State of the Science Evaluation:

Nonmonotonic Dose Responses as They Apply to Estrogen, Androgen, and Thyroid Pathways and EPA Testing and Assessment Procedures

U.S. Environmental Protection Agency
Jointly developed by:
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Office of Science Policy
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Office of Chemical Safety and Pollution Prevention
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At a Glance

Why we did this review
The risk assessment process is dependent upon the selection of doses and the characterization of dose-response relationships. There could be implications for selection of doses in toxicology studies and incorporation of results into risk assessments.

Background
Nonmonotonic dose responses are measured biologic effects with dose response curves that contain a point of inflection where the slope of the curve changes sign at one or more points within the tested range. They are of specific concern in the context of chemical testing and risk assessment because they do not follow the typically expected linear or threshold dose response, wherein increasing dose is associated with increasing frequency or severity of effect.

For further information contact
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The full report is at:
http://epa.gov/ncct/edr/nas-review.html

EPA's State of the Science Evaluation: Nonmonotonic Dose Responses as They Apply to Estrogen, Androgen, and Thyroid Pathways and EPA Testing and Assessment Procedures.

What We Did
Developed a state of the science document providing a judgment on the degree to which nonmonotonic dose-responses are evidenced in the scientific literature and to evaluate the extent to which they may impact USEPA's chemical testing and risk assessment.

What We Found
Nonmonotonic dose responses (NMDR) after exposure to xenobiotics do occur in biological systems but are generally not common. Where NMDRs were observed, biological endpoints closest to the molecular initiating event were more likely to identify a point of inflection (change of direction in slope) then those effects further downstream, including the apical adverse outcomes. The goal of chemical testing is to identify the potential for hazard after exposure to the xenobiotic of concern, not to identify and describe 100% of all the possible biological effects. As such, the current testing approaches perform this function successfully and, based on the current evaluation, are highly unlikely to mischaracterize a chemical that has the potential to adversely perturb the endocrine system due to an NMDR.

What We Concluded
- NMDRs do occur in estrogen, androgen, and thyroid systems in ecological and mammalian studies.
- NMDRs are not unexpected *in vitro* particularly when evaluating high dose levels and/or lower-order biological endpoints in estrogen androgen or thyroid systems.
- NMDRs 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.
- The nature of a dose response will vary over time, and nonmonotonicity due to compensation may be observed.
- NMDRs observed in endocrine endpoints may be biologically relevant and should be evaluated in context with the totality of the available scientific data, including epidemiologic and human studies.
- There is currently no reproducible evidence that the early key events involved in the expression of NMDRs that are identified at low dose are predictive of adverse outcomes that may be seen in humans or wildlife populations for estrogen, androgen or thyroid endpoints
- Therefore, current testing strategies are unlikely to mischaracterize, as a consequence of NMDR, a chemical that has the potential for adverse perturbations of the estrogen, androgen or thyroid pathways.

Foreward

Within the community of environmental and public health scientists, the discourse around the shape of the dose response curve has been energized in recent years especially as applied to chemicals that induce adverse health outcomes through interaction with endocrine systems. This topic and its relevance to the effectiveness of our chemical testing and evaluation strategies is of great interest to EPA; thus, in 2012 EPA's Office of Chemical Safety and Pollution Prevention (OCSPP) requested that the Office of Research and Development (ORD) conduct a rapid and high priority expert review of the state of the science on the specific topic of lack of monotonicity in dose-response for the endocrine disruptor mode of action. The particular interest of the EPA review was effects mediated by alterations in the estrogen, androgen and thyroid hormone systems. Several international agencies, including the Joint Research Centre of the European Commission and the European Food Safety Authority, recently conducted comparable reviews, each focusing on the areas most relevant to their mission.

In response to the request from OSCPP, senior scientists in ORD, together with other Agency colleagues and federal partners conducted a review of more than 2000 scientific documents over a six month period. ORD's approach to this rapid review was to capitalize on its extensive in-house expertise in estrogen, androgen, and thyroid hormone systems. To this end and to meet the timeline of this review, these expert groups initially worked independently drawing on their intimate knowledge of the most relevant literature in their areas of expertise, before gathering for the overall evaluation that led to the conclusions and responses to science questions. Our conclusions are, of course, bounded by the content of the available scientific literature, which was generally not developed with the express purpose of rigorously evaluating the nature of dose-response relationships for endocrine-related molecular initiating events. It should also be noted that the experimental animal literature is almost exclusively oriented toward single chemical exposures, whereas the real world environmental situation in which humans and wildlife are exposed is typically a complex mix of chemicals and other stressors, making comparisons among these disciplines challenging.

Finally, we at EPA are committed to further evolving our Path Forward objectives that reflects excellence in science, and is grounded in pragmatic, timely, relevant, and mission driven objectives. This exercise is a seminal case study in our progress as it reflects the agility and responsiveness of EPA science and scientific staff in the ability to address a complex topic of high import for EPA and the nation. The awareness of the importance of the issue motivated the request for this NRC review. On behalf of the Agency, let me thank you in advance for your efforts to evaluate our document and provide guidance on how we might improve it. We look forward to your engagement – acknowledging that your task is no less daunting and time-sensitive than ours was.

Robert Kavlock
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Executive Summary

The U.S. Environmental Protection Agency (USEPA) chartered a group of scientists to evaluate the impact of potential nonmonotonic dose response relationships (NMDRs), as they apply to estrogen-, androgen-, and thyroid-based modes of action and the Agency's testing and assessment procedures. The goals were to develop a state of the science document providing a judgment on the degree to which NMDR are identifiable in the scientific literature, and to evaluate the extent to which their existence may impact USEPA's chemical testing and risk assessment programs.

NMDRs are measured biologic effects with dose response curves that contain a point of inflection where the slope of the curve changes sign at one or more points within the tested range. NMDRs are of specific concern in the context of chemical testing and risk assessment because they do not follow expected dose response curves, wherein increasing dose is associated with increasing frequency or severity of effect. Although, the USEPA is concerned about the full domain of potential NMDR impacts, the current analysis focuses solely on effects caused by endocrine disrupting chemicals (EDC) that act via perturbation of the estrogen, androgen, or thyroid hormone systems. For the purposes of this document, an EDC is defined as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations WHO (2002). Consistent with existing USEPA and National Toxicology Program (NTP) activities and assessments, the current state-of-science document considered an effect to be adverse only if it displayed a change in morphology, physiology, growth, development, reproduction, or life span of a cell or organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences. Where appropriate, this document also distinguished adverse effects from adaptive responses, which has been defined as a process whereby a cell or organism responds to a xenobiotic but without impairment of function.

The USEPA has a well-documented and long standing interest in the shape of dose response curves, particularly at the lowest end of the range of exposures resulting in observed effects. USEPA has published guidance for dose response assessment, the step in risk assessment that estimates the likelihood of adverse effects at environmental levels of exposure. The USEPA is cognizant of the fact that the risk assessment process, therefore, is highly dependent upon the selection of dose levels in toxicology studies, the choice of measured endpoints and the ensuing characterization of dose-response relationships. The Agency has long recognized the importance of understanding the determinants that influence the biological response to exposures over a wide range of dose levels, including those that are environmentally relevant.

The USEPA also recognizes that if NMDRs are associated with low dose levels (i.e., the range of typical human exposures or doses lower than those typically used in standard testing protocols) of exposure to EDCs, there could be implications for the selection of dose levels in standard toxicology studies and incorporation of results from such studies into risk assessments.

In a June 15, 2012 memorandum, the USEPA's Office of Chemical Safety and Pollution Prevention's (OCSPP) Assistant Administrator requested the USEPA Office of Research and Development (ORD) "evaluate the impact of potential nonmonotonic dose response relationships, particularly as they apply to endocrine-based modes of action, on EPA testing and assessment procedures." In response, ORD convened a working group to develop this NMDR State of the Science Assessment. The working group included scientists, managers and technical experts from USEPA/ORD and OCSPP, the Food and Drug Administration (FDA), the National Institute of Environmental Health Sciences (NIEHS), and the National Institute of Child Health and Human Development (NICHD).

Although NMDRs have been reported in a wide range of biological systems, the current state of the science document is limited to an overview of biological and statistical concepts pertinent to NMDRs for adverse health effects of chemicals acting on the estrogen, androgen, or thyroid hormone systems. This assessment builds on existing scientific data, reports, and reviews but is not intended to cover all endocrine systems that may be disrupted by environmental exposures, to assess test methodologies for detecting EDCs, or to address specific EPA testing methodologies, risk assessments or risk management decisions. Rather, it evaluates the peer-reviewed scientific literature in which associations between chemical exposures and adverse outcomes have been demonstrated or hypothesized to occur via perturbation of estrogen, androgen, or thyroid hormone systems. The roles of pharmacokinetics, key molecular events, and modes of action as they impact the dose response curves for early developmental and reproductive endpoints and statistical analyses, are considered. We used an expert-driven approach as the most expedient way to accomplish our goal of a state of the science evaluation that cut across several disciplines.

We identified three central scientific questions to be addressed in the current state of the science assessment. The central scientific questions, and summarized responses, are these.

1. Do nonmonotonic dose responses (NMDRs) exist for chemicals and if so under what conditions do they occur?

Yes, we concluded that exposures to chemicals can result in NMDRs for specific endpoints. NMDRs arise from complex relationships between the dose of toxicant at its target site and the effect of interest WHO (2012). NMDRs are biologically plausible and

can arise when the biological system that is activated in response to toxicant exposure consists of at least two activities that can act in opposition to each other WHO (2012; Conolly and Lutz (2004). We determined that NMDRs are more frequently identified in these types of studies: *in vitro* studies, high-dose range studies, and short-term studies. Assays that provide data at a lower level of biological organization (such as proteomics or transcriptomics) are more likely to identify NMDRs than studies that provide data on apical adverse events further downstream from the molecular initiating event. Reproducibility of NMDRs is important in establishing plausibility of a response and its potential applicability as part of the hazard characterization. Factors that influence reproducibility include:

- Study design - dose selection, sample size, organism strain, diet, housing environment, statistical methods;
- Robustness of physiology – physiologic compensation producing changes in slope; and
- Competing processes– induction of metabolism, repair, or independent mechanisms.

2. Do NMDRs capture adverse effects that are not captured using our current chemical testing strategies (*i.e.*, false negatives)?

There are certainly adverse biological changes that may occur in a nonmonotonic manner that would not be captured using current testing strategies. No testing strategy is able to assess all potential adverse effects, for all biological systems, in all tissues, for all species, in all developmental time points.

As the work group progressed with its review and discussions of the science, it became clear that our second question needed to be further defined so that it could be more accurately and fully answered in the context of the science as evaluated. Thus, question 2 was further expanded for clarification.

- Are there adverse effects with NMDRs that are not being identified using the current chemical testing strategies?
- Are there NMDRs for adverse effects below the no observed adverse effect levels (NOAELS) or benchmark doses (BMD) derived from the current testing strategies?
- Do EPA chemical testing strategies detect relevant adverse effects for chemicals which produce NMDR for specific endpoints?

Chemicals that operate through endocrine modes of action (MoA) have multiple targets across organs, tissues, and cellular systems in various species, and across all life stages. It is not possible or feasible for chemical testing to measure or analyze all possible endpoints for all chemical MoA in all tissues. The objective of USEPA's chemical testing strategy is not to identify all possible adverse effects, but rather, to identify sensitive endpoints relevant to human or ecological health, providing confidence that adverse effects are not being induced at dose levels below what was determined to be a NOAEL.

For estrogen, androgen or thyroid MoA that provide adequate information to make an assessment, our evaluation shows that there is not sufficient evidence of NMDRs for adverse effects below the NOAELS or BMD derived from the current testing strategies. For some MoA, however, the scientific database remains too limited to conclude this with certainty.

While there are biological changes that may occur in a nonmonotonic manner in the low dose region, our review indicates that reproducible NMDRs for adverse effects occur in the high dose region of the dose response curve. Thus, the current testing approaches do not fail to identify or establish appropriate NOAELS in the low rose range of exposure, even if not all effects for every chemical are identified. The extensive evaluation conducted in the present review as well as almost two decades of experience with screening assays for hazard identification indicate that these assays do not fail to detect chemicals with endocrine activity for the estrogen or androgen hormone systems. Dose response assessment is not an issue for screening assays. NMDRs would be problematic only if a chemical with estrogen, androgen, or thyroid activity produced an effect *in vivo* at a dose below those used in screening, and the chemical had no effect on estrogen, androgen, or thyroid related endpoints at the higher screening dosage levels. Although, such NMDRs have been hypothesized they have not been demonstrated reproducibly, and none were found in the present evaluation.

Our assessment of the adequacy of the current testing assays concludes that a number of standardized short- and long-term assays are sensitive in detecting chemicals that interfere with the estrogen, androgen and thyroid signaling pathways. Specifically, the EDSP screening battery can detect disruption of these pathways using combined *in vitro* and *in vivo* assays in mammalian and aquatic models. Standard multigenerational test guidelines have measures that are sensitive to disruption of the estrogen and androgen signaling pathways. While these studies are considered the current standard for assessing the potential of a chemical to be a reproductive toxicant and for use in setting NOAELS, they are not without limitations. USEPA testing strategies are reviewed

periodically to assure they are incorporating the most sensitive and biologically relevant endpoints.

Further, if an objective of testing is to define the shape of the dose response curve and thereby identifying potential NMDRs, then the three treated groups and a control group used in current guideline studies may not be sufficient for this purpose. Modifications that could lead to a more clearly defined dose response characterization and increase the statistical power to detect low dose effects may be appropriate in specific instances.

3. Do NMDRs provide key information that would alter EPA's current weight of evidence conclusions and risk assessment determinations, either qualitatively or quantitatively?

Data from studies in which NMDRs are identified may be biologically relevant and as such should be evaluated in context with the totality of the available scientific data in weight of evidence (WoE) conclusions and risk assessment determinations. These data should be considered and analyzed, as all data are, and factored into the WoE based on standard criteria including, but not limited to, conduct of the studies, representation of biological processes that are relevant to the evaluation, biological plausibility, and reproducibility. NMDRs can have impact on both qualitative and quantitative risk assessments, but cannot be considered in isolation from other data for the chemical and biological response being considered.

In summary, nonmonotonic dose responses after exposure to xenobiotics do occur in biological systems but are generally not common. Where NMDRs were observed, biological endpoints closest to the molecular initiating event were more likely to identify a point of inflection; those effects further downstream, including the apical adverse outcomes, are most commonly monotonic in their dose-response. The goal of chemical testing is to identify the potential for hazard after exposure to the xenobiotic of concern, not to identify and describe 100% of all the possible biological effects. As such, the current testing approaches perform this function successfully and, based on the current evaluation, are highly unlikely to mischaracterize a chemical that has the potential to adversely perturb the endocrine system due to an NMDR.

Conclusions:

1. NMDRs do occur in estrogen, androgen, and thyroid systems as evidenced in ecological and mammalian studies.
2. NMDRs are not unexpected *in vitro* particularly when evaluating high dose levels and/or lower-order biological endpoints in estrogen, androgen, or thyroid systems.
3. NMDRs 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.
4. The nature of a dose response will vary over time, and nonmonotonicity due to compensation may be observed.
5. NMDRs observed in endocrine endpoints may be biologically relevant and should be evaluated in context with the totality of the available scientific data, including epidemiologic and human studies.
6. There is currently no reproducible evidence that the early key events involved in the expression of NMDRs that are identified at low dose are predictive of adverse outcomes that may be seen in humans or wildlife populations for estrogen, androgen or thyroid endpoints.
7. Therefore, current testing strategies are unlikely to mischaracterize, as a consequence of NMDR, a chemical that has the potential for adverse perturbations of the estrogen, androgen or thyroid pathways.

Acronyms

3β-HSD	3β-Hydroxysteroid Dehydrogenase
AGD	Anogenital Distance
ANOVA	Analysis of Variance
AOP	Adverse Outcome Pathway
AR	Androgen Receptor
ARE	Androgen Response Element
BDNF	Brain-Derived Neurotrophic Factor
BBP	Butyl Benzyl Phthalate
BMD	Bench Mark Dose
BPA	Bisphenol A
BW	Body Weight
cAMP	Cyclic adenosine monophosphate
CCB	1-Chloro 4-(Chloromethyl) Benzene
ChAT	Choline acetyltransferase
c-Kit	Protein on surface of many cell types
ClO4	Perchlorate
CNS	Central Nervous System
CYP	Cytochrome P450s
DBP	Dibutyl Phthalate
DCHP	Dicyclohexyl Phthalate
DEHP	Di-2-ethylhexyl Phthalate
DES	Diesthylstilberstrol
df	Degrees of Freedom
DHP	Dihexyl Phthalate
DHT	Dihydrotestosterone
DI1/DI2/DI3	Deiodinase 1, 2, or 3
DIBP	Diisobutyl Phthalate
DINP	Diisononyl Phthalate
DPeP	Dipentyl Phthalate
E	Estrogen
E2	Estradiol
EAS	Endocrine Active Substances
ED	Endocrine Disruptor
EDC	Endocrine Disrupting Chemical
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EE2	Ethynyl Estradiol

EFSA	European Food Safety Agency
ER	Estrogen Receptor
ERR	Estrogen Related Receptor
ETU	Ethylene thiourea
F1/F2/F3	1st, 2nd, or 3rd, Filial Generation
FSH	Follicle Stimulating Hormone
GD	Gestation Day
GLP	Good Laboratory Practices
GnRH	Gonadotropin Releasing Hormone
GSH	Glutathione
GSI	Gonadal Somatic Index
HCB	Hexachlorobenzene
HPG	Hypothalamic Pituitary Gonadal
HPT	Hypothalamic Pituitary Thyroidal
HRE	Hormone response element
Insl3	Insulin-like Factor 3
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
K_d	Equilibrium dissociation constant
KEDRF	Key Event Dose Response Framework
LABC	Levator Ani Bulbo-Cavernosus
LE	Long Evans
LH	Luteinizing Hormone
LOEL	Lowest Observed Effect Level
MAP kinase	Mitogen-activated protein kinase
MBP	Myelin Basic Protein
MCT	Monocarboxylate Transporter
MEHP	Methylethyl Hexyl Phthalate
MIE	Molecular Initiating Event
MMI	Methimazole
MoA	Mode of Action
MT	17α-methyltestosterone
NCTR	National Center for Toxicological Research
NIEHS	National Institute for Environmental Health Sciences
NIS	Sodium-Iodine Symporter
NMDR	Nonmonotonic Dose Response
NMDRC	Nonmonotonic Dose Response Curve
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level

NRC	National Research Council
NTP	National Toxicology Program
OATP	Organic Anion Transporting Polypeptide
OECD	Organization for Economic Cooperation and Development
OHF	Hydroxyflutamide
P	Parental Generation
PBPK	Physiologically Based Pharmacokinetic
PCB	Polychlorinated Biphenyl
PCR	Polymerase Chain Reaction
PHAH	Polyhalogenated Aromatic Hydrocarbon
PND	Postnatal Day
PNECs	Predicted no effect concentrations
PPS	Preputial Separation
PTI	1-methyl-3-propylimidazole -2-thione
PTU	Propylthiouracil
RIA	Radioimmunoassay
SARM	Selective Androgen Receptor Modulator
sc	Subcutaneous
SD	Sprague Dawley
SE	Standard Error
SEM	Semicarbazide
SERM	Selective Estrogen Receptor Modulator
SR-B1	Scavenger Receptor B-1
StAR	Steroid Acute Regulatory Protein
T	Testosterone
T3	Triiodothyronine
T4	Thyroxine
TB	Trenbolone
TBG	Thyroid Binding Globulin
TCDD	Tetrachlorodibenzodioxin
TDC	Thyroid Disrupting Chemicals
TH	Thyroid Hormone
TPO	Tyroperoxidase
TR	Thyroid Receptor
TRH	Thyroid Releasing Hormone
TSH	Thyroid Stimulating Hormone
TTR	Transthyretin
UGTs/UDGPTs	Uridine Diphophogluronyltranferases
VO	Vaginal Opening

VTG	Vitellogenin
WHO	World Health Organization
WoE	Weight of Evidence

1. Introduction

In June of 2012, USEPA chartered a group of scientists, managers, and technical experts to evaluate the existence and impact of nonmonotonic dose response (NMDR) relationships, as they apply to endocrine-based modes of action, within current USEPA testing and assessment procedures. The group was charged to develop a state of the science document providing a weight of evidence judgment on the degree to which NMDRs are identified in the scientific literature associated with evaluating the effects of endocrine active agents and the degree to which their existence may impact USEPA's chemical testing and risk assessment.

> Non Monotonic Dose Responses (NMDRs) – measured biological effects with dose response curves that contain a point of inflection where the slope of the curve changes sign at one or more points within the tested range.

Although USEPA is concerned about the full domain of potential NMDR impacts, the current analysis focuses solely on endocrine disrupting chemicals (EDCs) impacting the estrogen, androgen, or thyroid hormone systems. It is not intended to be a comprehensive treatise on any specific chemical, mode of action (MoA), or toxicological effect. The current analysis focuses primarily on effects observed in the developing organism or on reproductive function. Moreover, it does not address cumulative effects of chemical exposures, except very broadly in the context of MoA and observed dose-effect discussions.

> Endocrine Disrupting Chemical (EDC)– an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations. (WHO 2002)

This document relied upon publicly available, published literature. The authors of this document have commented in some places on statistical tests or interpretations of data in that literature. This state of the science evaluation did not include formal statistical or mathematical reanalysis of the published data.

A structured, consistent and formal systematic review of the literature, with defined criteria for study selection, may have been desirable. This has been recommended recently by both the National Research Council and our Science Advisory Board for exploring individual issues or single chemicals. Given the established timelines and availability of resources for this project, we used our expert-driven approach as the most expedient way to accomplish our goal of a

state of the science evaluation that cut across several disciplines. Guidelines for systematic review are evolving, and we look forward to applying new methodologies to complex questions such as those posed in this review.

In Section 5, *Conclusions,* the EPA work group has characterized its overall confidence in the extent and circumstances under which NMDRs are observed. Throughout the document the EPA workgroup has noted knowledge gaps, uncertainties, quality of data, and some scientific issues in the interpretation of dose response data. By doing so, we have conveyed the limitations of the current evaluation.

The data analyses and conclusions of this state of science assessment represent the authors' current thinking on NMDR. This document does not establish or necessarily reflect USEPA policy or test guidelines or guidance. Its purpose is to provide scientific information to aid USEPA senior leadership, program managers, and risk assessors in their effort to develop policies grounded, at a most fundamental level, in sound science.

For other general information about this assessment or other questions, the reader is referred to the USEPA, Office of Research and Development/Office of Science Policy at comments.nmdr@epa.gov.

1.1 Background

The USEPA has a well-documented and long standing interest in the shape of dose response curves, particularly at the lowest end of the range of exposures resulting in observed effects. USEPA has published guidance for dose response assessment (*e.g.,* U.S. EPA (2005a)), the step in risk assessment that estimates the likelihood of adverse effects at environmentally relevant levels of exposure. The dose response steps of the risk assessment process, therefore, are highly dependent upon the selection of dose levels in toxicology or exposure brackets in human studies and the ensuing characterization of dose-response relationships. The Agency has recognized the importance of understanding the determinants that influence the biological response to exposures over a wide range of dose levels, particularly those that are environmentally relevant.

Risk assessors extrapolate from effects observed in experimental systems to environmental exposures, in some instances relying on assumptions of low dose linearity or of an exposure associated with no adverse effect (*e.g.,* threshold). With few exceptions (such as essential elements), it is expected that lower exposures to biologically active chemicals will result in decreased measures of response. Responses that consistently increase or decrease, across a range of exposures, are said to exhibit monotonic dose responses.

This expectation has been subject to challenge e.g., Welshons et al. (1999); Nagel et al. (1997); vom Saal et al. (1997). According to these reports, NMDR were demonstrated when laboratory animals were exposed to "low dose" levels of putative EDCs. The question was raised whether these chemicals, by virtue of their ability to interfere with the endocrine system, were unique in their dose response relationships. Early on, other scientists were unable to replicate these findings Ashby et al. (1999); Cagen et al. (1999a), which created controversy and stirred debate as to the validity of the original reports of NMDRs.

NMDR are measured biological effects with dose response curves that contain a point of inflection where the slope of the curve changes sign at one or more points within the tested range. As described in Conolly and Lutz (2004), NMDRs are most commonly reported with either a decrease in the level of response observed in controls at low dose(s) followed by an increase at higher doses (called a U-shape or J-shape); or vice versa (an "inverted U" or β-shape) WHO (2012).

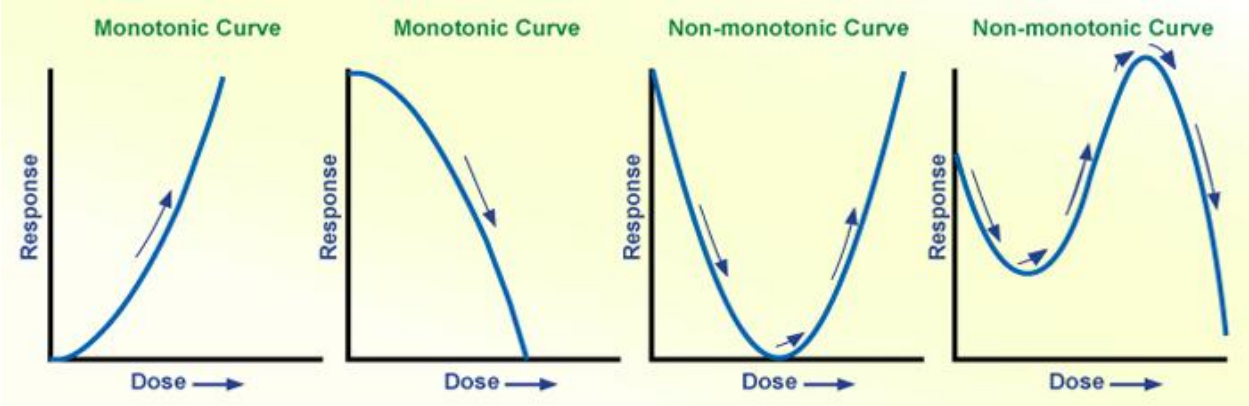


Figure 1.1: Examples of Monotonic and Nonmonotonic Dose Response Curves.

(Reproduced from Fagin (2012), *Nature* 490: 462-465)

The USEPA recognized that if NMDRs were associated with low-dose levels of exposure to EDC, there could be implications for the selection of dose levels in standard toxicology studies and the incorporation of results from such studies into risk assessments.

The USEPA convened the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), an expert panel to provide advice on the development of a screening program for endocrine disruptors. The panel addressed the "low dose hypothesis," as it was referred to at that time, in its 1998 report to EPA EDSTAC (1998). The EDSTAC report stated that if low-dose phenomena are reproducible, generalizable, and related to adverse effects, the implications for regulatory toxicity testing and risk assessment are profound. Evaluation of responses from exposure to EDCs at doses below traditional toxicology studies or at environmental levels is

important but different from determining the shape of the dose-response curve across many doses, including very low exposure levels. While an NMDR can coincide with low exposures, it can also be identified at any point along the dose-response continuum which is a separate issue from determining the lowest dose that can result in an effect. The report further advised that more research to address the nature of the dose-response curves for exogenous EDC was necessary to inform toxicology study designs, the use of data in risk assessments, and to resolve the then underlying uncertainties and controversy.

> Low Dose Effect– a biological change occurring in the range of typical human exposures or at doses lower than those typically used in standard testing protocols NTP (2001).

Responding to the EDSTAC conclusions is complicated by the fact that no single definition of "low dose" is consistently used by the scientific community. Low dose is a relative term that is evaluated in a chemical- and testing-specific context for environmental and biological relevance. Several different definitions have been proposed and used e.g., Welshons et al. (2006; Melnick et al. (2002; Brucker-Davis et al. (2001).

For Example:

- "Any biological changes occurring in the range of typical human exposures" NTP (2001)
- "Doses lower than those typically used in standard testing protocols, *i.e.,* doses below those tested in traditional toxicology assessments" NTP (2001)
- "A dose below the lowest dose at which a biological change (or damage) for a specific chemical has been measured in the past, any dose below the lowest observed effect level (LOEL) or lowest observed adverse effect level (LOAEL)" Welshons et al. (2006)

The current NMDR state of the science assessment uses the National Toxicology Program (NTP) definitions: a biological change occurring in the range of typical human exposures or at doses lower than those typically used in standard testing protocols NTP (2001).

Likewise there are multiple definitions of adverse effect, which are used by risk assessors. In 2002, EPA defined an adverse effect as "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" USEPA (2002). This NMDR state of the science document considers the concepts of mode of action, toxicity pathways and adverse outcome pathways (see section 2.2); in particular we consider the adaptive capability of organisms or populations that encounter environmental stress. To that extent we include the systems biology oriented description of adversity by Keller *et al.* (2012).

In 2000, an NTP panel concluded that the traditional multigeneration reproduction testing paradigm used by EPA has not revealed major reproductive or developmental effects in laboratory animals exposed to endocrine active agents at doses approaching the no observed adverse effect levels (NOAELs) set by the standardized testing protocols NTP (2001). The peer review panel evaluated data from major, selected studies that supported the presence or absence of low-dose effects in laboratory animals and that would be relevant for human health assessments. In particular, the NTP panel was interested in evaluating the scientific underpinnings of dose-response relationships for reproductive toxicants, including nonmonotonicity within the low dose range.

The NTP panel also recommended that EPA periodically review testing paradigms used for assessments of reproductive and developmental toxicity to see if changes are needed regarding dose selection, animal model selection, age when animals are evaluated, and the endpoints being measured following exposure to endocrine active agents. In 2002, the USEPA issued a statement that additional research was needed to support a better understanding of the low-dose hypothesis USEPA (2002). The statement noted that an improved understanding of the mechanisms of action by which endocrine-active agents exert their effects would determine whether existing testing protocols needed modification (http://www.epa.gov/endo/pubs/edmvs/lowdosepolicy.pdf). Further, the USEPA indicated that it would be monitoring the outputs of ongoing research for applicability to its Endocrine Disruptor Screening Program (EDSP). The statement left open the possibility that USEPA could require low-dose testing on a case-by-case basis if relevant information on specific chemicals became available.

There have been many other workshop reports, reviews, statements, and other publications on the issue of low-dose, NMDR, and endocrine disruptors e.g., Birnbaum (2013; DCED (2013; UNEP (2013; Birnbaum (2012; Vandenberg et al. (2012; Zoeller et al. (2012; Boettcher et al. (2011; Hirabayashi and Inoue (2011; EFSA (2010; Diamanti-Kandarakis et al. (2009; Kortenkamp (2008; Sekizawa (2008; Kamrin (2007; Kortenkamp (2007; Scholze and Kortenkamp (2007; Welshons et al. (2006; Owens and Chaney (2005; Haseman et al. (2001).

> Adverse Effect – a measured endpoint that displays a change in morphology, physiology, growth, development, reproduction, or life span of a cell or organism, system, or population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences Keller et al. (2012).

Congress also showed an interest in this topic. In 2009, the Endocrine Disruption Prevention Act (HR 4190 and S 5210) was introduced with the following statement: "Congress finds that…..traditional toxicology and risk assessment, which evaluate one chemical at a time, and only at high concentrations, have failed to sufficiently address the effects of low doses of chemicals…". At a 2010 hearing on EDCs in drinking water, Representative Jim Moran testified that even "infinitesimally low levels of exposure" to EDCs could cause adverse effects, quoting a 2009 statement from the Endocrine Society. In its response to Representative Moran, the USEPA committed to convening a workshop to bring together expert scientists to share their findings, characterize the state of the science, identify data gaps and determine the best way to address the issue.

This internal EPA 2011 workshop was convened for the following purposes: 1) to re-examine the state of the science with an eye toward determining whether current study designs and/or risk assessment approaches should be modified; 2) to determine whether there is a need for an international workshop, and if so, what its goals would be, which organizations could be potential co-organizers, and how the path forward should be mapped; and 3) to develop a statement summarizing the USEPA's perspective on NMDRCs and endocrine disruptors. This state of the science evaluation is an outgrowth of that workshop.

Contemporary reviews that deal in total or in part with NMDR have been prepared for different purposes. Vandenberg *et al.* (2012)reviewed over 800 papers with the objective of evaluating the literature for the existence of NMDR in the context of biological mechanisms for these responses. Based on their description of application of a weight of evidence (WoE) approach, they conclude that "when nonmonotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses. Thus, fundamental changes in chemical testing and safety determination are needed to protect human health." Zoeller *et al.* (2012) note their objective is to use principles of endocrinology to make recommendations for testing programs designed to identify EDC. The adequacy or relevance of testing and screening programs for EDC has also been addressed by the Danish Centre on Endocrine Disrupters DCED (2013), which concluded the following: "The current information requirements in REACH are not designed for the identification of endocrine disrupters, although certain endpoints and assays may give some indication of endocrine disrupting effects." By contrast to other reviews, this conclusion was not based solely on the observation of NMDR.

The European Commission Joint Research Centre (JRC 2013) states that the purpose of the European Union Endocrine Disrupters Expert Advisory Group is: "to provide detailed reflections on scientific issues relevant to endocrine disrupting substances, not specific to any regulatory framework, including advice/orientation on scientific criteria for the identification of endocrine disrupting substances." They agreed upon elements for identification of EDCs, but they were not able to provide a full evaluation of the adequacy of the currently available test methods. The JRC report relies in part on Kortenkamp et al. (2011).

EFSA (2013) reviewed existing information related to the testing and assessment of endocrine active substances (EASs) and EDCs. They set three criteria that define an EDC: "the presence of i) an adverse effect in an intact organism or a (sub)population; ii) an endocrine activity; and iii) a plausible causal relationship between the two". EFSA noted the lack of consensus in the scientific community on the reproducibility and relevance to risk assessment of NMDR; they stated that the quality and robustness of data for studies reporting NMDR should be assessed, as it is for any other studies. Finally, the WHO UNEP report UNEP (2013) was not focused on NMDR, but rather on the state of the science for links between EDC exposure and human disease, with a recommendation for reducing exposures to such compounds. However, section 1.2.4 of UNEP (2013) describes some instances and rationales for NMDR *in vitro*. Most of the above contemporary reviews suggest the need for additional review of dose response relationships for potential EDC.

> Weight of Evidence (WoE) – an integrative and interpretive process routinely used by EPA and other risk assessors to evaluate health and ecological toxicity in a manner that takes into account all relevant scientific and technical information.

In response to recommendations for continued review of nonmonotonicity, the USEPA convened a working group to generate this state of the science assessment of NMDRs for chemicals acting via the estrogen, androgen, or thyroid hormone systems, in support of USEPA's continued review of testing and risk assessment processes. Congruent with other contemporary reviews (*e.g.,* DCED (2013)) we based our approach to evaluating the literature on concepts common to WoE.

The principles and criteria for weighing and integrating different lines of evidence articulated in existing EPA documents U.S (2005, 2002, 1998, 1996, 1991) are applicable to evaluating data on endocrine active chemicals. USEPA refers to the WoE approach as "*…a collective evaluation of all pertinent information so that the full impact of biological plausibility and coherence is adequately considered.*" U.S (1999). In its recommendations to EPA, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) referred to the WoE approach as "*…a process by which trained professionals judge the strengths and weaknesses of a collection of*

information to render an overall conclusion that may not be evident from consideration of the individual data" EDSTAC (1998).

The USEPA uses a WoE process to make determinations on the hazard and risk from exposure to a chemical stressor; for an example see the WoE document on the EDSP website (www.epa.gov/endo). All WoE processes involve a number of common steps: assembling the relevant data; evaluating that data for quality and relevance; and an integration of the different lines of evidence to support conclusions concerning a property of the substance. WoE considers both negative and positive studies, but it is not a simple tallying of the number of plusses and minuses. The significant issues, strengths, and limitations of the data and the uncertainties that deserve serious consideration are presented, and the major points of interpretation highlighted.

In our evaluation we did not assign categories of likelihood for NMDR, as is done in many types of hazard identification (see for example descriptions of WoE categories for human cancer in U.S. EPA (2005a) and the WoE categories in DCED (2013)). Nor was there an attempt to design exclusion/inclusion criteria for studies uncovered in all literature searches; for reasons of resource limitation, this was done primarily for the description of the data on the thyroid pathway. For sections of our review, judgments of likelihood were based on relevant examples rather than a formal WoE. All discussions, however, considered instances wherein NMDR were demonstrated or were not substantiated; the description of the latter studies can be found in appendices to this document.

1.2 Scope of the Current Evaluation

NMDRs may be observed for specific endpoints in any biological system, but the scope of this state of the science document is limited to chemicals affecting the estrogen, androgen and thyroid pathways. Our review is narrowly focused on discussion of existence of, biological rationales for, and relevance of NMDRs to risk assessment. When identified, endpoints that may be affected by multiple modes of action or alternative pathways were considered in the analysis for compounds with primary modes of action via the estrogen, androgen, or thyroid systems. In these cases, alternative pathways were not specifically investigated. This state of the science document also does not consider cumulative stressors.

This evaluation builds on existing scientific data reviews and documents and is not intended to cover all endocrine systems that may be disrupted by environmental exposures; to assess test methodologies for detecting EDCs; or to address specific EPA testing methodologies, risk assessments or risk management decisions. Our document does not attempt to establish a link between endocrine active compounds and human disease, nor does it make a statement on the

likelihood of EDC to have effects at low or environmental exposure levels. This state of the science document does not deal generally with demonstrations of effects at low dose, but rather with documentation and explication of NMDR as defined in section 1.2.

1.3 Central Scientific Questions

Prior to the formation of the working group an NMDR Steering Committee identified three central scientific questions to be addressed in the current state of the science assessment. This state of the science evaluation addresses these three central scientific questions for the estrogen, androgen and thyroid pathways.

1. Do nonmonotonic dose responses (NMDR) exist for chemicals and if so under what conditions do they occur?
2. Do NMDRs result in *adverse effects* that are not captured using our current chemical testing strategies (*i.e.*, false negatives)?
3. Do NMDRs provide key information that would alter EPA's current weight of evidence conclusions and risk assessment determinations, either qualitatively or quantitatively?

The rest of the document deals in depth with the evaluation of information to answer the above questions.

2. General Biological Concepts and Statistical Considerations for NMDR

The concepts of Mode of Action (MoA) (described in Boobis et al. (2008; U.S (2005; Sonich-Mullin et al. (2001) guided the evaluation of the data related to NMDR in the present report. MoA is defined as a set of key events starting from interaction of a xenobiotic with a cellular receptor and proceeding to the apical effect. The roles of pharmacokinetics in determining the shapes of dose response curves are important in the interpretation of NMDRs. The concepts presented in this section are generally applicable to mammals including humans and to nonmammalian species (ecological toxicology) unless otherwise noted. Although exposure is not a focus of this evaluation, it should be noted that the concept of "low dose" implies some knowledge of actual or expected exposures.

2.1 Pharmacokinetic Processes

Many pharmacokinetic processes are saturable, such as energetically mediated transport across membranes, bioactivation, and detoxification. The rates of these kinds of processes are initially linear with concentration but become sublinear as saturation is approached. Pharmacokinetics can, thus, impart nonlinearities to the dose-response curve. Characterization of concentrations

or doses at which such nonlinearities occur, relative to the doses used in experimental work and environmental exposures, reduces uncertainty in dose-response characterization and in any associated risk assessments. Physiologically based pharmacokinetic (PBPK) models are important tools for these characterizations. Appropriately developed and validated PBPK models are useful for predicting pharmacokinetic behaviors for situations that lack experimental data, is often the case with interspecies and high-to-low dose extrapolations Mclanahan et al. (2012).

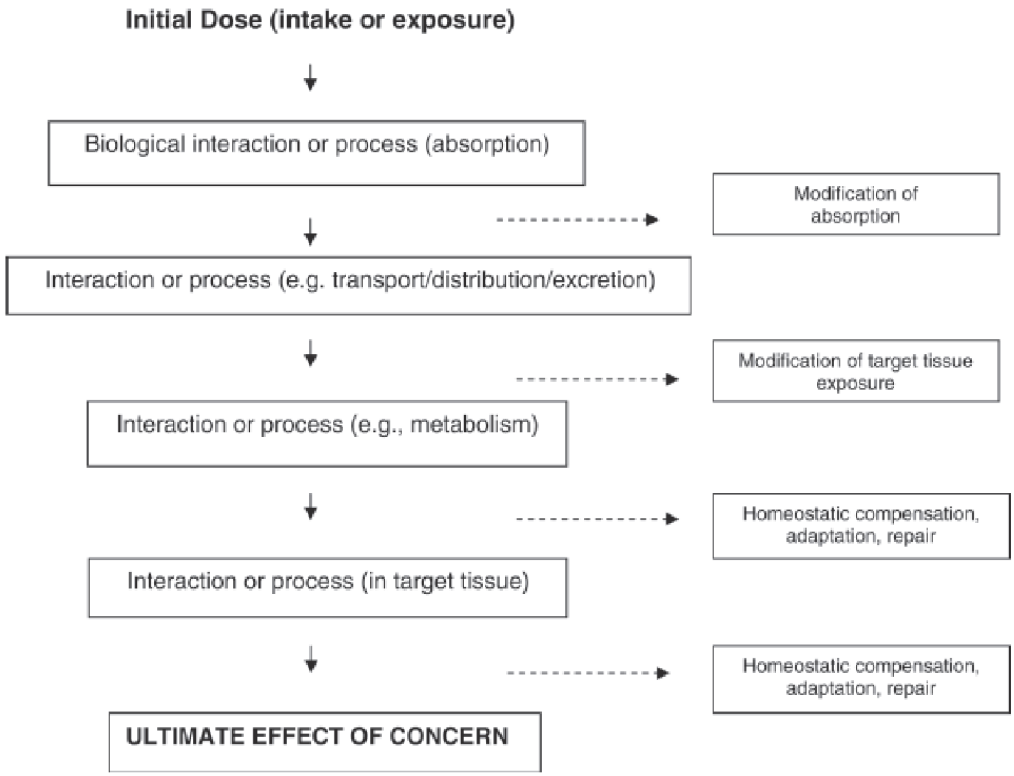


Figure 2.1: Key event dose-response framework organizes available information on the multiple kinetic and dynamic events that occur between an initial dose and the effect of concern. Events are indicated generically here; but, for a given pathway, many specific kinetic and dynamic events may occur. (Fig. 2 in Julien *et al.* (2009))

2.2 Modes of Action and Adverse Outcome Pathways

There has been a longstanding view that MoA analysis improves dose response evaluation and extrapolation to environmental and human exposures of interest Boobis et al. (2008; Boobis et al. (2006; U.S (2005). To ensure rigor and transparency in the evaluation of MoA, a framework was developed in conjunction with the International Programme for Chemical Safety (IPCS) Boobis et al. (2008; Boobis et al. (2006) and by the U.S (2005). This MoA framework provides a weight of evidence approach based on considerations for causality as originally articulated by

Sir Austin Bradford Hill for epidemiologic studies Hill (1965). These include considerations of dose response and temporal concordance; consistency, specificity, biological plausibility; and coherence. The Hill considerations provide a framework for consistency and transparency in evaluation of data and are used internationally including when evaluating endocrine disrupting chemicals WHO (2002). While the MoA framework was originally developed for human health effects, it has also been applied to ecological effects. In this context, the term adverse outcome pathway (AOP) has been used, but it is conceptually similar to the MoA Ankley et al. (2010). Another conceptually similar framework, the Key Event Dose-Response Framework (KEDRF) Julien et al. (2009) has the advantage of including a more explicit consideration of pharmacokinetics and dose (Fig. 2.1). For the purposes of this document, MoA, AOP, and KEDRF are used interchangeably.

