory and practice. This means that by applying the generally agreed principles of statistical testing in (eco) toxicology, a threshold can be determined in practice (with $\alpha = 0.05$).

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On the other hand, it has been asserted that thresholds cannot be established for endocrine effects because the zero-effect level is subject to limits of detection and other sources of uncertainty (Zoeller and Vandenberg 2015), an argument that relies on a mathematical definition of threshold as the lowest dose that produces a nonzero response (Slob 1999). A related argument is that because the endocrine system of an individual organism is already activated above zero response by endogenous hormones whose concentrations are low and fluctuate widely, small additions or subtractions of even a single molecule will exhibit "additivity to background," producing adverse effects (Hass et al. 2013). A third argument is that because the sensitivity and function of the endocrine system changes with growth and development, and different endpoints are differentially sensitive to the same hormone, it would be impossible to define a single threshold dose for an EAS, even if one existed (Zoeller and Vandenberg 2015).

Other scientists disagree, pointing to the definition of threshold as the lowest dose that produces a measurable physiological effect in an individual organism (Piersma et al. 2011; Rhomberg et al. 2011) and citing contradictions between this hypothesis and established principles of endocrine pharmacology and clinical epidemiology, the mathematical improbability of a single molecule altering occupancy at receptors, enzymes, or transporters (Borgert et al. 2013), and mechanistic concepts demonstrable from systems biology (Zhang et al. 2014).

In the 6 case studies evaluated as part of the SETAC Pellston Workshop, in nearly all cases threshold determination was straightforward. Studies that scored a Klimisch rating of 1 or 2 described experiments with apical endpoints and exposure concentration ranges that allowed thresholds to be resolved for adverse population-relevant effects. Examples are EE2, TRB, TBT, and propiconazole's impacts on fish reproduction (see Case Studies in Supplemental Data S1, Data S2, Data S3, and Data S4). For certain legislations such as REACH, defining thresholds is an important aspect of the process for an EDS. In terms of apical population-relevant endpoints, determining clear thresholds for endocrine-mediated adverse effects does not present a barrier to robust endpoint derivation for risk assessment. Indeed, the difficulties and uncertainties stated by the references above apply, not only to EDS, but to all chemicals. In general, the current methods in risk assessment allow us to tackle most of these to achieve sound decision making.

Environmental risk assessment is most often concerned with effects on populations, not individuals (reviewed in Marty et al. this issue). Resiliencies of populations (Hutchings 2011; Hazlerigg et al. 2012, 2014; Pittman et al. 2013; Debes et al. 2014) make it easier conceptually to define thresholds for many EDS in terms of adverse population-level effects. Several extended tests for EDS are available from the OECD tool box (discussed below and in more detail by Coady et al. [this issue]), which in combination with appropriate exposure concentrations–doses allows robust population-relevant thresholds to be determined. If these criteria for threshold determination are satisfied, we recommend that the environmental risk assessment process can proceed for an EDS.

IMPORTANCE OF TEST DESIGNS RELATED TO EAS

All the issues highlighted pertain to the ability to derive robust NOAELs required for sound risk assessment. Consequently, the availability and adequacy of test methods is crucial to resolve these uncertainties on a substance-by-substance basis. The OECD Conceptual Framework (CF) outlines the available validated tools (OECD 2012). Once a substance has been confirmed as an EAS, higher tier testing is typically required to confirm if the activity translates into an adverse effect, i.e., whether the substance is an EDS or not. The development of these tests (e.g., OECD CF levels 4 and 5 or tier II of the USEPA-EDSP) was largely driven by the perceived need to assess the potential of endocrine-mediated multigenerational effects. Validation and demonstration studies highlighted the fact that shorter designs were preferable, as the evidence for markedly lower effect levels with additional generations was not supported by the data (Janer, Hakkert, Slob et al. 2007; Piersma et al. 2011; Schulz et al. 2014). Indeed, this trend cannot only be seen in the move away from a medaka multigeneration test design to an extended one-generation design (OECD TG 240 and USEPA MEOGRT 890.2200), but also in the rat extended one-generation reproduction toxicity study (OECD TG 443). The extended one generation test designs do assess effects in the F1 offspring, and the design has focused on increasing replication of the breeding adult and F1 offspring assessments, which is analogous to the developments in the medaka-extended one-generation design. Therefore, within the tool box there are tests capable of adequately identifying EAS/EDS, and when necessary, providing robust NOAELs for risk assessment. These higher tier tests can capture potential delayed, multigenerational effects and NMDR if relevant.

However, test methods alone do not cover all elements of experimental design that should be optimized to address the substance-specific questions. These issues are discussed in detail in the companion article by Coady et al. (this issue). Interpreting the data from these tests also requires us to mechanistically link effects from different biological levels (biochemistry, histopathology, individual morphology, behavior, and reproduction) to conclude on the mode of action that may lead to adversity. This is no easy task and will require new tools to better understand the biological responses and what magnitude of change can lead to adverse apical effects.

DISCUSSION AND CONCLUSIONS

It is clear that for EDS, it is important to consider delayed effects and multigenerational effects when deciding which tests to perform for an environmental risk and/or hazard assessment. Standardized tests to assess EAS that include exposures over critical windows and follow organisms for

sufficient time (to determine whether there are delayed effects or effects on the next generation) are available for fish, birds, and mammals in the OECD test battery.

Nonmonotonic dose responses can occur with exposure to EDSs and other chemicals. These are observed more frequently in biomarker-type responses (nonapical mechanistic responses) and can often be explained by system adaptation and compensation in the early phases of exposure prior to re-establishing biological homeostasis. Some NMDRs are seen in apical endpoints and may often be explained by stimulation at low concentrations and toxicity at higher concentrations or by the existence of different mechanisms of action at low and high exposure ranges. However, NMDRs for apical endpoints are less common. If the apical-effect dose–response curve with the NMDR can be repeated, explained, and understood, then a robust threshold can be derived. If a threshold cannot be determined, risk assessment is not recommended.

Similarly, threshold derivation depends on the selection of appropriate exposure concentrations–doses used in the appropriate EDS test (from the OECD tool box). When the appropriate tests and concentration ranges are used, population-relevant robust thresholds can be derived for use in environmental risk assessment.

The OECD Conceptual Framework forms the basis of the toolbox to address the issues highlighted in this article. It should not be considered a test battery per se. It is not necessary to have data from all levels to conclude on potential endocrine disrupting properties of substances. The tools in the OECD Conceptual Framework should rather be used selectively to establish an adequate suite of experiments that should be performed to best address the identified EDS-related questions (e.g., delayed effects, multigenerational effects, NMDR, etc).

In conclusion, although EDS require detailed ecotoxicological test designs that consider critical windows of exposure, delayed effects, multigenerational effects, and sometimes nonmonotonic-dose–response curves, these factors should not prevent the robust determination of NOAELs for use in environmental risk assessment.

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Data availability—Data of the case studies used in the EHRA Pellston Workshop are available in the 4 Supplemental Data files published with this article.

SUPPLEMENTAL DATA

Data S1. Ethinylestradiol Case Study
Data S2. Trenbolone Case Study
Data S3. Tributyltin Case Study
Data S4. Propiconazole Case Study

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