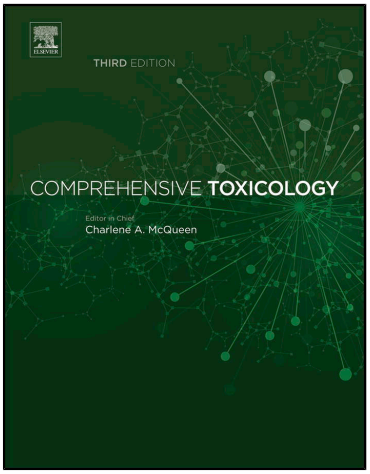

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1.02 Alternative Approaches to Dose–Response Modeling of Toxicological Endpoints for Risk Assessment: Nonmonotonic Dose Responses for Endocrine Disruptors

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1.02.1 Brief Introduction

Since the term "endocrine disruptor" was originally coined in the 1990s, significant attention has been given to this group of environmental contaminants. Researchers from a variety of disciplines have contributed to scientific knowledge about these chemicals and the mechanisms responsible for their health effects. Yet, considerable debate remains about whether endocrine-disrupting chemicals (EDCs) represent a public health concern. In this article, we review one issue that has been central to this debate—the question of dose–response shape and whether nonmonotonic dose responses could influence regulatory decision-making.

A number of recent reviews provide a good starting point for additional information on EDCs (Bergman et al., 2013b,c,d; Gore et al., 2015a,b; Vandenberg et al., 2012; Zoeller et al., 2012, 2014; Beausoleil et al., 2013; Heindel et al., 2015; Munn and Heindel, 2013; Birnbaum, 2012, 2013; Beronius et al., 2014a,b; Schug et al., 2011; Janesick and Blumberg, 2011). Some of these reviews provide excellent overviews of the issues discussed in this article including nonmonotonicity and how EDCs are addressed by regulators and risk assessors.

1.02.2 Overview of Endocrine-Disrupting Chemicals

In 1996, the US Environmental Protection Agency (US EPA) offered the first formal definition of an EDC as "An exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock et al., 1996). Other groups have subsequently defined EDCs using different language including the World Health Organization (WHO) and International Programme on Chemical Safety (IPCS): "An exogenous substance or mixture that alters function(s) of the endocrine system

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and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations. A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations" (IPCS, 2002) and the Endocrine Society: "An exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (Zoeller et al., 2012).

These different definitions bring with them divergent requirements for determining whether a chemical in question should be considered an EDC (Zoeller et al., 2014). For example, the US EPA's definition requires only that a compound be demonstrated to affect one or more actions of natural hormones in the body, whereas the WHO/IPCS definition requires that such actions be linked to a downstream adverse effect (Beronius and Vandenberg, 2016). The use of these different definitions in different regulatory frameworks has caused controversy because, based on the weight of the available evidence, a chemical could be labeled an EDC according to the US EPA but not an EDC according to the WHO/IPCS. Another important issue that has received significant attention is the use of the term "adverse" in the WHO/IPCS definition of an EDC (Vandenberg et al., 2013a; Bergman et al., 2013a). We discuss this issue in more depth later in this article.

1.02.2.1 Identification of EDCs

One important point that must be addressed when considering whether a compound should be classified as an EDC is the complexity of the endocrine system (Diamanti-Kandarakis et al., 2009). Although most attention relevant to the identification and study of EDCs has been given to hormones involved in reproduction, dozens of hormones are involved in coordinating the organs and systems of the body (Gore et al., 2006; Myers et al., 2009b). This complexity is rarely appreciated when chemicals are tested for their endocrine disruptive properties (Grun and Blumberg, 2009; Bergman et al., 2013b; Gore et al., 2006).

The US EPA developed a series of assays that can be used to ascertain whether chemicals interfere with estrogen, androgen, or thyroid hormone signaling (Borgert et al., 2011). The Endocrine Disruptor Screening Program (EDSP) is a two-tiered screen. In Tier 1, 11 assays are employed. These include cell fraction in vitro, whole cell in vitro, and whole animal in vivo tests (**Table 1**). Positive results in Tier 1 assays could lead to additional tests being conducted in Tier 2 including one-generation and two-generation in vivo toxicity tests. In a standard one-generation assay, adult male and female rodents are exposed to a test compound for 2 weeks prior to mating, and exposures continue through mating, pregnancy, and lactation (Tyl, 2009a). After weaning, the offspring continue to be exposed through puberty and adulthood. Traditional measures of toxicity are assessed including body weights, weights of organs, measures of clinical chemistry (serum cholesterol, measures of oxidative stress, etc.), and reproductive measures (length of pregnancy, number of live pups, sex of offspring). Other developmental measures including the incidence and type of birth defects and overt signs of neurotoxicity (seizures, abnormal posture) are also recorded. Although these types of endpoints are typically taken as reasonably adequate indictors of toxicity by toxicologists (Tyl, 2010), they have proven to be poor predictors of endocrine-disrupting properties because they are prone to false negatives (Zoeller et al., 2012; Markey et al., 2001). For example, the chemical bisphenol A (BPA) has been shown to bind to a large number of receptors including estrogen receptors α and β, membrane estrogen receptor, GPR30, thyroid hormone receptor, androgen receptor, estrogen-related receptor γ, and aryl hydrocarbon receptor (Vandenberg et al., 2009), earning it the third highest toxicological priority index of more than 300 chemicals evaluated (Reif et al., 2010). It tests positive in a number of EDSP Tier 1 tests, yet produces few effects in Tier 2 (Zoeller et al., 2012). It has also been argued that the Tier 2 assays do not adequately evaluate function of the endocrine system, and thus these measures do not map to the human diseases that have raised concern about the safety of EDCs (Myers et al., 2009a,b; vom Saal et al., 2005; Vandenberg et al., 2013c).

The assays used in the EDSP Tier 1 and Tier 2 include tests that were developed in the 1930s and 1940s; the rodent uterotrophic assay, for example, was first described in the 1930s as a method to screen for estrogenic substances (Bulbring and Burn, 1935; Dorfman et al., 1936). Additional evaluation tools using more state-of-the-art technology and scientific knowledge have been constructed to identify EDCs (Birnbaum, 2013; Birnbaum et al., 2012; Gore, 2010; Gore et al., 2006; Myers et al., 2009a,b). These high-throughput methods, including assays in the National Toxicology Program (NTP)'s Tox21 screens (Bucher, 2013; Betts, 2013;

Table 1 The EDSP tier 1 assays

Assay	*Type*	*Dose groups*
ER binding assay	Cell fraction	At least 5
ER transcriptional assay	In vitro	At least 5
AR binding assay	Cell fraction	At least 5
Steroidogenesis	In vitro	At least 5
Aromatase assay	Cell fraction	At least 5
Uterotrophic	In vivo	Control + 3 dose groups
Hershberger	In vivo	Control + 3 dose groups
Pubertal female	In vivo	Control + 2 dose groups
Pubertal male	In vivo	Control + 2 dose groups
Fish short-term reproduction	In vivo	Control + 3 dose groups
Amphibian metamorphosis	In vivo	Control + 3 dose groups

Attene-Ramos et al., 2013) and the US EPA's ToxCast (Kavlock et al., 2012; Knudsen et al., 2011; Dix et al., 2007), provide a large number of tools to test environmental chemicals. These include a variety of biochemical and cell-based high-throughput screening assays that measure the ability of chemicals to bind to, activate, or antagonize nuclear hormone receptors important for a substantial fraction of endocrine activity. This approach shows some promise for the estrogen and androgen receptors (Rotroff et al., 2013, 2014) and as a result is being proposed by EPA as a substitute for Tier 1 of the EDSP estrogenic assays (Browne et al., 2015). However, ToxCast assays perform very poorly for other nuclear receptors, such as the 9-cis retinoic acid receptors and the peroxisome proliferator-activated receptor gamma, producing numerous false-positive and false-negative results (Janesick et al., 2016). It is currently unknown why the results of high-throughput receptor activation assays correlate so poorly with the results from identical or similar assays performed in a laboratory setting (Janesick et al., 2016), but it is obvious that further refinement in the performance of ToxCast and Tox21 endocrine assays will be required before they can be robust tools for detecting EDCs. Beyond the Tox21 and ToxCast tools, a group of green chemists and academic environmental health scientists have collaborated to develop a robust, tiered approach to the identification of EDCs that improves the accuracy and reliability of EDC testing (Schug et al., 2013).