Interaction of the active form of the xenobiotic with its tissue and cell-specific target site constitutes the initial key event, or molecular initiating event, in the MoA. This leads to a sequence of changes that progress through time and levels of biological organization, leading finally to an endpoint of concern. Key events are defined as measurable and quantifiable rate limiting biological steps that lead to the development of an adverse consequence at the organ, organism, and/or population level. The set of key events is considered necessary for the development of the adverse effect, but none are sufficient by themselves to cause the adverse effect and, generally, are not considered, individually, as adverse effects.

Resilience, or adaptation, is an important consideration in evaluating a MoA or AOP. When the magnitude of the perturbation associated with the initial key event is small, cells and tissues exhibit resilience, consisting of adaptive responses that maintain homeostasis Andersen et al. (2005). Propagation of the initial effect along the sequence of key events to the final, apical event occurs when the adaptive capacity is overwhelmed by higher doses (Fig. 2.2). The tight regulation of steroid hormone levels is a good example of this kind of homeostatic control.

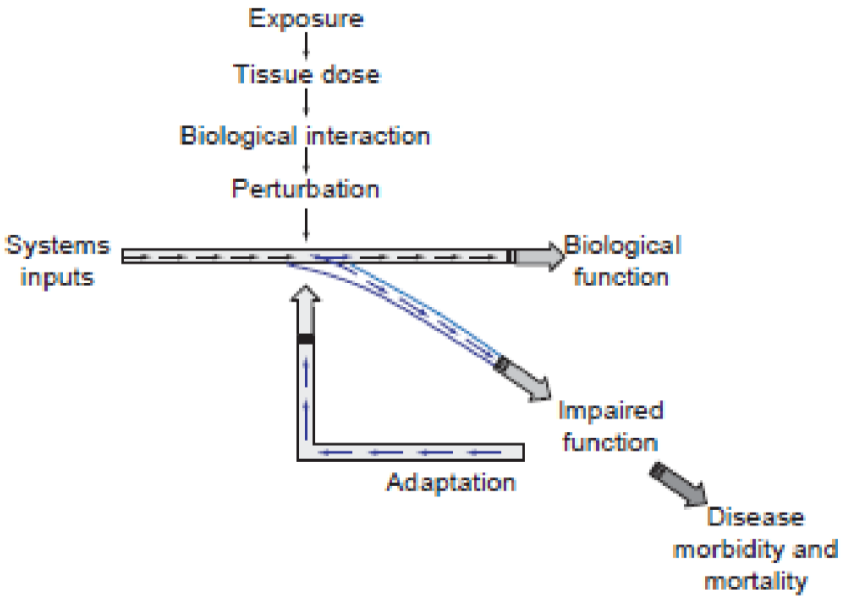


Figure 2.2: Perturbation by a xenobiotic of normal biological function may be overcome by adaptive responses (homeostasis), while higher doses of the xenobiotic overwhelm the adaptive capability, driving the system away from its normal biological functions to frank toxicity. (Fig. 5 in Andersen *et al.* (2005)).

2.3 Determinants of Nonmonotonic Dose-Response

Conolly and Lutz (2004) noted that "The first interaction of a toxic agent with its primary biological target molecule follows the law of mass action, which results in a monotonic dose response." Thus, nonmonotonic responses must arise from more complex relationships between the dose of the toxicant at its target site and the effect of interest. In studies using multiple hormone sensitive cell lines, NMDR can be produced from integration of two or more monotonic responses. One may see NMDR related to receptor down-regulation where the receptor number is inversely proportional to the amount or concentration of the ligand. NMDR may be identified when very high concentrations of the compound are toxic to the cells which would result in cell degeneration or killing and a damping of the response at high dose. NMDR may be identified due to differences in receptor affinity where multiple receptors bind a ligand resulting in complex dose response effects WHO (2012).

Conolly and Lutz (2004) developed computational models to illustrate several mechanisms that can generate NMDR:

1. The level of cyclic AMP as a function of the activation of adenosine A1 and A2 receptors by phenylisopropyladenosine.

2. Androgen-mediated gene expression in which combined exposure to native androgen and a synthetic analog interact competitively at the androgen receptor to form a series of homo- and heterodimers with differing abilities for promotion of gene expression.
3. DNA damage leading to induction of repair wherein the induced repair capacity also repairs DNA damage from background processes.
4. The rate of mutation from DNA damage when the damage activates checkpoints in the cell cycle, with the longer-duration checkpoints providing additional time for DNA repair before the replicative synthesis of DNA that fixes the mutation.

Table 2.1: Endpoints with nonmonotonic dose-response and causative influences that are dominant over different dose ranges*.

Endpoint with nonmonotonic dose-response	Toxicant or ligand	Dominant influence 1**	Dominant influence 2**
cAMP	Phenylisopropyladenosine	Adenosine A1 receptor, down regulation of cAMP (low dose)	Adenosine A2 receptor, up regulation of cAMP (high dose)
Androgen-mediated gene expression	hydroxyflutamide (+ 10-7 M dihydrotestosterone)	homodimers that promote gene expression (low and high dose)	heterodimers that inhibit gene expression (mid dose)
DNA damage	Xenobiotic that directly damages DNA	DNA adducts increase with increasing dose of xenobiotic (low and high dose)	induction of DNA repair reduces adduct level (mid dose)
Mutation	Xenobiotic that directly damages DNA (but no induction of repair)	replicative DNA synthesis converts DNA adducts into mutations (low and high dose)	cell cycle checkpoint delays replicative DNA synthesis (mid dose)

*Adapted from data presented in Conolly and Lutz (2004).
**In this context, "low dose" refers to the low dose end of the nonmonotonic dose-response curve, and "high dose" to the high dose end. "Mid dose" falls in between low and high dose.

It is possible, in these four examples (Table 2.1), to distinguish two classes of mechanisms that give rise to NMDR. Two of the examples involve adaptive responses of the exposed tissue –

induction of DNA repair and activation of cell cycle checkpoints. The other two examples, cAMP and androgen-mediated gene expression, do not involve adaptation but, rather, simply reflect constitutive biology. Since induction of an enzymatic repair process requires some minimum amount of time, the appearance in this case of the NMDR has a temporal aspect in that nonmonotonicity will not be seen if the interval between exposure and measurement of the relevant endpoint is too brief. In general however, for risk assessment, we are concerned with both short- and long-term exposures, so the dependence of some nonmonotonic responses on this kind of adaptation does not make them in any sense irrelevant.

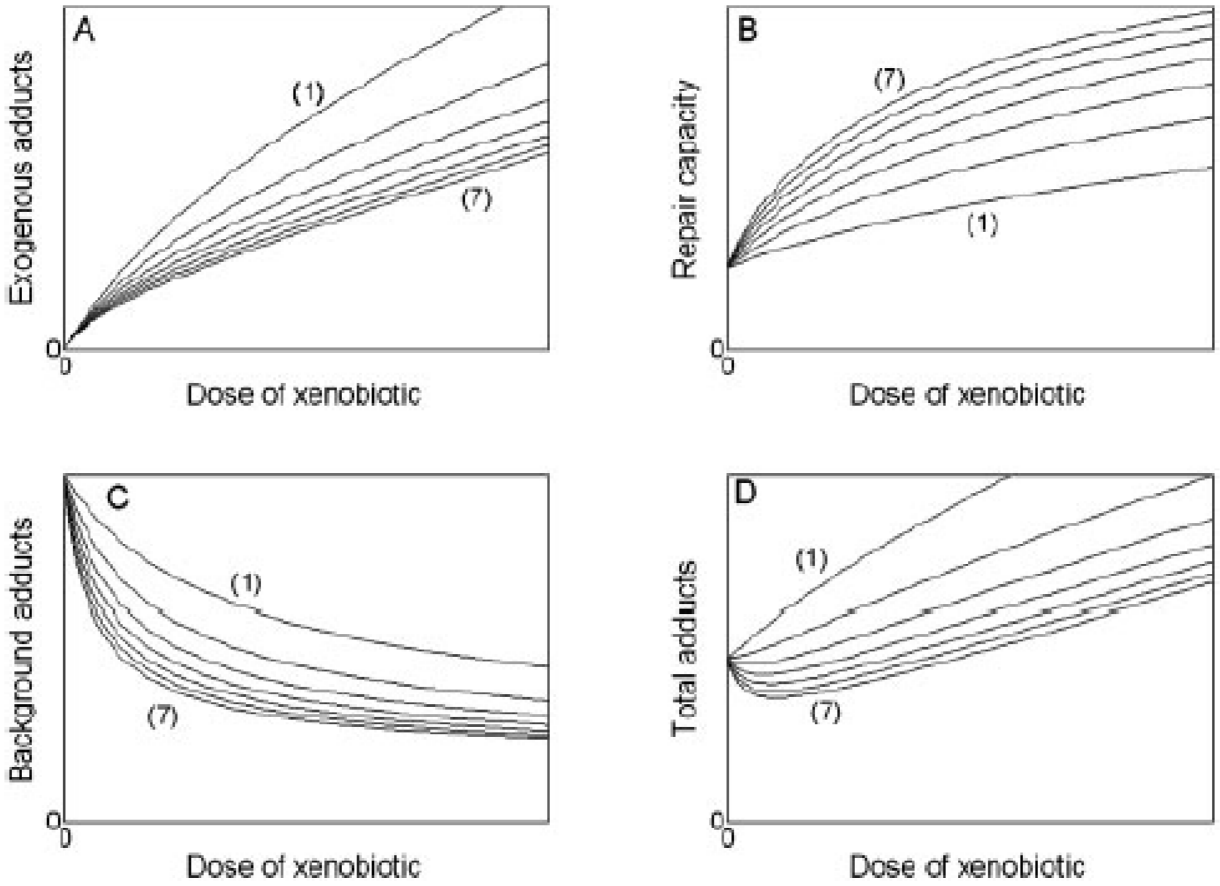


Figure 2.3: (A) Modeling of dose-response relationships for DNA-adduct levels as a function of dose of an exogenous xenobiotic. Numbers indicate increasing efficacy of saturable induction of DNA repair, as illustrated in B. (C) Decrease of background DNA adducts due to the induced repair. (D) Total adducts (background plus exogenous), obtained by superimposition of A and C.

The four examples, though diverse, are all characterized by the presence of more than one influence on the shape of the dose-response curve, with each influence dominant over a different range of doses (Fig. 2.3). Conolly and Lutz (2004) used "parameter sweeps" to characterize how these influences combine to generate nonmonotonicity. In these parameter

sweeps value of a key parameter was varied to produce a corresponding set of dose-response curves. For the case of nonmonotonicity due to induction of DNA repair, a sweep on the parameter determining the efficacy or degree of induction of DNA repair was conducted (Fig. 2.3). When the efficacy of induction was minimal, the dose-response curve was monotonic, while maximal efficacy generated a nonmonotonic curve (Fig. 2.3, panel D). Interestingly, intermediate efficacy leads to a dose-response curve where, at low dose, the increase in the adduct burden due to the xenobiotic is closely balanced by a decrease in adducts due to the induction of repair capacity, resulting in a threshold-like curve. It is important to note here that the threshold-like behavior is not due to a single action of the xenobiotic but rather to the addition of two opposing influences that happen to balance each other over a range of doses. Similar results were obtained by Conolly and Lutz (2004) for the other three cases that they examined; sweeping on a key parameter led from monotonic dose-response, through an intermediate, threshold-like regimen to a clearly nonmonotonic response. These results suggest that the conditions under which nonmonotonicity arises may be only subtly different from those generating monotonic responses, possibly involving no more than a quantitative difference in a single component of the system under study.

A general principle can be deduced from the forgoing examples. NMDR can arise when the biological system that is activated in response to chemical exposure consists of at least two activities that can act in opposition to each other. If we consider the nonlinear nature of signaling pathway biology e.g., Bhalla et al. (2002; Hoffmann et al. (2002), the ensemble of inducible cellular stress response pathways Simmons et al. (2009) and, in the case of the endocrine system, the tight homeostatic regulation of hormone levels, it seems quite possible that under some conditions and for some types of endpoints, NMDR relationships would be expected. The key concern is not so much about the existence of NMDR but, rather, whether or not nonmonotonicity occurs over dose-ranges that are relevant to humans and to nonhuman target species and for endpoints of regulatory concern.

2.4 The Hypothalamic-Pituitary-Gonadal and Hypothalamic-Pituitary-Thyroid Axes

The preceding discussion of determinants of the shapes of dose-response curves, whether monotonic or nonmonotonic, is relevant to EDCs but up to this point has been generic rather than focused specifically on endocrine endpoints. This section provides an overview of the biology of the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) axes with the goal of providing necessary background for the evaluations in Sections 2, 3, and 4 of *in vitro* and *in vivo* data on NMDR.

The HPG and HPT axes function as integrated systems to produce and tightly regulate blood and tissue levels of estrogen (E), testosterone (T), and the thyroid hormones (TH) (Figs. 2.4 and 2.5).

E and T bind to their respective high affinity cytoplasmic receptors, usually referred to as estrogen receptor (ER) and androgen receptor (AR). TH, specifically T3, binds to nuclear thyroid receptors (TR).

Estradiol 17β (E2) is a potent natural steroid produced primarily by gonadal tissues in most vertebrates. The "normal" physiological level of E2 varies greatly among different species, genders, life stages and reproductive status. Levels required for normal reproductive function at one stage of life can produce adverse effects at another stage of life. In adulthood, both higher and lower than "normal" E2 levels also can have adverse effects. The estrogens estrone and estriol also are normally found *in vivo* and are only slightly less potent than E2. These hormones affect many, if not all, tissues in the body in a tissue-specific manner via one of two nuclear receptors, ERα or ERβ, or less well characterized cell membrane receptors. Tissue-specific responses arise from differences in receptor levels, levels of coactivators and corepressors, E2 metabolism, receptor stability, different target gene estrogen response elements, gene silencing, and other factors.

The two highly related estrogen receptors, ERα and ERβ have different tissue distributions and activate different sets of genes. Although ERα and ERβ bind many ligands with similar affinity, a number of chemical structures have been shown to have selectivity towards one of the pair. Ligand binding to the ER or AR receptor induces a conformational change resulting in translocation to the nucleus for receptors located in the cytoplasm and receptor dimerization. Dimerization is required to form a complex capable of binding to specific DNA sequences, termed response elements, in the promoters of their target genes. The ligand-bound receptor homodimer has a 3-dimensional shape that varies from ligand to ligand, resulting in unique coregulator recruitment profiles and forming "interactomes" that moderate the endocrine activity of the transcriptional complex, thus potentially altering patterns of target gene expression O'Malley et al. (2012; Huang et al. (2010). The mRNA produced is translated into protein that then directs physiologic responses to maintain homeostasis, regulate growth, or control other specific processes. O'Malley et al. (2012) reported that interactomes associated with estrogen receptor binding to its natural ligand consist commonly of approximately 10 protein partners that combine into one functional unit. Human cells contain about 11,500 unique gene products that code for proteins that directly or indirectly regulate nuclear receptor function as components of interactomes. Much less is known about the signaling mechanisms following activation of the membrane-associated estrogen receptors, but these pathways do not involve direct transcriptional regulation. Tissue-specific responses arise from differences in levels of receptors, coactivators and corepressors, receptor stability, gene silencing and other factors. In addition, EDC-specific variations in the makeup of interactomes can induce gene expression patterns that differ from those induced by E2 itself.

The androgen signaling pathway shares many molecular and cellular traits with the estrogen signaling pathway. There is only one AR in mammals, and as the gene resides on the X chromosome, males will have only one copy Gao et al. (2005). In some species of fish there are two AR (ARα and Arβ) due to whole genome duplication that occurred at a point in evolutionary history Ogino et al. (2009). There are two physiologically active androgens in mammals; testosterone is the major regulatory steroid in many androgen-dependent tissues, while other tissues rely upon the conversion of testosterone to dihydrotestosterone (DHT) by 5α reductase Gao et al. (2005). There also are two active androgens in fish, testosterone and 11-ketotestosterone; however, the physiological relationship between the two in terms of function is uncertain. In the androgen hormone system, as with the estrogen hormone system, tissue-specific coregulators elicit tissue-specific responses.

Thyroid hormone is produced by the thyroid gland (Fig. 2.5) and is essential for normal development and growth. While TH production is tightly controls by the HPT axis, there are a large number of targets for thyroid disrupting chemicals that are outside this axis and also control circulating and tissue levels of THs (see also section 4.2.3.1). Importantly, there is a lack of detailed information on the temporal and dose-dependence of the multi-tissue feedback-control systems that regulate TH homeostasis.

The developing brain is one of the most vulnerable organs to thyroid hormone (TH) excess or insufficiency. THs regulate neuron proliferation and migration, synaptogenesis, synaptic plasticity and myelination in the developing brain (Williams, 2008). A key feature of TH action in brain is the temporal sequence of events it supports, a feature that increases the complexity of determining the impact of xenobiotic-induced alterations in thyroid function Howdeshell (2002).

In the circulation, TH is tightly bound to serum transport proteins for delivery to the many TH-dependent organs. The neuroendocrine control of thyroidal TH synthesis and secretion is very sensitive to negative feedback exerted by circulating TH and involves various molecular mechanisms such as specific expression patterns of TR genes, TH transporters and deiodinases Chiamolera and Wondisford (2009; Fekete and Lechan (2007; Fliers et al. (2006). T4 is a prohormone that is converted into the active form, T3, by type 2 5'-deiodinase, but may also have non genomic effects via membrane-bound receptors specific for T4. Nongenomic effects of TH have been reported for blood vessels, heart, and brain Davis et al. (2008) and may influence cell proliferation and cancer.

Endocrine activity in the context of this document refers to biological effects resulting from these hormones circulating through the blood to target tissues where they bind to their receptors to implement their respective activities (Fig. 2.6). An EDC may bind to ER or AR or TR

as a full or partial agonist, mimicking wholly or in part the ability of the endogenous hormone to activate the receptor. Alternatively, an EDC may be an antagonist, binding to the receptor but not activating it and thereby blocking the activity of the endogenous hormone. Differences in chemical structure between EDCs and their corresponding endogenous hormones can lead to differences in the conformational change of the receptor that occurs upon ligand binding. These conformational differences can lead to differences in the signaling downstream from the receptor, resulting in different patterns of gene expression (Fig. 2.6).

Patterns of gene expression can also be changed when EDC alter normal levels of endogenous hormones. This MoA involves the activation of nuclear receptors (*e.g.*, CAR, PXR) by the parent EDC or metabolite. Activation of these nuclear receptors results in an induction of Phase I, II, and III hepatic proteins, increasing hormone clearance and thereby lowering circulating levels Crofton and Zoeller (2005; Hill et al. (1998; Capen (1997). For example, methyl-tertiary butyl ether and wholly vaporized gasoline induce metabolism in the liver and other tissues of enzymes that catabolize endogenous estrogen Moser et al. (1998; Moser et al. (1997; Moser et al. (1996a; Moser et al. (1996b; Standeven and Goldsworthy (1994; Standeven et al. (1994b; Standeven et al. (1994a). Phase I CYP inducers include pesticides and pharmaceuticals Lake (2009; Martignoni et al. (2006) Phase II enzyme induction includes the enzyme family that metabolize thyroid hormone, UDP-glucuronosyltransferase enzymes, cytosolic sulfotransferases, and GSH S-transferase enzymes, as well as some Phase III cellular transporters Omiecinski et al. (2011; Martignoni et al. (2006).

Steroid hormone receptor activity can also be modified through allosteric interactions Kumar and Mcewan (2012). In this case receptor activity is modified not by classical "lock and key" ligand binding at the active site but by binding elsewhere on the receptor molecule that alters its three dimensional structure and, thereby, it's signaling behavior. For example, Joseph *et al.* (2009) describe "second site" ligands identified in pharmacological studies that antagonize the activity of AR. While allosteric interactions are a potential class of key events for environmental endocrine active xenobiotics, there do not currently appear to be documented cases of this mode of action.

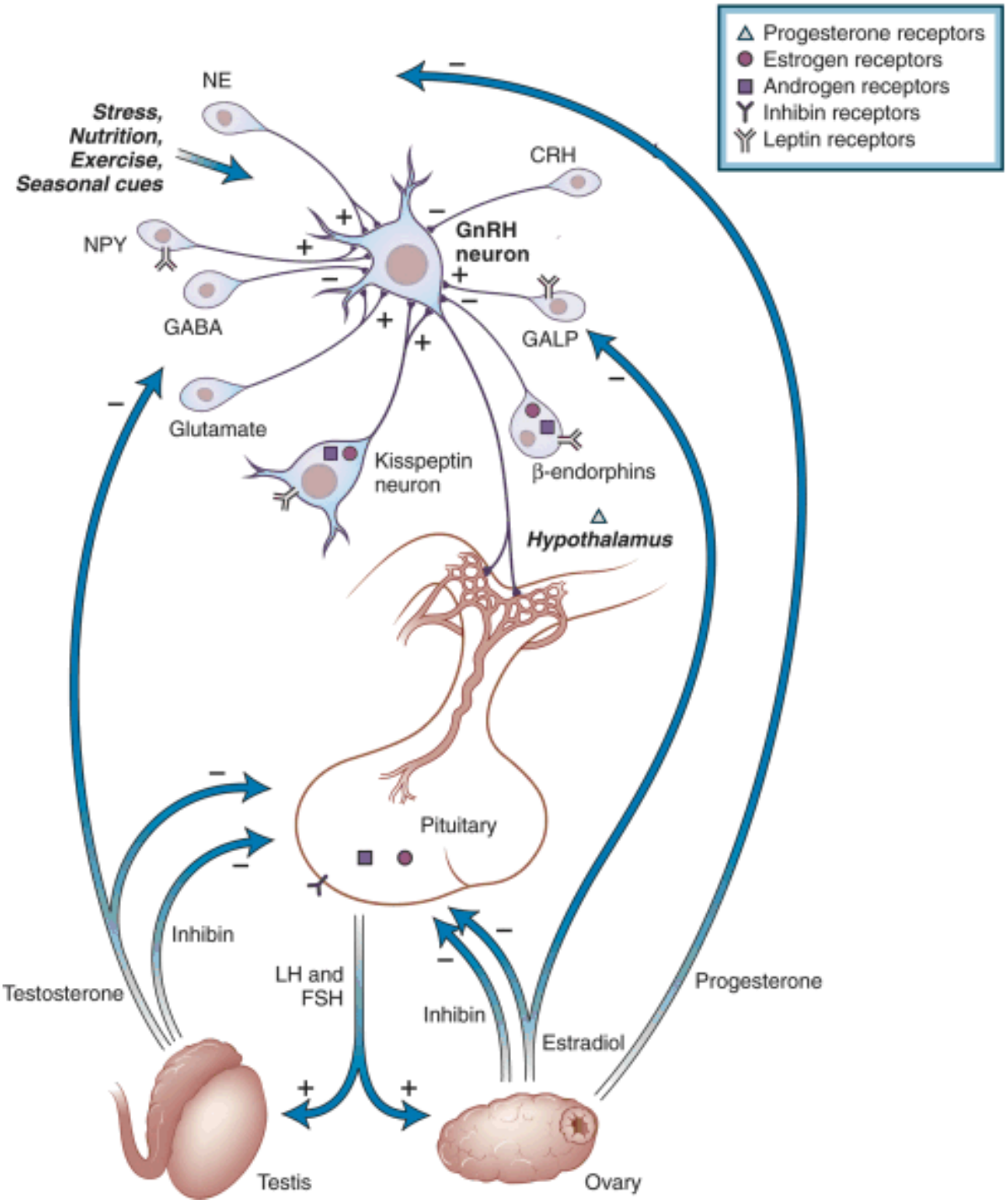


Figure 2.4: Hypothalamic-pituitary-gonadal (HPG) axis. Blood and tissue levels of sex steroids are tightly regulated in this multi-tissue, feedback-controlled system. The hypothalamus produces gonadotropin-releasing hormone (GnRH); the pituitary produces luteinizing hormone (LH) and follicle stimulating hormone (FSH), and the gonads (testis, ovary) produce testosterone and estrogen. (Fig. 2.4 from www.Health-7.com)

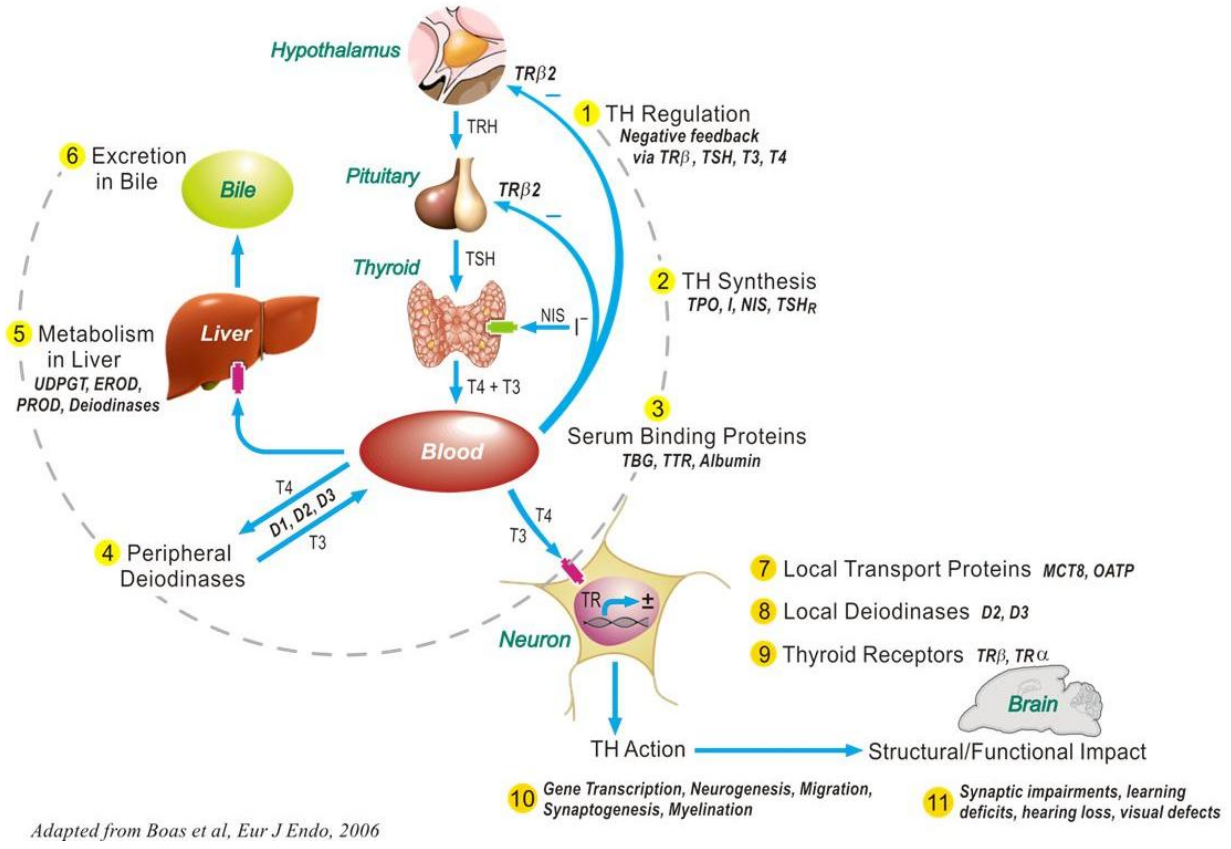


Figure 2.5: Regulation of thyroid hormone production, release, transport, metabolism and action. Control of thyroid hormone action in tissues is regulated by a combination of the HPT axis and other extrathyroid tissue processes. The HPT feedback process regulates synthesis and release via the hypothalamic production of thyrotropin-releasing hormone (TRH); the pituitary production of Thyroid Stimulating Hormone (TSH) and the Thyroid gland production and release of thyroid hormones (T4 and T3). In most mammals the majority of T3 is produced at the target tissue from deiodination of T4 and impacts T3 and T4 levels in both tissues and blood. The liver controls catabolism of THs and can thus indirectly regulate TH levels in the blood. Finally, local tissue regulation of transport proteins and receptors can regulate tissue level transcription. (Fig.2.5 from Gilbert et al. (2012))

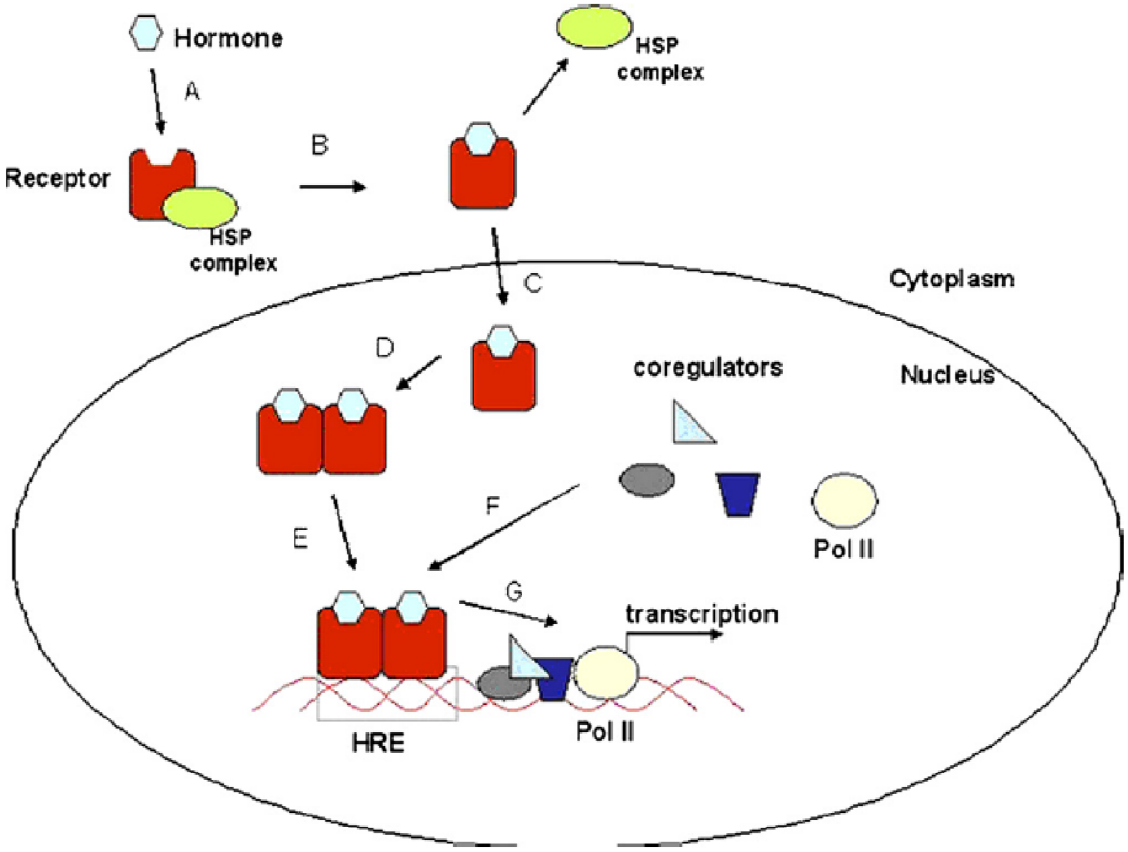


Figure 2.6: Schematic diagram of the activation of a cytoplasmic steroid hormone receptor. After hormone binding (A) the HSP complex dissociates from the receptor (B), the hormone receptor complex translocates to the nucleus (C), dimerizes (D) and binds to a hormone responsive element (HRE) in the promoter region of a specific gene (E). After binding to the HRE different coregulators of transcription are recruited (F), which are responsible for transcriptional activation (Fig. 2.6 from Riedmaier *et al.* (2009)).

2.5 Statistical Issues and Experimental Design

When determining the appropriate analytical approach, the underlying assumptions of the statistical tests, which often include normality, monotonicity, and balanced groups, need to be considered. In cases where these assumptions are violated, tests that have been developed to avoid these common assumptions should be used. New methods have been developed in many areas to overcome these challenges in order to improve estimation of nonmonotonic dose responses Bretz and Hothorn (2003); Takizawa et al. (2000); Chen (1999). In particular, Gaylor *et al.* (2004) described methods for the analysis of J-shaped dose-response curves for both continuous and quantal endpoints. J-shaped curves are NMDR in which the curve initially has a negative slope downward from a nonzero control level, which then inflects upward to a positive slope at higher doses. Gaylor *et al.* (2004) suggest experimental designs to identify

nonmonotonicity and include information regarding numbers of doses and group sizes. In general, they show that much larger sample sizes are needed to detect J-shaped effects at low doses, particularly for quantal data by comparison to continuous data. This work highlights that principles of statistical design of experiments need to be utilized when determining experimental doses and sample sizes to maximize the power to detect J-shaped curves. Since Gaylor *et al.* (2004) was restricted to J-shaped curves, its relevance to NMDR generally may be limited.

Multiple testing problems can arise and potentially confound identification of NMDR. Several methods have been developed to correct for multiple comparisons in order to control the familywise error rate. Familywise error rate is the term used in the multiple comparisons literature to mean that the overall alpha level is kept to 0.05 (or another pre-specified level) for all hypothesis testing performed. These methods range from the most conservative Bonferroni approach where the type I error rate is set to be alpha/n, where n is the number of tests performed, to more advanced computerized resampling methods, including bootstrapping and Monte Carlo simulations. As pointed out by Bretz and Hothorn (2003), a trend test is not useful if it does not "control the probability of incorrectly declaring a dose to be effective, when in fact it is not effective."

Limitations in experimental design, in particular the number of animals in each dose group, can lead to inaccurate interpretation of the data, including indicating nonmonotonicity when the true response is monotonic. Though small sample sizes may be unavoidable for logistical reasons, they may contribute to considerable random variation. Lutz *et al.* (2005) demonstrated that NMDRs can arise due to random variation in a quantal endpoint when the true underlying dose-response has a positive, linear slope.

Use of larger group sizes or replication of entire studies may not be practical for large-scale studies, such as carcinogen bioassays or multigeneration reproductive toxicology studies. However, when results are potentially significant for our understanding of toxicological mechanisms and their attendant regulatory implications, experiments should be evaluated for statistical power and replicated when possible. Jasny *et al.* (2011), in a special issue of *Science* on Data Replication and Reproducibility, noted that "Replication - the confirmation of results and conclusions from one study obtained independently in another - is considered the scientific gold standard." For large scale studies, where study replication or modification of study design is not practical, it is desirable to use ancillary studies of pharmacokinetics and the MoA to gain insights into expected dose-response behaviors.

The number of dose groups in current guideline studies is considered adequate to inform most risk-based decisions, but they may not be sufficient to fully describe the shape of an NMDR. It

is generally true that the shape of any dose response curve can be more clearly defined by using more dose groups and by adjusting group sizes using statistical power calculations OECD (2011; Blystone et al. (2010; Hotchkiss et al. (2008).

3. NMDRs from *in vitro* Studies

The occurrence of NMDR *in vitro* has been well documented in the scientific literature with examples presented in recent review articles (*e.g.*, Vandenberg et al. (2012); DCED (2013)). The current state of the science document does not include an additional exhaustive review of the *in vitro* literature, but rather discusses general concepts for nonmonotonicity *in vitro*, incorporates observations from recent review articles, and cites examples from primary literature, where applicable.

NMDRs have been reported for a variety of *in vitro* assays targeting endocrine receptors, and incidence of their observation is likely to increase with the recent impetus to develop and implement high-throughput toxicological screening technologies. To understand the implications of *in vitro* NMDR findings, it is useful to begin with a mechanistic understanding of common assays and potential causes of NMDRs. For the estrogen and androgen receptors, the great majority of the reported biological activity of xenobiotic chemicals (especially E and A signaling) is through binding to the ligand-binding site of the receptor, resulting in a modulation of the receptor activity manifested as agonistic, antagonistic or selective receptor modulation. The original *in vitro* assays targeting endocrine receptors measured direct binding of radiolabeled E and A ligands to preparations of crude, partially purified or highly purified receptors. Alternatively, binding of non-labeled ligands could be detected indirectly through competitive displacement of radiolabeled ligand. With such binding assays, NMDRs have not been reported except as artifacts. Binding of the ligands to nuclear receptors follows the law of mass action in which a ligand binds reversibly to the receptor and the fractional occupancy of the receptor is a function of the ligand concentration and the K_d, the equilibrium dissociation constant for the ligand/receptor pair. Thus, as measured in a radioligand binding assay, the response is a monotonic, competitive binding curve. When behavior of the assay deviates from this with a nonmonotonic response, it is characterized by decreased binding at high concentrations. Such effects are explained either by insolubility of the compound at higher concentrations or by assay interference artifacts, such as aggregation or denaturation, common at higher concentrations.

Binding of the receptor ligand to its binding site is the initial step in receptor activation. This alone, however, does not provide information about whether the compound has functional agonist or antagonist behavior. Functional cellular reporter gene assays, called transactivation assays, have traditionally been used to determine this behavior. These assays measure ligand-

bound receptor binding to a hormone-response element on DNA, recruitment of coregulator proteins, and changes in the expression of measurable reporter gene products. Major variations in transactivational assay approaches include: full-length receptor vs. ligand-binding domain; stable vs. transient transfection of the reporter gene constructs and receptor; endogenous vs. transfected coregulator proteins; and the specific reporter gene product. Finally, these assays can be run in agonist mode to measure the ability of the test compound to activate or increase receptor activity, or in antagonist mode performed in the presence of a single concentration of a receptor agonist, measuring the ability of the test compound to inhibit or reduce agonist-induced activity.

NMDRs can occur in transactivational assays due to cytotoxicity. Dose response effects caused by a chemical are often impacted by cell death at high levels. Since many compounds have multiple molecular targets that are affected at different concentrations, compounds that bind and activate steroid receptors at one concentration can interact with other cellular proteins at higher concentrations, leading to cytotoxicity. In a reporter gene assay, this is manifested as increasing reporter gene activity with increasing compound concentration up to a point at which there is significant cytotoxicity and concurrent loss of reporter gene activity. An example is the phytoestrogen genistein. Genistein binds and activates the estrogen receptor with a K_d of 20 nM but also affects a number of other important targets such as protein kinases and topoisomerase at higher concentrations, leading to cytotoxicity and cell death. Measuring a transactivation assay for genistein with the ER will result in a NMDR with loss of activity at higher concentrations.

Use of transactivation assays serve as surrogate assays for endogenous target gene expression and corresponding encoded protein levels, the primary biological activity regulated by nuclear receptors. Measurement of gene or protein expression in other types of *in vitro* systems suffers the same proclivity to NMDR effects as do transactivation assays. An illustrative example of this is provided by Schmieder *et al.* (2004) who examined induction of the egg yolk protein precursor vitellogenin (VTG) in rainbow trout liver slices exposed to several model ER agonists. They observed a significantly depressed induction of VTG at high versus low doses with a number of putative estrogens (o,p'-DDT, monohydroxy-methoxychlor, p-nonylphenol), a response that appeared to be attributable to overt toxicity of the chemicals to the liver cells. In that paper Schmieder *et al.* (2004) noted the importance of evaluating cell viability in order to accurately interpret responses to EDCs in this type of *in vitro* system.

In a recent review, Vandenberg *et al.* (2012) identified 80 examples from the literature, which provide evidence of nonmonotonicity *in vitro*. The NMDRs in these studies were from a wide range of chemical classes including natural and xenobiotic hormones, phytoestrogens, plastics, surfactants, metals, PAHs, PCBs, PBDEs, pesticides, and dioxin. The nonmonotonic effects were

predominantly seen in lower-order biological endpoints closer to the molecular initiating event. Included in these endpoints were measures of protein or hormone levels, gene expression, and cell number. Subsequent analysis of these 80 NMDRs by The Danish Centre on Endocrine Disruptors DCED (2013) revealed that cytotoxicity was the most commonly observed mechanism for causing NMDRs *in vitro*. Nearly half (45%) of the *in vitro* studies had NMDRs that were likely caused by cytotoxicity. Further, the authors established that an additional 22% were "false NMDRs" caused by study design (i.e., mixtures of chemicals, inappropriate statistical analyses for establishing nonmonotonicity). The remaining third of the studies either showed evidence of NMDRs (16%) or provide evidence that may or may not be due to NMDRs (17%). These study evaluations are provided in Table 3.1.

Table 3.1: *in vitro* NMDRs as evaluated by the Danish Centre on Endocrine Disruptors DCED (2013).