1.02.2.2 EDC Sources

To date, more than 1000 chemicals have been identified as EDCs, using relatively liberal criteria for characterization (TEDX, 2015; US FDA, 2010) and without a concerted effort to test all chemicals for endocrine activity. These chemicals are used in a variety of consumer products including personal care products, food and beverage containers, detergents, fragrances, electronics, upholstery and other furnishings, medical and sports equipment, and pharmaceuticals (Gore et al., 2015a; Bergman et al., 2013b). Many pesticides (herbicides, insecticides, fungicides, etc.) are also EDCs which is hardly surprising considering that these compounds are designed to interfere with biological processes. Some heavy metals including cadmium, copper, lead, nickel, and chromium have been shown to display hormonal activities; many of these can activate estrogen receptor (ER)α or progesterone receptor. Industrial chemicals, and compounds that are the byproducts of industrial reactions, can have endocrine-disrupting properties. Pharmaceuticals have been developed that can either mimic or block the actions of hormones. Although these are often used therapeutically (e.g., to treat hormone-dependent cancers or as birth control agents), because they are typically administered at high doses, they are excreted into the environment at appreciable concentrations where they can disrupt endocrine function in wildlife (Christen et al., 2010; Bhandari et al., 2015; Orlando and Ellestad, 2014). Finally, there are some naturally occurring EDCs including phytoestrogens, which are plant-derived estrogenic compounds. Examples of EDCs from each of these categories are provided in **Table 2**.

1.02.2.3 EDC Routes of Exposure and ADME

A major principle of toxicology is to consider how chemicals are *a*bsorbed, *d*istributed through the body, *m*etabolized (biotransformed), and *e*xcreted (ADME). These factors are similarly relevant when considering EDCs (Beronius and Vandenberg, 2016). As with other toxicants, EDCs are absorbed into the body via typical routes of exposure (Vandenberg et al., 2013c): (1) oral exposures, typically via ingested food, but many personal care products can also be ingested, as can dust; (2) inhalation exposures, via particles in air and vaporized chemicals including those found as a contaminant of water; (3) dermal exposures, via materials purposely placed on the skin such as personal care products and other compounds that inadvertently land on the skin; (4) intravenous exposures, via medical equipment; and (5) subcutaneous exposures, via implanted devices.

The distribution of EDCs throughout the body also follows the physiochemical laws of toxicology. Compounds that are highly lipophilic will be easily transported in blood lipids and stored in adipose tissue depots throughout the body. These compounds are most likely to bioaccumulate and thus can biomagnify in food chains. In contrast, hydrophilic substances are unlikely to accumulate in the body's tissues. Their distribution is typically limited.

Biotransformation (metabolism) of EDCs is also similar to the biotransformation of other xenobiotic agents. Chemicals are largely metabolized in the liver via chemical modifications. Induced xenobiotic biotransformation is regulated by the nuclear hormone receptors, pregnane X receptor (PXR), and constitutive androstane receptor (CAR) (Stanley et al., 2006; Xie et al., 2004). These receptors function as xenobiotic sensors that upregulate all three phases of xenobiotic metabolism when activated by the same xenobiotic ligands, including EDCs (Stanley et al., 2006; Xie et al., 2004). In Phase I biotransformation, compounds are hydroxylated by the cytochrome P450-dependent oxidase systems. In Phase II biotransformation, chemicals are conjugated with charged endogenous molecules including sulfate, glycine, glucuronic acid, or glutathione. These modifications alter the molecular weight, charge, and polarity of chemicals, making them more hydrophilic; this improves their transport to excretory organs such as the kidney. Importantly, biotransformation does not necessarily convert biologically active compounds to inactive compounds. In some instances, the products of biotransformation reactions (metabolites) are as active, or more active, than the original agent. Moreover, the ability of EDCs to induce or antagonize the activation of PXR and CAR will necessarily influence xenobiotic metabolism.

The excretion of chemicals occurs via two major routes: urine and feces. Chemicals (and their metabolites) that are hydrophilic are typically excreted in urine and/or bile. Some lipophilic substances are excreted via feces in a process that involves incorporation of the lipophilic substance into micelles and subsequent biliary excretion.

Table 2 Chemical categories and examples of EDCs

Category	*Examples of chemicals*
Pharmaceuticals	Diethylstilbestrol
	Ethinyl estradiol
	Trenbolone
	Tamoxifen
	Fluoxetine
	Fadrozole
Plastics	Bisphenol A
	Bisphenol S
	Phthalates (DEHP, DINP, DBP, etc.)
Detergents, surfactants	Octylphenol
	Propylphenol
	Nonylphenol
Heavy metals	Lead
	Cadmium
	Selenium
	Arsenic
Phytoestrogens and natural antioxidants	Genistein
	Coumestrol
	Daidezin
	Resveratrol
	Biochanin A
	Quercetin
Herbicides	Glyphosate
	Atrazine
	Simazine
Insecticides	Endosulfan
	Diazinon
	Dieldrin
	DDT
	Heptachlor
	Methoxychlor
	Chlorpyrifos
	Malathion
	Oxychlordane
Fungicides	Hexachlorobenzene
	Prochloraz
	Vinclozolin
Industrial chemicals	TCDD
	PCBs
Antimicrobials	Triclosan
Flame retardants	PBDEs
	Firemaster 500
	PBBs
Perfluorinated compounds	PFOA
	PFOS

1.02.2.4 Key Principles of Endocrinology

Just as EDCs can be studied and understood using the toxicological principles of ADME, because these compounds interfere with one or more actions of hormones, they must also be examined considering the principles of endocrinology (Myers et al., 2009b; Zoeller et al., 2012; Vandenberg et al., 2013a; Beausoleil et al., 2013; Gore et al., 2006). Five major principles dictate the study of hormones:

1. Hormones are responsible for coordinating the tissues of the body from conception until death (Diamanti-Kandarakis et al., 2009). As potent signaling molecules, virtually all actions of the body require hormones. Not only are these molecules responsible for reproduction but also they are essential for embryonic and fetal development, puberty, pregnancy, and aging.
2. Hormones act via highly specific binding to receptors (Norman and Henry, 2015). Cells that express these receptors are therefore responsive to the hormone, but cells that do not express the receptor are unaffected. Responses can be modulated by altering the concentration of hormones in blood or target tissues or by modulating the number of receptors.

3. Most endogenous hormones act at exceptionally low doses, typically in nanomolar to picomolar (part-per-billion or part-per-trillion) concentrations (Vandenberg et al., 2012; Vandenberg, 2014). In circulation, the majority of hormones are bound to transport proteins.
4. The effects of hormones depend on life stage (Wallen, 2009; Heindel and Vandenberg, 2015). The same hormone, at a given dose, will have different effects on adults than it will have on developing embryos, fetuses, or neonates. The effects of hormones on adults are termed "activational" because the individual is activated to respond when exposures occur, but the effects cease when exposures are terminated. Effects during development are termed "organizational" because they can permanently alter the organization—differentiation, proliferation, and so on—of cells, tissues, and organs.
5. Hormones rarely exhibit linear relationships between dose and effect over a wide range of doses (Welshons et al., 2003). Rather, nonlinear and nonmonotonic dose responses are very common. This is because there is a nonlinear relationship between hormone dose and number of receptors bound, as well as a nonlinear relationship between number of receptors bound and biological effect. This principle will be discussed in more depth throughout the remainder of this article.

In addition to these guiding principles, other factors that distinguish how endocrinologists understand hormones and EDCs are important to consider. One issue is the relationship between binding affinity and potency (Bergman et al., 2015; Zoeller et al., 2014). Binding affinity is a way to characterize the relationship between the concentration of the ligand that is required to maximally occupy the ligand-binding site of the receptor. EDCs that mimic endogenous hormones can be described by comparing their binding affinity to the binding affinity of the natural hormone. This is quite different from the potency of a compound; potency describes the concentration of a compound that is required to produce a biological effect at a given intensity. Potency is thus endpoint specific and may not necessarily be predicted by binding affinities. For example, two compounds could have binding affinities that differ by 1000-fold; for one endpoint, they could have potencies that also differ by 1000-fold whereas for another endpoint they could be equipotent.

Another important issue is the concept of a biological threshold (Bergman et al., 2013a, 2015). It is often presumed that toxicants have a "threshold dose," and it is assumed (but rarely demonstrated) that no biological effects occur below this dose, whereas above the threshold, biological effects are evident. In this view, all substances are considered to be toxicants differing only in their inherent potency. Yet, the concept of thresholds in many circumstances may not apply to endocrine active substances. This is because the endocrine system is already active in living organisms that use hormones. Therefore, rather than a substance having to activate a system that is inactive (and might be considered to have some threshold for activation), the endocrine system is active and can readily be disrupted by small amounts of chemicals that activate or antagonize it. Thresholds for chemicals that act as hormone receptor agonists and antagonists are difficult, if not impossible, to prove experimentally, and it has been argued that they do not exist (Zoeller et al., 2014; Zoeller and Vandenberg, 2015; Bergman et al., 2015). Others have shown that experiments, including test guidelines used to evaluate toxicity for regulatory purposes, often lack the statistical power to appropriately evaluate whether there are biological effects below "thresholds" because as the magnitude of an observed effect decreases, the sample size required to demonstrate such an effect increases (Scholze and Kortenkamp, 2007).

1.02.3 Dose–Response Relationships

During the risk assessment process, chemicals are first assessed for hazard (Beronius et al., 2009, 2014b). Hazard characterization can determine whether a compound is a reproductive toxicant, a developmental toxicant, a neurotoxicant, and so on. As discussed earlier in this article, the one-generation and two-generation assays are used in Tier 2 EDSP screening, along with other assays, to characterize hazards associated with exposures to test compounds (Borgert et al., 2011). After hazard identification has occurred, dose–response modeling is conducted. In this process, specific effects are attributed to specific doses.

1.02.3.1 Introduction to Dose–Response Curves

To construct a dose–response curve, a number of doses need to be examined for the same endpoint. The number of doses that are examined will influence how well the shape of the curve is characterized. When only a small number of doses are tested, confidence in the shape of the dose–response curve is limited, whereas the addition of more doses can allow for better resolution of the curve (see **Fig. 1** for an example).

The shape of the dose–response curve can be described using mathematical terms and thus can be described based on its mathematical features (Kohn and Melnick, 2002). Linear dose responses are observed when the biological response increases (or decreases) at a constant rate over the range of doses tested (**Fig. 2A**). Log-linear dose responses occur when the response increases (or decreases) at a constant rate over log-increases in doses. Truly linear dose responses are rare for biological processes, although linear responses are often observed within a limited portion of the dose range tested. More common are sigmoidal dose–response curves, which contain a linear component in the mid-range of doses tested (**Fig. 2B**). Sigmoidal curves are expected for many biological responses because a continued linear response at increased dose, to an infinitely high dose, is illogical; rather, a plateau in response is expected for almost all endpoints.

Both linear and sigmoidal responses are considered monotonic; monotonic dose responses are defined mathematically by having a slope (or multiple slopes) where the sign of the slope (positive or negative) does not change over the range of the doses

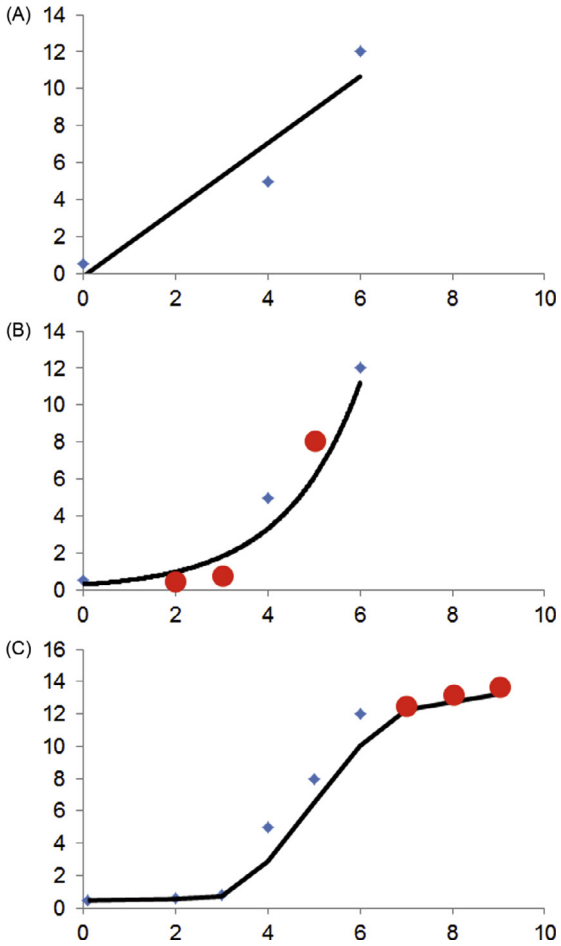

Fig. 1 Models of dose responses with increasing number of dose groups. Conclusions that can be drawn about the shape of the dose–response curve are influenced by the total number of dose groups examined. (A) With three dose groups (negative control plus two others), the response observed has characteristics of a linear response. (B) With the addition of three more dose groups (indicated by *red dots*), the response is more consistent with an exponential response. (C) When three additional dose groups are added (indicated by *red dots*), a sigmoidal curve is apparent.

examined. In contrast, nonmonotonic responses are defined mathematically as ones where the sign of the slope changes over the range of doses tested (Kohn and Melnick, 2002). Thus, nonmonotonic dose responses are often referred to as biphasic, U-shaped, or inverted U-shaped (**Fig. 2C**). Importantly, multiphasic dose responses, where the sign of the slope changes multiple times, would also be considered nonmonotonic (**Fig. 2D**).

Nonmonotonic dose responses are sometimes mistakenly referred to as examples of hormesis. Hormesis is a descriptive word that is used to describe dose responses where one range of doses is considered to have "beneficial" effects and another range of doses induces "toxic" effects (Calabrese, 2011). In some studies, hormesis is described as "low dose stimulation, high dose inhibition." To determine if a biological response is consistent with hormesis, experimenters typically graph the biological responses relative to the response of the negative controls (**Fig. 3**). Hormesis is perhaps best described in studies of ionizing radiation. Some studies have shown that low-level radiation decreases the number of deaths compared to the background population (a low-dose "benefit"), whereas high doses of radiation increase the number of deaths compared to background (a high-dose "toxicity") (Socol et al., 2014). However, other studies have not replicated these findings (Hwang et al., 2008).

Dose responses that are identified as examples of hormesis are probably better described as nonmonotonic because this term provides a mathematical explanation of the shape of the dose response curve without conflating curve shape with harm or benefit (Mushak, 2007). It should be noted that use of the term hormesis in studies of EDCs is not accepted by many endocrinologists because it has been argued that there is no evidence for "benefits" from altering hormone action (Thayer et al., 2005, 2006; Mushak, 2007, 2013; Weltje et al., 2005).

1.02.3.2 Nonmonotonicity

As described earlier, nonmonotonicity is a mathematical term used to describe dose responses where the sign of the slope changes (from positive to negative, or vice versa) along the range of doses tested (see **Fig. 2D**). Identifying a response as nonmonotonic is

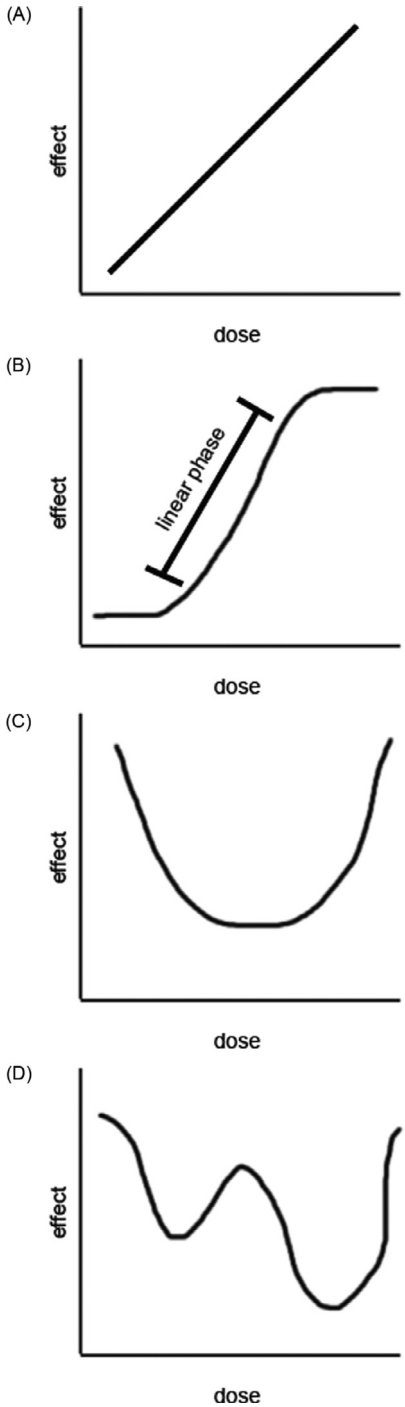

Fig. 2 Examples of different dose–response curves. (A) A linear relationship between dose and effect. (B) A sigmoidal dose–response curve. Here, the linear phase is indicated. (C) A nonmonotonic dose response. This example could also be described as "biphasic" or U-shaped. (D) A multiphasic dose–response curve.

therefore dependent on having a sufficient number of dose groups to properly characterize the slope of the dose–response curve over the entire dose range (Vandenberg and Bowler, 2014). Because three data points are typically required in mathematics to determine a function that describes the line ($y = mx + b$, where m is the slope of the line), at least five dose groups are needed to show that a response is nonmonotonic (Fig. 4).