Chemicals by chemical class	Nonmonotonic effect	Cell type	Refs.	Evaluation
Natural hormones				
17R-Estradiol	Cell number	MCF7 breast cancer cells	135, 716	Cytotox
	Dopamine uptake	Fetal hypothalamic cells (primary)	717	Maybe
	pERK levels, prolactin release	GH3/B6/F10 pituitary cells	41, 718, 719	NMDR
	R-Hexosaminidase release	HMC-1 mast cells	720	Cytotox?
	Cell number	Vascular smooth muscle cells	721	False
	Production of L-PGDS, a sleep-promoting substance	U251 glioma cells	722	Cytotox
5a-Dihydrotestosterone	Cell number	LNCaP-FGC prostate cancer cells	499	Cytotox
	Cell number, kinase activity	Vascular smooth muscle cells	721	False
5a-Androstenedione	Cell number	LNCaP-FGC prostate cancer cells	499	Cytotox
Corticosterone	Mitochrondrial oxidation, calcium flux	Cortical neurons (primary)	723	Cytotox
Insulin	Markers of apoptosis (in absence of glucose)	Pancreatic R-cells (primary)	724	Cytotox
Progesterone	Cell number	LNCaP-FGC prostate cancer cells	499	Cytotox
Prolactin	Testosterone release	Adult rat testicular cells (primary)	725	Cytotox
hCG	Testosterone release	Adult rat testicular cells (primary)	725	Cytotox
T_3	Rate of protein phosphorylation	Cerebral cortex cells (primary, synaptosomes)	726	NMDR
	LPL mRNA expression	White adipocytes (rat primary)	727	False
GH	*IGF-I* expression	Hepatocytes (primary cultures from silver sea bream)	728	False
Pharmaceutical hormones				
DES	Cell number	MCF7 breast cancer cells	716	Cytotox
	Prolactin release	GH3/B6/F10 pituitary cells	41	NMDR
Ethinyl estradiol	CXCL12 secretion	MCF7 breast cancer cells, T47D breast cancer cells	729	Maybe
R1881 (synthetic androgen)	Cell number	LNCaP-FGC cells	499	Cytotox
Trenbolone	Induction of micronuclei	RTL-W1 fish liver cells	730	NMDR
Plastics				
BPA	Cell number	MCF7 breast cancer cells	135, 716	Cytotox
	Dopamine efflux	PC12 rat tumor cells	40	NMDR
	pERK levels, intracellular Ca^{2+} changes, prolactin release	GH3/B6/F10 pituitary cells	41, 718	NMDR
	Cell number	LNCaP prostate cancer cells	731	False
DEHP	Number of colonies	*Escherichia coli* and *B. subtilis* bacteria	732	Maybe
Di-*n*-octyl phthalate	Number of colonies	*E. coli* and *B. subtilis* bacteria	732	Maybe

Chemicals by chemical class	Nonmonotonic effect	Cell type	Refs.	Evaluation
Detergents, surfactants				
Octylphenol	Cell number	MCF7 breast cancer cells	716	Cytotox
	Dopamine uptake	Fetal hypothalamic cells (primary)	717	Maybe
	pERK levels	GH3/B6/F10 pituitary cells	718	NMDR
	HCG-stimulated testosterone levels	Leydig cells (primary)	733	NMDR
Propylphenol	pERK levels	GH3/B6/F10 pituitary cells	718	NMDR
Nonylphenol	pERK levels, prolactin release	GH3/B6/F10 pituitary cells	41, 718	NMDR
	R-Hexosaminidase release	HMC-1 mast cells	720	Cytotox
	Cell number	MCF7 breast cancer cells	135	Cytotox
PAH				
Phenanthrene	All-trans retinoic acid activity	P19 embryonic carcinoma cells	734, 735	Maybe
Benz(a)acridine	All-trans retinoic acid activity	P19 embryonic carcinoma cells	734	Maybe
Naphthalene	hCG-stimulated testosterone	Pieces of goldfish testes	736	Cytotox
B-naphthoflavone	hCG-stimulated testosterone	Pieces of goldfish testes	736	Cytotox
Retene	hCG-stimulated testosterone	Pieces of goldfish testes	736	Cytotox
Heavy metals				
Lead	Estrogen, testosterone, and cortisol levels	Postvitellogenic follicles (isolated from catfish)	737	Cytotox
Cadmium	Expression of angiogenesis genes	Human endometrial endothelial cells	738	Maybe
Phytoestrogens and natural antioxidants				
Genistein	Cell number	Caco-2BBe colon adenocarcinoma cells	739	Cytotox
	CXCL12 secretion, cell number	T47D breast cancer cells	729	Maybe
	Cell number, cell invasion, MMP-9 activity	PC3 prostate cancer cells	740	Cytotox
	pJNK levels, Ca^{2+} flux	GH3/B6/F10 pituitary cells	719	NMDR
Coumesterol	Prolactin release, pERK levels	GH3/B6/F10 pituitary cells	719	NMDR
Daidezin	Prolactin release, pERK levels	GH3/B6/F10 pituitary cells	719	NMDR
	Cell number	MCF7 breast cancer cells	135	Cytotox
	Cell number	LoVo colon cancer cells	741	Maybe
Resveratrol	Expression of angiogenesis genes	Human umbilical vein endothelial cells	742	NMDR
Trans-resveratrol	pERK levels, Ca^{2+} flux	GH3/B6/F10 pituitary cells	719	NMDR
Artelastochromene	Cell number	MCF7 breast cancer cells	743	Maybe
Carpelastofuran	Cell number	MCF7 breast cancer cells	743	Maybe
Biochanin A	Induction of estrogen-sensitive genes	MCF7 breast cancer cells	744	Maybe
Licoflavone C	Induction of estrogen-sensitive genes	Yeast bioassay	745	Maybe
Quercetin	Aromatase activity	H295R adrenocortical carcinoma cells	746	Cytotox
	Cell number	SCC-25 oral squamous carcinoma cells	747	Maybe

Chemicals by chemical class	Nonmonotonic effect	Cell type	Refs.	Evaluation
Dioxin				
TCDD	Cell number, gene expression	M13SV1 breast cells	748	Maybe
PCB				
PCB-74	Cell viability, GnRH peptide levels	GT1-7 hypothalamic cells	749	Cytotox
PCB-118	Cell viability, GnRH peptide levels	GT1-7 hypothalamic cells	749	Cytotox
Aroclor 1242 (PCB mixture)	β-Hexosaminidase release	HMC-1 mast cells	720	Cytotox
POP mixture	Apoptosis of cumulus cells	Oocyte-cumulus complexes (primary, from pigs)	750	False
Herbicides				
Glyphosphate-herbicide (Round-Up)	Cell death, aromatase activity, ERβ activity	HepG2 liver cells	751	Cytotox
Atrazine	Cell number	IEC-6 intestinal cells	752	False
Insecticides				
Endosulfan	Cell number	IEC-6 intestinal cells	752	False
	β -Hexosaminidase release	HMC-1 mast cells	720	Cytotox
	ATPase activity of P-glycoprotein	CHO cell extracts	753	Maybe
Diazinon	Cell number	IEC-6 intestinal cells	752	False
Dieldrin	β -Hexosaminidase release	HMC-1 mast cells	720	Cytotox
DDT	Cell number	MCF7 breast cancer cells	144	Not evaluated
DDE	β-Hexosaminidase release	HMC-1 mast cells	720	Cytotox
	Prolactin release	GH3/B6/F10 pituitary cells	41	NMDR
3-Methylsulfonyl-DDE	Cortisol and aldosterone release, steroidogenic genes	H295R adrenocortical carcinoma cells	754	Cytotox
Fungicides				
Hexachlorobenzene	Transcriptional activity in the presence of DHT	PC3 prostate cancer cells	755	Cytotox
Prochloraz	Aldosterone, progesterone, and corticosterone levels; expression of steroidogenic genes	H295R adrenocortical cells	756	Cytotox?
Ketoconazole	Aldosterone secretion	H295R adrenocortical cells	757	False
Fungicide mixtures	Aldosterone secretion	H295R adrenocortical cells	757	False
PBDE				
PBDE-49	Activation of ryanodine receptor 1	HEK293 cell (membranes)	758	Cytotox
PBDE-99	Expression of GAP43	Cerebral cortex cells (primary)	759	Cytotox

In addition to cytotoxicity, other effects can result in NMDRs *in vitro*. Competition between ligands, ligand-induced down-regulation of receptors Alarid (1999), squelching due to limited availability of cofactors Bocquel et al. (1989), shutting-off of endocrine responses at high levels Sonnenschein et al. (1989), 1989), and mixed dimerization of ligands or receptors Chang et al. (2006; Wong et al. (1995) may lead to NMDRs. In general these mechanisms of nonmonotonicity have biological underpinnings that explain their occurrence.

In addition, NMDRs may be seen as an artifact of a specific *in vitro* assay and may not be repeatable in other transactivation systems. Increasing chemical concentrations may interfere with the reporter gene products by mechanisms such as chemical quenching of fluorescent signal or inhibition of a reporter gene product enzymatic activity. Sometimes the pharmacology of the assay system has not been optimized appropriately resulting in NMDR at high concentrations due to transcriptional squelching from rate-limiting steps in the signaling pathway. As an example of this, in transiently transfected assays utilizing recombinant receptors, a large excess of receptors relative to the more limiting endogenous coregulators could result in the generation of "unproductive transcription complexes" that lack sufficient coregulators and/or accessory proteins on the response elements to permit transcription. This occurs only at high concentrations of ligand and associated high receptor occupancy, whereas at low concentrations the relatively few active ligand-bound receptors have sufficient availability of coregulators and accessory proteins to signal appropriately.

An NMDR has been reported *in vitro* for the AR antagonist hydroxyflutamide (OHF, active metabolite of flutamide) via mixed agonist and antagonist activity. OHF binds AR and inhibits 4-dihydrotestosterone (DHT)-induced gene expression with montonic effects in the nanomolar to micromolar concentration range Wilson et al. (2002). At higher concentrations, however, OHF shows a loss of inhibition of DHT-induced activity beginning at around 10 micromolar, an activity attributable to agonist activity at this concentration as confirmed by activity in the absence of DHT Wong et al. (1995; Kelce et al. (1994). This mixed antagonist:agonist activity appears as a NMDR Wilson et al. (2002). The biological significance of these high concentration-effects remains unclear. The *in vivo* dose response data for flutamide provides no evidence that the androgenic activity seen *in vitro* is expressed *in vivo* Miyata et al. (2002; Mcintyre et al. (2001). In addition, several other AR antagonists also are androgenic *in vitro*, and for some of these chemicals, the concentration that produces AR antagonism of androgen in the test system media also induces AR-dependent gene expression when androgen is not present in the media. Most of these, however, appear to act as antiandrogens *in vivo*. A complete mechanistic understanding of these effects is lacking. Of note is the report that hydroxyflutamide can activate the MAP kinase pathway which would lead to ligand-independent activation of AR Lee et al. (2002). This would be consistent with polypharmacology of the compound occurring at concentrations higher than required for

receptor binding. An alternative proposal is an effect occurring only when receptor dimer subunits bind to hydroxyflutamide or vinclozolin and require high chemical concentrations Wong et al. (1995).

Estrogen may bind to additional receptors beyond the steroid nuclear receptors. The 7-transmembrane receptor GPR30 was found to bind estrogen with a K_d of approximately 5 nM, 10-fold higher concentration than the K_d for the ER itself Revankar et al. (2005; Thomas et al. (2005). This has also been noted for Bisphenol A (BPA), however, several other laboratories were unable to demonstrate estradiol binding to GPR30 or estradiol-activated signal transduction in GPR30-expressing cells. Binding to the GPR30 receptor has been reported to induce production of cAMP and intracellular calcium mobilization. Sensitive assay technologies exist for these endpoints and have been used to characterize GPR30 cellular activity. Some of these studies have resulted in apparent NMDR at relatively low concentrations. These studies are difficult to evaluate, however. The responses are often of very low magnitude, and, although statistically significant, have little biological validation. The GPR30 receptor was originally an orphan GPCR receptor discovered through DNA sequence analysis and only later found to bind estrogen. Further, it remains to be determined if estrogen is a physiological ligand for GPR30 regulating important *in vivo* functions. This receptor does not mediate estrogenic responses in reproductive organs in mice Otto et al. (2009). It is critical to consider the validity of the linkage of the in vitro activity to in vivo adverse effects for the evaluation of potential endocrine disrupting activity. Further, data gaps exist regarding the potential organizational and/or activational effects of GPR30 receptor binding and the significance of NMDRs.

Other components of the complex nuclear receptor signaling pathways could be manifested as NMDR in cellular assays. To this point the *in vitro* discussion has largely focused on binding to the ligand binding domain of the receptor, which results in conformational change that creates the coactivator binding site and permitting initiation of a functional transcriptional complex. However the amino-terminal region of the receptor is also transcriptionally active and not regulated directly by ligand-binding.

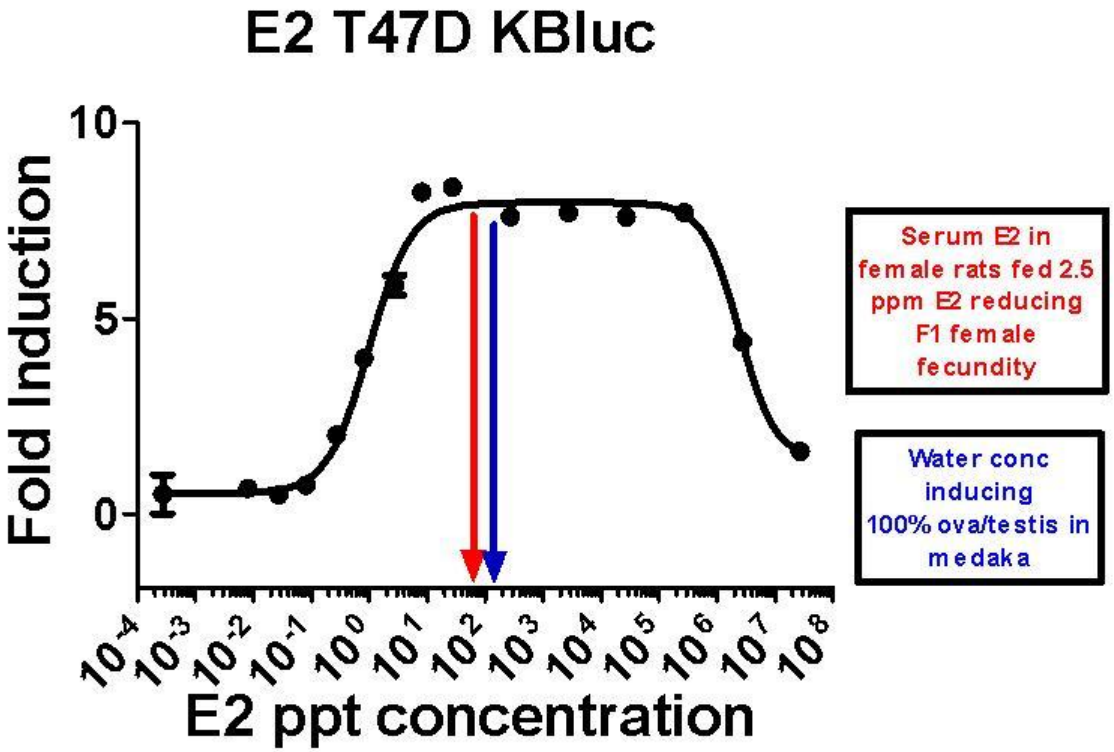
Other signaling pathways such as the MAP kinase pathway or tethering by the aromatic hydrocarbon receptor or other transcription factors such as AP-1 and STAT5, can modulate ER activity through this region and have been reported to generate NMDR. Again, although effects on receptor activity can be demonstrated *in vitro*, there is no evidence that this translates to an *in vivo* effect. As an example, Bulayeva and Watson (2004) show results for a group of reported xenoestrogens and estrogen on the MAP kinase signaling pathway. Effects on phosphorylation of ERK were shown at subpicomolar concentrations followed by a decline in activity, and then an increase in activity for some of the chemicals at higher concentrations. However, no positive

control for the MAP kinase pathway, such as epidermal growth factor, was used. Hence the marginal 20-40% maximal increase in signal over basal conditions in this in vitro assay system cannot be put into the context of effects *in vivo* for the endocrine or any other physiological system.

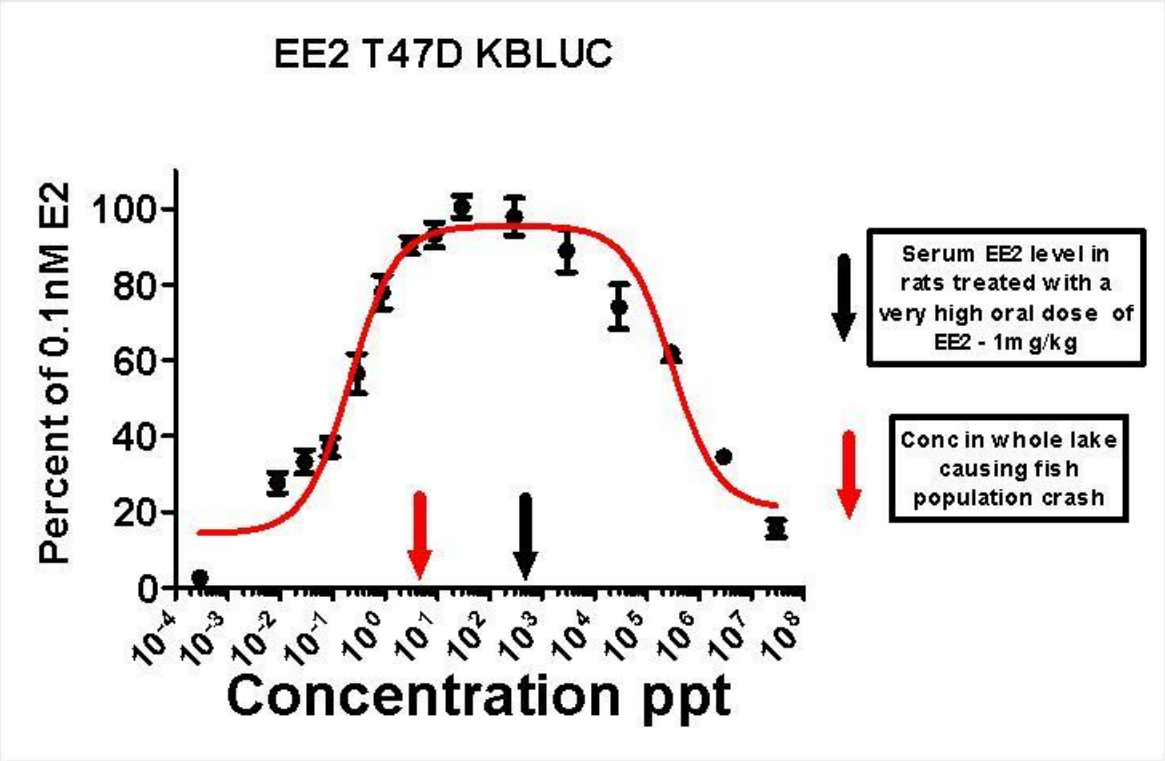
The point of inflection for *in vitro* nonmonotonic curves is typically seen at high doses above the dose range known to cause overt effects *in vivo* or that are measured in environmental samples (Figure 3.1). Therefore, a key question is how relevant is the *in vitro* concentration at the nonmonotonic point of inflection when compared to *in vivo* tissue levels or environmental exposures? A comparison of the *in vitro* concentration-response curve for E2-induced luciferase activity is presented in Figure 3.1a. Superimposed on the graph are the doses at which *in vivo* reproductive effects for apical endpoints have been seen in mammals Biegel et al. (1998b; Biegel et al. (1998a) and fish Hirai et al. (2006). In this example the reproductive effects occur at exposures more than three orders of magnitude lower than the *in vitro* high dose point of inflection at which the curve turns downward. Similar differences between *in vitro* points of inflection and *in vivo* or environmental exposure concentrations exist for many chemicals, including EE2, BPA, Trenbolone, and Testosterone (Figures 3.1b-e). In our assessment of the literature, no examples were identified wherein the point of inflection *in vitro* was near or below the concentration producing adverse effects in animals.

In summary, NMDRs are sometimes seen with *in vitro* assays measuring estrogen and androgen receptor signaling pathways. However, these are generally resulting from assay artifacts, cytotoxicity, or improperly controlled experimental conditions, in particular lack of appropriate positive controls. None of the reported *in vitro* effects relating to NMDRs appear to have significant evidence linking them to *in vivo* adverse outcomes. The majority of the evidence linking *in vitro* assays to *in vivo* effects is readily explained by the well described pharmacology of the steroid hormone receptors. Even the complex phenomena of selective receptor modulators follow law of mass action behavior and can be translated from *in vitro* systems to *in vivo* effects as exemplified by the selective estrogen receptor modulator (SERM) raloxifene, which shows *in vitro* agonistic activity for bone-derived cell types but antagonist activity for uterine and mammary cells, and tamoxifen, which shows antagonist activity towards mammary cells but agonist for uterine Shang and Brown (2002). *In vitro* systems do provide effective means for measuring potential *in vivo* activity for important endocrine targets. While one can identify specific cases where NMDRs could pose technical challenges for testing protocols, *e.g.,* testing for estrogenic activity only at a single very high concentration where assay interference (cytotoxicity or artifact) resulted in a false negative interpretation, these situations would be adequately addressed by testing a broad concentration-response that covered a range from picomolar to hundreds of micromolar.

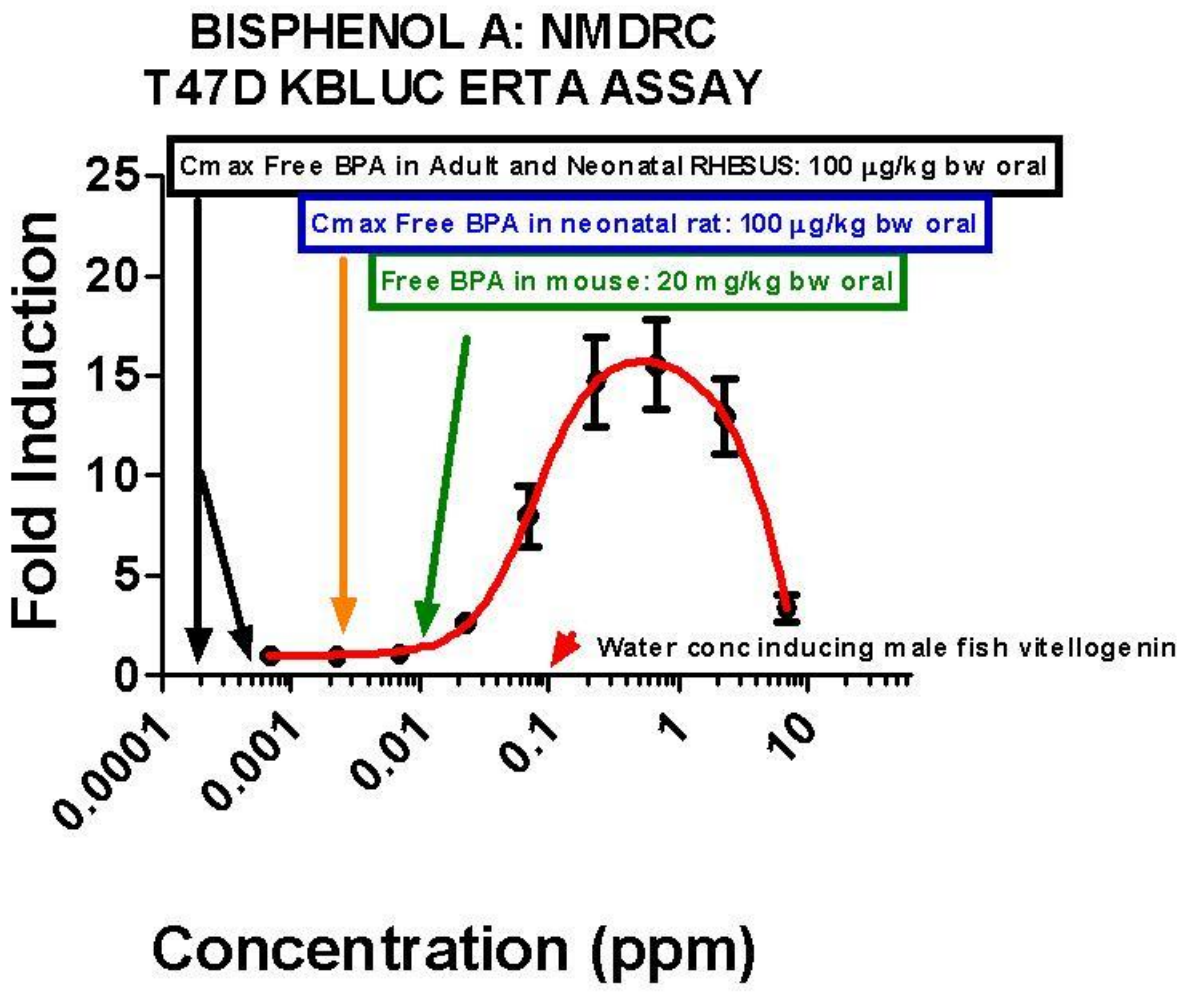
Figure 3.1: Comparisons of *in vitro* points of inflection and *in vivo* or environmental exposure concentrations using T47D KBluc cell luciferase activity induction.



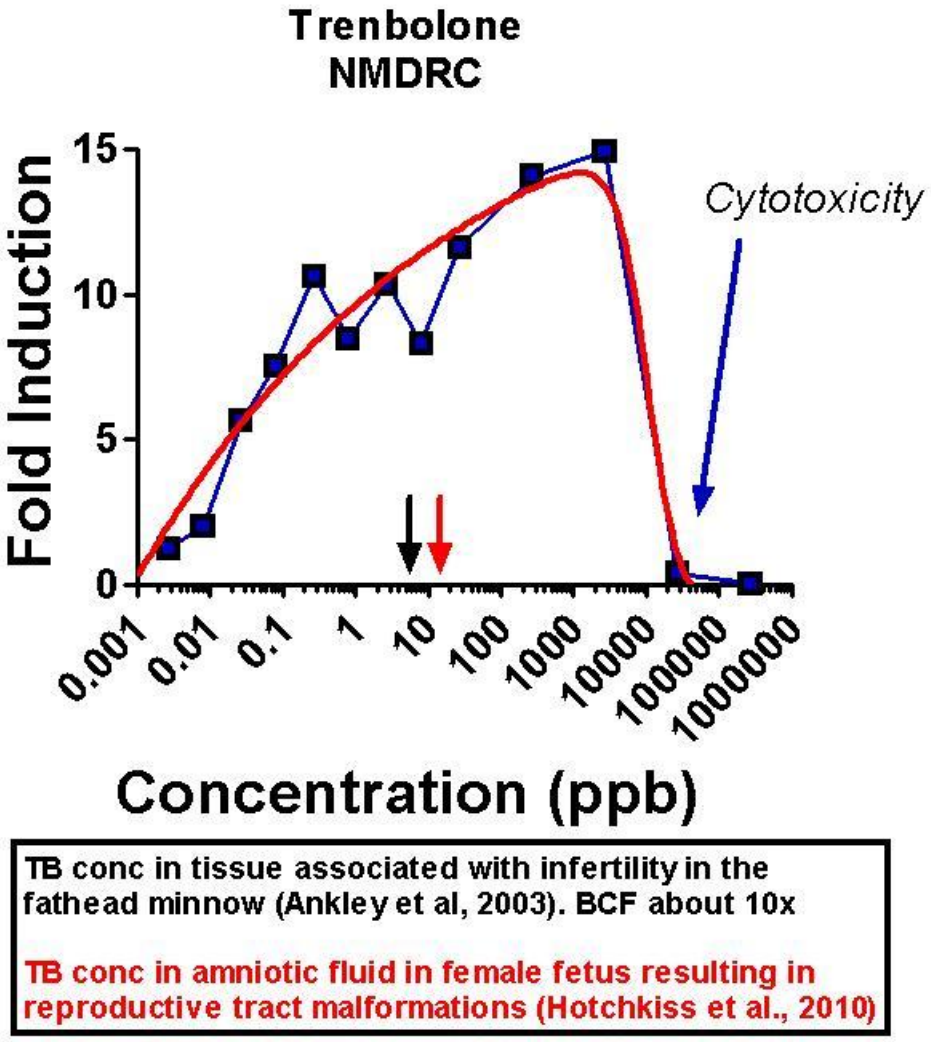
A. Comparison of the *in vitro* concentration-response curve for estradiol (E2) with concentrations found in the serum of acyclic female rats and water concentrations inducing ova-testes in 100% of the male fish. *In vitro* data from Wilson (personal communication), rat data from Biegel et al. (1998b); Biegel et al. (1998a) and medaka data from Hirai et al. (2006).



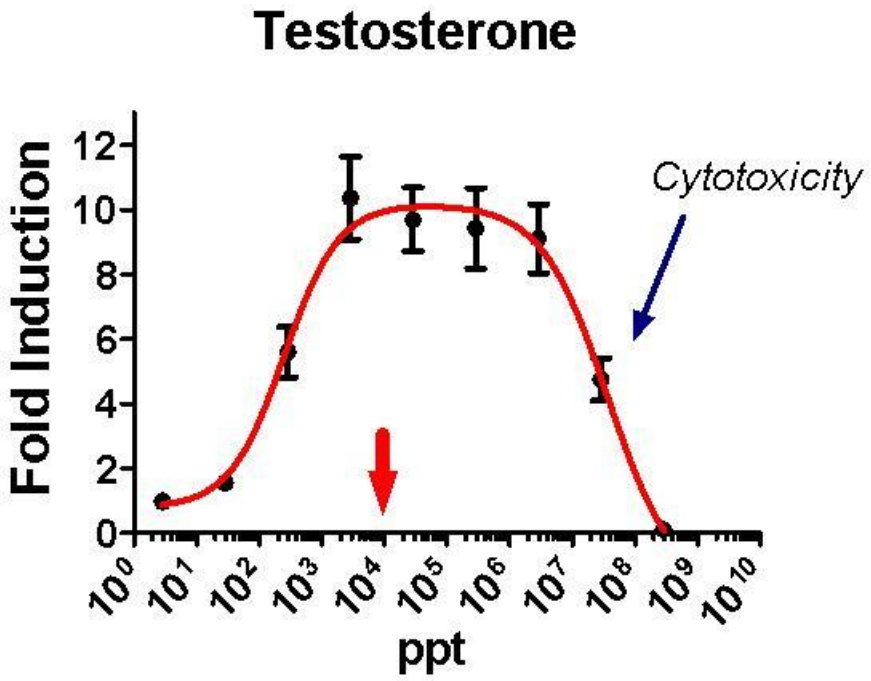
B. Comparison of the *in vitro* concentration-response curve for ethinyl estradiol (EE2) with concentrations found in the serum of rats treated with a very high dose of EE2 and water concentrations causing a population crash and near extinction of fathead minnows in a seven year whole lake study. *In vitro* data from Wilson (personal communication), rat data from Twaddle *et al.* (2003) and fathead minnow data from Kidd *et al.* (2007).



C. Comparison of the *in vitro* concentration-response curve (Wilson, personal communication) for free bisphenol A (BPA) with concentrations found in the serum of neonatal rats Doerge et al. (2010c; Doerge et al. (2010a) and neonatal and adult monkeys and mice Sieli et al. (2011; Doerge et al. (2010b) treated orally with a high doses of BPA (compared to human exposure levels) and water concentrations of BPA causing estrogenic effects in fathead minnow Ankley et al. (2010).



D. Comparison of the *in vitro* concentration-response curve for trenbolone (TB) with concentrations found in the amniotic fluid of rats treated with a dose of TB that induces reproductive tract malformations in female rat offspring Hotchkiss et al. (2010) and tissue concentrations in adversely affected adult fathead minnows exposed to TB in the water. *In vitro* data from Wilson (personal communication), rat data from Hotchkiss et al. (2010) and fathead minnow data from Ankley *et al.* (2010).



E. Comparison of the *in vitro* concentration-response curve for testosterone with concentrations found in the serum of pregnant rats on gestational day 19, treated with a dose of testosterone that induces reproductive tract malformations in female rat offspring Wolf et al. (2002).

4. NMDRs from *in vivo* Studies

4.1 Aquatic Models

The occurrence of NMDRs has been well documented in the field of ecotoxicology e.g., Kefford et al. (2008). And, as observed for some types of human health-oriented tests with EDCs, there are examples of NMDRs generated with endocrine-active chemicals in ecologically-relevant, non-mammalian species. In general, however, the occurrence of NMDRs in tests contributing to ecological risk assessments have not been as significant an issue as they are for human health assessments. There are multiple reasons for this. First, and probably most importantly, selection of concentrations for ecotoxicology testing typically includes actual or predicted chemical concentrations observed in the field. Thus, environmentally relevant dosing may be part of protocol development so that there is little need to extrapolate from high- to low-dose

effects; the latter are measured directly. Second, at least in some instances, there is less of a challenge in ecotoxicology relative to extrapolation of dose-response relationships across species. That is, it often is possible to directly evaluate the species of concern, or closely-related taxa, which is not the case for human health assessments. Finally, the occurrence of NMDRs in ecological risk assessments historically has been of lesser concern than for human health because a comparatively greater degree of uncertainty has generally been acceptable for the former. Tests that have been conducted with non-mammalian species lend insights as to the nature, frequency, and mechanistic underpinnings of NMDRs.

4.1.1 Literature Search and Analysis, Aquatic Species

We undertook a relatively focused literature search and associated analysis from which were identified illustrative examples of dose-responses relationships (including NMDRs) for different classes of HPG-active chemicals. These included those that interact with the estrogen and androgen receptors as well as inhibitors of enzymes involved in steroid synthesis, such as aromatase. For the present analysis we sought to identify studies encompassing full life-cycles or, when not available, longer-term experiments during portions of the life-cycle expected to be sensitive to endocrine-active chemicals. For example, in the present effort short-term lethality studies were not assessed. In addition, where possible we focused on studies that examined apical endpoints clearly related to endocrine function (*e.g.*, aspects of early development or active reproduction). Ideal study would have at least four treatment groups, however, in some instances studies with fewer doses were included for evaluation in order to include additional chemicals and pathways in the analysis.

To facilitate an efficient literature survey, we focused on a subset of 28 model chemicals (Table 4.1) that are known to interact with HPG pathways of concern for which, at least some data exist for non-mammalian species in the peer-reviewed literature. By far the best studied HPG-active chemicals in fish are ER agonists, xenoestrogens. The two most commonly tested compounds are 17α-ethynylestradiol (EE2) and BPA, both of which were included in our analysis. We also were able to identify a longer-term, multi-dose fish study with the SERM, tamoxifen. Chemicals selected as model ARagonists for the analysis were the synthetic steroids 17β-trenbolone (TB) and 17α-methyltestosterone (MT). Flutamide and vinclozolin were evaluated as prototype AR antagonists. Finally, fish studies with fadrozole, trilostane, prochloraz and ketoconazole are included as illustrative examples of the nature of dose-response curves with known inhibitors of sex steroid synthesis.

Table 4.1: Model Chemicals for Aquatic Models Assessment .

ESTROGEN SIGNALING PATHWAY	ANDROGEN SIGNALING PATHWAY

- Estrogens
  - Ethinyl Estradiol
  - Estradiol
  - Diethylstibestrol
  - Genistein
  - Zearalenone and Zeranol
  - Bisphenol A
  - Methoxychlor
  - Chlordecone
  - Octylphenol
  - Nonylphenol
- Selective Estrogen Receptor Modulators
  - Raloxifene
  - Tamoxifen
  - Lasofoxifene
- Aromatase Inhibitors
  - Fenarimol
  - Letrozole
  - Fadrozole
  - Anastrazole
  - Exemestane
- Androgen Receptor Antagonist
  - Flutamide
  - Vinclozolin
- 5-alpha reductase inhibitors
  - Finasteride
  - Semicarbazide
- Inhibitors of P450 enzymes in steroidogenesis
  - Prochloraz
- Androgen Receptor Agonists
  - Testosterone
  - Trenbolone
- Selective Androgen Receptor Agonists
  - Ostarine
  - Andarine
- Phthalates

To obtain representative literature for our analysis, a multi-phased literature search was conducted. Initially, a personal ProCite database maintained by one of the authors of the document (GTA) was searched for studies with non-mammalian species using the 11 chemicals identified in the previous paragraph. This database has been maintained for more than 20 years, and contains about 10,000 entries on different topics in environmental toxicology. To generate additional coverage, searches focused on effects of the 11 target chemicals, predominantly in fish, were conducted using PubMed, and the ECOTOX database U.S (2007). The ECOTOX database is comprised of greater than 40,000 papers concerning the toxicity of chemicals to, primarily, aquatic species. Prior to inclusion in the ECOTOX database, all papers and their data are reviewed as to their quality using a set of well-defined criteria U.S (2007).

The examples and the associated analyses described in Section 4 relative to HPG-active chemicals were restricted largely to studies with fish for practical considerations (i.e., available data), and because there is well-documented structural and functional conservation of most characteristics of the HPG axis across vertebrate classes (Norris 2006). Although there are examples of NMDRs for putative EDCs in invertebrate species e.g., BPA in snails; Oehlmann et al. (2006), there is substantial uncertainty as to whether chemicals known or suspected to impact HPG function in vertebrates operate via similar pathways in invertebrates. We also restricted the scope of our review and analysis to those chemicals with adequately described mode(s) of action as they pertain to the HPG axis. For example, due to uncertainties associated with the data from atrazine exposed amphibians and fish, we did not include those in our analysis Tillitt et al. (2010; Hayes et al. (2003).

Additional studies were reviewed to assess the occurrence of NMDRs associated with the thyroid hormone system of fish and amphibian species. Numerous chemicals have been investigated for thyroid effects in fish and amphibians. Among these are chemicals of environmental concern with relatively little information regarding thyroid activity, as well as recognized thyroid disrupting chemicals with relatively well established mechanisms of action. Among the established mechanisms of action operative in thyroid disruption, this review focused on those chemicals which have been shown to inhibit the sodium-iodide symporter (NIS) or the thyroid peroxidase (TPO) enzyme as the molecular initiating event. Both NIS transport and TPO activity represent critical steps in TH synthesis, and their inhibition can result in a hypothyroid state. Perchlorate is a well known competitive inhibitor of NIS, which concentrates iodide in the thyroid follicular cell. Methimazole and propylthiouracil (PTU) are well known inhibitors of TPO, the enzyme that covalently binds iodide to tyrosine residues of thyrogobulin and links two iodinated tyrosines to form iodinated thyronines, primarily as T4, in the thyroid follicular lumen.

Published papers were identified through electronic searches (PubMed and ECOTOX) using both chemical names such as, PTU, ethylene thiourea, propylthiourea, methimazole, perchlorate, and protein names such as, sodium iodide symporter, NIS, thyroperoxidase, and TPO. Searches were taxonomically limited to fish and amphibian species. References in acquired papers were also reviewed for relevant publications.

The endpoints used to determine thyroid disrupting effects typically include: circulating T3 and/or T4 concentrations, thyroid gland histology, and selected expression of genes associated with thyroid hormone homeostasis and action. Some studies included developmental endpoints. Nearly all studies evaluated the effect of the test chemical on survival and growth, measurements of weight and/or length, as a general indicator of toxicity. Studies that did not report thyroid-specific endpoints were excluded, as were studies with single concentrations of the test chemical. Statistical tests used in the studies were accepted as reported.

Table 4.1: Select studies from literature review displaying some evidence of non-monotonicity.

All studies are described in detail in the main text (pages 59-69).

Chemical	Species (Life Stage)	Concentration or Dose	Route	Test Type (Duration)	References
Ethinyl estradiol	Fathead minnow (all)	0.32, 0.96, 3.5, 9.6, 23 ng/L	Water	Full Life Cycle (150 days)	Parrott and Blunt (2005)
Bisphenol A	Zebrafish (adult)	0.01, 0.1, 1, 10, 100 µg/L	Water	Short Term (4 days)	Villeneuve *et al.* (2012)
Bisphenol A	Fathead minnow (adult)	0.01, 0.1, 1, 10, 100 µg/L	Water	Short Term (4 days)	Villeneuve *et al.* (2012)
Bisphenol A	Fathead minnow (adult)	1, 16, 160, 640, 1280 µg/L	Water	Partial Life Cycle (164 d)	Sohoni *et al.* (2001)
Tamoxifen	Fathead minnow (all)	0.01, 0.08, 0.18, 0.56, 0.64, 1.8, 5.12, 5.6, or 18 µg/L	Water	Partial (42 d) or Full Life Cycle (284 days)	Williams *et al.* (2007)
Trenbolone	Fathead minnow (adult)	0.005, 0.05, 0.5, 5.0, 50 µg/L	Water	Partial Life Cycle (21 days)	Ankley *et al.* (2003)
Methyl testosterone	Nile tilapia (juvenile)	3.75, 7.5, 15, 30, 60, 120, 240, 480, 600, 1200 mg/kg	Diet	Partial Life Cycle (28 days)	Phelps and Okoko (2011)
Methyl testosterone	Zebrafish (juvenile)	0.026, 0.05, 0.1, 0.26, 0.5, 1.0 µg/L	Water	Partial Life Cycle (40 days)	Orn *et al.* (2003)

Chemical	Species (Life Stage)	Concentration or Dose	Route	Test Type (Duration)	References
Vinclozolin	Goldfish (adult)	100, 400, 800 µg/L	Water	Partial Life Cycle (30 days)	Hatef *et al.* (2012)
Fadrozole	Fathead minnow (adult)	1.85, 5.55, 16.7, 50 µg/L	Water	Short Term (7 days)	Villeneuve *et al.* (2006)
Prochloraz	Zebrafish (juvenile)	32, 38, 75, 100, 150, 300, 320, 600 µg/L	Water	Partial Life Cycle (60 days)	Holbech *et al*. (2012)
Ketoconazole	Fathead minnow (adult)	6, 25, 100, 400 µg/L	Water	Partial Life Cycle (21 days)	Ankley et al. (2007)
Ammonium perchlorate	Zebrafish (adult)	18, 677 mg/L (as perchlorate)	Water	Partial Life Cycle (56 days)	Patino et al. (2003)
Ammonium Perchlorate	Fathead minnow (embryo)	0.85, 8.47, 84.7 mg/L (as perchlorate)	Water	Developmental (28 days)	Crane et al. (2005)
6-Propylthiouracil	Zebrafish (larvae)	2.5, 10, 25, 50 mg/L	Water	Partial Life Cycle (35 days)	Schmidt and Braunbeck (2011)
Methimazole	Fathead minnow (all)	32, 100, 320 µg/L	Water	Partial Life Cycle (84 days)	Crane et al. (2006)
Amitrole	Chinese rare minnow (juvenile)	1, 10, 100, 1000, 10,000 ng/L	Water	Partial Life Cycle (28 days)	Li et al. (2009)
Magnesium perchlorate	Chinese rare	5, 50 µg/L	Water	Partial Life Cycle	Li *et al.* (2011)

Chemical	Species (Life Stage)	Concentration or Dose	Route	Test Type (Duration)	References
	minnow (larvae and adult)	(as perchlorate)		(21 days)	
Ethylenethiourea	X. laevis (larvae)	1.0, 2.5, 10, 25, 50 mg/L	Water	Developmental (90 days)	Opitz et al. (2006)
6-Propylthiouracil	X. tropicalis (larvae)	2, 5, 10, 20, 75 mg/L	Water	Developmental (14 days)	Carlsson and Norrgren (2007)

4.1.2 NMDRs in the Estrogen Hormone System in Aquatic Species

4.1.2.1 Estrogen Receptor Agonists

A variety of estrogens have been studied *in vivo* in fish, with endpoints ranging from molecular and biochemical responses (*e.g.*, induction of the egg yolk protein precursor VTG in males) to apical developmental and reproductive outcomes more typically used in risk assessment. Many of these studies have focused on environmentally-relevant concentrations of the chemicals, sometimes for relatively extended periods of time (*i.e.*, multiple generations). For example, Kidd *et al.* (2007) conducted a field study in which a whole lake was treated with EE2 for three years (during the summer months), and impacts on the entire system (including fish populations) were assessed.