It is rare indeed for the slope of a dose–response curve to be calculated either when it is reported in the literature or when it is analyzed during a risk assessment. How can nonmonotonicity be demonstrated if such mathematical descriptions of the dose response are not calculated? Similarly, how can nonmonotonicity be demonstrated if fewer than five dose groups are included

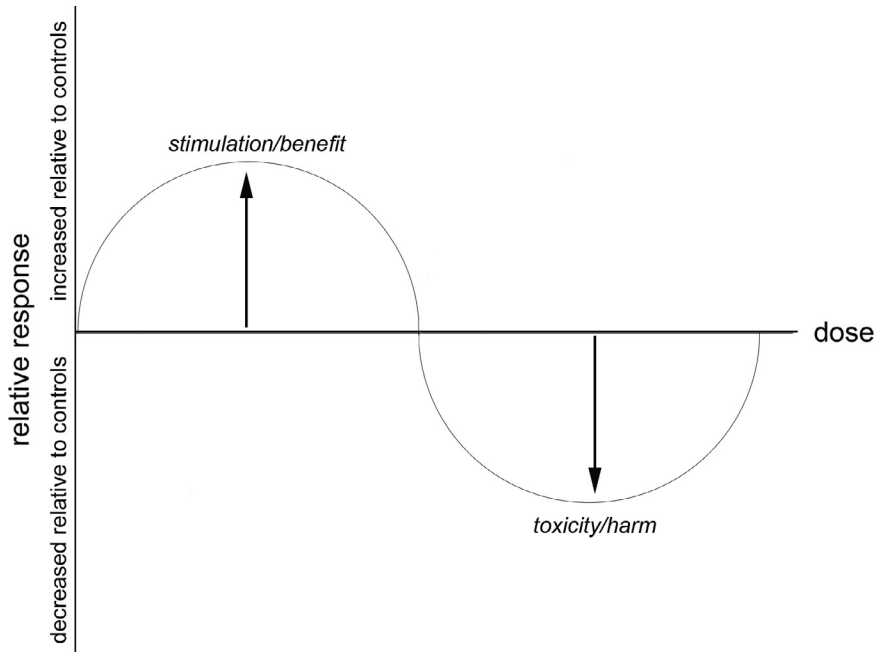

Fig. 3 A model of hormesis. To determine whether a dose response is consistent with hormesis, experimenters start by graphing the responses relative to the control, so responses that are increased or decreased relative to the untreated (or unexposed) group can be more easily visualized. The concept of hormesis proposes that low doses would induce responses that are often characterized as "stimulating" a "beneficial" response whereas high doses induce toxicity responses that are characterized as "harmful."

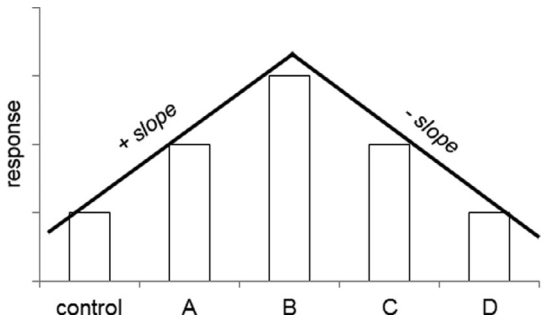

Fig. 4 Five dose groups are needed to mathematically demonstrate nonmonotonicity. To calculate the slope of a line, three data points are needed. Here, we demonstrate the minimum number of dose groups to calculate the slopes of two lines, as two slopes with different signs (positive or negative) must be present to draw conclusions about the presence of a nonmonotonic response. In this example, treatment dose B is the point of inflection and is used to calculate the slope of both lines.

in an experiment? Is the possibility of nonmonotonicity discounted or dismissed in this case? Dose responses that are consistent with nonmonotonicity have been identified using other criteria (Vandenberg and Bowler, 2014) including:

1. A significant difference is observed between the untreated control group and a mid-dose group. No significant difference is observed between the untreated control group and a high-dose group (**Fig. 5A**).
2. A significant difference is observed between the untreated control group and a mid-dose group. A significant difference is also observed between the untreated control group and a mid-dose group, but due to changes in the opposite direction (**Fig. 5B**).
3. A significant difference is observed between the untreated control group and a mid-dose group. A significant difference is also observed between the mid-dose group and a higher dose group (**Fig. 5C**).

1.02.3.3 Hormones, EDCs, and Nonmonotonicity

Consider the relationship between water and mortality: individuals that consume too little or too much water are more likely to die, whereas moderate consumption of water is needed to support healthy life (**Fig. 6**). Similar biphasic relationships are observed for many vitamins and essential nutrients; too little or too much can induce toxicity (Querfeld and Mak, 2010). Thus, nonmonotonic dose responses are well understood in the field of nutrition science.

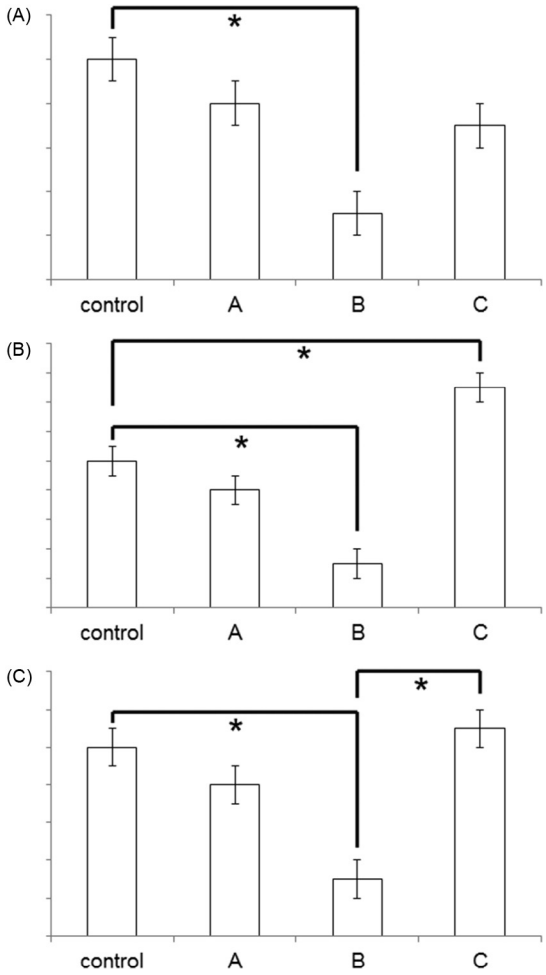

Fig. 5 Nonmonotonicity with imperfect data. Many dose–response experiments do not include sufficient dose groups for a direct assessment of the curve shape. For example, dose–response experiments with only four dose groups are common; experiments from the Endocrine Disruptor Screening Program Tier 1 assays often include only four dose groups. Thus, different criteria, other than the calculation of the slope of the dose–response curve, are needed to assess whether the responses are consistent with nonmonotonicity. These curves represent three criteria that can be used to determine whether a dose response is consistent with nonmonotonicity. These include: (A) a low or moderate dose is significantly different from the control, but a high dose is not. (B) A low or moderate dose is significantly different from the control, and higher doses are also significantly different from the control but due to a response in the opposite direction. (C) A low or moderate dose is significantly different from the control and a higher dose is also significantly different from the low dose, with a response in the opposite direction.

Similarly, nonmonotonicity has been observed for the relationship between hormones and a wide range of endpoints including frank diseases (Vandenberg et al., 2012; Zoeller et al., 2012). These nonmonotonic relationships have been observed at all levels of biological organization including cultured cells, laboratory animals, and human populations. For example, in cultured cells, an inverted U-shaped relationship has been observed for the effect of 17β-estradiol on total cell number for breast cancer cells (Shioda et al., 2006; Welshons et al., 1999). This inverted U-shaped response has also been demonstrated for the effects of 5α-dihydrotestosterone and 5α-androstenedione on prostate cancer cells (Sonnenschein et al., 1989). In laboratory animals, an inverted U-shaped relationship has been repeatedly observed for the effect of 17β-estradiol on growth parameters of the mouse mammary gland (Vandenberg et al., 2006; Wadia et al., 2007; Skarda, 2002; Skarda and Kohlerova, 2006). 17β-estradiol also has nonmonotonic effects on organ weights including the developing prostate and uterus (vom Saal et al., 1997; Shelby et al., 1996). In the epidemiology literature, nonmonotonic dose responses have been observed for the association between testosterone and the incidence of coronary events (Laughlin et al., 2010), thyroid-stimulating hormone and the incidence of Alzheimer's disease (Tan et al., 2008), leptin and mortality (Lieb et al., 2009), and cortisol and body mass index (Kumari et al., 2010).

Hundreds of examples of nonmonotonic dose responses have been reported for EDCs (Vandenberg et al., 2012; Vandenberg, 2013, 2014). Like those observed for natural hormones, nonmonotonic curves have been observed in experiments with cultured cells and laboratory animals. Strikingly, several dozen examples of nonmonotonic dose responses have been reported for EDCs in epidemiology studies (Vandenberg et al., 2012).

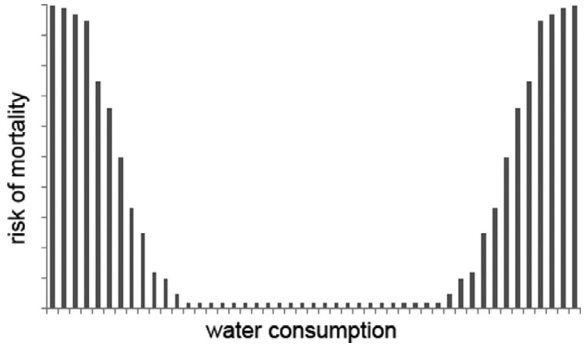

Fig. 6 A biphasic relationship between water consumption and mortality. There is a nonmonotonic relationship between water consumption and risk of mortality. Severe restriction in water consumption as well as extreme consumption of water are both associated with increased risk of death. Similar U-shaped relationships are observed for other essential nutrients and vitamins and their association with morbidity and mortality.

1.02.3.4 Mechanisms Underlying Nonmonotonicity

Considering how common nonmonotonic dose–response curves are in endocrinology, the mechanisms that underlie these phenomena have been well described for hormone-sensitive endpoints. Importantly, many studies that have identified nonmonotonic responses have not determined exactly which mechanism is responsible for the observed dose response, but this is not necessary to accept the phenomenon itself.

One mechanism that has been widely studied is the presence of competing monotonic dose responses that act on a single endpoint. An example is the relationship between hormone dose and cell proliferation, plus the relationship between hormone dose and cell death. These two distinct endpoints both map to total cell number; if increasing doses increase both proliferation and cell death, the resulting cell number will have an inverted U-shaped dose response curve (Welshons et al., 2003). These overlapping monotonic dose responses can occur for many endpoints, not just cytotoxicity (Liang et al., 2014).

It has also been noted that different cell types can have vastly different responses to hormones due to differences in the expression of single receptors (Couse et al., 1997), expression of different cofactors (Geck et al., 1997, 2000), and expression of different receptor proteins that bind the same hormone (e.g., ERα and ERβ) (Kuiper et al., 1997; Lemmen et al., 1999). Many hormones have different binding affinities for specific receptor proteins (Li et al., 2012). In some tissues, the proteins produced from related receptor genes can have competing biological activities. For example, estrogen action on ERα induces cell proliferation in the uterus, whereas estrogen action via ERβ induces apoptosis (Morani et al., 2008; Zhao et al., 2008).

Another mechanism that can contribute to nonmonotonicity is receptor downregulation. When estrogen receptors are bound by ligand, they translocate into the nucleus from the cytoplasm. Once in the nucleus, the receptor can bind to specific DNA elements and activate gene expression. Following binding, the receptor is degraded by means of the proteasome via the ubiquitin pathway (Ismail and Nawaz, 2005; Nawaz et al., 1999). As the dose of ligand increases, the production of new receptors cannot match the rate of receptor degradation. These competing receptor kinetics will lead to a decreased response to estrogen despite increasing dose, producing a nonmonotonic response (Modrall et al., 2001; Kinyamu and Archer, 2003; von Zastrow and Kobilka, 1994).

Similarly, some receptors can be desensitized after chronic binding of ligand. Nonmonotonicity can occur due to desensitization when the activation of a G-protein-coupled receptor is followed by the uncoupling of the activated receptor from its G proteins due to phosphorylation of its binding partners (Lohse, 1993; Bohm et al., 1997; Shankaran et al., 2007). This form of biochemical receptor inactivation is typical for peptide hormones (Bohm et al., 1997).

1.02.3.5 Controversies

A number of controversies have arisen in the study of EDCs that involve nonmonotonic dose responses. In a 2012 review of the literature, hundreds of examples of nonmonotonic curves were identified in studies that involved cultured cells, laboratory animals, and human populations (Vandenberg et al., 2012). Responses to this review included statements from the director of the US National Institute of Environmental Health Sciences, Dr. Linda Birnbaum, who wrote, "the question is no longer whether nonmonotonic dose responses are 'real' and occur frequently enough to be a concern; clearly these are common phenomena with well-understood mechanisms. Instead, the question is which dose–response shapes should be expected for specific environmental chemicals and under what specific circumstances" (Birnbaum, 2012).

Yet, other groups have responded by raising questions about the frequency of nonmonotonic dose responses, the likelihood that they are reproducible, whether nonmonotonic curves are relevant for human exposures, and others (Rhomberg and Goodman, 2012). Similarly, in 2013, the US EPA published a draft report which challenged the conclusion that nonmonotonicity is "common" for hormones and EDCs; the EPA report concluded that nonmonotonic curves "are not unexpected in vitro particularly when evaluating high dose levels and/or lower order biological systems" but that they are "not commonly identified in estrogen,

androgen, or thyroid systems in vivo and are rarely seen in apical endpoints after low dose and/or long-term exposure" (US EPA, 2013).

Following the publication of the EPA's draft report, the National Academy of Sciences was asked to review it. Although they did not evaluate the evidence on nonmonotonicity directly, they did consider the quality of the EPA's work and whether its conclusions were supportable by scientific evidence. In its assessment, the National Academy panel wrote that "the agency failed to establish (or enforce) a clear set of methods for collecting and analyzing the evidence" for nonmonotonicity (NRC, 2014). They also wrote, "Given its importance and its broad use, the committee judges that the [EPA's] evaluation should meet a higher standard of evaluation, particularly given the heated controversy surrounding this issue… Although it is clear that the [EPA] authors spent enormous time and energy in developing the evaluation, it is fundamentally compromised, at least in appearance" (NRC, 2014).

At this time, some of these controversies remain debated by different academic, government, and industry-funded groups (Lamb et al., 2014; Gore, 2013; Gore et al., 2013; Nohynek et al., 2013). It has been proposed that some of this "debate" is actually an example of manufactured doubt (Bergman et al., 2015; Oreskes et al., 2015), where groups with financial interests in a consumer product, industrial process, or chemical will utilize tactics to dismiss science that challenges the safety of their product. These tactics were first developed by the tobacco industry and have been successfully employed in many other fields (Michaels, 2005, 2006). Here, we briefly describe some of these points and, where available, evidence to suggest that some of these "concerns" can be dismissed.

1.02.3.5.1 The dose makes the poison

The concept of "the dose makes the poison" has been attributed to Paracelsus (1493–1541), who is often considered the father of the field of Toxicology. Yet, this quote is an oversimplification of what Paracelsus wrote in his Third Defense (Borzelleca, 2000). "All things are poison, and nothing is without poison; only the dose permits something not to be poisonous." This quote in no way detracts from the concept of nonmonotonicity; in the case of a nonmonotonic curve, it is the dose that determines whether harm is (or is not) seen. Thus, there is no assumption that this occurs at the highest (or lowest) doses and no inference of a requirement for monotonicity is possible. Paracelsus believed that diseases were caused by "poisons" brought by the stars. Further, he argued that poisons should not all be viewed negatively, because "evil" could be used to counteract "evil," and thus poisons could have beneficial effects (Conrad et al., 1995). Paracelsus's work in the fields of alchemy, astrology, and the occult are rather inconsistent with the modern view of him as a scientist.