Probably the most studied xenoestrogen in fish is EE2, which has been associated with feminization of males exposed to municipal waste water treatment plants Purdom et al. (1994) Routledge et al. (1998). A number of full and partial life-cycle tests have been conducted with EE2 in a variety of fish species, including fathead minnows, zebrafish and medaka, all well-characterized small fish models. There have been several independent analyses of these studies in an attempt to derive robust predicted no-effect water concentrations (PNECs) for reproductive effects of EE2, as a basis for generating protective water quality criteria for the estrogen Caldwell et al. (2012; Caldwell et al. (2008; U.S (2008). These study reviews involved critical evaluation of both the experimental designs employed (*e.g.*, use of multiple EE2 concentrations, including those relevant to the environment) and quality of the data generated (*e.g.*, analytical confirmation of EE2 concentrations in the test water, validity of methods used to measure endpoints, and nature of concentration-response relationships). Based on data from approximately 10 of the highest quality EE2 chronic fish studies, a predicted no effect concentrations (PNEC) for adverse apical effects of 0.1 ng EE2/L has been proposed, which is a concentration lower than that reported in effluents, but above that typically found in surface waters Caldwell et al. (2012). NMDRs were identified in these long-term studies, and one of them reported nonmonotonic data at an EE2 test concentration below 1 ng/L. Parrott and Blunt (2005) conducted a full life-cycle (150 day) test with the fathead minnow using EE2 water concentrations of 0, 0.32, 0.96, 3.5, 9.6 and 23 ng/L. Concentrations above 3.5 ng/L resulted in substantial reproductive effects, with essentially no viable eggs produced. Fecundity (production of eggs) exhibited an NMDR as it was significantly greater than control values in the 0.32 and 0.96 treatments, but fertility also was reduced in these treatments; these off-setting observations served to mitigate overall EE2 impacts in terms of total number of viable embryos (*i.e.*, number of fertile eggs). One complication relative to interpretation of the Parrott and Blunt (2005) study was that EE2 test concentrations were not confirmed analytically in the 0.32 and 0.96 ng/L treatment groups. Overall, considered in isolation, it would be difficult to derive

a robust PNEC based on the shape of the low-end dose-response data in the Parrott and Blunt (2005) study, however, when combined with other available chronic fish data, Caldwell et al. (2012; Caldwell et al. (2010) felt that the derived value of 0.1 ng EE2/L would be protective of effects on reproduction.

BPA acts both as an ER agonist and AR antagonist in fish Ekman et al. (2012), but most of the work conducted concerning endocrine-related effects of BPA in fish has focused on the chemical's estrogenic properties. Like EE2, BPA is commonly detected in a wide variety of environmental matrices for a review see Staples et al. (1998). BPA is much less potent than EE2 relative to effects in fish but, nonetheless, estrogenic responses (*e.g.*, induction of VTG) have been observed at the low µg/L water concentrations of BPA occasionally encountered in the environment Kang et al. (2007). Villeneuve et al. (2012) reported a relatively unusual dose-response relationship for BPA in studies with the fathead minnow and zebrafish. In those experiments, reproductively-active male and female fish were exposed for 4 d to 0.01, 0.1, 1, 10 or 100 µg BPA/L water, following which ovarian gene expression (determined using microarrays) and plasma VTG concentrations were assessed. In both zebrafish and fathead minnows, NMDRs were observed for expression of ovarian genes. Specifically, the greatest number of differentially-expressed genes, by far, was observed in the 10 µg/L treatment group. In the fathead minnow, the second largest number of differentially-expressed genes after the 4-d exposure was seen in fish exposed to 0.01 µg BPA/L. In contrast to the nonmonotonic effects of BPA on gene expression, there was a concentration-dependent induction of VTG in BPA-exposed males of both species, with the threshold for response occurring in the 10 µg/L treatment group. Plasma VTG also was induced in a monotonic fashion in fathead minnow females; there was no indication of increased VTG in zebrafish females.

There also have been longer-term experiments concerning the effects of BPA on endocrine-mediated responses and reproductive success in the fathead minnow Mihaich et al. (2012; Sohoni et al. (2001). Both studies exposed reproductively-active adult fish to BPA for 164 d at water concentrations of 1, 16, 160, 640, and 1280 µg/L by Sohoni et al and at 1, 16, 64, 160, and 640 µg/L by Mihaich *et al.* (2012). Reductions in egg production were observed only at 1280 µg/L; however, changes in the relative gonad weight (gonadal-somatic index; GSI) and histology also occurred at lower test concentrations. Plasma VTG in both sexes was induced at BPA concentrations of > 64 µg/L. Most endpoints in the two studies exhibited monotonic concentration-response relationships. One clear example of an NMDR, however, was the GSI of females in the Sohoni *et al.* (2001) study. After 43 d of exposure GSI was slightly, but significantly greater than controls in after treatment with 1, 160 and 640 µg BPA/L, but was not affected in animals exposed to 16 or 1280 µg BPA/L. The cause of this is uncertain, but it is noteworthy that by conclusion of the assay (164 d) there was a significant monotonic (negative) relationship between BPA exposure concentration and female GSI. Another example of a

possible NMDR from the Sohoni *et al.* (2001) study involved the relative distribution of testicular male sex cell types across the various treatment groups at 164 d, however, Mihaich *et al.* (2012) questioned the functional significance of this observation since successful egg production and fertility occurred at all but the very highest BPA concentration.

4.1.2.2 Selective Estrogen Receptor Modulator (Tamoxifen)

One fish study was identified in which the long-term toxicological effects of the SERM tamoxifen were examined Williams et al. (2007). Partial (42–d) and full-life (284 d) life-cycle tests were conducted with fathead minnows exposed to tamoxifen concentrations in the water ranging from 0.01-18 µg/L (0.01, 0.08, 0.18, 0.56, 0.64, 1.8, 5.12, 5.6, or 18 ug/L, depending on stage in the lifecycle). Very limited monitoring data (from the UK) suggested that tamoxifen could occur in surface water at a concentration as high as 0.2 µg/L. In the 42-d study, conducted with sexually mature fish, tamoxifen decreased plasma VTG concentrations in females to about the same degree at test concentrations of 0.56 µg tamoxifen/L and higher, which is consistent with antagonism of the estrogen receptor. However, in the full life-cycle test, tamoxifen did not affect VTG concentrations in F0 or F1 females at test concentrations up to, and including 5.12 µg/L. Interestingly, the SERM induced VTG in a dose-dependent manner in F0 males exposed to 0.64 and 5.12 µg tamoxifen/L, as well as in F1 males in the 5.16 µg/L treatment group. The drug did not affect any endpoints related to reproduction (egg production, hatching success) at concentrations < 5.12 µg/L. There were some effects of tamoxifen on larval (F1) size (weight, length) in the higher dose groups (0.08, 0.64, 5.12 µg/L), but this effect was transitory in that it was observed 28 d post-hatch, but not at 112 d. Larval size at 112 d was smaller in fish exposed to 0.01 µg tamoxifen/L than in controls or the other treatment groups. It is uncertain, however, whether this seeming nonmonotonic growth response should be considered biologically significant Williams et al. (2007), and/or whether it is related to estrogen receptor-mediated responses to tamoxifen in the fish.

4.1.3 NMDRs in the Androgen Hormone Pathway in Aquatic Species

4.1.3.1 Androgens

Compared to chemicals that interact with estrogen receptors (primarily agonists), less is known concerning the toxicological effects of AR agonists in long-term fish tests. Although fewer in number, we identified several full and partial life-cycle tests with fish exposed to the synthetic steroids Trenbolone and methyl-testosterone, including some examples of NMDRs.

Ankley et al. (2003) describe a study in which effects of the steroidal androgen TB, a growth-enhancer used for livestock production, was evaluated at multiple biological levels of

organization in reproductively-active fathead minnows, resulting in NMDRs for some biochemical endpoints but monotonicity of apical endpoints. The fish were exposed to water-borne TB concentrations spanning 5 log units (0.005, 0.05, 0.5, 5.0, or 50 µg/L) for 21 days. Based on studies at beef feedlots, the lower two test concentrations would be considered environmentally-relevant Durhan et al. (2006). Consistent with the anabolic nature of TB, body weight of exposed female fathead minnows was increased in a concentration-dependent manner. The androgen also caused morphological masculinization of the females, resulting in a concentration-dependent induction of male-like dorsal nuptial tubercles. Exposure to TB had relatively rapid and profound effects on cumulative egg production in the fish, with monotonic response. In contrast to these apical responses, several key biochemical measures of HPG function monitored in TB-exposed female fathead minnows exhibited a U-shaped dose-response relationship, with decreased responses occurring at the higher test concentrations. There was some evidence of this relative to plasma VTG concentrations, with much more pronounced nonmonotonic relationships for plasma 17β-estradiol (E2) and, especially, testosterone (T) concentrations (Fig. 4.1a-c). The fact that these three endpoints are functionally related to one another lends veracity to the nature of the U-shaped dose-response curve. Specifically, T is the metabolic precursor to E2 (via a reaction catalyzed by cytochrome P450 aromatase [CYP19]), and E2 activation of hepatic estrogen receptors is responsible for VTG production in fish. The basis of the U-shaped dose-response relationship(s) is uncertain, but the response is suggestive of some sort of compensatory mechanism in the fish. However, further exploration of this possibility would require measurements of basic endocrine function and signaling (*e.g.*, temporal changes in gonadotrophins) not included in the original study.

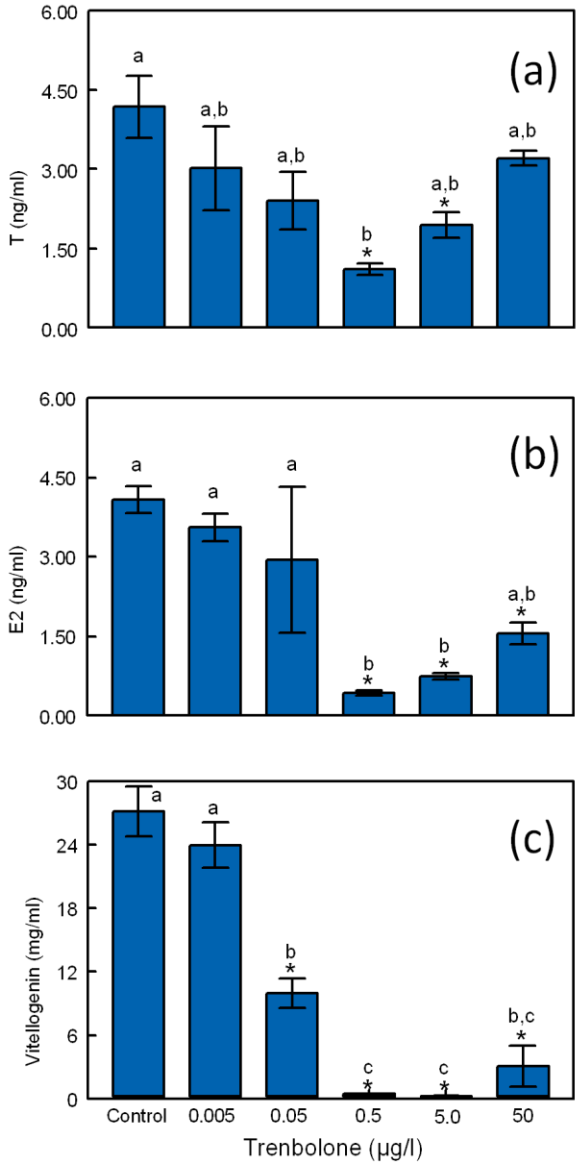


Figure 4.1: Effects of a 21-d water-borne exposure to 17b-trenbolone on plasma concentrations of (a) testosterone (T), (b) 17β-estradiol (E2) and vitellogenin in female fathead minnows.
Ankley et al. (2003)

Following the work by Ankley et al. (2003) several other researchers have also used TB as a model androgen in fish studies. For example, Seki et al. (2006) described a study in which adult male and female fathead minnow, Japanese medaka and zebrafish were exposed to TB for 21-d at water concentrations of 0.05, 0.5 and 5 µg/L. Consistent with expectations, the androgen masculinized female fathead minnows and medaka, and depressed (female) plasma VTG concentrations in all three species. Absolute sensitivity relative to impacts of TB on these endpoints was species-specific, but all responses identified were monotonic. Hemmer *et al.* (2008) also conducted a 21-d study with the sheepshead minnow, an estuarine species,

exposed to 0.005, 0.05 and 5 µg TB/L water. There were no significant effects noted at the lower two TB concentrations, but 5 µg/L significantly reduced egg production. Decreased egg production corresponded with reduced plasma VTG concentrations in female sheepshead minnows although, due to among individual variability, this response was not statistically significant. Cripe *et al.* (2010) assessed the effects of TB on sheepshead minnow reproduction in a three-generation (42 week) experiment that started with reproductively mature adults (F0) and concluded with an assessment of reproductive success of F2 animals. Target water concentrations were 0.01, 0.04, 0.2, 1.0 and 5.0 µg TB/L. Some of the somatic measurements exhibited nonmonotonic relationships. For example, F1 and F2 fish from the two lowest TB treatments were longer than the controls by 90 or 28 dph, respectively, and the GSI of F0 females was larger in fish from the 1.0 than 5.0 treatment group than in untreated animals. However, these types of variations did not seem to be systematic. Apical endpoints directly related to reproduction were monotonic when different from control in the study by Cripe *et al.* (2010). Although there were differences in the absolute sensitivity of the fish across the three generations (i.e., in terms of lowest observed effect concentrations of TB), effects on cumulative fecundity, fertility, embryo hatch, and percentages of abnormal embryos and infertile eggs all exhibited monotonic dose-response relationships. Cripe *et al.* (2010) also noted depressions in plasma VTG concentrations in F0 females from the highest two TB treatment groups.

Effects of TB also have been evaluated in sexual development assays with zebrafish. Holbech *et al.* (2006) exposed newly-fertilized zebrafish eggs to target concentrations of 0.005, 0.05, 0.5 or 5 µg TB/L for 59 d, following which they evaluated phenotypic (gonadal) sex of the fish. In all but the lowest treatment group 100% of the zebrafish were scored as males at the end of the test—there was no indication of non-monotonicity in the responses. Morthorst *et al.* (2010) conducted an experiment and published a set of results qualitatively similar to that of Holbech *et al.* (2006), except that they monitored sex of the fish (held in clean water) for up to 170 d post-exposure. Effects of the TB on sexual differentiation were irreversible in that timeframe.

Steroidal chemicals, both estrogens and androgens, have been used for many years in fish culture to produce mono-sex populations with desirable attributes such as enhanced growth. Although a substantial amount of work has been done in this area, the types of studies conducted to support aquacultural practices have limited relevance to assessing potential toxicological properties and risks of endocrine-active chemicals for a number of reasons: (1) exposure often is dietary, rather than via the water, leading to uncertainties as to actual dose (supporting, for example, extrapolation of results to field settings); (2) concentrations of chemicals added to the diet typically are quite large—much higher than could possibly occur in the environment; and (3) many of the steroids used for aquaculture have not been implicated as environmental contaminants.

A recent study by Phelps and Okoko (2011) reflects all of these characteristics, but nonetheless is quite interesting in terms of producing a relatively novel NMDR. In that study Nile tilapia fry were fed diets containing 3.75, 7.5, 15, 30, 60, 120, 240, 480, 600, or 1200 mg/kg of methyltestosterone (MT, a very commonly used steroid in aquaculture) for 28d, following which the animals were sexed by examination of the gonads. A robust inverted U-shaped dose-response was observed, wherein intermediate doses produced completely male populations, while in the control and high-dose groups fish exhibited a normal sex ratio of around 50:50 (Figure 4.2). Past studies with MT have shown that it can be converted to methylestradiol in fish (via CYP19), thereby producing seemingly "paradoxical" estrogenic and androgenic responses simultaneously Hornung et al. (2004; Ankley et al. (2001). However, Phelps and Okoko (2011) did not think that this adequately explained the unusual dose-response they observed. Further work would be required to determine the mechanistic basis of this NMDR.

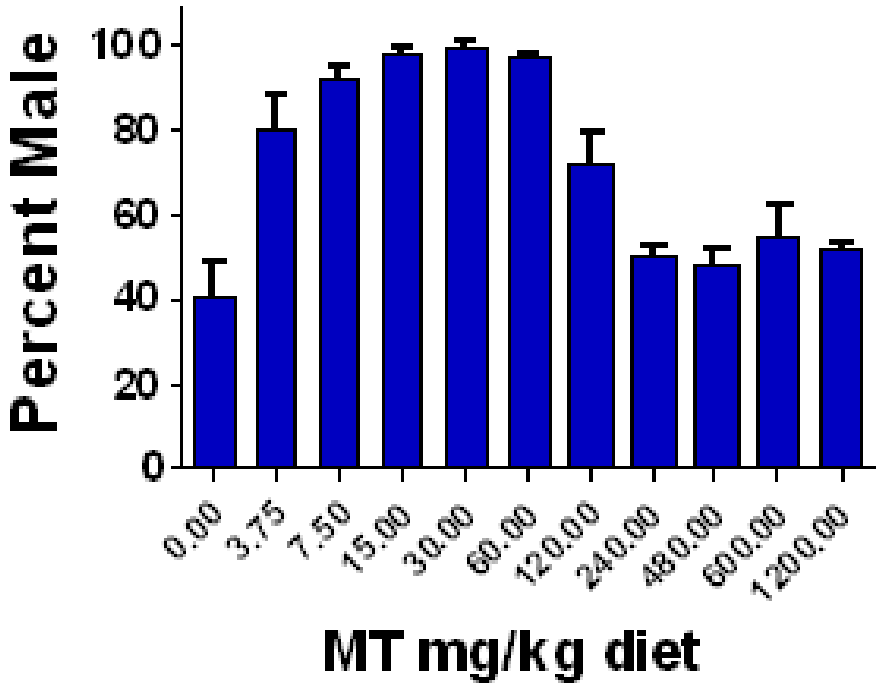


Figure 4.2: Effects of a 28-d dietary exposure to methyltestosterone on sex ratio in Nile tilapia.
Phelps and Okoko (2011))

Other more toxicologic studies with MT have not typically produced NMDRs. For example, Pawlowski *et al.* (2004) exposed adult fathead minnows to water concentrations of 0.1, 1, 5 and 50 µg MT/L for 21 d during gonadal recrudescence (moving from a "winter-type" non-spawning condition to a "summer-type" condition, through temperature and photoperiod manipulation),

following which a number of endpoints related to HPG function were measured. Consistent with previous studies (*e.g.*, Ankley et al. (2001)), MT exhibited dual endocrine activities, masculinizing females (production of male secondary sex characteristics) and feminizing males (inducing VTG). Exposure to MT also inhibited egg production in fish subsequently moved to clean water for 21 d. Effects of MT on secondary sex characteristics, VTG, fecundity and fertility were all monotonic. Kang *et al.* (2008) exposed adult Japanese medaka to 0.0225, 0.0468, 0.0881, 0.188 and 0.380 µg MT/L of water for 21 d. Fecundity and fertility of the fish, and VTG concentrations in females were depressed in a concentration-dependent manner at concentrations > 0.0468 µg/L. The relative gonad weight of females was increased to a relatively similar degree in the four higher treatment groups. Seki *et al.* (2004) conducted a two-generation experiment, also with medaka, at nominal water concentrations of 0.00031, 0.00098, 0.00313, 0.01 and 0.032 µg/L. The test was initiated with newly-fertilized eggs, continued through reproduction by the F0, and concluded after 60 d post-hatch of the F1 generation. The highest test concentration strongly skewed the F0 gonadal sex toward males by 61 d post-hatch. The lower test concentrations did not cause additional marked effects during the F0 generation, but in F1 animals MT induced hepatic VTG in males (0.01 µg/L group), and reduced hepatic VTG in females, to about the same extent, in all the remaining lower treatment groups (i.e., 0.00031-0.01 µg/L).

One toxicologically-relevant study with MT did exhibit a NMDR. Orn *et al.* (2003) exposed juvenile (20 days post-hatch) zebrafish to MT water concentrations ranging from 0.026, 0.05, 0.1, 0.26, 0.5, or 1 µg/L for 40 d, following which gonad morphology and whole body VTG concentrations were assessed. Complete sex reversal occurred in all fish exposed to MT, with no ovaries observed in animals from any treatment group. Whole-body VTG concentrations in the fish exhibited a "U-shaped" dose-response relationship, with smallest values occurring in the intermediate treatment groups. This response could plausibly be linked to the conversion of methyl testosterone to methylestradiol, and subsequent activation of the estrogen receptor at the higher exposure concentrations.

4.1.3.2 Androgen Receptor Antagonists

No fish full life-cycle tests with the AR antagonists flutamide or vinclozolin were identified. However, endocrine-related effects of the two chemicals have been examined in multiple partial life-cycle tests with fish. Bayley *et al.* (2002) fed a diet amended with 0.01 or 1 mg flutamide/kg to guppies from birth to adulthood (ca. 26 wk) and evaluated several endpoints. They found that flutamide caused a skewing of sex ratio toward females (60-70% of the total population) at both doses. Sexual maturation of the males was delayed in a dose-dependent manner, and size of males at maturation was significantly reduced in the high flutamide treatment. Flutamide also caused a decrease in gonopodium length (a male secondary sex

characteristic) in both treatment groups. In a reproduction study with flutamide, Jensen *et al.* (2004) exposed adult fathead minnows via water to 50 or 500 µg flutamide/L for 21-d. Egg production was significantly reduced in the high treatment group, and the flutamide caused slight alterations in plasma sex steroid and/or VTG concentrations in both sexes, but all of these changes were monotonic.

Bayley *et al.* (2002) also examined the effects of the pesticide vinclozolin on development of guppies fed a diet with 0.1 or 10 mg/kg for 26 wk starting at birth. Responses observed were sex ratios biased toward females, delayed maturation and growth in males, and decreased gonopodium length. Vinclozolin also caused a dose-dependent decrease in sperm count in the guppies. In a follow-up study, Bayley *et al.* (2003) reported that male guppies fed a diet containing 0.1, 1 or 10 mg vinclozolin/kg for 6 wk exhibited concentration dependent decreases in sperm count and number of first-clutch juveniles sired. Martinović *et al.* (2008) assessed the effects of vinclozolin on reproductively-active fathead minnows exposed via the water to 60, 255 or 450 µg/L. They found a dose-dependent decrease in egg production in the fish, as well as a depression in normal secondary sex characteristics in males. Other vinclozolin-induced changes in plasma VTG concentrations, sex steroid production and/or expression of transcripts for the androgen receptor occurred in male or female fish, but none of these alterations exhibited NMDRs. Hatef *et al.* (2012) exposed goldfish to 100, 400 or 800 µg vinclozolin/L for 1 month. They noted a significant decrease in sperm quantity and quality in males from the high treatment group. Plasma concentrations of 11-ketoteststerone (a fish-specific androgen) in the males exhibited an NMDR, with significantly elevated levels of the steroid in the 100 µg/L treatment group and depressed levels in the 800 µg/L fish. The increase in 11-ketoteststerone at the lower vinclozolin concentration observed by Hatef *et al.* (2012) might reflect a compensatory response in the fish resulting from reduced androgen signaling. The authors suggested that this increase in 11-ketoteststerone could be responsible for a lack of adverse effects on sperm quantity and quality in the 100 µg/L treatment group.

4.1.3.3 Steroid Synthesis Inhibitors

A number of model chemical inhibitors of sex steroid synthesis have been tested in short-term or partial life-cycle assays with fish. For example, several fish studies have been conducted with the drug fadrozole, a relatively specific inhibitor of CYP19 A, which originally was developed to treat estrogen-dependent breast cancers in humans. In a short-term study, Villeneuve *et al.* (2006) exposed sexually-mature female fathead minnows for 7 d to 1.85, 5.55, 16.7 or 50 µg fadrozole/ L water. They found that fadrozole reduced aromatase activity in both the brain (CYP19B) and ovaries (CYP19A) of the fish. Effects in the brain followed a monotonic dose-response relationship, whereas inhibition of aromatase activity by fadrozole in the gonad exhibited an inverted U-shaped dose response. Fadrozole also produced a concentration-

dependent increase in expression of CYP19A in the ovaries, leading Villeneuve *et al.* (2006) to speculate that the basis of the NMDR was due to interplay between the increased CYP19A transcripts (a compensatory response) and direct inhibition of the enzyme. By contrast, Ankley *et al.* (2002) saw no evidence of NMDRs in a longer-term, partial life-cycle study with fadrozole in adult fathead minnows, conducted at water concentrations of 2, 10 and 50 µg/L. Over the course of the 21 d reproduction test, the drug caused concentration-dependent reductions in egg production in the fish, as well as decreased plasma E2 and VTG concentrations in females. In a study focused on sexual development, Kwon *et al.* (2000) exposed Nile tilapia genetic females to fadrozole via the diet for 30 d starting 7 d post-hatch, and assessed effects on phenotypic sex ratios. They found a dose-dependent increase in the incidence of males at doses ranging from 0 to 200 mg fadrozole/kg food; at dietary concentrations from 200 to 500 mg/kg, the percentage of males remained constant as about 92-96%.

Fish partial life-cycle studies also have been conducted with prochloraz, trilostane and ketoconazole, all known inhibitors of sex steroid synthesis in mammals. Ankley *et al.* (2005) described a 21-d fathead minnow reproduction test with prochloraz, an imidazole designed to inhibit fungal CYP14α-demethylase (CYP51), which catalyzes a key step in ergosterol biosynthesis supporting cell wall formation. Prochloraz, like many other fungicides designed to inhibit fungal CYP51s, also inhibits a variety of vertebrate CYPs involved in steroid production, including CYP19 and CYP17 (hydoxylase/lyase). Fathead minnows exposed to 30, 100 and 300 µg prochloraz/L water exhibited a concentration-dependent decrease in egg production over the course of 21 d, a response coincident with monotonic decreases in plasma E2 and VTG in the female fish Ankley et al. (2005). Similarly, Zhang *et al.* (2008) reported a dose-dependent decrease in hepatic expression of VTG transcripts and egg production in adult Japanese medaka exposed for 7 d to 3, 30 or 300 µg prochloraz/L.

The effects of prochloraz also have been assessed in studies focused on sexual differentiation and development in fish. Kinnberg *et al.* (2007) exposed zebrafish to 16, 64 or 202 µg prochloraz/L water for 60 d starting at 24 h post-hatch. The authors reported a significant shift in sex ratio in fish from the 202 µg/L treatment, with a slight bias toward males. Similar to observations made by Ankley *et al.* (2005) and Zhang *et al.* (2008), prochloraz decreased (whole-body) concentrations of VTG in female zebrafish. Concentrations of VTG in males were depressed in the 202 µg/L group, but they were slightly elevated in the two lower treatment groups. Although this endpoint displayed an NMDR, the biological significance of the response is uncertain as VTG levels in all males from the study were very low (several orders of magnitude below females), and VTG has no known functional role in male fish. Holbech *et al.* (2012) describe an effort focused on standardization of a test protocol for endocrine-active chemicals, in which the effects of prochloraz on sexual development were evaluated in zebrafish and the fathead minnow, in a ring test conducted by five labs. Animals were treated

with prochloraz from days 0 through 60 post-hatch to concentrations of 32, 38, 75, 100, 150, 300, 320, or 600 µg/L water. In both species prochloraz caused a significant skewing of the sex ratio towards males, and a depression of VTG concentrations in females. With one exception, all of the effects data were monotonic. One of the labs testing zebrafish did not observe statistically significant masculinization at the highest prochloraz concentration tested but did see significant masculinization at the next two lower test concentrations. However, two other labs involved in the ring test reported significant masculinization of zebrafish at concentrations comparable to the high treatment group in the first lab Holbech et al. (2012).

Trilostane is a human pharmaceutical designed to inhibit 3β-hydroxysteroid dehydrogenase (3βHSD), which catalyzes the conversion of pregnenolone to progesterone. Villeneuve *et al.* (2008) evaluated the effects of trilostane on endocrine function in several studies with the fathead minnow. Initial studies showed that trilostane effectively inhibited the *in vitro* production of E2 by ovary explants in a dose-dependent manner, while a 21-d *in vivo* exposure at 60, 300 and 1500 µg trilostane/L indicated a concentration-dependent depression of plasma VTG concentrations in females, and a significant decrease in egg production in the high treatment group.

Ankley *et al.* (2007) described a series of fathead minnow studies with ketoconazole, a fungicide capable of inhibiting multiple steriodogenic CYPs in mammals, most notably CYP17 and CYP11A (CYP cholesterol side-chain cleavage). In an initial 7-d range-finding experiment, ketoconazole depressed plasma VTG concentrations in female fish, a response consistent with inhibition of steroid synthesis. In a subsequent 21 d reproduction study, fish were exposed to 6, 25, 100 and 400 µg ketoconazole/L water; *ex vivo* production of T by gonad explants from both sexes was decreased in the three highest treatment groups. However, this did not translate into decreased plasma concentrations of T or E2 in either sex, or VTG in females. The unexpected lack of impact of ketoconazole on these *in vivo* functional measures of HPG status appeared to be due to compensatory responses in the fish, manifested as increased GSI, altered gonad histopathology (*e.g.*, interstitial cell proliferation in males), and up-regulation of genes coding for steriodogenic proteins, including CYP17 and CYP11A. An NMDR was observed relative to effects of ketoconazole on production of eggs, which was significantly depressed in the 25 and 400, but not 100 µg/L treatment groups. It is uncertain whether the compensatory molecular and histological responses were the cause of the nonmonotonic effect of ketoconazole on egg production. Zhang *et al.* (2008) conducted a 7 d reproduction test with 3.0, 30, and 300 ug/L ketoconazole in the Japanese medaka. They reported a significant decrease in egg production at the highest concentration tested (300 µg/L), with no evidence of a nonmonotonic relationship at two lower test concentrations (3 and 30 µg/L).

4.1.3.4 Role of Compensatory Processes in NMDR – Examples from Fish Time-Course Studies with Three EDCs

Some of the fish EDC studies summarized in the previous section are suggestive of the occurrence of NMDRs due to compensatory mechanisms. To explore this hypothesis more directly, data from a series of intensive time-course studies with fathead minnows exposed to different classes of EDCs Ankley et al. (2009a) were reassessed. In the experiments described below, the animals were exposed to fadrozole, prochloraz or TB (Villeneuve Ekman et al. (2011; Ankley et al. (2009b; Villeneuve et al. (2009). Although the three chemicals operate via different molecular initiating events, past studies have shown that they all effectively depress T and/or E2 synthesis and plasma concentrations of VTG in female fathead minnows, ultimately resulting in depressed egg production Ankley et al. (2010).

The time-course studies with fadrozole, prochloraz and TB utilized the same basic experimental design Ankley et al. (2009a). Sexually mature fathead minnows (of both sexes) were continually exposed to two different, analytically confirmed, concentrations of the chemicals via water in a continual-flow system. The two test concentrations used for each chemical were indexed to data from 21-d reproduction studies Ankley et al. (2003; Ankley et al. (2002), such that the high concentration was associated with substantial reproductive impacts (*e.g.*, total cessation of egg production), while the low concentration was expected to have slight (or no) effects on egg production. Fish were sampled 1, 2, 4 and 8 d after initiation of the chemical exposure, and 1, 2, 4 and 8 d after ceasing exposure (*i.e.*, animals were held in constantly-renewed control water). A variety of measurements indicative of HPG function were made, including plasma VTG concentrations, *ex vivo* gonadal production and plasma concentrations of T and E2, and gonadal expression (determined via real-time quantitative polymerase chain-reaction [PCR]) of several genes coding for proteins involved in endocrine signaling and steroid production. Specifically, NMDRs were defined as those wherein one (or both) of the treatment groups differed significantly from the control at a given sampling time, but the relationship of the two treatment groups to one another did not exhibit a monotonic rank order. Studies with more than two test concentrations per time point would have, of course, been preferable for the analysis, but the data from these studies nonetheless illustrate the role of compensatory processes in producing unanticipated dose-response relationships.

Consistent with inhibition of CYP19A, exposure of fish to fadrozole depressed *ex vivo* ovarian E2 production and plasma concentrations of E2 and VTG in female fathead minnows Villeneuve et al. (2009). Reductions in E2 were significant, and concentration-dependent, on days 1 and 2 of the exposure in both the 3 and 30 µg/L treatment groups. While plasma E2 remained depressed in the high treatment throughout the course of the 8 d exposure, in the 3 µg fadrozole/L treatment, E2 concentrations showed significant recovery during the exposure

phase of the test to levels comparable to controls by 8 d. Upon cessation of the fadrozole exposure, plasma E2 concentrations in the 30 µg/L treatment group returned (within 2 d) to control levels, but in the low fadrozole treatment there appeared to be an "overshoot" in plasma E2 concentration relative to controls on day 1 post-exposure, such that there was an NMDR. The behavior of plasma E2 concentrations, especially in the low-dose treatment was indicative of compensation in the female fish. A component of this compensatory response appeared to be related to up-regulation of genes coding for several key proteins involved in steroidogenesis, including CYP19A, CYP11A and steroid acute regulatory protein. There were instances over the course of the study in which expression of one or more of these genes did not vary in a monotonic fashion. For example, on day 1 of the recovery phase of the test, expression of CYP19A and CYP11A transcripts were higher in fish from the 3 µg/L treatment than in controls, while levels of the two gene products in fish from the 30 µg/L treatment group were intermediate to those of the control and 3 µg/L treatment groups.

Prochloraz appears to depress steroid synthesis in fish via inhibition both of CYP19A and CYP17 Ankley et al. (2005). In a time-course study, ovarian *ex vivo* E2 production and plasma E2 concentrations in females both were decreased by exposure to prochloraz Ankley et al. (2009b). After 1 d of exposure, the two test concentrations of the pesticide (30 and 300 µg/L) had depressed plasma E2 concentrations in a dose-dependent manner. Plasma E2 concentrations remained depressed in the high treatment during the 8 d exposure phase of the test, but in the 30 µg/L group, E2 recovered to levels comparable to or exceeding the controls by day 4 of the exposure. *Ex vivo* E2 production by ovarian explants displayed a similar pattern relative to plasma responses observed on day 4, in that E2 synthesis in the low dose group was significantly greater than controls, while production in females exposed to 300 µg prochloraz/L was lower than control values. Hence, the plasma and *ex vivo* E2 data from the day 4 females both exhibit nonmonotonic relationships due to the compensatory overshoot-type response in the 30 µg/L treatment. Interestingly, during the exposure phase of the test, plasma concentrations of VTG were depressed in the high- but not low-dose prochloraz treatment. Up-regulation of several ovarian genes coding for proteins involved in steroid production, including CYP19A, CYP11A and CYP17, were observed in prochloraz-exposed animals Ankley et al. (2009b).

Past experiments have shown that TB decreases plasma T and E2 concentrations in female fathead minnows, presumably through feedback inhibition of steroidogenesis (Fig. 4.1a-c; Ankley et al. (2003)). In the time course study, exposure of females to both test concentrations of TB (0.05, 0.5 µg/L) depressed *ex vivo* E2 production and plasma E2 concentrations within 1-2 d Ekman et al. (2011). Plasma E2 levels in fish from the low-dose group exhibited what was thought to be a compensatory response, rebounding during the exposure phase of the test to concentrations comparable to (or even perhaps exceeding) the controls, while in the high dose

group plasma E2 concentrations in the female fish returned to control levels only after cessation of TB exposure. Plasma VTG concentrations were slightly decreased in the 0.05 µg TB/L group during the exposure, returning to control levels within 1 d of depuration; VTG concentrations in the 0.5 µg/L females were strongly depressed during the chemical exposure, but also recovered to control levels by the latter part of the recovery phase of the experiment. Relative expression of several ovarian genes involved in HPG function changed over the course of the TB exposure. Expression of transcripts for CYP11A in fish from the low dose group were lower, higher and lower than corresponding control values on exposure days 2, 4, and 8, respectively. On each of these occasions, the dose-response relationship between the control and treatment groups was nonmonotonic. There were also several occurrences of NMDR between treatment level and expression of gene products for VTG and the AR in the ovaries of TB-exposed females.

The fathead minnow studies with fadrozole, prochloraz and TB are a subset of a larger group of time-course experiments with different endocrine-active chemicals, several of which show comparable results to those described herein Ankley et al. (2009a). For example, similar response patterns, including the occurrence of NMDRs for endpoints reflecting gene expression and sex steroid status, have been observed with ketoconazole (an inhibitor of multiple steriodogenic CYPs other than, or in addition to, CYP19A) and trilostane, a specific inhibitor of 3β-hydrosysteroid dehydrogenase, which converts pregnenolone to progesterone in the steriodogenic cascade (Ankley *et al*. 2011; 2012). Although these different time-course studies were not designed specifically to generate or examine the basis of NMDRs, they nonetheless are illustrative of the important role of compensation in generating NMDR. Although there were variations among the time-course studies with fadrozole, prochloraz and TB, several common patterns were observed. For example, plasma E2 concentrations (Figs 3b; 4b; 5b) exhibited a consistent depression early (1-2 d) at both the low and high test concentrations. Subsequent to this, but still during the chemical exposure, plasma E2 in the low-dose groups (i.e., at water concentrations associated with minimal effects on egg production in a 21-d reproduction test) exhibited compensatory behavior, returning to (and occasionally exceeding) control levels of E2. In the high dose groups (i.e., at concentrations associated with substantial impacts on egg production over 21 d), plasma E2 concentrations tended not to return to levels comparable to controls until after the chemical exposure had ceased. It was during these periods of active compensation and early recovery when NMDRs for endpoints related to steroid production and/or gene expression were most commonly observed. This pattern suggests, perhaps, that NMDRs may be more prevalent in shorter-term assays, especially during periods of system disequilibrium. Further, these data clearly highlight the time-dependent nature of dose-response relationships.

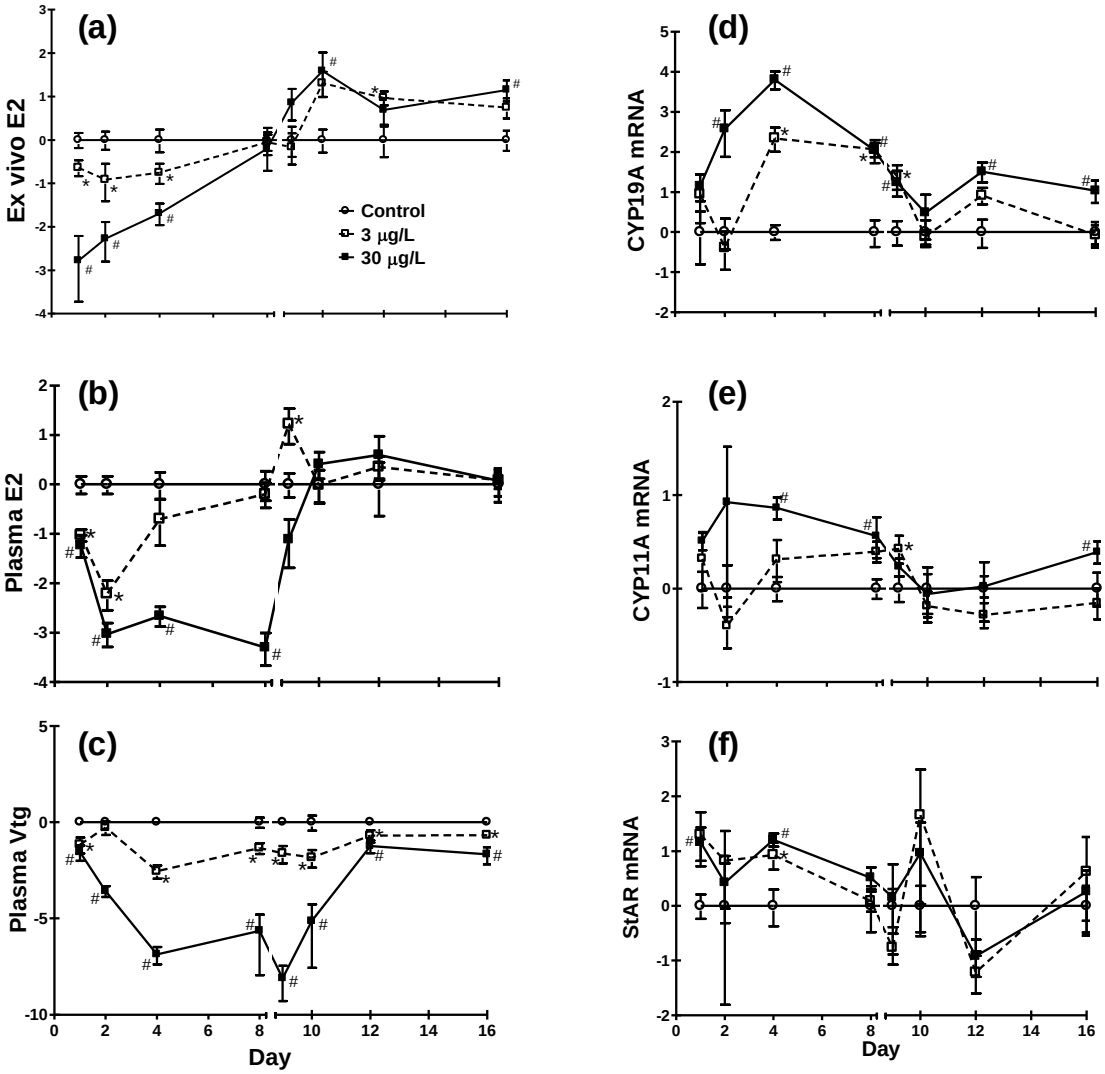


Figure 4.3: Time-course (8-d exposure and 8-d recovery) effects of fadrozole on female fathead minnow.

(a) ovarian synthesis of 17β-estradiol (E2); plasma concentrations of (b) E2 and (c) vitellogenin (Vtg); and ovarian expression of mRNA for (d) cytochrome P450 (CYP) 19A, (e) CYP11A, and (f) steroid acute regulatory protein (StAR). Differences from control are indicated by "#" and "*" from Villeneuve et al. (2009).

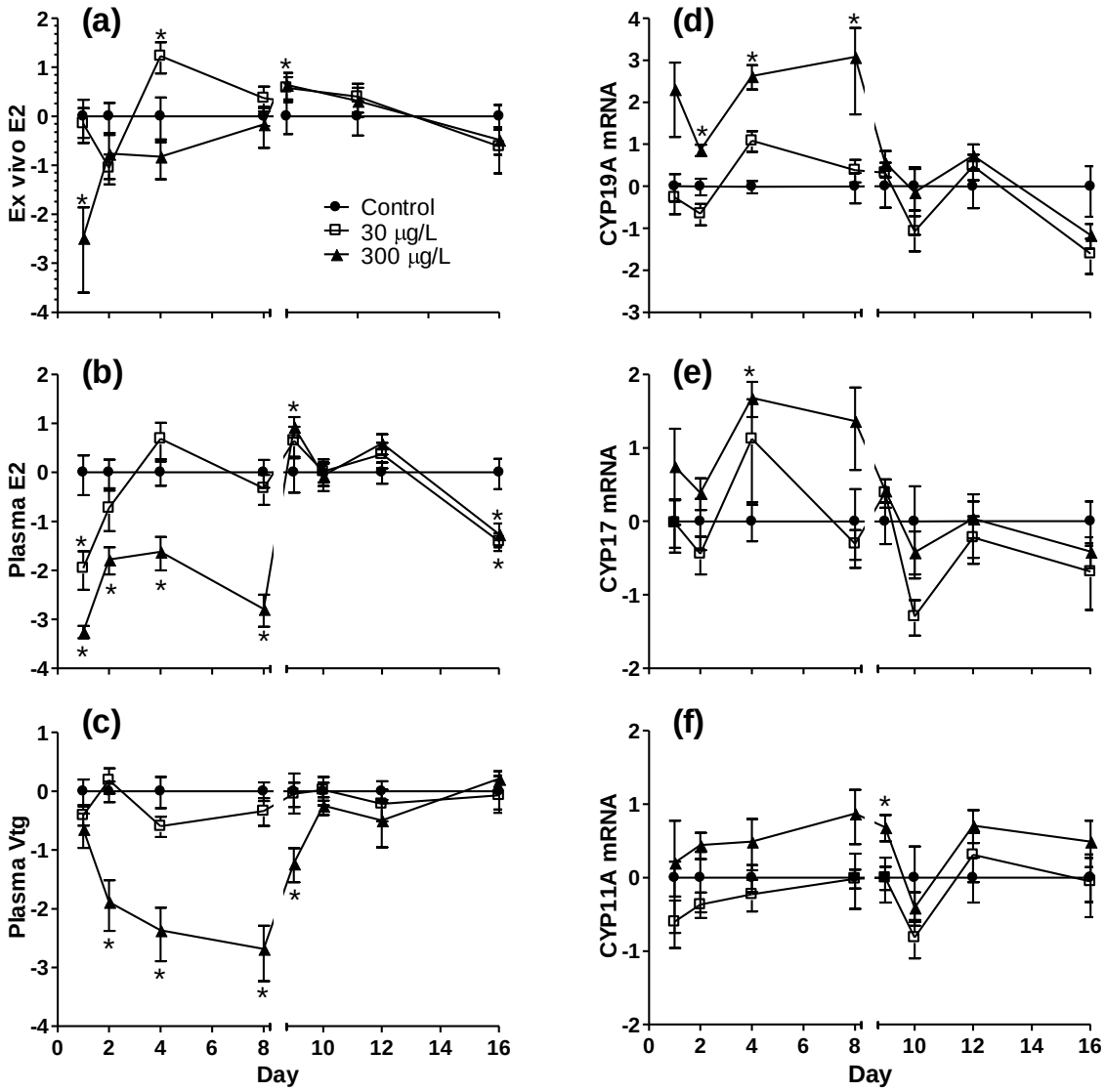


Figure 4.4: Time-course (8-d exposure and 8-d recovery) effects of prochloraz on female fathead minnow.

(a) ovarian synthesis of 17β-estradiol (E2); plasma concentrations of (b) E2 and (c) vitellogenin (Vtg); and ovarian expression of mRNA for (d) cytochrome P450 (CYP) 19A, (e) CYP11A, and (f) CYP17. Differences from control are indicated by asterisks from Ankley et al. (2009b).

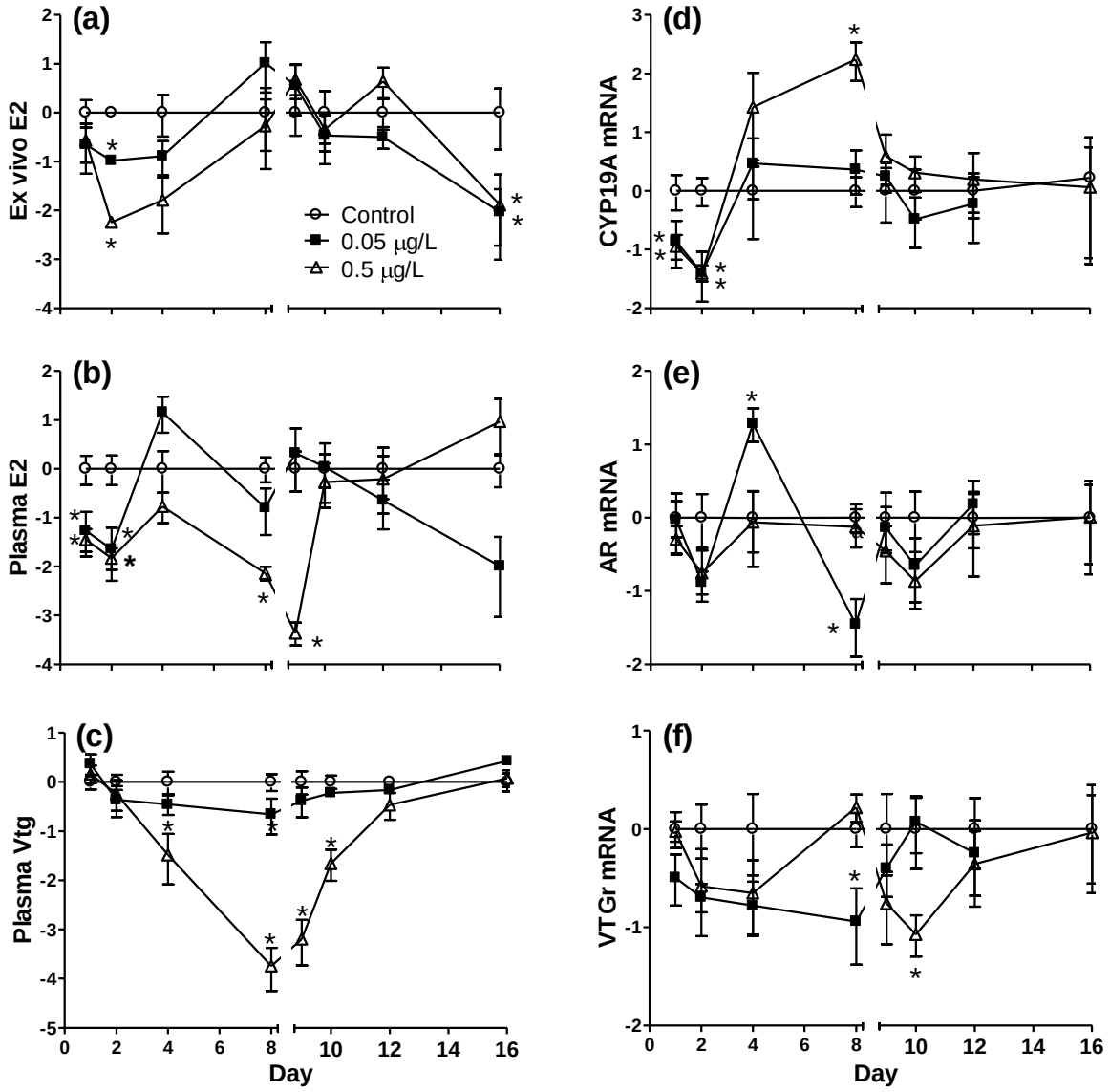


Figure 4.5: Time-course (8-d exposure and 8-d recovery) effects of trenbolone on female fathead minnow.

(a) ovarian synthesis of 17β-estradiol (E2); plasma concentrations of (b) E2 and (c) vitellogenin (Vtg); and ovarian expression of mRNA for (d) cytochrome P450 (CYP) 19A, (e) androgen receptor (AR), and (f) Vtg receptor. Differences from control are indicated by asterisks (from Ekman et al. (2011)).

The time-course experiments also provide insights as to the types of endpoints that might be expected to exhibit NMDRs. For example, in the fadrozole, prochloraz and TB time-course studies there were several instances where changes in gene expression were nonmonotonic. There also were examples of either plasma steroid concentrations or *ex vivo* steroid production exhibiting nonmonotonic relationships with dose. Based on this we hypothesize that responses at molecular and biochemical levels, such as those involved in feedback regulation of homeostasis (including compensation), may more commonly exhibit nonmonotonic characteristics than more integrated, downstream endpoints. Notably, this is consistent with the results of other studies described above, such as the 21-d fathead minnow reproduction test with TB, where NMDRs were observed for a number of biochemical endpoints in females, but not apical responses such as changes in weight, induction of male secondary sex characteristics or egg production Fig. 1a-c; Ankley et al. (2003). Villeneuve *et al.* (2012) and Osachoff *et al.* (2012) further highlight the variability that can be observed in terms of the nature of dose-response relationships for changes in gene expression. As described above, Villeneuve *et al.* (2012) noted NMDRs for the effects of BPA on global gene expression (measured via microarray) in the ovaries of zebrafish and fathead minnows. Osachoff *et al.* (2012) conducted time-course microarray experiments with Chinook salmon exposed to different concentrations of sewage effluent which, depending upon the gene examined, produced a variety of monotonic and nonmonotonic relationships that varied over time in livers of the animals.

Finally, and most significantly, although NMDRs were observed in the short-term time course studies with fadrozole, prochloraz and TB for gene expression and steroid endpoints, longer-term (21-d) studies with these same three chemicals, in the same dose range used for the time-course work, did not produce NMDRs for apical endpoints such as changes in secondary sex characteristics, growth, fertility or fecundity Ankley et al. (2005; Ankley et al. (2003; Ankley et al. (2002).

4.1.4 NMDRs in the Thyroid Hormone Pathway in Aquatic Species

4.1.4.1 Fish

There are no existing test guidelines routinely used to assess the effects of chemicals on thyroid function in fish. Therefore, the literature reviewed here is comprised of studies that include various chemicals, species, protocols, and endpoints. The most commonly studied species was zebrafish, and fewer studies used stickleback, fathead minnow, Chinese rare minnow, eastern mosquitofish, coho salmon, and sea lamprey. A total of 38 studies were evaluated. Five were eliminated due to use of a single test concentration. Fourteen studies were eliminated based on the use of chemicals with insufficiently defined MoA. Two studies were eliminated due to

lack of thyroid-specific endpoints. Of the remaining 17 studies, 14 included the NIS inhibitor, perchlorate, and 6 included a TPO inhibitor.

There were 14 studies in fish using perchlorate anion, which included more than 1 exposure concentration and included thyroid specific endpoints. These include studies with embryos, larvae, juveniles, subadults, and adults. The number of exposure concentrations ranged from 2 to 7 and the durations of the different studies ranged from 3 days to 16 weeks. Thyroid histology was the most commonly used endpoint, followed by plasma and whole body measurements of T4 and/or T3.

Thyroid histological changes included follicular cell hypertrophy and hyperplasia, changes in follicular size and colloid characteristics, and overall thyroid gland size and sensitive endpoints of thyroid hormone disruption commonly incorporated into toxicology studies Grim et al. (2009); metrics used to report these endpoints are often variable. In most studies examining this endpoint in fish, thyroid histology was affected monotonically.

Apparent NMDRs for histological changes were generally confounded by overt toxicity of the test compound, poor study design, or failure to normalize changes in gland size to body weight. For example, Patino et al. (2003), showed increased follicular cell hyperplasia and colloid depletion at 18 ppm perchorate as compared to the controls, whereas 677 ppm perchlorate had no effect. General toxicity and lethality was observed in the 677 ppm treatment, and samples were taken at different times at the two dose levels (4 weeks earlier in high dose vs the low dose group). Similarly, Crane *et al.* (2005) evaluated the effects of ammonium perchlorate on fathead minnows and reported an apparent NMDR with follicular cell height where exposure to 1 and 10 ppm were significantly larger than the controls, but exposure to 100 ppm resulted in significantly reduced follicular cell heights compared to the 10 ppm exposure. However, this study also observed significant reductions in growth at 10 and 100 ppm of about 40 and 60 % respectively, indicating that the two higher exposure concentrations were at toxic concentrations.

Finally, Schmidt and Braunbeck (2011) evaluated the effects of 2.5, 10, 25, and 50 mg PTU/L water on zebrafish and reported that histological effects on thyroid follicles and follicular cells occurred in a concentration dependent manner, as did the reduction in whole body T4 concentrations. By contrast, morphometric analysis of the pituitary suggested an NMDR in total pituitary area and adenohypohyseal area, which was highest at an intermediate PTU treatment. However, the normalized area of the adenohypophysis, using the ratio of the adenohypophysis: neurohypophysis, increased in a monotonic manner. Indexing of morphometric data is commonly used to avoid scaling errors associated with differences in body size, so it remains unclear if this observation is meaningful based on the un-indexed data.

Circulating concentrations of T4 and T3, are also used as indicators of thyroid toxicity in aquatic species but they are often impractical measurements due to the relatively small size of the test organisms and thus limited blood volume. Some studies have evaluated thyroid hormones using whole body extracts. However, whole body T4 measurements are largely inconclusive and highly variable for technical reasons Crane et al. (2006; Park et al. (2006). Crane et al. (2006) used both whole body and circulating T4 measurements to evaluate the effects of methimazole on fathead minnows, depending on the size of the organism at the time of sampling. Circulating thyroid hormone levels at 84 days were not statistically analyzed in the females due to sample size problems, but there were no significant differences observed in the circulating T4 and T3 measurements in the males. However, an apparent NMDR was observed in the 28-day measurements of whole body T4 concentrations, where T4 was significantly reduced at 32 and 100 ug/L but not at 320 ug/L methimazole. At 56 days, whole body T4 was increased at 320 ug/L only. Whole body T3 was reduced at 320 ug/L at 28 days, but a slight decrease was observed only at the 100 ug/L at 56 days. The authors suggest that the NMDRs in whole body thyroid hormone measurements could be due to either compensatory mechanisms or from altered thyroid hormone metabolism. No data are presented in support of either suggestion. The inconsistent direction of change and the previously noted potential problems with whole body thyroid hormone measurements contribute to uncertainty about these observations. Furthermore, studies in zebrafish with PTU did observe monotonic reductions in circulating T4 and T3 van der Ven et al. (2006) and whole body T4 concentrations Schmidt and Braunbeck (2011).

Finally, two studies in fish evaluated thyroid hormone-dependent gene expression in response to two thyroid disruptors, ammonium perchlorate and amitrole (Li, Zha et al., 2011)Li et al. (2009). For the most part, gene expression exhibited montonic concentration-responses, with one time point exhibiting an NMDR. Li, Zha et al, 2011 evaluated the effects of 5 and 50 ppb perchlorate on the expression of genes for the iodothyronine deiodinase enzymes (DI1, DI2, and DI3) and sodium iodide symporter (NIS) in the brain and liver of adult Chinese rare minnows. They also measured DI2 and NIS in larvae. Expression of these genes was analyzed following 7, 14, and 21 days of exposure. Circulating T4 and T3 were measured in adult plasma at the end of the 21 day exposure. Changes in DI2 and NIS expression in the larvae were monotonic within each sampling event, though the direction of change was inconsistent among the different sampling times. Effects on hepatic expression of DI1, DI2, DI3, and NIS were monotonic in males within each sampling event, though direction of change was again inconsistent among the sampling times. DI3 and NIS expression in female livers were monotonic at 7 and 21 days, but nonmonotonic at 14 days. Effects on brain expression of DI2, DI3, and NIS were monotonic in females, with DI3 showing strong and consistent reductions at most time points. The same three genes in males exhibited both monotonic and nonmonotonic

responses of varying magnitude and direction, depending upon the sample time. The gene expression response patterns in this study are complicated, and the observation of apparent nonmonotonic responses was dependent on when the samples were taken and were not consistent throughout the duration of the study.

Li et al. (2009) evaluated the effects of 1 to 10,000 ng/L amitrole using juvenile Chinese rare minnows exposed for 28 days. Expression of DI1, DI2, transthyretin (TTR), and thyroid hormone receptor alpha (TRα) were measured in liver and brain. The only gene showing a possible NMDR was that of hepatic DI2, which was significantly elevated in the low and intermediate concentrations, but was above or at control levels in the highest concentrations. However, hepatocyte degeneration was also observed at the high concentration, indicating that cytotoxicity was occurring.

4.1.4.2 Amphibians

Numerous studies have been conducted with amphibians regarding thyroid disruption, as amphibian metamorphosis is a well described thyroid hormone-dependent process. Many studies have used single exposure concentrations and, therefore, were not considered in this review. Recently, however, several multiple concentrations studies have been published. These were largely executed in the development of a test guideline for regulatory purposes OECD (2009; U.S. EPA (2009). Some studies are now appearing in the literature based on the relatively new test guidelines. The most commonly studied species is *Xenopus laevis*.

The amphibian data reviewed here showed little evidence of nonmonotonic behavior in developmental endpoints, thyroid histology, thyroid hormones, and gene expression. Potential nonmonotonic responses were observed in histological endpoints of two studies. Opitz et al. (2006) evaluated the developmental, histological, and molecular effects of ethylene thiourea (ETU) exposure on *X. laevis* larvae using 1.0, 2.5, 10, 25, and 50 mg ETU/L for up to 90 days. Metamorphic development and most of the morphological and histological endpoints changed monotonically with the exception of follicular cell height, which was significantly decreased at 10 mg/L, but increased at 25 and 50 mg/L. This apparent NMDR was not interpreted further in the Opitz et al. (2006) discussion. In any case, the prevalence of the remainder of the histological observations, including follicular cell hypertrophy, increased monotonically with ETU concentration. Indeed, five of the seven histological endpoints were affected at 10 mg/L. Thus, despite an internal contradiction in the data, the overall histological profile indicates a monotonic response.

Carlsson and Norrgren (2007) evaluated the effects of 2, 5, 10, 20, and 75 mg/L 6-PTU on development and thyroid gland histology of *X. tropicalis* larvae in a 14 day exposure. The

developmental and histological effects were observed to be monotonic, with the exception of follicular lumen area, which increased through 10 mg/L but began to decrease at 20 mg/L. This apparent NMDR is attributable to collapse of the follicle associated with the depletion of colloid in the higher dose; an observation made in other studies of TH synthesis inhibitors. Reinforcing this explanation is the fact that the follicular lumen areas at the 75 mg PTU /L concentration were excluded from analysis due to severe follicular lumen collapse, which prevented accurate measurements.

NMDR in fish and amphibians exposed to TPO and NIS inhibitors were infrequent. Where they were observed, they were identified to be the result of toxicity at high doses, associated with temporal sampling which may not have adequately represented the toxicodynamics of the response (including compensation), resulting from highly variable and inconsistent measurements, and/or normal observations associated with specific phenomena. In all cases the apparent nonmonotonic responses were identified in isolated, single studies.

4.2 Mammalian Models

4.2.1 Literature Search and Selection Strategy for E and A Pathways

A large database of journal articles and other reports was examined resulting in the review of numerous dose response curves for different EDC mechanisms of toxicity leading to disruption of the estrogen or androgen hormone system. Each article was evaluated to determine if an NMDR occurred. The focus of this review was on *in vivo* EDC effects with NMDRs identifiable from a broad range of doses, including the low dose range. The evaluation determined if the observed NMDRs were consistent among similar studies on the same chemical, and identify if the observed NMDR were robust and reproducible. The evaluation, when possible, attempted to identify whether or not NMDR were clearly adverse effects or were upstream endpoint causally linked to an adverse effect. Where possible there was an attempt to identify uncertainties in the data base on EDCs with respect to NMDRs.

The general approach taken was to review the *in vivo* literature for well characterized low dose effects reported for drugs, pesticides and toxic substances that disrupt the estrogen or androgen signaling pathways. If these chemicals induced NMDR effects in the low dose range, then it is possible that other chemicals displaying the same mechanisms of toxicity also would induce NMDR.

A multipronged strategy was utilized to obtain references for this evaluation. A list was produced that included chemicals that directly disrupt E or A signaling pathways at key points, including AR or ER nuclear receptor binding or by altering steroid hormone synthesis. Articles

and documents evaluated included peer reviewed literature, US and international regulatory agency risk assessments, as well as peer review committee and agency review articles and summaries. A second approach evaluated key articles that were cited in the above documents or guideline reports. Appendices A and B contain the review and evaluation of each individual study.

The preference for inclusion was for studies with six or more dose groups by oral exposure to the test chemical because these provide more robust information on the shape of the dose response curve than do studies with fewer dosage levels. However, excluding studies with four or five dose levels would have severely restricted the numbers of studies available for review and would have also excluded many of the studies cited by others as displaying NMDRs (Vandenberg *et al*., 2012). Therefore, we also included in the evaluation *in vivo* studies of EDCs, which encompassed a broad dose-range and have at least four or more groups (a control and three treated groups).

In addition to the standard phenotypic endpoints, some genomic studies that utilized a subcutaneous dosing regimen are included since they examined the effects of hormonally active chemicals over a very broad dose response range with a comprehensive assessment of EDC-induced altered gene expression in reproductive tissues. Studies using subcutaneous dosing also are included when there were few, if any, oral studies with a specific estrogen or androgen MoA.

The literature reviewed for the A pathway contains a very robust data base for some of the chemicals. Some of the identified studies provided unique insights on how often one can identify NMDRs, the conditions under which they occur, the potential relevance of NMDRs in risk assessment and the general shape of the dose response curve. In addition to laboratory animal studies, several dose-response studies have evaluated the beneficial and adverse effects of androgens and selective androgen receptor modulators (SARMS) in both young and aged men.

The studies were evaluated based on the robustness of the data available in the publication and relative to other publications reporting on the same chemical.

More detail on study selection is provided on a chemical-by-chemical basis in Appendices A and B. In total, more than 70 studies were reviewed, which had six or more dose groups and over 200 with four or more dose groups.

4.2.1.1 Specific Considerations in Reviewing the Literature on E and A

Evaluation of the data was done to determine the robustness of the NMDR. An NMDR was considered robust if it was reproducible and biologically plausible. In some cases, even though the NMDR was not reproduced in additional studies because they had not been done, it was still considered well supported base on its biological plausibility. There were a number of situations in evaluating the studies that would suggest that the NMDR was an incidental finding, was not biologically plausible, or while it might have been reported by the study authors was not considered to be supported by the data by the authors of the current document. These cases included the following: identification of the frequency of one effect declining as a more severe one develops; a failure to correct for multiple comparisons or other inappropriate statistical analyses; reporting of NMDR at dosage levels below background levels of the xenobioticl. In some cases the study authors interpreted the data as an NMDR based on the shape of the curve but in the absence of statistically significant differences from control. And finally some NMDR were identified that were associated with overt toxicity or adverse effects at lower doses. In this evaluation of E and A pathways we paid particular attention to the issues in evaluating potential endocrine and other systemic effects in the overall WoE for the observation of NMDR.

Several robust examples of NMDR were identified for adverse effects, but these were not common. Exposure to androgenic chemicals has been shown to produce robust NMDRs *in utero* and during adult life in female and male rats, respectively. For example, Robaire *et al.* (1979) found that administering increasing doses of testosterone to adult male rats resulted in a biphasic response in testis weight and sperm production. This response also has been demonstrated repeatedly in young adult male rats Dykman (1981; Robaire et al. (1979; Ewing et al. (1977; Walsh and Swerdloff (1973), rabbits Ewing et al. (1973), and rhesus monkeys Ewing et al. (1976). Similar NMDR effects on the testis and sperm production have not been reported for human males; however, administration of high doses is precluded by the onset of adverse effects, especially in aged men. Administration of testosterone to mature intact male rats, rabbits, and rhesus monkeys causes a reduction in LH, followed by declines in testis androgen levels, sperm production and testis weight without causing increases in serum testosterone or androgen- dependent organ weights. However, as testosterone dosage levels are increased above the nadir of the NMDR, testis weight and sperm production levels are partially restored due to increasing levels of intratesticular testosterone from the serum (Figure 2, from Robaire et al. (1979), redrawn below, Appendix B.6.b.1).

These are cases in which an effect displays an NMDR that peaks in the mid-dose range of a study and then reverses direction at a higher dosage level as one effect is replaced by another more biologically important effect. In the example below (Figure 4.6), glans penis weight

reaches a nadir in the mixture group exposed *in utero* to 50 % of the highest dose of dibutyl phthalate (DBP) plus procymidone, whereas males exposed to the highest dose of the mixture display organ weights greater than, not less than, control values (Appendix B.1.c.6). This results from the fact that in the mid-dose group the glans was reduced in size but was not malformed; by contrast almost all of the tissues from the high dose group were too malformed to weigh. These kinds of NMDRs are not uncommon with chemicals that induce malformations or histopathological alterations.

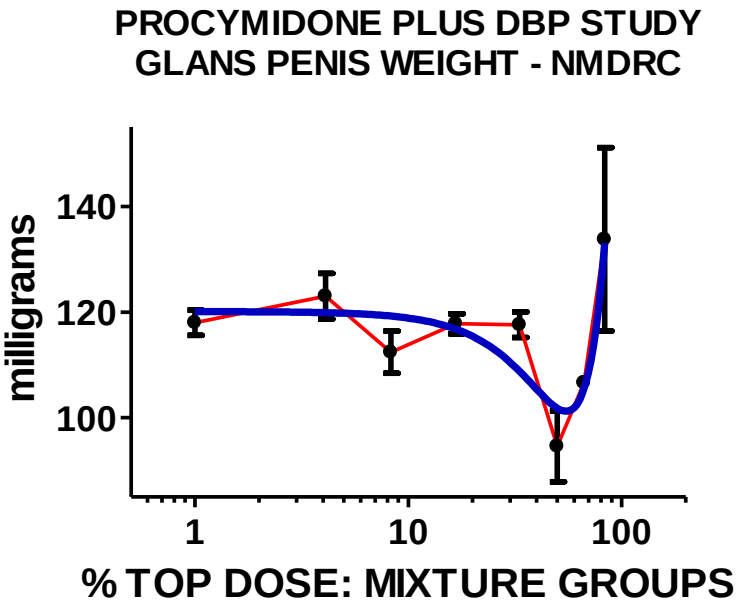


Figure 4.6: Glans Penis Weight after exposure to a mixture of procymidone and dibutyl phthalate.
Glans penis weight reaches a nadir in the mixture group exposed in utero to 50 % of the highest dose of DBP plus procymidone whereas males exposed to the highest dose of the mixture display organ weights greater than the control Hotchkiss et al. (2010).

There were occasions wherein an NMDR was identified by the study authors based on what may be considered inappropriate statistical tests. This includes, for example, failure to account for multiple comparisons by adjusting the p-value required for statistical significance in studies with large numbers of endpoints and dosage levels. The NTP/NCTR five generation studies with four dose levels of the estrogenic chemicals genistein (Appendix A.1.d.1) and EE2 (Appendix A.1.a.1) included hundreds of endpoints (examination of both sexes, frequent measures of growth and food consumption, multiple organ weights and histopathology, landmarks of puberty, fertility, estrous cyclicity, etc). More dose levels of each chemical were included in the one-generation dose range finding studies. Even if these chemicals had no endocrine or other toxicity at the dosage levels used, one would reasonably expect to detect falsely as positive some treatment related effects if one inappropriately compared each dose group to the control

value for every effect with a t-test, or an analysis of variance (ANOVA) accepting a p-value of 0.05 as a significant difference. In some of these cases the NMDR was not consistently identified across experiments. In the NCTR/NTP EE2 dose range finding study NTP (2010), two endpoints among all those measured in F1 males displayed NMDRs; age at preputial separation (PPS) (Figure 4.6) and dorsolateral prostate weight (Figure 4.8). However when examined in the F1 and F2 generations of the five generation study EE2 did not induce NMDRs in the same dose range. Further increasing the lack of support for a true NMDR is lack of concordance with other effects in the study and lack of consistency with other studies. Accelerated onset of PPS at 25 ppb EE2 suggests that EE2 is androgenic or that it induces an earlier onset of increased androgen levels. However, no other androgen-dependent tissue was "androgenized" at this dose and these effects have not been seen in any other EE2 study or with other estrogenic chemicals.

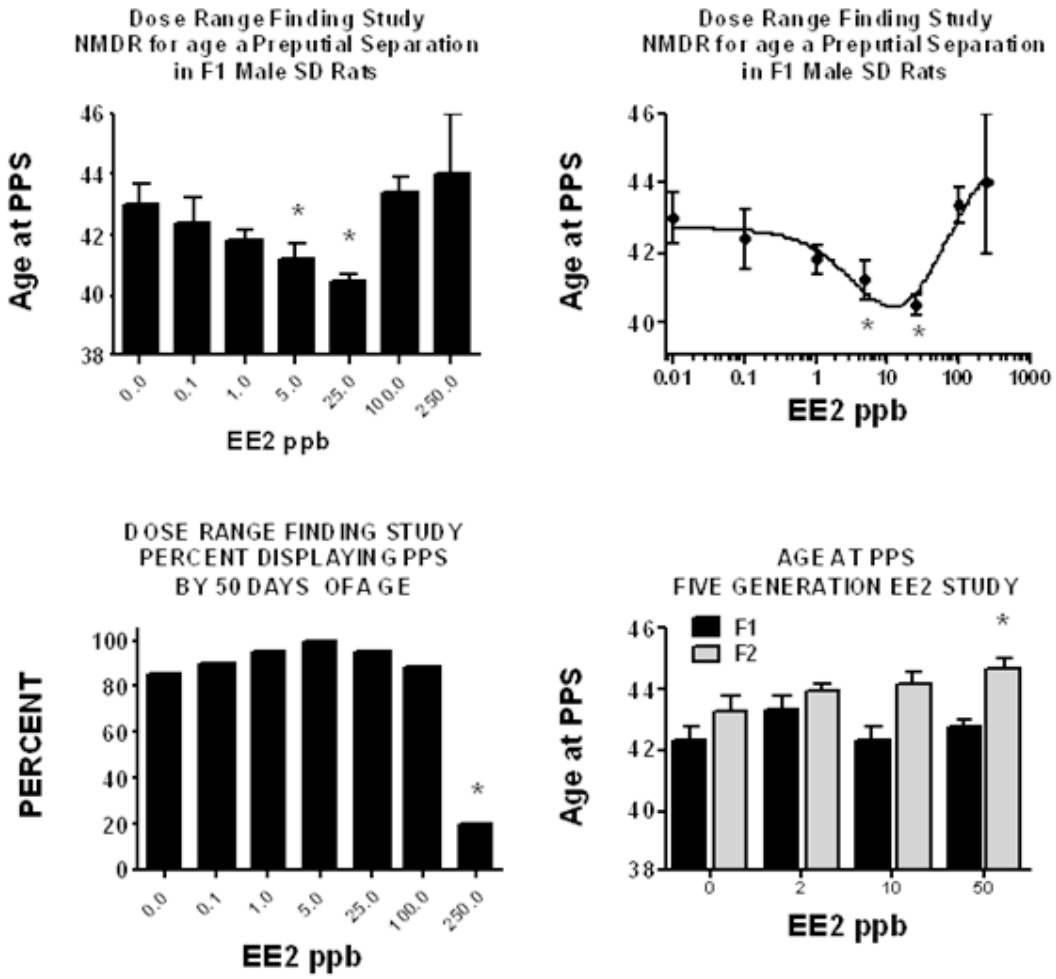


Figure 4.7: Comparison of results for preputial separation in male rats after exposure to EE2. An NMDR was identified for PPS in the dose range finding study, that dose range did not accelerate PPS in similarly exposed males from the F1 and F2 multigenerational study. In the

multigenerational study, several endpoints (out of the hundreds measured over 4 generations) displayed NMDRs; that is they were increased or decreased in a statistically significant fashion versus control and an intermediate dose but not at a higher dosage level NTP (2010; Delclos et al. (2009).

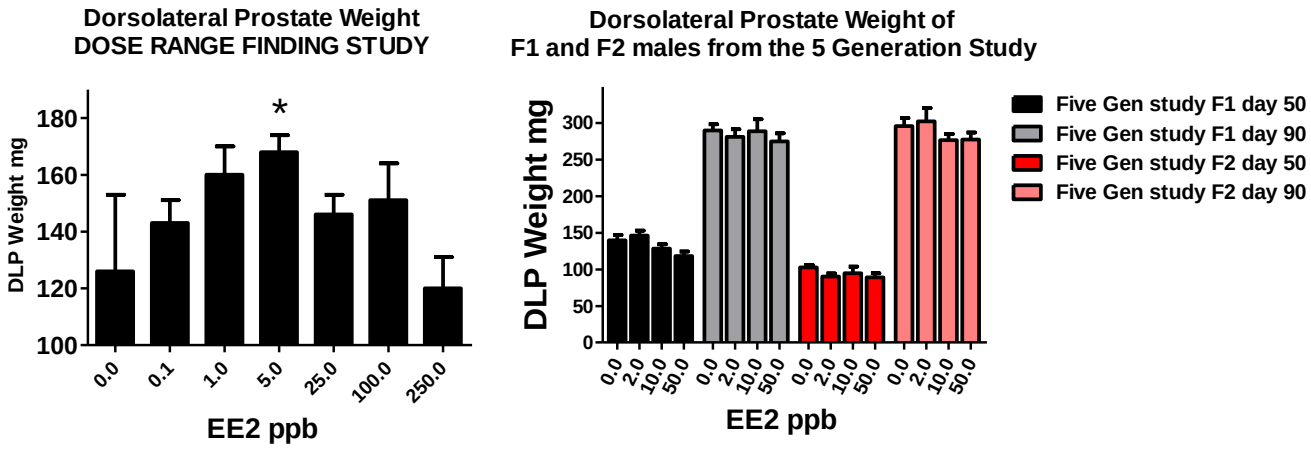


Figure 4.8: Comparison of results for dorsolateral prostate weight in male rats after exposure to EE2.

An NMDR was identified for dorsolateral prostate weight in the dose range finding study but not in similarly exposed males from the F1 and F2 multigenerational study NTP (2010; Delclos et al. (2009).

Another finding that raised uncertainty in the identification of a NMDR was statistical analyses in studies using multiple pups from the same litter or repeated measures on the same endpoint on the same pup at different ages. This error can inflate the error degrees of freedom and underestimate the error mean square, resulting in inflated significant F and t-values. An example would be of a small study wherein3 pregnant rats per dose group are treated with DEHP at four dose levels (control and three treated groups. If F1 serum testosterone is recorded for 3 pups/litter repeatedly (5 observations/F1 male offspring over a lifetime) an ANOVA analysis that fails to account for litter effects and repeated measures would have an F value for a main effect of DEHP on serum testosterone levels based upon 176 degrees of freedom in error; this is by contrast to 11 degrees of freedom in error given corrections got litter effects and repeated measures.

An additional confounding variable occurs for certain xenobiotics that happen to be ubiquitous in the environment, and thus routinely contaminate feed and bedding in rodent studies. Several of the chemicals discussed in this current evaluation are ubiquitous in the environment and are found in a majority of environmental and human urine samples. Some are present in

human diets and, not surprisingly, they also are found in animal diets; this can be at concentrations orders of magnitude above those used by investigators in what is suggested to be a low dose study. Some study design and analysis and interpretation of the data have not taken those background levels into consideration. For example, several publications have reported that phthalates are found in animal's diets at low ppm concentrations and are reported at even higher concentrations in the litter used for cage bedding. One study reported that 100% of the animal diets and beddings contained DBP, BBP and DEHP and another reported that these exposures produced measurable phthalate metabolite levels in the tissues (Kondo et al. (2010) (Appendix B.2.a). In the case of the phthalates, the validity of the results from studies reporting NMDRs at dose levels below the reported levels in diets and beddings would likely be of questionable value.

In figure 4.9, the administered levels of DEHP fed to pregnant mice in this particular study falls well within the range of phthalate concentrations identified in a survey of control animal diets (Appendix B.2.e.2). Failure to control for this dietary confounder raises uncertainty about the actual exposure levels in the study. Thus, there would be uncertainty about the NMDR conclusion as the F-value for a treatment effect was not significant, and the reported effect is a small increase in anogenital distance (AGD), whereas the phthalate syndrome phenotype is characterized by shortened AGD in newborn male rats. Do *et al*., (2012) (Appendix B.2.e.2) orally administered DEHP from GD 9 to 18 at 0, 0.5, 1.0, 5, 500, 50,000, and 500,000 µg/kg/d and examined maternal and fetal (1M position males only) hormones on GD 18. In figure 4.6, the range of the estimated daily phthalate intake from the control animal's diets is shown in red (estimate from Kondo *et al*., 2010, Appendix B.2.a). The fraction of total phthalate exposure contributed by the diet and unaccounted for in the study could be considered substantial and raises questions regarding an NMDR in such a study.

Animal diets may also contain heavy metals, mycotoxins, phytoestrogens, dioxins and PCBs and other contaminants at varying levels (www.fda.gov/AnimalVeterinary/Products/AnimalFoodFeeds/Contaminants/default.htm). These additional contaminants may interact with the test material.

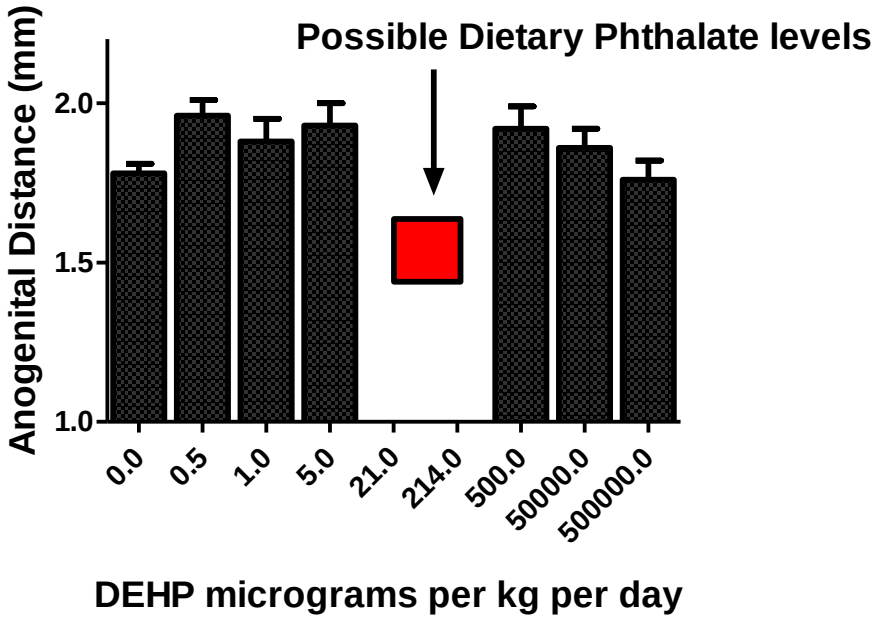


Figure 4.9: Representation of possible background dietary phthalate concentration. Possible dietary phthalate levels (red box) based on calculation from Kondo et al (2010) related to doses as provided to mice in Do et al. (2012).

In some cases NMDR have been reported when no treated group is statistically significantly different from control, and observation of NMDR appears to be based on inspection of the data. For example, Vandenberg *et al.* (2012) reported that DEHP data from Grande *et al.* (2006) displayed NMDRs for the ages at vaginal opening (VO) and first estrus in female rats (Appendix B.2.a.3). In contrast, Grande *et al.* (2006) reported "A significant delay in the age at vaginal opening (approximately 2 days) at 15 mg DEHP/kg bw/day and above, as well as a trend for a delay in the age at first estrus at 135 and 405 mg DEHP/kg bw/day"; the study authors did not report NMDRs (see figure 4.10).