1.02.3.5.2 The frequency of nonmonotonic dose responses

One major question that remains in the field is to understand the frequency of nonmonotonic dose–response curves. In their 2012 review, Vandenberg and colleagues wrote that these responses were "common" for hormones and EDCs and erred in not describing what was meant by common (Vandenberg et al., 2012). A 2013 follow-up suggested that the term "common" was meant to describe that hundreds of examples had been identified across multiple levels of biological organization and experimental design (Vandenberg et al., 2013a).

But how "common" are nonmonotonic dose responses? Addressing this question is not easy, as it would require a systematic review of the EDC literature to identify all relevant dose–response data, or at least a truly representative sample. Such analyses (unbiased, systematic) have been conducted in the general toxicology literature, and those estimates indicate that biphasic curves (U- or inverted U-shaped nonmonotonic dose responses) occur in 12–24% of all dose–response studies (Davis and Svendsgaard, 1994). The authors note that there is a bias in some scientific communities against publishing nonmonotonic dose responses and conclude that such approximations are likely to be underestimates (Davis and Svendsgaard, 1994).

In a more recent analysis, 109 in vitro studies of the EDC BPA were examined for dose–response data (Vandenberg, 2013). Ninety-three of these studies contained a total of 250 dose–response curves, and 26% of these dose–response curves were characterized as nonmonotonic. Thirty-four percent of all publications with dose–response data included at least one that was consistent with nonmonotonicity.

1.02.3.5.3 Distinguishing nonmonotonic dose responses from statistical flukes

Statistical analysis is often used as a way to separate significant and nonsignificant effects. Importantly, within the Popper Framework, statistics are used to determine whether observed effects are distinguishable from the null hypothesis. A *p*-value is used to describe the likelihood that an effect is observed by chance. Within this framework, there should always be attention paid to the possibility of type I errors (a "false positive" where the null hypothesis is mistakenly rejected) and type II errors (a "false negative" where the null hypothesis is not rejected, even though it is false). One concern that has been raised is that nonmonotonic curves might represent false positives, or statistical flukes, where significant differences are observed for some doses but not others, simply by chance. This kind of criticism could be made for any scientific finding, including a monotonic dose response, and is defeated by appropriate statistical analysis and sound study design, including large enough sample sizes to evaluate effects when the magnitude of such effects are small.

It has also been suggested that studies reporting nonmonotonicity could be poorly designed compared to studies reporting monotonic dose responses (Rhomberg and Goodman, 2012); the idea put forward was that studies that report nonmonotonic responses might examine fewer doses over a shorter dose range. Yet, one examination of the literature revealed that studies that are most likely to reveal nonmonotonicity included six or more dose groups (Davis and Svendsgaard, 1994). Another report found that studies with nonmonotonic dose–response curves examine more doses, over a wider range of concentrations, compared to

studies that report monotonic responses (Vandenberg, 2013). These findings suggest that many examples of monotonicity could be undetected nonmonotonic dose responses that are not revealed because of undersampling, rather than the opposite.

1.02.3.5.4 Adverse outcomes and levels of biological organization

One criticism that was raised by Rhomberg and Goodman (2012) and the US EPA (2013) is whether nonmonotonic dose responses have been observed for adverse outcomes. These groups have suggested that nonmonotonic responses might occur, but for endpoints that are either not toxicologically significant, or that there are adaptive responses that prevent these responses from manifesting at higher levels of biological organization. An example would be a nonmonotonic relationship between dose and expression of a transcription factor; this could be argued to be not toxicologically relevant. Further, if the increased expression of the transcription factor does not influence downstream expression of genes that manifest in an acknowledged adverse outcome like organ weight, then it does not raise concern.

The question is what is meant by adverse and who determines whether an outcome can be characterized as such? The IPCS defined an adverse effect as "a change in morphology, physiology, growth, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increase in susceptibility to the harmful effects of other environmental influences" (IPCS, 2004). The US EPA's definition is "a biochemical change, functional impairment, or pathologic lesion that affects the performance of the whole organism, or reduces an organism's ability to respond to an additional environmental challenge" (US EPA, 2012). Other risk assessment agencies including the US Food and Drug Administration and the European Food Safety Authority do not have a definition for adversity, leaving decisions about whether a specific endpoint is (or is not) adverse to the judgment of risk assessors.

Numerous groups have argued that these definitions leave open too much room for uncontrolled variability in decision-making and a lack of transparency (Woodruff et al., 2008). Others have noted that the definitions do not allow for clarity about whether specific endpoints such as reduced fecundity or altered circulating hormone concentrations would be considered adverse (Vandenberg et al., 2013a). Further, the use of "expert judgment" is concerning because expertise in generalized toxic responses is insufficient to assess the biological basis for many nonmonotonic dose responses (Zoeller and Vandenberg, 2015). Moreover, much of what passes for toxicological analysis of EDCs has been described as equivalent to diagnosing whether a car is performing correctly by disassembling it and weighing the parts; toxicity is measured via alterations in organ weight, whereas disrupted neurobehaviors, alterations to tissue organization, and increased sensitivity to hormones or carcinogens are summarily dismissed as "not toxicologically relevant" (Myers et al., 2009a,b).

It is hard to argue that many of the endpoints that have demonstrated nonmonotonicity do not represent adverse outcomes. Nonmonotonicity has been observed for sexual behaviors, memory, sex ratios, embryo number, aggressive behaviors, fertility, body weight, weight of reproductive organs, responses to allergens, timing of puberty, and survivorship, among others (Vandenberg et al., 2012, 2013a). Further, nonmonotonicity has been observed in guideline studies that are used to assess the toxicity of chemicals (Karaman et al., 2011; York et al., 2010; Biesemeier et al., 2011). Nonmonotonicity has also been observed in the EDSP Tier 1 guideline assays (Vandenberg and Bowler, 2014). Unfortunately, these responses are often dismissed as biologically irrelevant or "not dose dependent," rather than being analyzed for their underlying significance.

1.02.4 Low Dose Effects

Any discussion of nonmonotonicity and EDCs must also acknowledge a related issue, the concept of "low dose effects." Unfortunately, these concepts are often conflated. Yet, they have distinct definitions that should make it clear that these two concepts must be considered separately.

The term "low dose" is often used in the scientific literature without an explanation of what is meant. In 2002, an expert panel assembled by the US NTP and US EPA defined "low doses" as (1) doses below those typically examined in traditional toxicology studies, for example, doses below the toxicological no observed adverse effect level (NOAEL) or (2) doses in the range of typical human exposures (Melnick et al., 2002). Others have proposed additional definitions including (3) doses that replicate in animals the concentrations of a chemical that circulate in human bodies (Vandenberg et al., 2012). This latter definition acknowledges that toxicokinetic differences exist between humans and many model organisms used to assess hazard. For example, rodents typically require much higher administered doses compared to human exposure levels to achieve the same serum concentrations as are observed in humans, when dose is compared on a body weight basis (e.g., mg/kg body weight). The use of body surface area as the dose metric (e.g., mg/m^2 body surface), rather than body weight, generally provides for a much better correlation between administered dose and blood concentration.

The term "low dose effects" thus refers to the biological responses that are observed in the low-dose range (Beausoleil et al., 2013) and does not provide any information about the shape of the dose response curve. Low-dose effects could be linear, nonlinear monotonic, or nonmonotonic.

1.02.4.1 Low-Dose Effects Are Dismissed Because They Are Not "Adverse"

What distinguishes low-dose effects from typical measures of toxicity? Most endpoints that are examined at low doses are not overt signs of toxicity (such as significant reductions in body weight, increased mortality, and abnormal shifts in organ weight). Rather,

they represent subtle effects that are more consistent with intermediate steps between an initiating event (e.g., the binding of a ligand to a receptor) and a disease outcome (vom Saal et al., 2005, 2007; Welshons et al., 2003; Vandenberg et al., 2012). For example, numerous studies have shown that developmental exposures to xenoestrogens can induce proliferation of mammary epithelial cells, increase the number of structures where neoplasias typically develop, alter the histological architecture of the mammary gland, disturb gene expression in this tissue, and alter the response of the gland to hormones and carcinogens (Vandenberg et al., 2013b; Soto et al., 2008, 2013; Crain et al., 2008). Thus, these xenoestrogens may not induce mammary carcinomas by themselves [although newer evidence suggests that they might (Acevedo et al., 2013)], but they induce changes that are found along a continuum of effects between binding to the ER and the disease itself.