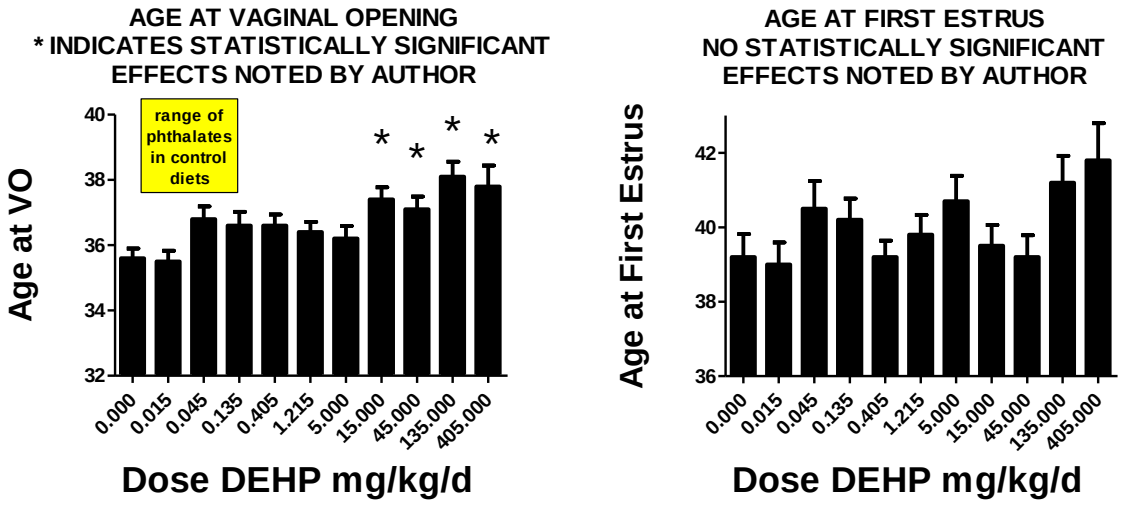


Figure 4.10: Histograms illustrating effects on vaginal opening and age at first estrus in DEHP exposed rats.

Grande et al. (2006). The histogram of age at first estrus has the appearance of indicating an NMDR although none of the doses are statistically different from control. Yellow box indicates calculated background phthalate exposures from feed based on Kondo et al (2010).

Identification of a NMDR after high dose exposure associated with overt toxicity or adverse effects that occur at doses below the NMDR introduces uncertainty. Chemicals that induce overt toxicity also may indirectly disrupt the endocrine system and secondarily induce reproductive and endocrine alterations. For example, Vandenberg *et al.* (2012) listed semicarbazide (SEM) as an EDC that disrupted male rat pubertal development in an NMDR fashion (Appendix B.4.C). SEM acts as an osteolathyrogen, and induces osteochondral and vascular lesions in young rats due to impaired cross-linking reactions of collagen and elastin (Appendix B.4.b). In addition, teratogenic effects such as induction of cleft palate and aortic aneurysms also have been reported (Appendix B.4.a). However, the literature provides little support for this chemical as an EDC; moreover the identified NMDR is observed at a dose level that produces a severe reduction in weight gain during dosing and is higher than doses of SEM that produces lesions of the joints and vascular system.

In summary, in this evaluation of E and A pathways we paid particular attention to the above issues in evaluating potential endocrine and other systemic effects in the overall WOE for the observation of NMDR.

4.2.2 Estrogen Hormone System

In addition to natural steroidal estrogens, many chemicals including plant and fungal estrogens, pharmaceuticals in the environment, toxic substances and pesticides also bind the estrogen receptors of mammals. Other than some of the estrogenic pharmaceuticals the xenoestrogens bind ERs with affinities orders of magnitude lower than naturally occurring 17β estradiol (E2). For this reason relatively high exposure levels are required to produce estrogenic effects for many xenoestrogens. For example, oral administration of E2 and ethinyl estradiol (EE2) induce increases in uterine weight at low µg/kg dose levels whereas bisphenol A (BPA), bisphenol AF and methoxychlor require doses four to five orders of magnitude higher to induce a uterotrophic response of similar magnitude. In some cases the endocrine activity may be displayed only at doses equal to or above the induction of some other systemic toxicity; for example, with the tremorogenic pesticide chlordecone (Kepone), estrogenicity is observed only at doses equal to or above those inducing some other form of systemic toxicity. The interaction of a chemical with ER or induction of an estrogen-dependent response does not enable one to predict with certainty what other estrogenic effects may occur, the dose that will produce an effect, or the shape of the dose response curve in each tissue. Tissue-specific responses arise from differences in these factors: receptor levels, levels of coactivators and corepressors, E2 metabolism, receptor stability, different target gene estrogen response elements, gene silencing, and other factors. The studies that identified NMDR are described below with additional evaluations of a broader collection of chemicals and studies presented in Appendix A.

Table 4.2: NMDR From Studies Evaluating the Mammalian Estrogen Hormone System.

Chemical	Species	Group Size	Doses Given	Dosing route	Reference(s)	Appendix location
Ethinyl estradiol	rat	5	0.1, 1.5, 5, 25, 100 or 200 ppb	feed	NTP (2007)	A.1.a.1
Ethinyl estradiol	rat	?	2, 10, or 50 ppb	feed	Delclos et al. (2009)	A.1.a.1
Ethinyl estradiol	rat	19-43 dams	0.05, 0.15, 0.5, 1.5, 5, 15, or 50 μg/kg	gavage	Howdeshell *et al.* (2008)	A.1.a.4
Ethinyl estradiol	mouse	10-12 dams	0.002, 0.02, 0.2, 2, 20, 200 μg/kg	gavage	Thayer *et al.* (2001)	A.1.a.5
Ethinyl estradiol	rat	10, 5 per block	0.01, 0.05, 0.2 mg/kg	gavage	Andrews et al. (2002)	A.1.a.9
17β Estradiol	mouse	10	0.005, 0.05, 0.5, 2.5, 5, 10, or 50 ppm	feed	Tyl *et al.* (2008a)	A.1.b.2
17β Estradiol	mouse	25	0.001, 0.005, 0.05, 0.15, or 0.5 ppm	feed	Tyl *et al.* (2008c)	A.1.b.3
Diethylstilbestrol	mouse	?	0.002, 0.02, 0.2, 2, 20, and 200 ng/g	gavage	vom Saal *et al.* (1997)	A.1.c.1
Diethylstilbestrol	mouse	10	1, 5, 10, or 15μg/kg	gavage	Nagao et al. (2012)	A.1.c.2
Diethylstilbestrol	rat	10	0.2, 1.0, or 5.0 μg/kg	gavage	Kim et al. (2002b)	A.1.c.6
Diethylstilbestrol	rat	10	10, 20, or 40 μg/kg	gavage	Shin *et al.* (2009)	A.1.c.7
Genistein	rat	10 dams	5, 25, 100, 250, 625, or 1250 ppm	feed	Delclos *et al.* (2001)	A.1.d.1
Genistein	rat	10	5, 25, 100, 250, 625, or 1250 ppm	feed	NTP Tox Report (2007, 08)	A.1.d.1
Genistein	rat	9 dams	5, 50, 500, 1000 ppm	feed	Akingbemi et al. (2007)	A.1.d.6
Genistein	rat	~17	5, 100, 500 ppm	feed	Dalu et al. (2002)	A.1.d.2
Nonylphenol	rat	25	2, 10, or 50 mg/kg	gavage	Nagao et al. (2001)	A.1.H.3
Bisphenol A	rat	28	0.01, 0.1, 1.0, 10 ppm	water	Cagen et al. (1999b)	A.1.k.2
Bisphenol A	rat	20 dams	0.015, 0.3, 4.5, 75, 750, or 7500 ppm	feed	Tyl *et al.* (2002)	A.1.k.3
Bisphenol A	mouse	28	0.018, 0.18, 1.8, 30, 300, or 3500 ppm	feed	Tyl *et al.* (2008b)	A.1.k.4
Bisphenol A	rat	25	0.2, 2, 20, or 200 μg/kg	gavage	Ema *et al.* (2001)	A.1.k.5
Bisphenol A	rat	10	0.33, 3.3, or 33 ppm	feed	Kobayashi *et al.* (2012)	A.1.k.7
Bisphenol A	rat	24	0.15, 1.5, 75, 750, or 2250 ppm	feed	Stump *et al.* (2010)	A.1.k.8
Tamoxifen	rat	10	10, 50 or 200 μg/kg	gavage	Kim et al. (2002b)	A.2.b.1
Tamoxifen	rat	10	5, 30, or 200 μg/kg	gavage	Kennel *et al.* (2003)	A.2.b.2
Exemestane	rat	8 or 30	2, 4, 5, 10, 20, 40, 50, 90, 100, 125, 200, 250, 500, 810, 1000 mg/kg	gavage	Beltrame *et al.* (2001)	A.3.b.1

4.2.2.1 Ethinyl Estradiol (EE2) [A.1.a][1]

There are several robust, well designed, comprehensive, low to high dose, multigenerational and transgenerational EE2 studies conducted with rats. In addition, there are a number of shorter-term, mechanistic studies that examined the effects of EE2 over a broad dose range that included upstream endpoints. The literature on the estrogen EE2 provides a rich data base to address questions about dose response, sensitivity of endpoints and critical life stages for induction of adverse effects of estrogenic chemicals. The NTP executed a series of studies with EE2 and other well characterized estrogenic chemicals, specifically to examine the shape of the dose response curve and to identify endpoints sensitive to estrogens for potential inclusion in multigenerational studies of chemicals displaying estrogenicity in screening assays.

The NTP executed a series of studies with EE2 and other well characterized estrogenic chemicals specifically to examine the shape of the dose response curve and to identify endpoints sensitive to estrogens for potential inclusion in multigenerational studies of chemicals displaying estrogenicity in screening assays Delclos et al. (2009; Latendresse et al. (2009) [A.1.a.1]. These studies included measurement of fertility, fecundity, maternal and litter measures, repeated observations of growth and food consumption throughout the life cycle, AGDat birth, neonatal developmental landmarks, pubertal landmarks, estrous cyclicity, organ weights, histopathology, ovarian oocyte counts and sperm counts over 5 generations.

The first study was a dose range finding none generation study with 7 dose levels (0, 0.1, 1.5, 5, 25, 100 or 200 ppb) in the feed. Exposure began on GD 7 and continued through gestation, lactation and directly to F1 in the diet until necropsy at 50 days of age. Dietary exposure of the dams continued through lactation. Pups from five litters, culled to eight per litter with an equal sex distribution on postnatal day (PND) 2 were maintained on the same dosed feed as their mother after weaning until sacrifice at PND 50. Maternal body weight gain was reduced during pregnancy by about 50% in the 200 ppb dose group and by 30% at 100 ppb and mean pup weight at birth also was reduced in these two dose groups. The percentage of F1 males displaying PPS at 50 days of age was dramatically reduced from 85% in controls to 20% in the 200 ppb dose group. The age at PPS displayed an NMDR, being accelerated by about 1.6 days at 5 and 25 ppb. In F1 males, terminal body weight, ventral prostate weight, testis sperm count and testes weights were reduced at 200 ppb, whereas the dorsolateral prostate weight was identified as a NMDR with a significant increase only at 5 ppm. Histopathological alterations were noted in reproductive and nonreproductive organs at 100 and 200 ppb and mammary gland ductal hyperplasia was present at doses of 25 ppb and higher dose levels, all monotonic responses Latendresse et al. (2009) [A.1.a.1]. In F1 females, VO was accelerated in the 25, 100,

[1] The bracketed alpha-numeric identifier corresponds with the specific location in the appendix.

and 200 ppb groups and body and ovarian weights were reduced at 200 ppb and ovarian, uterine and vaginal tissues displayed histological abnormalities at 200 ppb; however no NMDRs were identified.

The data from the dose range finding study was used to select dosage levels for a 5 generation study (n>30 mated pairs per group) wherein 0, 2, 10, 50 ppb was fed in the diet. In the multigenerational study, vaginal opening was accelerated at 50 ppb in the F1, F2 and F3 generations, but not in the F4 generation (unexposed) Delclos et al. (2009) [A.1.a.1]). Ethinyl estradiol accelerated the attainment of puberty in females provided 50 ppb under continuous exposure conditions (F_1 and F_2) and in the F3 where dosing was terminated at weaning. Perturbation of the estrous cycle (prolonged cycles, aberrant cycles, time in estrus) in young females after VO and prior to mating was observed in the F_1 and F_2 generations. In males, statistically significant inductions of male mammary gland hyperplasia (F_0 through F_3 generations) and mild mineralization of renal tubules (F_1 and F_2 generations) were observed. The majority of these effects in male and female rats were observed at 50 ppb. Sporadic NMDRs that did not repeat in similarly exposed groups of rats from different generations were identified in this very large and comprehensive study of a model estrogenic chemical. Although PPS displayed an NMDR in the dose range finding study, that dose range did not accelerate PPS in similarly exposed males from the F1 and F2 multigenerational study. In the multigenerational study, several endpoints (out of the hundreds measured over 4 generations) displayed NMDRs being increased or decreased significantly versus control and an intermediate dose but not at a higher dosage level. None of these effects were consistent across similarly exposed generations. Although dorsolateral prostate weight displayed an NMDR in the dose range finding study, that dose range did not increase the weight of this tissue in similarly exposed males from the F1 and F2 multigenerational study. In addition, no histopathological lesions were detected in this tissue.

Howdeshell *et al.* (2008)[A.1.a.4] studied the effects of gestational and lactational oral gavage exposure to 0.05, 0.15, 0.5, 1.5, 5, 15, or 50 □g EE2/kg on the postnatal development and reproductive function of the male rats. The numbers of live pups at weaning was increased at 0.15 and 1.5 µg/kg/d but not at higher dosage levels. Increased survival is not typically considered an adverse effect.

Thayer *et al.* (2001)[A.1.a.5] treated pregnant mice with 0.002, 0.02, 0.2, 2, 20, or 200 □g EE2/kg by gavage and found body weight at necropsy and AR per prostate in male offspring, exhibited NMDRs following exposure.

Administration of 0.01, 0.05, 0.2 mg EE2 /kg by gavage to adult female and male rats (OECD Test Guideline no. 407) produced adverse effects at relatively high dosage levels as compared

to dosage levels producing effects in developing rats. Thyroid stimulating hormone (TSH) levels displayed an NMDR in the first block, being statistically significantly increased at 50 µg/kg/d, but not at a higher dose. However, this effect was not seen in the second block Andrews et al. (2002)[A.1.a.9].

4.2.2.2 17β Estradiol (E2) [A.1.b]

Mice were exposed to 0.005, 0.05, 0.5, 2.5, 5, 10, or 50 ppm E2 in feed during a 2-week pre-breeding period, mating, gestation and lactation in a one generation study Tyl et al. (2008a)[A.1.b.2]. Of the 100 or so measurements (with multiple contrasts for each versus control values) only seminal vesicle weight in the P_0 generation displayed an apparent NMDR, being increased versus the control group at 0.005 and decreased at 5 ppm and above. In a companion two generation study, mice were fed 0.001, 0.005, 0.05, 0.15, or 0.5 ppm E2 during an 8 week pre-breeding period, mating, gestation and lactation Tyl et al. (2008c)[A.1.b.3]. The NMDR on P_0 seminal vesicle weight in the one generation study at 0.005 ppm was not a statistically significant effect in the latter study. In the latter study, thyroid weight was the only endpoint among more than 60 to display an NMDR, being decreased only at 0.005 ppm; however, this effect was not seen in the former study. In summary, 2 of 160 endpoints displayed apparent NMDRs in these two E2 studies with mice; and neither effect was replicated.

4.2.2.3 Diethylstilbestrol (DES) [A.1.c]

DES is a recognized developmental toxicant in humans. It was synthesized in 1938 for treatment of menopausal symptoms in women. Studies that followed shortly thereafter identified that *in utero* administration of DES produced uterine malformations in female rat offspring and that long-term treatment was carcinogenic. DES is still an environmental estrogen of concern, as it is used today in aquaculture in some countries, in order to induce sex reversals and control growth in some fish species.

vom Saal *et al.* (1997)[A.1.c.1) treated pregnant mice with 0.002, 0.02, 0.2, 2, 20, and 200 ng DES/g/d by gavage and reported increased prostate weights versus controls at 0.02 and 2 ng/g/d with a statistically significant decline at 200 ng/g/d in mice following prenatal exposures. In similar studies by Ashby *et al.* (1999)[A.1.c.1] and Cagen *et al.* (1999a)[A.1.k.2] neither identified the prostate weight change in mice exposed to 0.2 ng DES/g, which was the only dose of DES provided in these two studies as it was used as a concurrent positive control.

Nagao et al. (2012)[A.1.c.2] examined placental function and gene expression after oral treatment with 1, 5, 10, or 15 µg DES/kg by gavage to pregnant mice on GD 4 to 8. All effects

displayed monotonic dose responses except expression of ERα mRNA in the placentas of male (but not female) embryos; ERα mRNA was expressed only in male embryos of dams exposed to DES at 5 µg/kg/d and in no other dose group. ERβ, ERRβ and ERRλ mRNA were not statistically significantly affected at any dosage level.

Kim et al. (2002b)[A.1.c.6] exposed 21 to 41 day old female rats to 0.2, 1.0, or 5.0 µg DES/kg by oral gavage and NMDRs were identified for liver and kidney weights which were decreased only at 1 µg/kg/d. Other reported effects demonstrated monotonic dose responses.

Shin *et al.* (2009)[A.1.c.7] studied the effects from oral gavage administration of 10, 20, or 40 µg DES/kg on pubertal development in male rats, exposed from PND 33 to 53. Among the endpoints measured, liver weight showed an NMDR, increasing at 10 and 20 but declining at 40 µg/kg/d concurrent with a 20% reduction in terminal body weight. In other studies of perinatal exposure of rats or mice to DES Ashby et al. (1999)[A.1.c.1]; Cagen et al. (1999a, b)[A.1.k.2]; Odum et al. (2002)[A.1.c.5]; Nagao and Yoshimura (2009)[A.1.c.2]; Ohta et al. (2012)[A.1.c.4]), no effects displayed NMDRs.

4.2.2.4 Genistein [A.1.d]

Genistein is one of several phytoestrogens found at high levels in many animal and human diets. Thus, a series of studies were conducted by the NTP. Rats were administered 5, 25, 100, 250, 625, or 1250 ppm genistein in the diet, which spanned the levels found in human diets. Of the >60 individual dose response curves generated in the dose range finding study one showed an apparent NMDR; that was for the effects of genistein on the righting reflex in F1 female (but not male) pups Delclos et al. (2001)[A.1.d.1]). Three different dose levels, 50, 100, and 500 ppm, were administered in the multigeneration study NTP (2008)[A.1.d.1]). This study resulted in more than 200 individual dose response curves of which a few were NMDRs. The age at VO was accelerated in an NMDR fashion at 5 ppm in females from the F3 generation, but not in any other generation. The percent time spent in different stages of the estrous cycle also displayed NMDRs in some cases; this effect was not consistent from generation to generation. Female adrenal weights were reduced in only the 5 ppm group of the F2 generation (but not F0, F1 or F3 generations); pituitary gland weight was increased only in the 100 ppm group of the F0 generation (but not F1, F2 or F3 generations); spleen weight was increased in the 5 ppm group of the F1 generation (but not F0, F2 or F3); and thymus weight was decreased in the 100 ppm group of the F3 generation (but not F0, F1 or F2). None of these NMDRs were consistently expressed across generations, sexes, or doses. The NMDR of genistein reported by Akingbemi *et al.* (2007 (A.1.d.6]) on F1 male AGD, body weights at weaning and testis weights, were not replicated in this study in neonatal males from any of the filial generations that were exposed to 5, 50, 500, 1000 ppm genistein *in utero* and during lactation.

Three NTP studies NTP (2007)[A.1.d.1]) administered genistein in the diet of rats from the time of conception, throughout pregnancy (0, 0.5, 9, or 45 mg/kg), and through lactation (0.7, 15, or 75 mg/kg). One study continued exposures for 20 weeks post birth (0.4, 8, or 44 mg/kg to females and 0.4, 7, or 37 mg/kg to males) and another one continued exposures for 2 years (0.3, 5, or 29 mg/kg to females and 0.2, 4, or 20 mg/kg to males). There were more than 200 dose responses that were evaluated; 40 tissues were examined histologically and many tissues were weighed in the 3 studies. Effects exhibiting NMDRs were observed, however, the same response was not seen in both sexes or in more than one of the three cohorts. The NMDR identified were for these effects: reduced incidence of mammary gland fibroadenomas in the 5 ppm group in one of 3 cohorts; reduced incidence of pituitary adenomas in male rats in the 5 ppm group in one of 3 cohorts; increased incidence of preputial gland squamous cell carcinoma in male rats in one of 3 cohorts at 100 ppm; reduction in benign neoplasms in all organs in the 100 ppm group in one of 3 cohorts; reduced incidence of uterine stromal polyps in 1 of 3 cohorts in the 5 ppm group; variations in body weight; increased brain weight in females in 1 of 3 cohorts in the 100 ppm group; increased pituitary weight in females in 1 of 3 cohorts in the 100 ppm group; and increased spleen weight in females in 1 of 3 cohorts in the 5 ppm group. Some NMDR were observed in effects that might not be considered adverse, such as reduced tumor incidence.

An NMDR was identified from a two-generation reproduction study in rats given 5, 100, 500 ppm genistein in feed (Dalu et al. (2002)[A.1.d.2]). This was for ERα in the dorsolateral prostate at 100 ppm (F1 but not F2) in the cohort exposed through their mothers and then directly to genistein (G/G) but not in the cohort switched to a control diet at the time of weaning (G/C). An NMDR was also identified for ERβ protein levels in the ventral prostate of the F1 but not the F2 at 100 ppm in the G/C but not G/G cohorts. The NMDR for serum testosterone, which Akingbemi et al. (2007)[A.1.d.6]) reported as increased at 5 ppm but not at higher dosage levels in rats given 5, 50, 500, 1000 ppm genistein in the feed was not replicated in the Dalu et al. (2002)[A.1.d.2] study.

The study by Akingbemi et al. (2007)[A.1.d.6]) reported a number of findings that were not seen in other studies of genistein at similar exposure levels. Akingbemi et al. (2007) did not administer genistein, but rather they fed pregnant LE rats casein-based diets containing whole soybean as sources of protein with isoflavone concentrations of 0, 5, 50, 500, or 1000 ppm from GD 12 to PND21. They measured serum concentrations of free and conjugated insoflavones in male rats and dams at 21 d postpartum. NMDRs were identified for increased body weights and longer AGD in male rats at postnatal day (PND) 5 (at 5 and 50 ppm but not higher). In addition, serum testosterone levels and *in vitro* Leydig cell testosterone production were increased only at 5 ppm in prepubertal males, and serum luteinizing hormone (LH) was

increased only at 50 ppm in adult male offspring (fed the same diet as the dam until PND 90). They noted that testis weight was reduced at all dose levels, which has not been reported other studies of genistein.

4.2.2.5 Bisphenol A [A.1.k]

The selection criteria for studies reviewed herein on BPA used the approach described in the scientific reviews by governmental and regulatory agencies. The studies included for discussion in this document were selected from those identified by these groups as being "adequate for evaluation" and of "high utility" in the respective evaluations Chapin et al. (2008). The current state of the science evaluation does not revisit conclusions on hazard characterization for BPA. Rather we review the shapes of the dose response curves only from adequate, high utility studies that used oral dosing, with a broad range of dosage levels (at least 3 treated levels and a negative control). In most perinatal and adult studies in rats and mice exposed to BPA (Kim et al. (2002a)[a.1.k.11]; Tinwell et al. (2002)[A.1.k.1]; Ashby et al. (2003)[A.1.k.13]; Chitra et al. (2003b); Chitra et al. (2003a)[A.1.k.14]; Yamasaki et al. (2003)[A.1.k.12]; Howdeshell et al. (2008)[A.1.k.9]; Kobayashi et al. (2010)[A.1.k.6]; Ryan et al. (2010a); Ryan et al. (2010b)[A.1.k.10]) hundreds of endpoints that were measured but did not display NMDR. The specific examples of NMDR are summarized below.

Cagen et al. (1999b)[A.1.k.2] conducted a study in rats given 0.01, 0.1, 1.0, 10 ppm in water to examine the effects of prenatal and lactational BPA exposure on reproductive development of rats. Of all of the endpoints examined only male sex ratio was affected in an NMDR manner, being increased in the 0.1 ppm group (56.7% males versus 48.4% in control) but not at any other dose level. However, the same effect was not seen in other studies of BPA in rats in this dose range.

Tyl et al. (2002)[A.1.k.3] performed a multigenerational study of BPA in Sprague Dawley rats given 0.015, 0.3, 4.5, 75, 750, or 7500 ppm BPA in the feed. NMDRs were identified for several measures across the study. There were no reproducible NMDRs across generations in the current study on any endpoint. Of the 52 dose response comparisons for body and organ weights in this set of studies, relatively few had identifiable NMDR. Male rats had NMDR for liver, kidney, testes, and seminal vesicle with coagulating gland weights seemingly randomly across the generations. Female rats had NMDR for liver, ovary, and uterus weight across the generations as well. There did not appear to be any consistency across gender; for example, the liver weight NMDR inflection point was in the 0.3 ppm group in the F2 males whereas it was the 0.015 ppm group in the F2 females. Of the 11 reproductive parameters evaluated in male and female rats across the 4 generations, two NMDR were identified: the number of implantation sites and the total pups per liter both in the 0.3 ppm group of the F3 generation.

NMDR were identified for only 3 of the 17 reproductive development parameters reported in this paper: the number of live pups/liter in the F3 generation exposed to 0.3 ppm; AGD in females across the 0.3-170 ppm groups in the F2 generation; and the age of PPS in males across the 0.3-75 ppm groups in the F2 generation. Given the large number of statistical comparisons, it could be suggested that the number of NMDRs identified in the study does not exceed what could occur by chance variation.

BPA was administered in the diet of mice at 0.018, 0.18, 1.8, 30, 300, or 3500 ppm in a two generation reproductive toxicity study Tyl et al. (2008b)[A.1.k.4]). The NMDRs identified for seminal vesicle weight, 21 day survival, and thymus weight were not consistent across the generations or cohorts.

Ema *et al.* (2001)[A.1.k.5] conducted a multigenerational reproductive toxicity study administering 0.2, 2, 20, or 200 μg BPA/kg via gavage to rats. Numerous measurements were reported across the 3 generations evaluated in this study. Several NMDR were identified. Of the 50 separate measurements reported for AGD in males and females (different ages across two generations) occasional NMDR responses were identified; however, NMDR seen in the F1 were not replicated in the F2. NMDR for negative geotaxis was identified in F2 males (but not F2 females or F1 males or females); NMDR related to accelerated mid-air righting reflex was identified for F1 males (but not F1 females or F2 males or females). Of the approximately 60 organ weights taken at necropsy of F1 and F2 weanlings, two NMDR were identified in males but not females. Of the approximately 24 sperm measures taken in F1 and F0 males, an NMDR was identified for one; that is, a decrease in abnormal sperm from 1.6% in controls to 0.6% in a mid-dose group in F1 but not F0 adult males. In addition, an NMDR was identified in F1 adult males of a reduction in testis weight. However, there were no consistent NMDR across generations or genders.

Kobayashi *et al.* (2012)[A.1.k.7] treated pregnant SD rats with 0.33, 3.3, or 33 ppm BPA in the diet from GD6 to PND21. F1 pups were not exposed to BPA directly after weaning and were necropsied at 5 weeks or 3 months of age. An NMDR was noted for increased body weights of F1 males, but not females, at 0.33 ppm at 12 and 13 weeks of age (2 of 11 measurements of body weight) and in F1 females at 6 and 11 weeks of age at 33 and 0.33 ppm, respectively.

Stump *et al.* (2010)[A.1.k.8] reported on the potential of BPA to induce functional and/or morphological effects to the nervous system of F1 offspring from dietary exposure of 0.15, 1.5, 75, 750, or 2250 ppm during gestation and lactation. In that study NMDR were identified for some endpoints. Body weights were measured more than 15 times in P0 males and females, and NMDRs were identified at 5 preweaning ages in the 75 ppm group in males but not in P0 females. These effects did not persist after weaning. Weight gain was recorded for both sexes

for four time periods. In one of the eight measures of F1 weight gain during lactation male, but not female, weight gain was increased at 0.15 and 75 ppm. There is an indication of an NMDR in the Biel Water Maze as a low-dose effect in males, but not female F1 rats. The interpretability of some of the results in this study have been questioned. A Euopean Food Safety Assessment Panel concluded that "the study by Stump et al. (2010) cannot be used for the assessment of the effects of BPA on learning and memory due to methodological limitations. A number of studies addressing other neurobehavioural endpoints (*e.g.*, learning and memory behaviour, anxiety-related behaviour and gender-specific behaviour) were considered invalid or inadequate for risk assessment purposes by the Panel. The Panel does not consider the currently available data as convincing evidence of neurobehavioural toxicity of BPA" EFSA (2010)[A.1.k.8].

4.2.2.6 Selective Estrogen Receptor Modulators (SERMs) [A.2]

SERMS are synthetic drugs used as an option for the treatment of breast cancer, and osteoporosis in women, and for other conditions arising from adverse effects of estrogens or their lack. In this regard, SERMs have been developed and studied *in vitro* using cell lines from different estrogen-responsive tissues. They have also been studied in rat models (orchidectomized males and female rats) for their ability to have selective, beneficial effects on tissues like bone, without inducing potentially adverse effects on other tissues (for example, breast and prostate in females and males, respectively). These chemicals can act as estrogens in some tissues and as antiestrogens in others. The first SERM approved for pharmaceutical use was tamoxifen, but due to its stimulatory effect on the endometrium, which in some patients was determined to be a negative response, it is currently not indicated for osteoporosis.

4.2.2.7 Tamoxifen [A.2.b]

Kim et al. (2002b)[A.2.b.1]) performed the USEPA guideline EDSP pubertal female assay with several EDCs including tamoxifen given by gavage at 10, 50 or 200 μg/kg. NMDRs were identified for thyroid gland weight, thymus weight, estradiol, TSH, and T3. These effects were related to pituitary-thyroid function (thyroid weight, serum TSH, and serum T3) and ovarian estradiol production. Kennel et al. (2003)[A.2.b.2] administered 5, 30, or 200 μg tamoxifen/kg by oral gavage for 28 days to young adult male and female rats in an OECD 407 assay. Liver weight displayed an NMDR in one block, being increased at 5 μg/kg/day, but this was not repeated in the other block. In addition, uterine weight (with cervix) displayed an NMDR, being heavier after 5 μg/kg/day and lighter at 30 and 200 μg/kg/day (all groups differed statistically significantly from control). However, a one generation reproduction study in rats treated with 0.12, 0.6, or 3 μg tamoxifen/kg by gavage did not demonstrate any NMDR for any endpoints measured Yamasaki et al. (2005)[A.2.bl.3].

4.2.2.8 Aromatase Inhibitors (AI: block androgen to estrogen synthesis) [A.3]

Aromatase is a cytochrome P450 enzyme that converts androgens to estrogens by aromatizing the A ring of the steroid molecule. It is found in the ovary, brain and other tissues in the body. As a result of the structural similarity of this enzyme to other cytochrome P450 enzymes in the steroid synthesis pathway (among other pathways), aromatase inhibitors also can affect the synthesis of other hormones and disrupt multiple signaling pathways. In general, aromatase inhibitors have been developed as drugs for treatment of breast cancer and ovarian cancer in postmenopausal women.

Few multigenerational animal studies with continuous exposure were found for chemicals that act as aromatase inibitors. The aromatase inhibitors all appear to induce dystocia and to delay delivery due to inhibition of estrogen synthesis at this critical stage of pregnancy. In order to avoid the induction of dystocia, multigenerational studies terminate dosing several days before the end of pregnancy in the rat and reinitiate dosing at birth of the pups.

4.2.2.9 Exemestane [A.3.b]

Beltrame *et al.* (2001)[A.3.b.1]) describe a large number of parameters evaluated in developmental toxicity as well as male and female reproductive toxicity studies of exemestane with reported doses of 2, 4, 5, 10, 20, 40, 50, 90, 100, 125, 200, 250, 500, 810, 1000 mg/kg by gavage. The male/female sex ratio displayed an NMDR with an increased percentage of males in two mid-dose groups in one cohort in one of the two studies but not the other three cohorts. When F1s were mated to produce the F2, a sex ratio also displayed an NMDR with a reduced sex ratio in a midrange dose group. A few effects with NMDRs were identified in the Beltrame et al. (2001) study at doses above those that produced adverse effects. These received less weight in the evaluation of NMDR as a common finding (section 4.2 literature search and evaluation).

4.2.3 Androgen Hormone System [B]

The androgen signaling pathway shares many molecular and cellular traits with the estrogen signaling pathway. However, there are also some notable differences. Unlike the E pathway which has several ligand activated nuclear receptors there is only one wildtype androgen receptor (AR) in mammals. In addition, there are two physiologically active androgens. While testosterone is the major regulatory steroid in many androgen-dependent tissues, other tissues rely upon the conversion of testosterone to dihydrotestosterone (DHT) by the enzyme 5α

reductase. In the A pathway, as with the E pathway, there are tissue-specific cofactors that imbue tissue-specific responses and multiple forms of the androgen response elements (AREs) on different genes. AREs fall into two general classes, one of which is specific for AR and others that also are activated by the progesterone receptor and the glucocorticoid receptor.

Table 4.3: NMDR From Studies Evaluating Androgen Hormone System.

Chemical	Species	Group Size	Doses Given	Dosing route	Reference(s)	Appendix location
Vinclozolin	rat	10 dams, 25 pups	3, 12, or 200 mg/kg	gavage	Hellwig *et al.* (2000)	B.1.b.2
Vinclozolin	rat	20	40, 200, 1000 ppm	feed	Matsuura *et al.* (2005)	B.1.b.3
Vinclozolin	rat	25 dams	4, 20, 100 mg/kg	feed	Schneider *et al.* (2011)	B.1.b.7
Procymidone	rat	8 or 16 dams	5, 10, 25, 50, 100, or 150 mg/kg	gavage	Metzdorff *et al.* (2007)	B.1.c.4
DEHP	rat	17 pairs (P0)	1.5 (control), 10, 30, 100, 300, 1000, 7500, and 10,000 ppm	feed	Blystone *et al.* (2010)	B.2.a.1
DEHP	rat	11-16 dams	0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135, and 405 mg/kg	gavage	Grande *et al.* (2006)	B.2.a.3
DEHP	rat	11-16 dams	0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135, and 405 mg/kg	gavage	Grande *et al.* (2007)	B.2.a.4
DEHP	rat	20	0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135, and 405 mg/kg	gavage	Andrade *et al.* (2006a)	B.2.a.5
DEHP	rat	10-12	0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135, and 405 mg/kg	gavage	Andrade *et al.* (2006b)	B.2.a.6
DEHP	rat	6-15 dams	3, 10, 30, 100, 300, 600, or 900 mg/kg	gavage	Christiansen *et al.* (2010)	B.2.a.8
DEHP	rat	10-12 dams	20, 100, or 500 mg/kg	gavage	Dalsenter et al. (2006)	B.2.a.7
DEHP	rat	40	10, 500 or 750 mg/kg	gavage	Ge *et al.* (2007)	B.2.c.3
DEHP	rat		1, 10, 100, 200 mg/kg	gavage	Akingbemi *et al.* (2001)	B.2.c.1
DEHP	rat	50	5, 50, 500, or 5000 ppm	feed	Poon et al. (1997)	B.2.c.5
DEHP	rat	4	10, 100, 500 mg/kg	gavage	Vo *et al.* (2009)	B.2.d.2
DEHP	mouse	10 dams	0.05, 5, or 500 mg/kg	feed	Pocar *et al.* (2012)	B.2.e.1
DEHP	mouse	9-21 dams	0.5, 1.0, 5, 500, 50,000, or 500,000 μg/kg	gavage	Do *et al.* (2012)	B.2.e.2
DBP	rat	20 dams	50, 250, or 500 mg/kg	gavage	Zhang et al. (2004a)	B.2.b.4
DBP	rat	6-8 dams	20, 200, 2000, or 10,000 ppm	feed	Lee *et al.* (2004)	B.2.b.5
DBP	rat	?	4, 20, 100 or 500 mg/kg	gavage	Mahood et al. (2007)	B.2.b.6
DBP	rat	7 dams	0.1, 1.0, 10, 30, 50, 100, or 500 mg/kg	gavage	Lehmann *et al.* (2004)	B.2.b.7
DBP	rat	20	0.1, 1.0, 10, 100, or 500 mg/kg	gavage	Bao et al. (2011)	B.2.d.1
Semicarbazide	rat	10	250, 500, 1000 ppm	feed	Takahashi *et al.* (2009)	B.4.b

Chemical	Species	Group Size	Doses Given	Dosing route	Reference(s)	Appendix location
Semicarbazide	rat	20 dams	40, 75, or 140 mg/kg	gavage	Maranghi *et al.* (2010)	B.4.c
Semicarbazide	rat	20 dams	40, 75, or 140 mg/kg	gavage	Maranghi *et al.* (2009)	B.4.c
Prochloraz	rat	8 dams	3.9, 7.8, 15.6, 31.3, 62.5, or 125 mg/kg	gavage	Blystone et al. (2007a)	B.5.a.2
Testosterone priopionate	rat	4 dams	0.1, 0.5, 1, 2, 5, or 10 mg	sc	Wolf *et al.* (2002)	B.6.b.2
Testosterone enanthate	Human male	10-14	25, 50, 125, 300, and 600 mg/week	im	Gray et al. (2005)	B.6.b.12
SARM JNJ28330835	rat	10	2.5, 1, 30 mg/kg	gavage	Allan et al. (2007)	B.7.f

4.2.3.1 Vinclozolin [B.1.b]

Most perinatal studies conducted in rats or mice with exposure to vinclozolin (Ostby et al. (1999)[B.1.b.1]; Flynn et al. (2001)[B.1.b.6]; Owens and Chaney (2005)[B.1.b.8]; Hass et al. (2007)[B.1.b.4]; Metzdorff et al. (2007)[B.1.b.4, B.1.c.4]; Christiansen et al. (2009)[B.1.b.5]) did not identify effects that displayed NMDRs. Those that did are presented below.

Hellwig *et al.* (2000)[B.1.b.2]) administered vinclozolin by oral gavage of 1, 3, 6, 12, or 200 mg/kg to Long Evans (LE) and 3, 12, or 200 mg/kg to Wistar rats from day 14 of pregnancy until day 3 of lactation, and they monitored the reproductive development of all the male offspring from every litter through adulthood. In the Wistar strain, terminal body weight (at 3 mg/kg/d), testes weight (at 12 mg/kg/d), epididymal weight (at 3 and 12 mg/kg/d) and coagulating gland weight (at 3 mg/kg/d) displayed slight, but statistically significant, NMDRs. However, none of these NMDRs were reproduced in the LE strain. Additionally, it is not clear whether the statistical analysis of the organ weight data was adjusted for litter effects; failure to make such an adjustment can affect statistical significance, as the error degrees of freedom (df) is increased, and the standard error (SE) is decreased. NMDRs were identified for body and organ in one rat strain weights but not the other study, nor were they seen in the study by Ostby et al. (1999)[B.1.b.1]) in LE rats given 3.125, 6.25, 12.5, 25, 50, or 100 mg vinclozolin/kg by gavage.

Matsuura *et al.* (2005)[B.1.b.3] examined the effects of 40, 200, 1000 ppm vinclozolin in the diet on male and female rats of vinclozolin administration over two generations. There were more than 200 dose response curves, each with 3 group comparisons with the control for a total of 600 statistical comparisons. An NMDR was identified in liver weights for males but not females at 40 ppm, and in diestrus uterine weight for the P0 generation only. These were not seen in subsequent generations, or both sexes in the same generation (for liver weight).

In a one generation study by Schneider *et al.* (2011)[B.1.b.7]), vinclozolin was administered in the diet for nominal dosing of 4, 20, or 100 mg/kg for two (P0 females) to four (P0 males) weeks prior to mating through PND 21. After weaning, F1 animals were maintained on the diet through PND 70. The effects observed that displayed NMDR were these.

- Percent pup survival from PND 4 to 21 was reduced by 11%; however, litter sizes at PND 4 and 21 were not reduced
- Two of six measured antibody titers were reduced and displayed NMDRs

4.2.3.2 Procymidone [B.1.c]

Procymidone is a dicarboximide fungicide like vinclozolin and displays AR antagonism *in vitro* and *in vivo*. There is a reasonably comprehensive published data set on procymidone including *in vitro* AR binding and gene expression assays, short-term *in vivo* assays with some genomic data, and multigenerational studies. The dose levels used in these studies range from low mg/kg/d to hundreds of mg/kg/d. Most of the studies used in regulatory agency risk assessments, including several with relatively low procymidone exposure levels are summarized in the assessment documents; however, the dose response data are not available for public review. An NMDR was noted for left and right testis weights in male rat offspring exposed during gestation to 5, 10, 25, 50, 100, or 150 mg procymidone/kg by gavage with weights reportedly increased at 10 mg/kg/d but reduced at 100 and 150 mg/kg/d; the weights differed from the control value by only 1.3 and 0.9 mg, with standard errors of about 1.2 mg Metzdorff et al. (2007)[B.1.c.4]). In other perinatal studies rats were exposed to procymidone at 5, 10, 25, 50, 100, or 150 mg/kg by gavage Hass et al. (2007)[B.1.c.5]), 3, 10, 30, and 100 mg/kg by gavage Owens et al. (2007)[B.1.c.1]). None of the other endpoints that were measured displayed effects with NMDR.

4.2.3.3 Phthalates [B.2]

Some phthalate esters administered to pregnant rats *in utero* cause male reproductive tract abnormalities, fetal loss, abortions and skeletal malformations. The reproductive effects in the fetal male rats arise from abnormal testicular androgen and insulin-like factor 3 (insl3) hormone synthesis. By contrast, these chemicals apparently induce pregnancy loss by reducing maternal ovarian progesterone synthesis. In young pubertal males, these same phthalates disrupt Sertoli cell function, hormone synthesis and induce testicular atrophy in a wide range of mammalian species.

The list of phthalate esters that induce these effects continues to grow as more research is performed on this chemical class: DEHP, DBP, butyl benzyl phthalate (BBP), diisobutyl phthalate (DIBP), dipentyl phthalate (DPeP), dicyclohexyl phthalate (DCHP), dihexyl phthalate (DHP), diisononyl phthalate (DINP); and methylethyl hexyl phthalate (MEHP). Of these DPeP is the most potent, and DINP is only weakly positive for induction of the above effects. Studies also demonstrate that some phthalates do not produce reproductive effects at any dose. When mixture studies are conducted with phthalates they act in a dose-additive manner Howdeshell et al. (2008) where the phthalate mixture effect is a function of the dose and relative potency of each chemical.