Just as nonmonotonic dose responses have been dismissed by some scientists and regulators as not representing adverse outcomes, low-dose effects have similarly been disregarded or ignored (Borgert et al., 2013; Beronius et al., 2014a; Lamb et al., 2014; Rhomberg and Goodman, 2012; Tyl, 2009a,b). Others have argued that low-dose effects represent adaptive responses (Rhomberg and Goodman, 2012). Adaptive responses are difficult to define and identify. A committee of scientists from the chemical and pharmaceutical industries, governmental agencies, and academia has recently developed criteria to help distinguish adaptive and adverse effects (Keller et al., 2012). An adaptive response was defined as "the process whereby a cell or organism responds to a xenobiotic so that the cell or organism will survive in the new environment that contains the xenobiotic without impairment of function."

There are three important points that should be considered when evaluating whether the effects of an EDC (or another environmental factor) are adaptive versus adverse: (1) even when hormones are involved in the maintenance of homeostasis, the shifting of "set points" can have adverse effects on an individual. For example, individuals with type 2 diabetes have an abnormal homeostatic function where cells become resistant to insulin, leading to higher blood glucose levels (Grun and Blumberg, 2009; Schug et al., 2011). (2) Alterations of endocrine function during development cannot be considered adaptive, as embryos and fetuses do not have "homeostatic" controls (Gore et al., 2006; Heindel and Vandenberg, 2015). During these periods of development, hormones are responsible for the development and differentiation of tissues, organs, and systems. Even small alterations to this process can have significant, permanent effects that manifest later in life. (3) As noted by Keller and colleagues (Keller et al., 2012), an individual that "adapts" must do more than survive, it must do so without impairment of function. The issues that arise are when a creature "adapts" to one environment but finds itself in another environment. An example of this came from the work of David Barker and colleagues, who described the long-term health outcomes associated with early-life malnutrition (Barker, 2007). Individuals that are malnourished during gestation experience alterations in tissue organization of organs including the liver and kidney which allow for greater blood flow to the brain (Barker and Hanson, 2004). If these individuals are born and continue to live in a world with reduced caloric availability, they are primed to survive—they will have adapted to this restrictive environment. Yet, what Barker and others have demonstrated is that when individuals experience growth restriction in early life, but have normal or high caloric availability in later life, they develop metabolic diseases including diabetes, heart disease, liver disease, and stroke (Hales and Barker, 2001; Painter et al., 2006a,b; Sayer et al., 1997). Thus, the same event (fetal malnourishment) is adaptive in some individuals (those that continue to experience malnourishment) and adverse in others (those that have plentiful caloric availability). It is evident that claims that an effect might be adaptive must be supported by evidence to this effect and should consider whether an adaptive response in one context might be adverse in another (Vandenberg et al., 2013a; Zoeller et al., 2012, 2014; Keller et al., 2012).

1.02.4.2 Low-Dose Effects in Human Epidemiology Studies

By definition, human epidemiology studies examining EDC exposures and health outcomes in the general population are low-dose studies (Melnick et al., 2002). To date, there are thousands of epidemiology studies that suggest associations between EDCs and disease outcomes. Of course, by nature, most epidemiology studies cannot demonstrate causal relationships between chemicals and health effects. Yet, these studies can be viewed, in toto, to draw a stronger picture about the roles that EDCs may play in disease etiology. Furthermore, many of the effects observed in human studies can be replicated in controlled laboratory studies using rodents, nonhuman primates, and other animals.

Where are the strongest data? In the 2012 State of Science on EDCs published by the WHO and United Nations Environment Programme (UNEP), human data linking EDCs to diseases was identified for a number of health outcomes including cryptorchidism (undescended testicles), breast cancer, attention deficit hyperactivity disorder, and thyroid cancer (Bergman et al., 2013b). Additional associations between EDCs and decreased sperm count, changes to the timing of puberty, and metabolic diseases have also been explored.

1.02.4.3 Human Disease Trends Implicate EDCs and Other Environmental Factors

The 2012 UNEP/WHO report on EDCs also notes that many endocrine-related diseases and disorders are increasing in prevalence (Bergman et al., 2013b). These include infertility (male and female), male genital tract malformations, adverse pregnancy outcomes including preterm birth and low birth weight, neurobehavioral disorders including attention deficit hyperactivity disorder and autism, endocrine-related diseases including breast, ovarian, prostate, testicular and uterine cancers, early puberty in females, obesity, and type 2 diabetes. Improved detection and changes in diagnostic criteria may account for some, but not all, of these increases. Some commentaries and reviews have challenged the assumptions, approaches, and conclusions in the

2012 UNEP/WHO report and other evaluations (Lamb et al., 2014, 2015; Rhomberg et al., 2012; Nohynek et al., 2013; Borgert et al., 2013; Dietrich et al., 2013). This has led to a long series of responses and rebuttals in the literature which have clarified the points by both groups (Bergman et al., 2013a, 2015; Zoeller et al., 2014; Gore et al., 2013; Trasande et al., 2016; Bourguignon et al., 2016; Kortenkamp et al., 2016).

Genetic changes are not sufficient to explain the increases in disease incidence that have been observed over three to four decades. Other environmental factors including changes in diet and exercise patterns are likely to be involved in some of these diseases. Yet, EDCs can induce these conditions in laboratory animals, and, as discussed above, epidemiology studies suggest associations between exposures and these outcomes. While it is possible that this increase in prevalence of endocrine-related diseases has no connection to the increasing number and amount of hormonally active chemicals in our diet and environment, it is far more likely that there is a causal link.

1.02.5 Nonmonotonicity and Risk Assessment

The risk assessment process for EDCs and other chemicals involves four steps. First, hazards are characterized, typically through the use of guideline assays. Second, exposure levels are inferred based on modeling routes of exposure, use of the compound in consumer products (or elsewhere), and biomonitoring studies. Next, dose–response data are used to determine which effects are observed at what doses, then the doses at which hazards are observed are compared to inferred exposure levels. Finally, data and information from these three steps are integrated during a risk characterization step. During this step, risk assessors determine whether exposures exceed the derived "safe" doses and calculate the risks associated with such exposures. They also draw conclusions about the margin of safety.

1.02.5.1 The Use of Dose–Response Data in Risk Assessment

During the risk assessment process, dose–response data play an important role in the identification of the toxicity range for a compound. High doses that kill treated animals (e.g., the lethal dose that kills 50% of treated animals, the LD50) are examined as are doses that induce some toxicity but do not kill (e.g., the maximum tolerated intake or maximum tolerated dose). Lower doses continue to be tested to identify the lowest observed adverse effect level (LOAEL, the lowest dose that still induces significant adverse effects) and the NOAEL (the highest dose that does not induce significant adverse effects). The determination of the NOAEL and LOAEL relies on the definition of "adverse," but it should be assumed that these doses are relevant to endpoints that are widely acknowledged to be toxicologically relevant (e.g., mortality, significant loss of body weight, altered organ weight, induction of severe behavioral responses like seizures, severe histopathological lesions, etc.) and not necessarily endpoints that are relevant to diseases or disruption of hormone signaling pathways (e.g., increased body weight, expression of stereotypic behaviors, abnormal growth of the mammary gland at puberty, increased sensitivity of organs to hormones, altered gene expression, decreased fecundity, diabetes, etc.)

The NOAEL dose is considered a "threshold," below which no adverse effects should be observed. However, it should be noted that the determination of a NOAEL is dependent on the endpoints examined, and whether there is sufficient power in the experimental design to detect true effects, even if they are limited to guideline endpoints of toxicological relevance. It is also important to note that the NOAEL dose is an estimated value and should not be considered a "true" no-adverse-effect level (Filipsson et al., 2003). Further, the NOAEL dose is always tested in an experiment, and thus dose selection will significantly influence the dose identified as a NOAEL; smaller experiments tend to have higher NOAELs.

In recent years, the NOAEL has been replaced by a benchmark dose (BMD). The BMD is defined as the dose that corresponds to a specific change in an adverse response compared to the response in untreated animals (Crump, 1995). The level of uncertainty in the data is considered when calculating the BMD, and benchmark dose lower bound (BMDL), defined as the lower confidence limit on the BMD, is used as a point of departure in risk assessment. The use of the BMD has been described as a more powerful statistical tool than the NOAEL, but to date, the EPA databases continue to report NOAELs (US EPA, 2014) because many datasets are not amenable to BMD modeling (e.g., those that rely on incomplete data or have poor adherence to models).