There are numerous robust multigenerational and pubertal studies of phthalates, providing a relatively comprehensive data base for examination of the shape of the dose response curves

over a broad range of doses. A few examples of apparent low-dose NMDRs have been reported in the literature, none of which were replicated across studies.

Low-dose phthalate studies that failed to monitor background phthalate levels in control diets and bedding are difficult to interpret. Some authors have reported effects at dosage levels that were likely below the background phthalate exposure levels reported in control animals. For example, the control dietary values determined by measurement of background contamination levels reported by Blystone *et al.* (2010) is 1.5 ppm (equivalent to about 0.12 mg/kg/d); this value exceeds the levels of DEHP administered in several low-dose studies (Andrade et al. (2006c)[B.2.a.5], Andrade et al. (2006b)[B.2.a.5, B.2.a.6], Andrade et al. (2006c)[B.2.a.5]; Grande et al. (2006)[B.2.a.3]; Grande et al. (2007)[B.2.a.3, B.2.a.4] and Do et al. (2012)[B.2.e.2]). Similarly, Kondo *et al.* (2010)[B.2]) found DBP and DEHP in each of 12 untreated rodent diets tested (0.14 to 1.41 ppm) as well as all of 13 bedding materials (0.02 to 7.6 ppm) examined. The data, when further analyzed to produce a daily intake results in the background level consumed by rodents that can be as much as 1.4 µg/g diet consumed. If a pregnant rat eats about 20 g food/d then the resulting daily consumption can be as high as 84.7 µg/kg body weight/d. Pregnant mice eat about 5 g per day resulting in as much as 213.6 µg/kg body weight/d Kondo et al. (2010). Phthalates also were found in cereals, fat, oil, and other products in levels up to 10 ppm by Wormuth et al. (2006) and up to 58 ppm by Jarosova *et al.* (2009). Therefore, it is likely that tissues from control animals contain phthalate and phthalate metabolites Jarosova et al. (2009). The existence of phthalates as background contamination confounds the interpretation of studies that failed to control for background phthalate exposure and attempt to expose test animals to levels of phthalates falling below that background level.

Since several of the phthalate esters disrupt development in rats via a common mechanism of toxicity the following descriptions have been organized by species (rat then mouse), developmental period (*in utero* and lactation then pubertal) and then by chemical (DEHP then DBP).

4.2.3.3.1 Effects of Phthalates Esters in Rats: *In utero* and lactational studies: DEHP [B.2.a]

Grande *et al.* (2006)[B.2.a.3], Grande *et al.* (2007)[B.2.a.3, B.2.a.4]) and Andrade et al. (2006c); Andrade et al. (2006a)[B.2.a.5]) studied the effects of 0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135, and 405 mg DEHP/kg by gavage on Wistar rat offspring that were exposed by oral gavage from GD 6 to PND 2. The low concentrations in these studies are in the reported range for background feed levels Kondo et al. (2010). Body weights of females necropsied at PND 1 (2 per litter) displayed an NMDR, being increased at 0.045, 1.125 and 5 mg/kg/d, and liver weights

were increased in the two highest dosage groups Grande et al. (2006)[B.2.a.3]). The effects on body and liver weights seen on PND 1 were not present in females necropsied at weaning on PND 22. Several effects were reported as having NMDRs in the review by Vandenberg *et al.* (2012); however, these conclusions are not consistent with the study authors' analysis Grande et al. (2006)[B.2.a.3]). Grande *et al.* (2006)[B.2.a.3]) reported that all maternal and offspring lactational indices, litter sizes and body weights were unaffected by DEHP treatment at any dose level**.** Body weights of females necropsied at PND 1 (2 per litter) displayed an NMDR, being increased at 0.045, 1.125 and 5 mg/kg/d and liver weights were increased in the two highest dosage groups. The effects on body and liver weights seen on PND 1 were not present in females necropsied at weaning on PND 22. AGD and nipple retention in F1 females on PND 22 also were not affected by DEHP treatment. They did detect a dose related delay in VO in 15-405 mg/kg/d dose groups, all being delayed by about 2 days, but the age at first estrus was not affected. Several effects including "vaginal opening, and first estrous" were reported as NMDRs by Vandenberg *et al.* (2012)(Table 7), however, these conclusions are not consistent with Grande *et al.* (2006) analyses of the same data.

Maternal kidney weight displayed an NMDR, being increased in dams treated with DEHP at 0.045 mg/kg/d Grande et al. (2007)[B.2.a.3, B.2.a.4]). Similar to F1 females Grande et al. (2006)[B.2.a.3] and Andrade et al. (2006c)[B.2.a.5] reported that body weights of males necropsied at PND 1 (2 per litter) displayed an NMDR, being increased at 0.045, 1.125 and 5 mg/kg/d. Liver weights were also increased in the two highest dosage groups. Also similar to their female siblings, the effects of DEHP on body and liver weights at PND 1 were not present in males necropsied at weaning on PND 22.

In Andrade et al. (2006c)[B.2.a.5] an NMDR was identified for AGD at PND 22, being increased in the 0.015 mg/kg/d DEHP group versus controls and decreased at 405 mg DEHP/kg/d. An NMDR for testis weight was identified, and it was statistically significantly increased at 5, 15, 45 and 135 mg/kg/day on PND 22. This observation qualitatively differed from exposure to higher doses in Andrade et al. (2006c)[B.2.a.5]. These effects also differ from testis weight data from other studies in prepubertal male rats: studies wherein animals were exposed to DEHP at 3, 10, 30, 100, 300, 600, or 900 mg/kg Christiansen et al. (2010)[B.2.a.8]) and to 10, 100, 300, or 900 mg;kg Noriega et al. (2009)[B.2.c.4]) by gavage. The lack of an NMDR on the age of PPS is consistent with the observations of Noriega *et al.* (2009)[B.2.c.4]. Noriega *et al.* (2009) did not replicate the NMDR identified at 1 and 10 mg DEHP/kg/d which was reported in Ge *et al.* (2007)[B.2.a.5 and B.2.c.3]) who treated rats with 10, 500, or 750 mg/kg by gavage. Andrade et al. (2006a)[B.2.a.5] measured a number of endpoints, some of which displayed NMDRs at doses consistent with reported background dietary DEHP levels Kondo et al. (2010).

Dalsenter et al. (2006)[B.2.a.7] evaluated the effects of DEHP on rats treated with 20, 100, or 500 mg/kg by gavage for reproductive function and sexual behavior of F1 male rat offspring rats after *in utero* and lactational oral gavage treatment. Weaning weight displayed an NMDR being increased only at 100 mg/kg/d, the intermediate dose administered.

Christiansen *et al.* (2010)[B.2.a.8] administered 3, 10, 30, 100, 300, 600, and 900 mg DEHP/kg/day from GD 7 to PND16 by oral gavage in two blocks. NMDRs were seen but were generally not reproducible from block to block. The NMDR that were identified in this study were in F1 males (but not females) as the weaning weight was increased only after treatment with 100 mg/kg/d in block 1 but not block 2. Post-implantation loss was increased only after treatment with 10 mg/kg/d, in block 2 but not block 1. The diagnosis of "Mild" genital dysgenesis (on a score of 0 to 3, mild=1) was increased in 16 day old males in all dose groups except 30 mg/kg/d. The biological significance of this change is uncertain since malformation of the external genitalia has not been described for this dose in adult F1 animals. NMDRs were identified for the gene expression of PCB C3 and ODC in block 2 but not 1. NMDR were also identified for some organ weights across the study. Right but not left testis weight was reduced in block 1 (but not 2) after treatment with 100 mg/kg/d; this was not statistically different from control after treatment with 300 mg/kg/d. Levator ani bulbo-cavernosus (LABC) weight was decreased in block 1 in all treated groups except 600 mg/kg/d. In block 2, 10 and 30 mg/kg/d slightly reduced LABC weight, but 100 mg/kg/d did not. Adrenal weight was reduced in block 1 after treatment with 10, 100 and 900 mg/kg/d but not in the other dose groups; the effects at 10 and 100 mg/kg/d were not replicated in block 2. Many of these NMDR were no longer apparent when the two blocks were pooled and analyzed together.

4.2.3.3.2 Effects of Phthalates Esters in Rats: *In utero* and lactational studies: DBP [B.2.b]

Several perinatal studies with DBP exposure in rats treated with 250, 500, or 750 mg/kg Mylchreest et al. (1998)[B.2.b.3], 100, 250, 500 mg.kg Mylchreest et al. (1999)[B.2.b.2],.5, 5, 50, or 100 mg/kg Mylchreest et al. (2000)[B.2.b.1], and 10, 50, or 500 mg/kg (Wyde et al. (2005)[B.2.b.8]) by gavage did not identify any effects with NMDRs. Those studies that did are presented below.

Zhang et al. (2004b)[B.2.b.4]) administered 50, 250, and 500 mg/kg DBP by oral gavage from GD 1 to PND 21, and the male offspring were examined through adulthood. Of the 20 endpoints measured (with 60 potential statistical comparisons with the control group), prostate weight had an NMDR, being reduced only at 250 mg/kg/d, a dose at which several other endpoints also were statistically significantly affected in a monotonic manner.

In a study by Lee *et al.* (2004)[B.2.b.5], pregnant rats were treated with 20, 200, 2000 or 10,000 ppm DBP in the diet from GD15 to PND21. Several NMDRs were identified in this study. The number of live pups was reduced and body weights were increased in the low dose group only at PND2. The age at puberty in males occurred 1.3 days earlier at 200 ppm than controls but not at higher doses. Pituitary weight in males at week 11 was increased at 20, 200 and 2000 ppm, but not in females or in males at other ages. Prostate weight was increased at week 11 in the 200 ppm group but not at 20 weeks. An NMDR was also identified for the severity of histological alterations in male mammary gland of the low and middle dose groups at 11 and 20 weeks. No mammary gland effects were present at weaning or in females.

In a study by Mahood et al. (2007)[B.2.b.6] pregnant Wistar rats were gavaged daily with 4, 20, 100 or 500 mg DBP/kg from GD 13.5 to either GD20.5 (fetal samples) or GD21.5 (postnatal tissue), and the male fetuses and F1 adult male offspring were examined. All of the effects displayed monotonic responses. The frequency of mild Leydig cell clusters in GD 21.5 testes showed an NMDR, however, the severity of the testis alterations progressed from medium at 100 mg/kg/d to large at 500 mg/kg/d.

Lehmann *et al.* (2004)[B.2.b.7] administered 0.1, 1.0, 10, 50, 100, or 500 mg DBP/kg by oral gavage to pregnant rats on GD 12 to 19. Fetal testes were isolated on GD19, and changes in gene and protein expression and testicular testosterone concentration were measured. The protein expression and testosterone concentrations displayed monotonic responses, and these effects were statistically significant at 50 mg/kg/day and higher. Of the mRNAs evaluated, SR-B1, 3β-HSD and c-Kit displayed NMDRs, being significantly reduced at the lowest dosage levels (0.1 and 1 mg/kg/d) but not at 10 mg/kg/d. Attempts to replicate the low dose NMDR effects of DBP on SR-B1 and 3β-HSD were unsuccessful, whereas some of the reductions reported at the higher dosage levels were replicated (LE Gray personal communication with author).

4.2.3.3.3 Effects of Phthalates Esters in Rats: Peripubertal exposure effects of DEHP on male rat reproductive development [B.2.c]

Phthalate treatment during peripubertal development causes testicular lesions in several mammalian species including the rat, ferret, guinea pig, and some strains of mice and hamsters; the sensitivity of the species varies considerably. Some effects have also been reported for male and female marmosets. The constellation of effects of peripubertal treatment in the young males includes testicular atrophy, seminiferous tubule hypospermatogenesis, reduced sperm counts, infertility, delayed puberty, reduced androgen-dependent tissue weights and transient alterations in serum androgen levels.

Results from multigenerational studies demonstrate that some of the effects of phthalate treatment, such as delayed puberty, are consistently seen only when the treatment continues after weaning through the peripubertal period, rather than with *in utero* and lactational exposure alone. Multigenerational studies with pubertal dosing of 250, 500, or 1000 mg DBP/kg or 200, 400, 100 mg/kg, have not reported NMDRs for PPS Wolf et al. (1999).

An examination of the literature on the effects of DEHP on pubertal development indicates that two of the studies have reported NMDRs on pubertal measures in young male rats treated with 10, 500, or 750 mg/kg Ge et al. (2007)[B.2.c.3]; Vo et al. (2009)[B.2.d.2]) for the same endpoints. These NMDRs are not consistent with other data from the same laboratory (Akingbemi et al. (2001)[B.2.c.1]; Akingbemi et al. (2004)[B.2.c.2]) or with other laboratories (Noriega et al. (2009)[B.2.c.4]; Andrade et al. (2006c)[B.2.a.5]; Wolf et al. (1999)[B.5.b.4].

Ge *et al.* (2007) did studies in the same laboratory as those by Akingbemi *et al.* (2001)[B.2.c.1] and Akingbemi *et al.* (2004)[B.2.c.2]. In their study, Ge *et al.* (2007)[B.2.c.3] dosed rats with DEHP by oral gavage at 10, 100, or 200 mg/kg Akingbemi et al. (2001)[B.2.c.1], 10 or 100 mg/kg Akingbemi et al. (2004)[B.2.c.2]), and 10, 500 or 750 mg/kg Ge et al. (2007)[B.2.c.3]; this was done by gavage for 28 days from PND 21 to 49. This study reports an NMDR for the age at puberty in treated males, with a 1.8 day acceleration at 10 mg/kg/d; no statistically significant effect at 500 mg/kg/d; and a 6.9 day delay at 750 mg/kg/d. The authors also reported that body and seminal vesicle weights and serum T levels were increased at 10 mg/kg/d. It appears that the authors randomly assigned rats to treatment groups rather than controlling for weaning body weight as recommended in the EPA EDSP test guideline. This may introduce a confounder in the pubertal male assay, and failure to control for this confounder could explain the acceleration in PPS and other effects attributed to this 10 mg/kg/d dosing. In the Akingbemi *et al.* (2001)[B.2.c.1] study, this dose did not cause an increase in body weight in similarly exposed males, whereas LH was increased, an effect not seen by Ge *et al.* (2007)[B.2.c.3]. There is difficulty in interpreting whether serum hormone changes are adverse in that the two studies from the same laboratory report serum T and LH levels that differ by two fold from the control group across the studies; this is greater than the reported DEHP effects at 10 mg/kg/d. In addition, Noriega *et al.* (2009)[B.2.c.4]) did not see accelerated puberty or increased serum T levels in males exposed to DEHP at 10 mg/kg/d, whereas statistically significant delays in puberty were seen at 300 mg/kg/d and above. Similar studies with DBP given at 0.1, 1.0, 10, 100 or 500 mg/kg by gavage (Bao et al. (2011)[B.2.d.1]) a phthalate with the same mechanism of toxicity and mode of action as DEHP, did not identify any of the hormonal changes reported by Ge *et al.* (2007)[B.2.c.3].

When Ge *et al.* (2007)[B.2.c.3]) dosed rats with DEHP for 14 days from PND 21 to 34 none of the low dose effects from exposure on PND 21-49 were noted at 10 mg/kg/d. The authors also

reported that MEHP *in vitro* produced an NMDR on Leydig cell T production, increased above baseline at 10^{-4} and 10^{-3} M and then decreased at 10^{-2} M.

While effects on PPS and body and reproductive organ weights with an NMDR were observed in the Ge *et al.* (2007)[B.2.c.3], they were not seen by Noriega *et al.* (2009)[B.2.c.4] in either of two rat strains studied, and some of the other effects reported in Ge *et al.* are not consistent with findings from other publications.

4.2.3.3.4 Pubertal effects of DEHP in male rat [B.2.c]

Results from a study wherein rats were dosed with DEHP from PND 23 until necropsy at 56 or 98 days of age reported no NMDR Noriega et al. (2009)[B.2.c.4]. However, Poon et al. (1997)[B.2.c.5] performed a 90 day study with 5, 50, 500, or 5000 ppm DEHP in the diet in young male and female SD rats; this study conformed to OECD Guidelines under GLP. Of the endpoints measured and effects observed, NMDR were identified for two of six serum biochemistry indices in female, but not male, rats.

4.2.3.3.5 Pubertal effects of DBP in male rat [B.2.d]

In Bao et al. (2011)[B.2.d.1]) five week old male SD rats were administered DBP orally for 30 days, and reproductive organ weights, testicular histopathology and serum hormonal levels were measured at necropsy. In addition, proteomic analysis was performed on testes to determine if any proteins were affected by DBP treatment; however, these analyses were not conducted in the 100 and 500 mg/kg/d groups. Several nonmonotonic effects were identified.

- Serum E2 was increased at 0.1 and 500 mg/kg/d but not in other dose groups.
- Serum LH was increased in all groups, but the increase was not statistically significant at 1.0 mg/kg/d.
- Of the twenty proteins from the testis reported to be affected in the low dose groups (10 mg/kg/d and below) the dose response data are only shown for 4 of these, one of which (vimentin) displayed an NMDR (n=3 per group).

In addition, peripubertal DBP-treatment did not induce a NMDR on serum testosterone at low doses, in contrast to some of the aforementioned reports where DEHP was administered during puberty.

Vo *et al.* (2009)[B.2.d.2] administered 10, 100, and 500 mg/kg DEHP by gavage to rats from PND 21 to 35. Some authors have identified NMDR for seminal vesicle weight, epididymal weight, and testicular expression of steroidogenesis genes as measured by rtPRC Vandenberg et al. (2012). In reviewing the Vo *et al.* (2009) study we authors of the current evaluation note that

the data suggest no alteration of the three steroidogenesis genes measured, StAR, CYP11a1 and HSD3β1,by DEHP treatment at any dose level (Fig 4, a,b,c in Vo *et al.* (2009)[B.2.d.2]). However Vo *et al.* (2009) [B.2.d.2] do identify an additional set of 2 genes evaluated in the testis, whichdo express NMDRs. Vo et al. (2009) considered these to be markers of exposure. In summary NMDR were identified for two reproductive organ weights and for mRNA expression levels for two testis genes. The small sample size (n=4/group) and lack of concordance of the organ weight effects reported here with several other studies raises uncertainty about the biological significance of these results.

4.2.3.3.6 Effects of Phthalates Esters in Mice: *In utero* and lactational exposure effects of DEHP on male rat reproductive development [B.2.e]

NMDRs have been described at low dose levels for some endpoints after *in utero* DEHP exposure in mice. However, there is uncertainty in interpreting some of these results as they report administered DEHP at levels below the published background levels in rodent diets of this ubiquitous contaminant. In addition, the effects are small and are not necessarily adverse. At present there is debate in the scientific community about the ability of phthalates, such as DEHP, to demasculinize fetal mice as is seen in the fetal rat. The link between the changes seen in the fetal mouse to postnatal reproductive outcomes is not established.

DEHP was administered to mice at 0.05, 5, and 500 mg/kg in the diet during pregnancy and lactation, and the male and female offspring were examined and necropsied at PNDs 21 and 42 Pocar et al. (2012)[B.2.e.1]. DEHP induced an NMDR response on testis weight, being reduced only in the low dose group. The analysis of these data does not appear to be adjusted for litter effects, which can inflate the statistical significance of the reported effects. *In vitro* fertilization studies identified NMDR associated with reductions in the cleavage and blastocyst rates in the low dose group. It is not reported how many times the *in vitro* assays were replicated as the analyses appear to be based upon a single pool of oocytes and fertilized ova for each dose group. However, the expression of genes related to steroidogenesis were altered in the ovary, testis, and pituitary of the offspring in a dose related manner.

Do *et al.* (2012)[B.2.e.2]) administered 0.5, 1.0, 5, 500, 50,000, or 500,000 μg/kg DEHP by gavage from GD 9 to 18 orally to mice and examined maternal and fetal (1M males only) hormones on GD 18. Although the study was conducted in three blocks (incomplete block design) only the pooled data are presented in the paper. Data for fetal males from intrauterine positions other than 1M is not presented. The effects are small, and none of the effects were statistically significantly altered by DEHP-treatment (all F values from the ANOVA are non-significant; >0.05). The study authors, however, report several of these effects as NMDR. The biological significance of the reported effects (small increases in serum T), is not clear as T

changes dramatically in the dam and fetal male during this stage of pregnancy. There is uncertainty as to comparison of the administered doses of DEHP to reported background levels in the animal's diet and bedding. Some of the administered doses are below the levels of phthalates reported in animal diets and bedding Kondo et al. (2010).

4.2.3.4 Semicarbazide [B.4]

Semicarbazide is a metabolite of the banned antibiotic nitrofurazone and a breakdown product of azodicarbonamide, which is used as a blowing agent in plastic gaskets EFSA (2005). Semicarbazide is known to inhibit enzymes, such as lysyl oxidase, semicarbazide-sensitive amine oxidase and glutamic acid decarboxylase. Semicarbazide acts as osteolathyrogen, and induces osteochondral and vascular lesions in young rats due to impaired cross-linking reactions of collagen and elastin Takahashi et al. (2009)[B.4.b]). In addition, teratogenic effects, such as induction of cleft palate and aortic aneurysms, have also been reported. It has been described as an example of an EDC that displayed an NMDR for the age at puberty in male rats.

Semicarbazide was administered at 250, 500, and 1000 ppm in the diet to 6 week old male and female rats for 90 days Takahashi et al. (2009)[B.4.b]. The study included a large number of endpoints, a few of which displayed NMDRs at doses above the NOAEL; namely one of the >40 hematology measures and food consumption in male rats.

Semicarbazide was administered at 40, 75, and 140 mg/kg to 23 day old male and female rats by oral gavage for 28 days Maranghi et al. (2010; Maranghi et al. (2009)[B.4.c]. Puberty in males was accelerated at 40 and 75 mg/kg/d but delayed at 150 mg/kg/d. Given all the lesions seen with semicarbazide in the lowest dose level, taken together with what is known about the mechanism of toxicity for the bone and vascular lesions, we authors of this state of the science evaluation do not consider semicarbazide an EDC. The NMDR effect on puberty at high dosage levels is of uncertain biological releavance.

4.2.3.5 Multiple Molecular Initiating Events [B.5]

It has been shown that a number of environmental chemicals disrupt the androgenic signaling pathway in an antiandrogenic manner impacting multiple toxicity pathways. Pesticides such as linuron and prochloraz act as AR antagonists and inhibitors of fetal T synthesis. In contrast to the phthalates (discussed above), prochloraz and linuron reduce fetal T production without inhibiting mRNA expression of steroidogenic enzymes or the insl3 hormone. Although the chemicals that disrupt androgen signaling in the fetal male rat produce some malformations in common, the specific profiles of effects are chemical specific.

4.2.3.5.1 Prochloraz [B.5.a]

In the first of a series of experiments (Blystone et al. (2007a)[B.5.a.1], Blystone et al. (2007b)[B.5.a.2]), SD weanling male rats were dosed by gavage with prochloraz at 31.3, 62.5, or 125 mg/kg/day of from 23 to 42 or 51 days of age. There was a statistically significant delay in PPS at 125 mg/kg/day PCZ and several of the androgen-dependent organ weights were decreased significantly. At both ages, serum testosterone levels and *ex vivo* testosterone release from the testis were statistically significantly decreased whereas serum progesterone and 17a-hydroxyprogesterone levels were statistically significantly increased at dose levels below those that affected PPS or reproductive organ weights. The hormone results suggested that PCZ was inhibiting CYP17 activity. None of the effects displayed an NMDR; however, the dose response curves varied statistically significantly among the groups necropsied at 42 and 51 days of age, as did the LOEL for organ weight reductions being 31.3 mg/kg at 42 days and 62.5 at 51 days of age. In the second pubertal study rats were dosed by gavage with prochloraz at 0, 3.9, 7.8, 15.6, 31.3, or 62.5 mg/kg/d. Serum testosterone levels and ex vivo testosterone production were significantly reduced at 15.6 mg/kg/d whereas ex vivo androstenedione production was significantly reduced at 7.8 mg/kg/d with 3.9 mg/kg/d being a NOEL. Two related endpoints showed NMDRs in this study as body weight at necropsy and glans penis weights were increased at 3.9 mg/kg/d. In order to determine if prochloraz displayed AR antagonism in vivo independent of its effects on testosterone synthesis, castrated immature male rats were dosed with androgen and 0, 15.6, 31.3, 62.5, or 125 mg/kg/day PCZ for 10–11 days (Hershberger assay). In this assay, androgen-sensitive seminal vesicle and LABC weights were significantly decreased at 125 mg/kg/d. Liver weight was increased at 31.3 mg/kg/d and above and serum LH was decreased at 125 mg/kg/d. These effects were all dose related. In this study, glans penis weight displayed an NMDR being reduced only at 15.6 mg/kg/d, a response in the opposite direction from the NMRD seen in the pubertal study. Effects described in other studies from prenatal or pubertal exposures to prochloraz displayed monotonicity, and no other effects with NMDRs were seen Noriega et al. (2005)[B.5.a.4]; Christiansen et al. (2009)[B.5.a.3].

4.2.3.6 Androgen Receptor Agonists [B.6]

There appears to be no publically available rodent multigenerational study with administered androgen agonist in the diet or by oral gavage over a broad dose range. Most data sets have been developed for submission for pharmaceutical product approval. The dietary and oral gavage studies on the synthetic androgen trenbolone have been reviewed, and detailed summaries are available on the internet. These do not, however, allow one to examine the shapes of the dose response curves.

This class of chemicals provides some examples of NMDRs for clearly adverse effects by several androgens including testosterone, Trenbolone, and methyltestosterone in mammals and fish. The NMDRs identified with androgens in the mammalian studies administered the chemical either by subcutaneous injection or through the use of implants. The relevance of these routes of administration to human health can be debated. They were not considered to be useful for the present evaluation based on the literature search and evaluation criteria (section 4.2 above). When effects were compared in the Hershberger Assay between injection versus oral administration for androgens, the spectrum of effects are similar, but the oral route was approximately 80 fold less potent Wilson et al. (2002)[B.6.a.1]). One study wherein an NMDR was identified administered testosterone sc during pregnancy and evaluated the male and female offspring Wolf et al. (2002)[b.6.b.2]. In these studies, adverse effects were identified after exposure at doses below which the NMDR were identified; thus, the characterization of the NMDR would not affect determination of a NOAEL in the studies.

In addition to rodents, the effects of androgens have been studied extensively for decades in many vertebrate species, including humans. In this section of the review, a number of experimental studies on the dose-related effects of androgens in humans are presented. It is clear from these studies that few if any NMDRs are identifiable associated with testosterone administration to humans.

4.2.3.6.1 Trenbolone [B.6.a]

Trenbolone binds the AR and is a potent androgen agonist both *in vitro* and *in vivo*. Extensive dose response studies with trenbolone have been conducted in a number of mammalian species including rats, domestic animals, and nonhuman primates. Some of these studies are summarized in an FDA document (NADA-138-612 Finaplix at http://www.fda.gov/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/FOIADrugSummaries/ucm111214.htm). Summaries from a multigeneration and supplementary hormonal study conducted in rats (James, Huntington Research Centre), a preliminary oral toxicity study in young male and female Cynomolgus monkeys (Sortwell, Huntington Research Centre), and a hormonal study in female Rhesus Macaques (Hess, Oregon Regional Primate Research Center) report effects on reproductive or hormonal endpoints following exposure to trenbolone acetate; however, they do not indicate NMDRs. The actual data are not available for review.

In other studies, prenatal trenbolone exposure to 0.1, 0.5, 1.0, or 2.0 mg/kg Hotchkiss et al. (2007)[B.6.a.1] by gavage and 12.5, 25, 50, 100, or 200 µg IP (Wilson et al. (2002)[B.6.a.2]) resulted in androgenic effects on developmental and reproductive endpoints that were all dose related except that pup weight was reduced in the high dose group at birth but not at 13 days of age (Wilson et al. (2002)[B.6.a.2]). Findings from Hershberger Assays showed monotonic

dose related changes from oral administration of 0.3, 1.5, 8, and 40 mg/kg trenbolone by gavage Owens et al. (2007)[B.6.b.4]); however, sc administration produced an NMDR at high dose on body weight gain over the ten-day dosing period.

4.2.3.6.2 Testosterone [B.6.b]

Investigators have demonstrated pronounced effects with NMDRs from sc or implants of testosterone on the testis. It has been repeatedly demonstrated in young adult male rats Robaire et al. (1979; Ewing et al. (1977; Walsh and Swerdloff (1973), rabbits Ewing et al. (1973), and rhesus monkeys Ewing et al. (1976) that increasing doses of testosterone can result in a reduction in LH. This is followed by declines in testis androgen levels, sperm production and testis weight without causing increases in serum testosterone of androgen-dependent organ weights. However, as testosterone dosage levels are increased above the nadir of the NMDR, testis weight and sperm production levels are partially restored due to increasing levels of intratesticular testosterone from the serum.

In addition, Wolf *et al.* (2002)[B.6.b.2] administered 0.1, 0.5, 1, 2, 5, or 10 mg testosterone priopionate sc on GD 14-18. Although most effects displayed monotonic, dose-related changes, three effects displayed pronounced NMDRs; these were for uterine weight, incidence of uterine hydrometrocolpos, and survival of female offspring after puberty. Other prenatal Hotchkiss et al. (2007)[B.6.b.3] or Hershberger studies Owens et al. (2006)[B.6.b.4] exposing rats to testosterone sc, did not produce effects that displayed NMDRs.

In a series of studies, the dose-related effects of testosterone were described for a number of physiological processes in healthy, eugonadal men, 18–35 years of age. No effects with NMDRs were identified (Bhasin et al. (2001)[B.6.b.5]; Singh and Nocerino (2002)[B.6.b.7]; Sinha-Hikim et al. (2002)[B.6.b.6]; Storer et al. (2003)[B.6.b.8]; Coviello et al. (2005)[B.6.b.10]; Bhasin et al. (2005)[B.6.b.11]). In a study to determine if sexual function in older men (libido, sexual activity, and erectile function) was affected by graded doses of testosterone (Gray et al. (2005)[B.6.b.12]), some effects did display NMDRs; these included overall sexual function score and waking erection frequency.

4.2.3.7 Selective Androgen Receptor Modulators (SARMS) [B.7]

Over the last ten years SARMS have been developed for oral treatment of androgen-responsive tissues, which provide the beneficial effect with minimal side effects. None of the currently available SARMS are completely selective for the desirable anabolic effects on muscle and/or bone without producing undesirable androgenic side effects in sensitive tissues such as the prostate gland. SARMs have been developed that are orally active without causing liver

damage; in contrast to testosterone, more potent and selective SARMs have been developed with enhanced tissue-selectivity. In elderly men with osteopenia or osteoporosis, it is desirable to have a SARM targeting bone and muscle tissue but with lesser or no activity on the prostate or testes. A SARM for women would ideally stimulate bone retention or libido and other sexual function, without negative side effects such as masculinization, increased LDL/HDL ratios, or liver dysfunction.

Since the typical target populations of SARMS are aging men and women, there are few multigenerational test guideline-type studies. The animal models in use in the pharmaceutical industry to evaluate the SARMS are often the adult castrated male or female rat. In addition, as is the case for SERMS, *in vitro* assays have been useful to screen chemicals for tissue specific effects using cell lines derived from the different steroid-responsive tissues; this is because some of the selective responses of different tissues to SARMS arise from differences in tissue specific coactivators or corepressors. In addition, differential metabolism of the different steroidal androgens can also significantly alter the toxicity profile of the chemical (*e.g.,* aromatizable or not or activated or inactivated by 5α reductase). The studies that were available for review presented typical monotonic dose responses to exposure and are described in Appendix B.7.

4.2.4 Thyroid

Thyroid hormones are responsible for regulating day-to-day function of many biological systems in mammals and other vertebrates, and they are essential regulators of early development. As briefly described in Section 2, thyroid hormone levels and thyroid hormone action are regulated by multi-tissue feedback-control systems, including the classic HPT neuroendocrine steroid hormone feedback system that controls TH production and release. TH affects gene transcription through nuclear receptors encoded by two genes, TRα or TRβ and TH-mediated gene transcription is particularly important for brain development Williams (2008). A key feature of TH action in brain is the temporal sequence of events it supports, a feature that increases the complexity of determining the impact of xenobiotic-induced alterations in thyroid function. Transport of TH in the blood is controlled by serum binding proteins, thyroid binding globulin (TBG) and transthyretin (TTR) have the highest affinity for THs. Specific transporter proteins (monocarboxylate transporter, MCT and organic anion-transporting polypeptide, OATP) located on blood vessels, astrocytes, and neurons actively take up T3 and T4 (Williams, 2008; Bernal, 2011). Tissue concentrations of THs are also controlled by a number of cellular mechanisms, most notably the expression and activity of deiodinase enzymes which provide an additional means of control over TH-dependent gene regulation Bernal (2011; Williams and Bassett (2011). In addition to the direct molecular action of TH binding to target receptors, non-genomic actions of TH have also been identified Davis et al. (2008). T4 and its presumed

iodothyronamine metabolites, have rapid physiological action both *in vitro* and *in vivo* indicating that TH may also act at non-nuclear sites.

4.2.4.1 Environmental Contaminants and Thyroid Disruption

Environmental contaminants that cause adverse outcomes by acting as thyroid disrupting chemicals (TDCs) (Fig 2.7) have been shown to affect one or more pathways in the thyroid hormone system, which can then lead to an adverse outcome Murk et al. (2013; Crofton (2008; Köhrle (2008; Brucker-Davis (1998; Capen (1997). Current knowledge indicates that the majority of thyroid disrupting effects are mediated by molecular intiating events (MIEs) at sites other than direct interference with thyroid receptor (TR) binding in the target tissue (Figure 4.11). This includes both thyroidal and extrathyroidal sites of action Capen (1997) that extend well beyond the HPT axis. Several compounds are known to inhibit thyroperoxidase (TPO), a critical enzyme for the synthesis of T3 and T4 in the thyroid gland, as the molecular initiating event. There are also several anions (*e.g.*, bromate, chlorate, nitrate, perchlorate, thiocyanate,) that compete with iodine transport by the sodium-iodine symporter (NIS) Eskandari (1997; Wolff (1964) Dohan et al. (2003). The NIS is essential for concentration of sufficient amounts of iodine in the thyroid gland for adequate TH production. Competition for transport between these environmental contaminants and iodine reduces iodine uptake into the thyroid gland, resulting in a decrease of available iodine for production of TH, decreased TH production and secretion, and a compensatory increase in circulating TSH. A variety of extrathyroidal mechanisms also affect TH levels by: altering binding to hormone transport proteins, which may change tissue availibity of TH, increased hepatic clearance of TH or TH catabolites, which will lower cirrulating levels of TH, or inhibition of deiodination within peripheral tissues, which will decrease tissue levels of T3 and decrease transcriptional processes Murk et al. (2013; Crofton (2008; Capen (1997). The primary downstream consequences of most of these these effects are to alter TH-directed transcription via changes in circulating or tissue concentrations of THs (Figure 4.11).

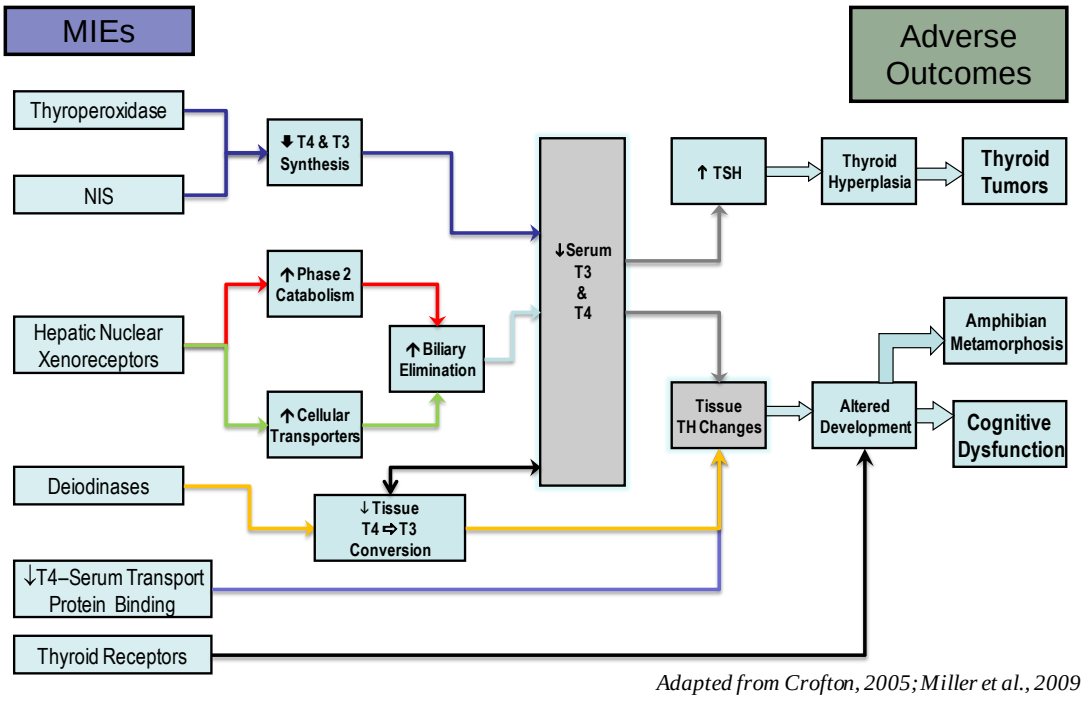


Figure 4.11: An adverse outcome pathway model for the effects of TDCs. Abbreviations: MIE, molecular initiating event; NIS, sodium iodine symporter: T3, triiodothyronine; T4, thyroxine; TH, thyroid hormone; TSH, thyroid stimulating hormone; TTR, transthyretin; UGT, uridine diphosphate glucuronyltransferase (modified from Miller et al. (2009); Crofton and Zoeller (2005).

4.2.4.2 Literature Search and Analysis

To evaluate the possibility that nonmonotonic dose-response relationships exist for many thyroid active chemicals, two approaches were used:

1) An in depth review was performed for a chemical representative of one of three widely accepted MIEs for which an extensive literature was available. The MIEs included thyroperoxidase (TPO) inhibition; inhibition of the sodium iodide symporter (NIS); and nuclear receptor activation induced upregulation of TH metabolism. These MIEs were represented by propylthiouracil (PTU), perchlorate (ClO_4^-), and polyhalogenated aromatic hydrocarbons (PHAHs), respectively. These specific chemicals were chosen because each is characterized by a rich database that encompasses studies from many laboratories, contain a diversity of endpoints, and a provide data from a broad range of doses and dosing scenarios.

2) The second approach adopted to evaluate the incidence and impact of NDMR for the thyroid system was an extensive review of the published literature for evidence of NMDRs for thyroid endpoints. The specifics of the literature search can be found in Appendix C. Briefly, evaluation of 1153 papers ceded a total of 814 relevant references and 2060 chemical-studies, of which 229 were non-mammalian in vivo chemical-studies and 1831 were mammalian in vivo or in vitro chemical-studies. Chemical-studies were defined as independent determinations of a dose or concentration-response for a chemical (see Appendix C for details). The bulk of which were mammalian based studies.

The publications containing the 1831 mammalian and in vitro chemical-studies were then evaluated for the presence of NMDR effects at low doses that would impact regulatory action (e.g, lowest effective dose for a chemical). The evaluation was conducted according to criteria outlined in the 'Decision Tree' in Figure 1 of Appendix C. Briefly, the criteria for inclusion were:

Filter 1: Minimum of 3 dose levels plus a control evaluated
Filter 2: Evidence of a statistically significant NMDR on any thyroid endpoint
Filter 3: Absence of observations at lower dose levels in the study that would have been used to determine the LOEL/LOAEL
Filter 4: a) Absence of other published reports on this chemical where effects were observed at low levels.
b) Absence of other published reports for effects on other endpoints that would have been used to determine the LOEL/NOEL below the dose identified as an NMDR.
c) Absence of study quality concerns or statistical power issues that weakened confidence in the NMDR observation.

4.2.4.3 Review of Three Major Targets for Nonmonotonic Thyroid Pathway Disruption

4.2.4.3.1 TPO Inhibition to Reduces TH Synthesis – Propylthioruacil Case Study

Several chemicals, including methimazole and PTU are known to inhibit TPO, an enzyme in the thyroid gland critical for the synthesis of T3 and T4 Gardner et al. (1986; Cooper et al. (1983). In peripheral tissues, PTU also inhibits deiodinases that convert T4 to its active form, T3 Cooper et al. (1983). PTU has been used extensively as an experimental tool to study the impact of TH disruption on physiological systems. As such, we have focused on PTU for a thorough review as a representative classic thyroid disruptor because of the breadth of its database in addition to the wide array of outcome measures that have been quantitatively examined in a dose-response manner.

Given the essential critical role of TH in early development and the greater susceptibility of the developing fetus and neonate to TH insufficiencies, PTU has been utilized in a multitude of studies as a model compound to alter thyroid status during a critical period of brain development. Although the majority of these are restricted to a single high dose of PTU that induced a severe state of hypothyroidism, a number of papers (~20) were identified that evaluated a range of doses. Reviewed here are studies that evaluated the impact of graded levels of thyroid disruption induced by PTU on serum markers and a variety of outcome measures in response to adult, neonatal, or fetal exposure to PTU.

A number of early reports administering multiple concentrations of PTU to adult male or female laboratory rats in the drinking water Cooper et al. (1983; Männistö et al. (1979), food O'Connor et al. (2002; Hood et al. (1999; O'Connor et al. (1999), or by oral gavage Cho et al. (2003) focused on the impact of PTU on serum TH profiles. Studies varied not only in route, but also in durations of exposures (days to weeks) and dose ranges, but in all cases three to five PTU concentrations were used. For all of these reports, monotonic dose-responses were most commonly observed for serum T3, T4 and TSH. Longer duration exposures led to more robust declines in serum T3 and T4, and reductions were evident at lower doses with increasing time of exposure. The lowest dose tested in these studies was the equivalent of 0.025 mg/kg/day delivered in the food and for which a 20% decline in serum T4 was observed that was further reduced with increasing dose O'Connor et al. (1999). Slightly higher doses were accompanied by declines in serum T3 and increases in serum TSH. Higher doses for the longer exposure periods were often accompanied by body weight deficits and increases in liver and thyroid weights.