Although the BMD is not considered a threshold, per se, it is used with the assumption that there will not be a nonmonotonic response below it or the BMDL. The same is true when risk assessors use the NOAEL as a point of departure; effects are not expected at lower doses (Fig. 7). In both cases, lower doses are typically not examined. Furthermore, both the NOAEL and BMD approaches rely on traditional endpoints of toxicity, while other endpoints that are consistent with disease outcomes are not tested.

During the risk characterization step, the NOAEL is divided by a number of uncertainty factors. These uncertainty factors can account for differences between adults and children, differences between animal species and humans, and genetic variability that can influence chemical metabolism or responses. The final number that is derived from these calculations is considered the reference dose (RfD) or the acceptable daily intake (ADI). These are the doses that are compared to known or anticipated exposure levels to determine the margin of safety. A somewhat different approach is used if a BMD is derived instead of an NOAEL; here, a curve is fitted to the available data, often using the maximum likelihood approach, which allows for extrapolation to the low-dose range.

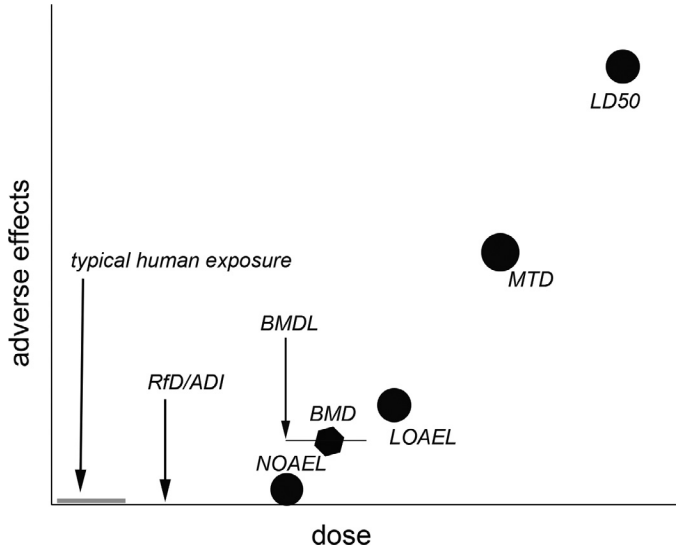

Fig. 7 Nonmonotonicity in the context of the no observed adverse effect level (NOAEL) and benchmark dose (BMD). During the risk assessment process, high doses, which induce overt toxicity, are examined. These include the LD50 and the maximum tolerated dose, a dose that induces toxicity without inducing death. The lowest observed adverse effect level (LOAEL) and NOAEL doses are also determined. In some risk assessments, the NOAEL dose is used directly to calculate the reference dose (RfD) or acceptable daily intake (ADI). In this case, the NOAEL is divided by uncertainty factors. Alternatively, some risk assessments use the BMD, a dose corresponding to a specific change in an adverse response compared to what is observed in untreated animals. The BMD includes uncertainty (indicated by the *line* through the BMD) and the lower bound of this confidence limit (the benchmark dose lower bound) is used as a point of departure in risk assessment. The RfD/ADI can be compared to human exposure levels to determine the margin of exposure. Yet, if nonmonotonic responses occur at or below the BMD or NOAEL doses, the extrapolation to the RfD/ADI is inaccurate.

1.02.5.2 Nonmonotonic Dose Responses in the Low-Dose Range Challenge Current Risk Assessment Procedures

If nonmonotonic dose responses are observed above the NOAEL (or BMD), they are not expected to influence the identification of an RfD/ADI, because extrapolations from the NOAEL are likely to be appropriate. However, the presence of a nonmonotonic response below the NOAEL suggests that an RfD/ADI cannot be accurately calculated, because the NOAEL dose does not accurately reflect, "no effect." Furthermore, the existence of nonmonotonic dose responses that occur below the RfD/ADI suggest that the entire process of extrapolating from the NOAEL to a "safe" dose should not be used.

The question then is whether nonmonotonic dose responses are ever observed below the NOAEL or the RfD/ADI. If so, the next issue to address is whether these endpoints represent adverse outcomes. One example of a nonmonotonic response occurring between the RfD (0.005 mg/kg/day) and the NOAEL (5 mg/kg/day) comes from a study of the pesticide permethrin, where moderate doses (1.5 mg/kg/day) of permethrin altered dopamine transport in mice, whereas higher (3 mg/kg/day) and lower (0.4 and 0.8 mg/kg/day) doses did not (Bloomquist et al., 2002). Considering the importance for dopamine transport in neurobehavior and mental health (i.e., dopamine transport is considered to be an important target for pharmaceutical manipulation), it is possible that altered dopamine transport could be an adverse outcome, although this is not a traditional toxicological endpoint. Another example comes from the EDC BPA (NOAEL: 50 mg/kg/day, RfD: 0.05 mg/kg/day), where a dose of 0.5 mg/kg/day altered weight of the gonadal and renal fat pads in male mice, but lower and higher doses were ineffective (Angle et al., 2013).

The presence of nonmonotonic dose responses below the RfD/ADI are much easier to document, as almost all environmental epidemiology studies revealing nonmonotonic responses would fall in this range (Lee et al., 2014; Vandenberg et al., 2012, 2013a). Because epidemiology studies typically focus on adverse health outcomes, or markers of disease, it is difficult to argue that these studies are not good examples of low-dose nonmonotonicity. Furthermore, it has been suggested that exposure mischaracterization could be responsible for nonmonotonic responses reported in human studies (Rhomberg and Goodman, 2012). However, since most of these responses have been observed with exposures to persistent chemicals, this is unlikely (Lee et al., 2014).

1.02.5.3 Systematic Evaluation of Nonmonotonic Dose Responses

One of the criticisms of the EPA's draft report on nonmonotonicity (US EPA, 2013) that was raised by the National Academy of Sciences was the EPA's failure to use systematic criteria in the assessment of dose–response data (NRC, 2014). The National Academy panel stated that the EPA "failed to establish (or enforce) a clear set of methods for collecting and analyzing the evidence on [non-monotonic] curves to ensure that the groups conducted their assessments in a clear, consistent, and therefore replicable manner." The National Academy panel also wrote that "EPA's approach to evaluating whether [non-monotonic] curves exist for

endocrine disruptors was not systematic, consistent, or transparent" and concluded that "[a]lthough it is clear that the [EPA] authors spent enormous time and energy in developing [their] evaluation, it is fundamentally compromised."

In 2015, Lagarde and colleagues developed a qualitative tool to assess the quality of nonmonotonic dose–response curves that were identified via a systematic review of the literature (Lagarde et al., 2015). Of the 148 nonmonotonic curves they analyzed, 82 were scored as having "moderate" or "high" plausibility. Further, Lagarde et al. examined only dose–response curves from studies of BPA. A total of 43 nonmonotonic dose responses were examined and 20 were considered to have "moderate," "high," or "very high" plausibility.

Some criticisms have been raised of the Lagarde method (Zoeller and Vandenberg, 2015). One point that was raised is that methods used to assess the quality of nonmonotonic dose responses should similarly be used to assess the quality of monotonic responses; many studies reporting significant effects for monotonicity are poorly designed and may represent undetected nonmonotonic responses based on the examination of a small number of doses, or doses over a limited dose range. Another criticism notes that a mechanism need not be fully understood to accept an observed phenomenon, thus the Lagarde decision tree should not rely on the identification of such mechanisms.

1.02.6 Conclusions

In this article, we have reviewed the evidence for nonmonotonic dose responses in studies of hormones and EDCs. We have also described how these phenomena can influence—or in some cases prevent—the use of standard risk assessment processes.

In a 2012 editorial, Dr. Linda Birnbaum noted, "Dose–response studies should include a range of doses to distinguish between linear monotonic and non-monotonic responses. Nonlinear relationships should not be dismissed… It is time to start the conversation between environmental health scientists, toxicologists, and risk assessors to determine how our understanding of low-dose effects and non-monotonic dose responses influence the way risk assessments are performed for chemicals with endocrine-disrupting activities. Together, we can take appropriate actions to protect human and wildlife populations from these harmful chemicals and facilitate better regulatory decision making" (Birnbaum, 2012).

See also: 4.22. Risk Assessment Studies: Epidemiology. 5.04. Pharmacokinetics and PBPK Models. 8.22. Estrogenic Endocrine Disruptors: Molecular Characteristics and Human Impacts. 9.12. Toxicology Assessment of Endocrine Active Substances. 9.13. Reproductive and Developmental Toxicity Studies.

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