O'Connor et al. (2002) administered PTU in food (0, 0.025, 0.25, 1.0 and 10 mg/kg/day) and reported that thyroid weight and thyroid histopathology appeared to be more sensitive than serum hormones in short duration exposures (15 vs 30 days), with significant deviations from control values evident in these measures in the lowest dose group tested (0.1 mg/kg/day). All measures impacted by PTU, however, followed a monotonic dose-response pattern. In ovariectomized female rats administered PTU for only 5 days, TH changes were restricted to the highest dose group (10 mg/kg/day). Body weight, liver weight and reproductive hormones were not affected with this shorter dosing regimen.

Hood et al. (1999) examined two TPO inhibitors, PTU and methimazole (MMI), with extensive 7-point dose groups in adult male rats. PTU or MMI were administered in food at concentrations of 0, 1, 3, 10, 30, 100 and 300 ppm (equivalent to 0.06-18 mg/kg/day) for 21 days. Monotonic dose-dependent reductions in total and free T4, and increases in TSH and thyroid weight were observed and were first evident at a dose of 0.8 mg/kg/day; observations consistent with the

findings of O'Connor et al. (2002) described above. However, an NMDR was seen for a related TPO-inhibitor, methimazole, for which a slight, but significant, increase in total T4 and free T3 were seen at the lowest dose tested, followed by monotonic declines as the dose increased.

In a developmental dosing paradigm, Blake and Henning (1985) reported on serum TH and growth in rat pups exposed to 1, 5, 10, 50 and 100ppm PTU through the drinking water of the dam beginning at birth of the pups and continuing throughout lactation. Serum T4 measured at weaning was reduced at the lowest dose (~0.09 mg/kg/day), and body weight deficits were evident at 5ppm (0.4 mg/kg/day) and above. Goldey *et al.* (1995) also assessed hormones, growth, motor activity and auditory function in offspring of dams exposed to PTU (0, 1, 5, 25 ppm in drinking water) beginning in late gestation (GD17) and continuing to weaning. No effects on body weight were seen at doses less than 25ppm. Serum hormones were reduced in a dose-dependent manner at the 5 and 25 ppm. Motor activity, acoustic startle and auditory threshold were altered at 5ppm and above.

Using a similar dosing paradigm, Sawin et al. (1998) reported comparable effects on serum TH and growth parameters resulting from PTU exposure. Brain weights were reduced in adult male offspring at the two highest doses. Brain weight in female offspring, however, was increased at the 5ppm dose with no change at the higher dose levels, suggestive of an NMDR. The weight difference was small and may not be biologically relevant, in the light of the following observations: control brain weights were more variable than the other groups; decreases rather than increases in brain weight are expected from TH insufficiency; and no effects were seen in brain/body weight ratios. Choline acetyltransferase (ChAT), an enzyme necessary for synthesis of the neurotransmitter, acetylcholine, was reduced in the prefrontal cortex of the adult brain at the 15ppm dose only, also suggestive of an NMDR, whereas reductions were seen in the hippocampus at all dose levels. No changes were seen in either brain region using another cholinergic marker, hemicholinium binding, or in ChAT activity assessed at PND25. PTU-induced reductions in ChAT at earlier ages followed a monotonic dose response pattern and were significantly reduced at the two highest doses.

A lower dose range of PTU was examined by Axelstad et al. (2008) using oral gavage administration of PTU (0, 0.8, 1.6, and 2.4 mg/kg/day) to pregnant rat dams beginning in early (GD7 as compared to GD17 in studies described above) and terminating prior to weaning on PND17. Dose-dependent reductions in serum TH were seen in dams on GD15 and in pups on PND16, and the reduction was evident in neonates at the lowest dose assessed. A series of apical measures of neurotoxicity including motor activity, spatial learning, and auditory function were negatively impacted at one or both of the highest dose groups assessed with no evidence of non-monotonicity.

Neurological effects were reported at slightly lower doses achieved through drinking water exposure to pregnant dams from GD6 to weaning of the pups on postnatal day (PND) 21 Sharlin et al. (2010). Abnormalities in white matter composition of oligodendrocytes and astrocytes in the corpus callosum and anterior commissure were detected at 2 and 3ppm PTU (0.18 and 0.27 mg/kg/day equivalents) and were linearly related to a monotonic decrease in serum T4 Sharlin et al. (2008). Expression of DI2 and transporter protein MCT8 were increased in the hippocampus in a dose-dependent manner and taken as evidence of induction of compensatory responses to protect against thyroid hormone insufficiency Sharlin et al. (2010). However, there was no evidence of nonmonotonicity in any of these brain-derived molecular and structural endpoints.

Two apical neurotoxicity endpoints, synaptic physiology in the hippocampus and a test of associative learning (trace fear conditioning) were evaluated in adult euthyroid offspring following developmental PTU exposure Gilbert (2011). Serum T4 in pups declined in a monotonic manner with significant decreases in all dose groups. Declines in dam serum T4 were restricted to the two highest dose groups. Excitatory and inhibitory transmission in the hippocampus was dose-dependently reduced at the two highest doses. Synaptic plasticity, was impaired at all dose levels, but to a greater extent at the intermediate dose level. Trace fear conditioning was also altered, with apparent "improved learning" evident at low doses and no change at the highest dose tested. The authors hypothesized that "improved learning" at the lower doses may reflect increased anxiety in developmentally hypothyroid animals that served to augment ''freezing'' behavior, the dependent measure in fear learning. The reversal of this impairment at the higher doses may reflect a learning impairment that counteracts the increased tendency to freeze. Observations of learning impairments at these levels of TH insufficiency induced by PTU are in agreement with other reports of cognitive deficits at the higher dose levels Axelstad et al. (2008; Gilbert and Sui (2006).

Lasley and Gilbert (2011) reported a monotonic effect on serum T4 accompanied by a nonmonotonic pattern in expression of brain-derived neurotrophic factor (BDNF) in the hippocampus and cortex of adult offspring of 1, 2, 3, and 10 ppm PTU drinking water exposed dams. Decreases in BDNF protein were observed at low doses (1 and 2ppm, ~0.09 and 0.18 mg/kg/day in pregnant rat), a return to control at 3ppm (~0.27mg/kg/day), and exceeding control levels in females at the highest dose level of 10ppm (~0.9 mg/kg/day). No such change was observed in another TH-responsive brain region (the cerebellum), nor were alterations observed in any of these three regions in the brain of the neonate. Neurotrophins are central to many aspects of CNS development Lewin (1996), but elevated levels of BDNF and other neurotrophins also accompany brain trauma and injury where they promote neuroprotection, recovery, or repair Di Fausto et al. (2007; Chen et al. (2005). The authors suggest the

neuroprotective induction of BDNF serves to mask the effects of TH insufficiency evident at lower doses and results in the observed biphasic dose-response pattern.

4.2.4.3.2 Sodium-Iodide Symporter (NIS) – Perchlorate Case Study

Iodine is an essential element for TH synthesis. Several anions (*e.g.*, perchlorate, thiocyanate, nitrate, bromate) compete with iodine transport by the sodium-iodine symporter (NIS) Dohan et al. (2003; Eskandari (1997; Wolff (1964). NIS ion transport is essential for concentration of sufficient amounts of iodine in the thyroid gland to secure adequate TH production and competition for transport iodine transport by environmental contaminants reduces iodine uptake into the thyroid gland, resulting in decreased available iodine for production of TH. When gland-level iodine deficiency is of sufficient magnitude to result in a decrease in TH production and secretion, a compensatory increase in circulating TSH may occur with subsequent enlargement of the thyroid gland. A large number of studies have been conducted to evaluate the effects of these molecules in laboratory rodents, humans, and other species.

The best studied compound in the group of NIS inhibitors is perchlorate (ClO_4^-). Perchlorate is a ubiquitous environmental contaminant, with measurable levels found in ground and surface waters, and has both natural and manmade sources Dasgupta et al. (2006). Perchlorate exposure to humans can be through water or food Murray et al. (2008; Kirk et al. (2005). In 2005, a committee of the National Research Council (NRC) reviewed the state of the science on perchlorate NRC (2005) to inform the USEPA's Integrated Risk Information System (IRIS) Assessment for perchlorate U.S. EPA (2005b). The NRC Committee suggested a point-of-departure for quantitative risk assessment of 0.007 mg/kg-day based on inhibition of NIS thyroidal iodide uptake. More recently, Vandenberg *et al.* (2012) reviewed the perchlorate literature and, while they found weak evidence of low-dose effects of perchlorate~~;~~, they did not report evidence of NMDR associated with perchlorate exposure. Determination of the perchlorate dose that may induce an adverse effect, how much of a decrease in TH is a concern, and in what population, are all issues still surrounding the toxicity of perchlorate.

Perchlorate represents an environmental thyroid disruptor for which a very large experimental database exists. This database was extensively reviewed by EPA U.S. EPA (2005b) and the National Research Council NRC (2005) as part of regulatory determination of perchlorate human health hazard risks. Overall, the shapes of the dose-response curves associated with the early events following inhibition of uptake of iodine at the NIS are typically monotonic in nature that exhibits a threshold that has been used as the point of departure to characterize the hazard (*e.g.*, U.S. EPA (2005b); Yu et al. (2002)). In our NMDR review, only a limited number of perchlorate studies reporting NMDR were identified (summarized in Appendix C).

Männistö et al. (1979) administered 0, 10, 50, 100, and 500 mg/L potassium perchlorate in drinking water for 5 days to male rats and found a statistically significant decrease in serum T3 at 50 and 100 mg/L and a return to near control values at 500mg/L. However, a decrease in serum T4 was seen at all doses greater than or equal to 50 mg/L.

Thuett et al. (2002a); Thuett et al. (2002b) administered low doses (0, 1nM, 1uM, 1mM; approximately 0.01, 0.1 and 1.5 µg/kg-day) of ammonium perchlorate in drinking water to male and female mice during mating and throughout gestation and lactation. The pups were euthanized at PND21 and measurements taken. A statistically significant increase in plasma T4 was observed in the low and mid dose groups, and no change was observed at the high dose in PND21 mice. Thyroid histopathology analysis found a decrease in active follicles at the low and high dose, and the middle dose did not result in a statistical difference from control. The changes noted in this paper are inconsistent with each other and what is known about the thyroid axis, as well as what is seen in the majority of the thyroid literature for perchlorate.

One other NMDR was reported for serum hormones in a two-generation rat study completed by York *et al.* (2001). In this study slight increases in serum T4 or T3 were observed in low dose groups (0.3 and 3.0 mg/kg-day with declines as the dose was increased York *et al.* (2001). However, the lowest effect associated with an NMDR in this study was not the sole determinant of the study wide NOEL. York et al. (2004) also reported an NMDR in the F1 male generation of rats born to dams treated with 0, 0.1, 1.0, 3.0, or 10.0 mg/kg-day perchlorate from gestation day 0 to lactation day 10. A statistically significant decrease in follicular lumen area was reported at the low (0.1 mg/kg-day), second intermediate (3.0 mg/kg-day), and high (10 mg/kg-day) dose in F1 male pups; however, no change in follicular lumen area was found at the 1st intermediate dose (1.0 mg/kg-day). Additionally, the standard deviation for the 1.0 mg/kg-day dose that did not show a decrease in follicular lumen was very large, almost 3-times the SDs for the other dose groups, and data from this study was previously subjected to re-analysis by outside experts and a significant main effect of treatment on lumen size for all doses at PND5 was found U.S. EPA (2005b).

Gilbert and Sui (2008) evaluated the dose-response for perchlorate in a developmental study. They assessed serum hormone levels as well as a apical functional endpoints (excitatory and inhibitory synaptic transmission, synaptic plasticity). Monotonic dose-response patterns were observed for the functional measures, but an NMDR was seen for serum TSH in pups on PND14, with an increase at the intermediate dose but no change at the highest dose. This pattern was not seen in pups at PND21 or dams, nor was it accompanied by any change in serum hormones in pups at any age. Dam T4 was dose-dependently decreased at all dose levels and TSH increases were limited to the high dose group.

Overall, considering the large number of studies in which extensive dose-response relationships for perchlorate are been described, the report of NMDR curves is rare. In cases in which NMDR were reported, these were often isolated instances of low magnitude, occurred at doses above those that were defined as the critical determinants of the study or overall chemical NOEL. Furthermore, several findings were not consistent with what is known about the thyroid axis biology, and in the case of York et al. (2004), a re-review of study results U.S. EPA (2005b) concluded that the purported NMDR histopathology was in fact monotonic in nature.

4.2.4.3.3 Activation of Nuclear Receptors/ Altered Hepatic Metabolism –Polyhalogenated Aromatic Hydrocarbons Case Study

A number of xenobiotics decrease circulating TH as a consequence of their induction of metabolizing enzymes in the liver. This particular MoA involves the activation of nuclear receptors in the liver (*e.g.*, CAR, PXR) when the the compound or metabolite acts as a ligand. Activation of these nuclear receptors results in an induction of a series of Phase II and III hepatic proteins increasing TH clearance and resulting in lower circulating levels of serum TH Crofton and Zoeller (2005; Hill et al. (1998; Capen (1997). Phase II induction includes the enzyme family that metabolize and eliminate thyroid hormone, UDP-glucuronosyltransferase (UGT) enzymes, cytosolic sulfotransferases, and GSH S-transferase enzymes, as well as some Phase III cellular transporters Martignoni et al. (2006); Omiecinski et al. (2011). The increased metabolism and excretion of T4 by UGT in the liver, results in decreased circulating T4, a required key event that leads to an adverse outcome Crofton and Zoeller (2005; Hill et al. (1998). As with the other thyroid disruptors, the key events and adverse outcomes associated with this mode of action tend to follow a monotonic dose-response.

The class of chemicals known as polyhalogenated aromatic hydrocarbons (PHAHs), include a number of groups and mixtures of chemicals that have been shown to disrupt thyroid hormone homeostasis by interacting with nuclear receptors and stimulating induction of enzymes Crofton and Zoeller (2005). In studies performed in rats that were exposed to individual or mixtures of PHAHs (*e.g.*, PCBs, dioxins, furans) there was evidence for NMDR in some endpoints evaluated, including serum T3 and T4. Li et al. (2001; Desaulniers et al. (1997; Gray et al. (1993; van Raaij et al. (1993). In a study by Zoeller et al. (2000) pregnant rats were treated with PCBs throughout gestation and lactation and a number of endpoints were examined in offspring including serum TH and myelin basic protein (MBP) mRNA in the brain. The expression levels of MBP exhibited nonmonotonicity, but all other endpoints, followed a typical monotonic dose response with the lowest effect level provided by the changes in T4.

There were additional studies that used an extensive number or range of doses. NMDR were not found in an *in vitro* study that employed 7 concentrations of Aroclor 1254 in COS-7 cells transfected with thyroid receptor beta (TRβ) and a concentration range from 1 pM to 100 uM Bogazzi et al. (2003). An *in vivo* rat study of 18 PHAHs (including two dioxins, four dibenzofurans, and twelve PCBs) evaluated 7-9 dose groups and dose ranges designed to cover human exposures; no evidence of NMDR was found Crofton et al. (2005). In two additional reports, there are some instances of NMDR at lower concentrations of exposure to one PCB congener, PCB77. Desaulniers et al. (1997) reported increased serum T4 at 0.06, 0.6 and 6 ug/kg/day, followed by a decrease at the highest dose (60 ug/kg-day).

4.2.4.4 Results of Literature Analysis

As noted in section 4.2.3.2, four filters were used to select studies from the literature review for more in depth analyses of NMDR results (further described in Appendix C). From the total 1153 references, 814 were deemd relevant. These 814 references yieled 2060 chemcial- studies, of which there were 1005 mammalian and in vitro chemical-studies with 3 or more dose groups and a control (Filter 1). Of these, only 46 NMDR chemical-studies from 42 different papers were identified, representing 38 chemicals (Table 4.4 and Appendix C). Two important conclusions can be made from this analysis. First, the number of NMDR reports is small compared to the entirety of the reviewed papers (only 46 out of 1005 chemical studies). Second, is that a large portion of the extant literature is comprised of reports with too few dose groups to make a determination of the existence of NMDR (Appendix C.5). Almost half of the papers (45%) used only 1 or 2 dose groups; only 6% of the *in vivo* chemical-studies used 5 or more dose groups. A synopsis of each of these 46 chemical-studies (from 42 papers) and justification for the filtering applied in each instances is provided in Appendix C. Of these 46 chemical-studies, 28 were eliminated based on the Filter 3 (effect is not the sole driver of the study-wide LOAEL/NOAEL) yielding a total of 18 chemical-studies papers with evidence of a nonmonotonic low dose effects on some aspect of thyroid function. Based on the criteria for Filter 4, only 8 of these 18 chemical-studies remained. This amounts to a small percentage of total number of chemical-studies that contained mammalian thyroid-related endpoints and 3 or more dose groups (8 of 1005, or 0.8%) and even smaller proportion of the total number of chemical-studies from all of published literature that was reviewed (8 of 2060, or 0.38%).

When observed, the most common nonmonotonic effect was an increase in serum T4 or T3 at low doses followed by return to control levels (*i.e.*, chloromethyl benzene, tamoxifen) or declines at higher doses (tert-butyl methyl-phenol, methimazole, PCB110, PCB77). The MIEs identified for the few chemical that produced NMDR were TPO inhibition (MMI) and nuclear receptor activated up-regulation of hepatic metabolism (chloromethyl benzene, PCB77, PCB110, thioxpyr). The vast majority of reports for chemicals that act via these MIEs failed to

report NMDRs for any TH related endpoints. The MIEs for the chemicals described in the three papers were not readily discernible (propylparaben and tertbutylmethylphenol), although tamoxifen is a well known estrogen receptor antagonist. The nature of the NMDRs of the suite of chemicals found in this review did not readily lend themselves to categorization based on the proposed MIE. A full description of the findings on these papers is included in Appendix C, and each are briefly summarized below.

CCB (1-chloro-4-(chloromethyl)benzene, 0, 10, 50, 250 mg/kg-day) was administered to male and female SD rats via oral gavage daily for 28 days, beginning at 8 weeks of age (Yamasaki *et al.*, 2012). A slight (~20%) but statistically significant increase in serum T4 was observed at the middle dose in female rats, with no change in male rats. No other changes in serum T3, TSH or thyroid histopathology were noted in either sex after exposure to chloromethyl benzene. The absence of decreases in serum T4 at high CCB concentrations is inconsistent with a proposed hepatic mode of action for this compound. Interestingly, the authors concluded that no endocrine-mediated effects, including thyroid dysfunction were observed in any groups of rats treated with 1-chloro-4-(chloromethyl) benzene Yamasaki et al. (2008). In this same publication, a statistically significant monotonic dose-dependent increase in serum T4 was reported in response for a related benzene derivative (1,3-diethyl benzene). No other published studies on any effects, endocrine or otherwise, of this chemical could be found.

This same group of investigators Yamasaki et al. (2008), using an identical study design, reported on the thyroid effects of oral exposure to 4,4'-butylidenebis-(2-tert-butyl-5-methylphenol (0, 5, 25, 125 mg/kg-day). A statistically significant increase in serum T4 (15%) was observed at the low dose, no change at the middle dose, and a decline in serum T4 (17%) at the highest dose tested. Unlike the effects on serum T4 reported above for CCB exposure, this alteration in serum hormone was detected in males but not females. Increases in TSH were detected in both sexes at the high dose. Thyroid histopathology was performed only in the high dose animals, and no abnormalities were noted. The authors compared these effects to PTU, but provided no explanation of the increases in T4 at the lowest dose. No other published reports on endocrine effects of this compound were found.

Hood et al. (1999) administered MMI or PTU (0, 1, 3, 10, 30, 100, 300 ppm) to adult male rats in the diet for 21 days. Both MMI and PTU inhibit the synthesis enzyme TPO in the thyroid gland. Statistically significant increases in serum total T4 (~30%, RIA) and free T3 (~25%) were observed at the 3ppm dose level (~0.18mg/kg/day) followed by monotonic dose-dependent declines at higher doses. A similar pattern, although less robust and below the level of statistical significance, was also observed for PTU. Important to note in this study was the wide dose range employed (1 – 300 mg/kg/day) and used 6 dose groups. This type of design is rare

for published *in vivo* studies and makes it easier to discern possible low-dose NMDR effects. No additional studies were located for this dose of MMI or lower.

A similar profile for serum T4 was seen after a 13-week dietary exposure of young female rats to PCB77 (3,3',4,4'-tetrachlorobiphenyl, 0, 10, 100, 1000, 10,0000 ppb) Desaulniers et al. (1997). Statistically significant increases in serum T4 were observed at 10, 100, 1000ppb (20-45%) with declines (50%) at the highest dose tested. The declines in serum T4 at the highest dose were accompanied by slight increases in serum TSH and up-regulation of UDP-GTs in the liver. These food concentrations correspond to dose of 0.06, 0.6 and 6 ug/kg/day and represent some of the lowest doses for which an effect on thyroid or any other *in vivo* endpoint has been reported. A related chemical, PCB28 (2,4,4'-trichlorobiphenyl) assessed in the same study did not alter any of the thyroid endpoints assessed.

PCB110 (0, 8, 32, 48, 96 mg/kg/day) was administered alone or in a mixture contaminated with PCB126, ip, in corn oil vehicle, to juvenile female SD rats on PND21 and 22 Li et al. (1998). As seen with PCB77 Desaulniers et al. (1997), a statistically significant increase in serum T4 was observed at the lowest dose, followed by a monotonic decrease in T4 at higher dose levels. Hepatic microsomal enzymes were not induced at lower dose of PCB110, which may have permitted mobilized T4 to reach higher serum levels. This conclusion is supported by induction of metabolic pathways at all dose levels of the PCB110 plus PCB126 mixture where a monotonic dose-response pattern was observed for serum T4.

Contrasting with previous studies, three additional chemicals with NMDR were identified, which exhibited decreases in serum T4 (propylparaben) or T3 (thiazopyr, tamoxifen) at lower doses with return to control levels or reductions at higher doses. Vo et al. (2010) reported on a number of thyroid and reproductive endpoints for a series of paraben antimicrobial agents. Propylparaben was administered to young male and female rats by oral gavage (0, 62.5, 250, 1000 mg/kg/day) for 20 days. A statistically significant decrease in serum T4 (42% based on a commercial ELIZA kit) at 250 mg/kg of propylparaben was observed, but not at higher or lower doses, and there were no effects on any other parameter assessed (thyroid weight, liver weight, or female reproductive endpoints). The only NMDR thyroid effect reported for propylparaben was restricted to this nonmontonic decrease in serum T4, while the other five paraben compounds assessed resulted in effects in a number of thyroid and other reproductive endpoints, but none of these effects was nonmonotonic. No other studies of propylparaben were found.

An NMDR for serum T3 was reported for the pre-emergent herbicide thiazopyr Hotz et al. (1997). Thiazopyr was administered (0, 10, 30, 100,300, 1000, 3000 ppm) to adult male SD rats for 56 days. Significant reductions in serum T3 (~20%, RIA) were detected at the mid-dose of

30ppm with increases in T3 at the highest dose tested. Effects on serum to T4 and TSH were limited to reductions and increases, respectively, at the highest dose of 3000ppm. However, within this study, the high dose increases in serum T3 were not consistently observed, while increases in TSH, liver and thyroid weights were replicated at 3000 ppm. As such, a reduction in serum T3 at the 30ppm dose level constitutes the nonmonotonic effect of concern. UGTs were increased in liver but only at the two highest doses. A decrease in serum T3 at the mid- dose level in the absence of effect on T4 is not consistent with an upregulation of hepatic metabolism. No other thiazopyr studies were found.

The estrogen receptor antagonist, tamoxifen was administered (0, 10, 50, 200 µg/kg) by oral gavage to immature female SD rats for 20 days and thyroid glands and blood collected 24 hours after the last dose Kim et al. (2002b). Thyroid gland weights were increased at the middle two doses but were not significantly different from controls at the high dose. Both serum TSH and T3 were also increased at these intermediate doses, but no change was detected in serum T4 at any dose level. Increases in TSH are consistent with increases in thyroid weight, but not with increases in serum T3. Another report in the literature using a very similar dosing regimen did not replicate these observations Kennel et al. (2003). In this report male rats were unaffected at any dose of tamoxifen (5, 30 or 200 µg/kg/day), and females exhibited a decrease in serum T4 with no changes in other serum markers or in thyroid gland weight.

In summary, a very limited number of publications were identified with NMDR characteristics at the lower end of tested dose ranges. The nature of NMDR was not generally consistent with categorization of the chemicals based on the proposed MIE. The most common nonmonotonic effect identified was an increase in serum T4 or T3 at low doses followed by return to control levels (i.e., chloromethyl benzene, tamoxifen) or declines at higher doses (tert-butyl methyl-phenol, methimazole, PCB110, PCB77). Although there are no well-documented mechanistic explanations for the small increases in serum hormones found, there are some plausible, and testable, hypotheses that could explain the results. The small increases in THs could result from the activation of homeostatic mechanisms to maintain euthyroid serum hormone levels, including increased sensitivity to TSH during initial response, modulation by deiodinases, recovery of T3 metabolites, and enterohepatic circulation (*e.g.*, Brabant et al. (1992). Overall, the magnitude of increase in serum TH was typically small, occurred infrequently, and may represent the transitory adjustments of a dynamic system in flux. The biological significance of these changes is open to debate.

4.2.4.5 Conclusions for Thyroid Studies

Both the case-study approach and literature review of TDCs identified a very small percentage of studies with evidence of NMDR for thyroid disruption at the low end of the dose-response curve. In approximately half of the cases where nomonotonicity was detected, it was not for an endpoint that defined the critical effect or represented the sole determinant that would drive the NOAEL or LOAEL for risk assessment. As discussed above, only eight instances were identified where the NMDR profile was at the lowest dose levels reported in the study. The reported effects were most often on serum T3 or T4 and in a direction inconsistent with the proposed MoA identified for those chemicals. Furthermore, the NMDR was often inconsistent with the known downstream adverse effect of primary concern, or the effect seen for this same endpoint at higher dose levels. The declines in serum TH observed at low doses in some studies were inconsistent with other thyroid related measures or with other reports in the literature. Replication of many of these effects is recommended before consideration for use in regulatory decisions. Overall, a more complete understanding of the temporal dynamics of compensatory processes in the mutli-tissue regulation of thyroid hormones and thyroid hormone action would aid interpretation of the biological significance of many of the NMDRs reported here.

Table 4.4: From a thyroid literature review of more than 1153 references, 1831 mammalian or in vitro chemical-studies were evaluated for the presence of NMDR. A filtering protocol developed to help evaluate NMDR data was applied to these studies. Approximately one-half of these studies were eliminated as they had fewer than 3 dose groups and a control. This table summarizes the 42 papers that report NMDR curves for thyroid related endpoints. Detailed information on each of these studies is available in Appendix C. The four filtering criteria used to determine NMDR are described in the text and the Appendix, and only 8 chemical studies from 8 separate papers passed all filtering criteria (Filter Applied=Discuss). Each of these appears in the Appendix and is also discussed further in the text (Section 4.2.3.4).

Chemical	Species	Group Size	Doses Given	Study Type	Filter Applied	Reference(s)
17α-methyltestosterone	Rat	9-10	5, 20, 80 mg/kg-day	Gavage	3	Okazaki et al. (2002)
17β-estradiol (E2)	Mouse	16-25	0.001, 0.005, 0.05, 0.15, 0.5ppm	Feed	3	Tyl et al. (2008c)
1-chloro-4-(chloromethyl)benzene	Rat	5	10, 50, 250 mg/kg-day	Gavage	Discuss	Yamasaki et al. (2012)
1-methyl-3-propylimidazole -2-thione (PTI)	Rat	15	5, 10, 25, 75 mg/kg-day	Gavage	3	Biegel et al. (1995)
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	Rat	12	6.25, 12.5, 25, 50, 100 µg/kg	Gavage	4a	Potter et al. (1986)
4,4'-butylidenebis(2-tert-butyl-5-methylphenol)	Rat	10	5, 25, 125 mg/kg-day	Gavage	Discuss	Yamasaki et al. (2008)
5-ethylidene-2-norbornene	Rat	10	52, 148, 359 ppm	Vapor	3	Ballantyne et al. (1997)
Amiodarone	In vitro	3[#]	0.5, 1, 5, 10, 15 µM	In vitro	4c	Freitas et al. (2011)
Chlorpyrifos	Rat	12 pups	1, 10, 100 mg/kg	Gavage	3	Jeong et al. (2006)
d-d-T80-prallethrin	Rat	24	120, 600, 3000 ppm	Feed	3	Seki et al. (1987)
Dibromoacetonitrile	Rat	10	0.1, 1, 10, 100 ppm	Water	3	Poon et al. (2003)
Diethylstilbestrol (DES)	Rat	10	10, 20, 40 µg/kg-day	Gavage	3	Shin et al. (2009)
Ethylenethiourea (ETU)	Rat	5	5, 25, 125, 250, 500 ppm	Feed	4c	Graham et al. (1975; Graham et al. (1973)*
Furan	Rat	12	0.03, 0.12, 0.5, 2.0, 8.0 mg/kg	Gavage	3	Gill et al. (2010)
Hexachlorobenzene (HCB)	Rat	3-5	3, 10, 30, 100, 300, 1000 ppm	Gavage	3	van Raaij et al. (1993)
Imidazole	Rat	8-10	20, 60, 200 ppm	Water	3	Comer et al. (1985)
Methimazole (MMI)	Rat	4-5	3, 10, 30, 100, 300, 1000 ppm	Feed	Discuss	Hood et al. (1999)
Methoxychlor	Rat	9-10	20, 100, 500 mg/kg	Gavage	3	Okazaki et al. (2001)
Parabens						Vo et al. (2010)
Propylparaben	Rat	10	62.5, 250, 1000 mg/kg	Gavage	Discuss	
Isobutylparaben	Rat	10	62.5, 250, 1000 mg/kg	Gavage	3	

Chemical	Species	Group Size	Doses Given	Study Type	Filter Applied	Reference(s)
Isopropylparaben	Rat	10	62.5, 250, 1000 mg/kg	Gavage	3	
Butylparaben	Rat	10	62.5, 250, 1000 mg/kg	Gavage	3	
Pentabromodiphenyl ether (DE-71)	Mouse	6	0.8, 4.0, 20, 100, 500 mg/kg	Gavage	4a	Fowles et al. (1994)
Pentachlorodiphenyl ethers						
PCDE 35	Rat	6 dams	25, 50, 100 mg/kg-day	Gavage	4c	Rosiak et al. (1997)
PCDE37	Rat	6 dams	50, 75, 100 mg/kg-day	Gavage	4c	
Perchlorate (ClO_4^-)	Mouse	10 dams	1 nM, 1mM, 1mM	Water	4c	Thuett et al. (2002a); Thuett et al. (2002b)*
Perchlorate (ClO_4^-)	Rat	30 dams	0.3, 3.0, or 30.0 mg/kg	Water	3	York et al. (2001)
Perchlorate (ClO_4^-)	Rat	25 dams	0.1, 1.0, 30, 10.0 mg/kg-day	Water	4c	York et al. (2004)
Perchlorate (ClO_4^-)	Rat	5-6	10, 50, 100, 500 mg/L	Water	3	Männistö et al. (1979)
Perchlorate (ClO_4^-)	Rat	16-27 litters	30, 300, 1000 ppm	Water	3	Gilbert and Sui (2008)
PCB Aroclor 1254	Rat	15	5, 50, 500 ppm	Feed	4b	Collins and Capen (1980)
PCB Aroclor 1254	Rat	6 dams	1, 4, 8 mg/kg	Feed	3	Zoeller et al. (2000)
PCB110	Rat	4-11	8, 32, 48, 96 mg/kg	i.p.	Discuss	Li et al. (1998)
PCB149	Rat	5-9	8, 32, 96 mg/kg/day	i.p.	4c	Li et al. (2001)
PCB77	Rat	10	10, 100, 1000, 10000 ppb	Feed	Discuss	Desaulniers et al. (1997)
Potassium Bromate ($KBrO_3$)	Rat	50	0.02, 0.1, 0.2, 0.4 g/L	Water	3	Wolf et al. (1998)
Propazine (DACT)	Rat	15	16.7, 33.8, 67.5, 135 mg/kg/day	Gavage	3	Laws et al. (2003)
Propylthiouracil (PTU)	Rat	10-14	1, 2, 3 ppm	Water	3	Gilbert et al. (2012)
Propylthiouracil (PTU)	Rat	21 dams	1, 2, 3, 10 ppm	Water	3	Lasley and Gilbert (2011)
Propylthiouracil (PTU)	Rat	5	5, 15, 25 ppm	Water	3	Sawin et al. (1998)
Propylthiouracil (PTU)	Rat	12	2.5, 5, 10, 25 ppm	Water	4a	Gordon et al. (2000)
Saisentong	Rat	10	5, 10, 15 mg/kg-day	Gavage	4c	Zhang et al. (2010)
Tamoxifen	Rat	10	10, 50, 200 µg/kg	Gavage	Discuss	Kim et al. (2002b)
Thiazole-Zn	Rat	10	40, 100, 200 mg/kg-day	Gavage	3	Yang et al. (2013)
Thiazopyr	Rat	20	10, 30, 100,300, 1000, 3000 ppm	Feed	Discuss	Hotz et al. (1997)
Triclosan	Rat	8-15	3, 30, 100, 200, 300 mg/kg-day	Gavage	3	Zorrilla et al. (2009)

4.3 Human Studies and Epidemiology

4.3.1 Context for Human Studies in this Review

A major focus of this document (as seen in sections 4.1 and 4.2) has been on *in vivo* animal data, with an emphasis on what can be learned about frequency of NMDR in reproducible toxicology studies. Given the impact and relevance to current test rules, a particular effort has been made to review those toxicology reports that conform to test guidelines and Good Laboratory Practices. However, concerns about the adequacy of these tests and testing strategies have largely been prompted by findings in the epidemiologic literature and in human studies that show health effects of EDCs at low doses and environmentally relevant concentrations.

Resources for this EPA review did not support a systematic literature search and review of human studies. However, a number of recent publications and reports from international health organizations provided relevant insights into the state of the science and were helpful in informing the Agency's understanding of the literature. These included two separate reports in 2013 from the EFSA Scientific Committee and the European Joint Research Centre (JRC) that provided guidance on identification, evaluation, and hazard assessments of endocrine active and endocrine disrupting compounds EFSA (2013; JRC (2013), as well as the World Health Organization (WHO) report, State of the Science of Endocrine Disrupting Chemicals (2012). This WHO report is an update of the scientific knowledge related to concerns about potential adverse health effects of endocrine disrupting chemicals on humans and wildlife and does not focus on NMDR *per se* WHO (2012). In addition the Vandenberg *et al.* (2012) review was helpful in identifying topics for discussion of some of the considerations listed below.

4.3.2 Interpreting Epidemiological Evidence

There are increasing numbers of reports in the epidemiological literature showing possible health effects associated with EDCs at low doses Vandenberg et al. (2012; WHO (2012). In these settings, low dose is usually meant to describe doses at environmentally relevant levels. As such, epidemiological studies provide a context for evaluating and understanding associations between low dose chemical exposures and human health, and together with other human studies they have provided much of the basis for further exploration into evaluating health effects at low doses. Epidemiological studies have made important contributions to understanding the impact of various exposures on multiple health endpoints and are a critical link between toxicological testing and evaluating adverse effects in humans.

Historically, WoE approaches have been used to incorporate multiple data streams from different study types to inform risk and hazard assessments. But currently, there is no consensus guidance on how epidemiological or human studies might be used to shape or alter the design of a toxicity testing study. This is primarily due to profound differences between epidemiological and toxicological studies that both enrich and complicate the state of the science. Some of these issues are highlighted below.

4.3.2.1 Multiple Exposures (Combined or in Sequence)

Standard toxicological tests are designed to assess the impact of individual chemicals for hypothesized health impacts using controlled experiments. Epidemiological studies, on the other hand, attempt to find causal associations between a health outcome and an exposure. However, in observational and non-interventional studies, those exposures typically occur concurrent exposure to other chemical and non-chemical stressors that may or may not modify the observed effect. In addition, the observed population may have experienced prior exposures to the same or different sets of stressors that could also affect the nature and magnitude of the observed effect to current exposures. Because most epidemiological studies are not designed to include control populations, it is difficult to provide a definitive attribution of causation to the specific exposure of interest EFSA (2013; Vandenberg et al. (2012).

Moreover, many exposures are highly correlated, introducing methodological issues; such as collinearity, high dimensionality, and potential synergistic or inhibitory effects. This further exacerbates the problems with treating environmental exposures observed in epidemiological studies as single entities JRC (2013). Several advances have been made in this area and methods have been designed to identify subsets of mixtures Gennings et al. (2010), to assess mixtures that are also affected by a limit of detection Herring (2010), to accommodate joint analysis of high-dimensional biomarker data Zhang et al. (2012), and to model interactions Yeatts et al. (2010; Moser et al. (2005; Charles et al. (2002).

4.3.2.2 Exposure Assessment

Another consideration in human studies is that when they are measured, exposures are evaluated in environmental or biological media. While these measurements are more realistically representative of environmental exposures, in comparing these human studies to animal toxicology studies, there are typically two kinds of challenges. First it is difficult to extrapolate from the measured exposures to internal exposures or dose delivered to the target organ, which are the exposures of interest in typical toxicology tests. This is particularly important for EDCs, as they produce tissue-specific effects WHO (2012). Second, there may be

important discrepancies in how the data are measured and reported, including the way in which measurement error is handled, this may affect how the study results are interpreted.

For example, biomarkers are increasingly used for biological assessment of exposure. When exposure levels are low, measurement sensitivity becomes increasingly important, and often results in a large number of measurements taken that are reported to be below the limit of detection. Although measurements are produced both above and below this cut point, in many cases all points below the limit of detection are reported as ''not detected.'' The way in which data below the limit of detection are handled in epidemiological studies is not always clearly specified; this may introduce bias into the exposure assessment and has implications for the understanding and identification of both low dose effects and NMDRs (Schisterman Schisterman et al. (2006; Lubin et al. (2004; Richardson and Ciampi (2003). Approaches have been developed for more accurate and efficient estimation when dealing with data subject to a limit of detection Herring (2010; Schisterman and Little (2010; Vexler et al. (2008; Perkins et al. (2007).

4.3.3 Moving Forward – Converging Evidence

While methodological issues may create difficulties for interpreting their findings in the context of toxicological data, population and epidemiological studies are essential to identifying adverse effects of EDCs in human populations EFSA (2013; JRC (2013). Observational studies allow for the study of various exposures that otherwise would be unethical to deliver in a randomized fashion to humans. Several criteria or principles have been proposed to guide causal inference from epidemiological studies Rothman et al. (2008; Hill (1965). These principles include important considerations such as biologic plausibility and temporality. In particular, the ability to see consistent results across studies of different designs and populations is critical for inferring causality. New large longitudinal epidemiologic studies are being undertaken to further evaluate relationships between EDCs and relevant health endpoints Buck Louis et al. (2011b; Buck Louis et al. (2011a). Moreover, these studies are increasingly using validated biomarkers of exposure and are allowing for the study of chemical mixtures to further our understanding in this area.

The Endocrine Society has noted strong evidence for adverse reproductive outcomes after exposure to EDCs for both males and females Zoeller et al. (2012; Diamanti-Kandarakis et al. (2009). Others have also recognized that EDCs have been shown to affect a wide array of fecundity and fertility endpoints Mendola and Buck Louis (2010; Buck Louis et al. (2006; Toft et al. (2004). Alhough effects have been observed for several intermediate endpoints, there have been limited data published on human exposure and fecundity endpoints, including gynecologic disorders. However, emerging evidence shows that EDCs are associated with a higher risk of

endometriosis, including these examples: select phthalate concentrations Buck Louis et al. (2013); urinary concentrations of benzophenone-type UV filters Kunisue et al. (2012); persistent organochlorine pollutants Buck Louis et al. (2012a; Cooney et al. (2010); and perfluorinated chemicals Louis et al. (2012). In addition, persistent environmental chemicals Buck Louis et al. (2013), metals Buck Louis et al. (2012b), and PBDEs Harley et al. (2010) have been observed to be associated with reduced fecundity in humans, and PCBs with pregnancy loss Pollack et al. (2011). These studies overcome several important limitations from previous studies, including prospective assessment of reproductive outcomes, reporting biomarkers of exposure, and assessment of chemical mixtures.

4.3.4 Summary

As noted previously, this assessment did not include a systematic evaluation of the open scientific literature or data for epidemiologic evidence for NMDR of EDCs in humans. The authors of this Agency review, however, recognize that the data from new and emerging epidemiology and human studies suggest a narrative that may be different from that which can be constructed from data from toxicology tests. Clearly there is a need, within the community of environmental health scientists, to examine and reconcile these differences, and to develop guidance for synthesizing the evidence from and strengthening the designs of future human studies and toxicity tests EFSA (2013; WHO (2012). Ideally, epidemiologic studies of high quality and adequate statistical power need to be integrated with the *in vitro* and *in vivo* laboratory aquatic and mammalian animal toxicological studies to fit into the overall regulatory database. This is particularly important in the investigation of common MoA for chemicals across different levels of biological organization. This approach will help identify relevant adverse outcome pathways for the putative agent(s) and contribute to discerning the plausibility and coherence of the associations in the findings. Although epidemiologic data are limited, they have direct relevance to our understanding of associations between low dose chemical exposures and human health. Consideration of the measurement process, including limit of detection and chemical mixtures, can improve our ability to obtain unbiased estimates of the associations between EDCs and human health outcomes to facilitate their use in risk assessment EFSA (2013; JRC (2013; WHO (2012).

5. Conclusions

5.1 General Conclusions

The literature on NMDRs has been extensively and critically evaluated for estrogen, androgen and thyroid hormone pathways. Based on these evaluations, the WoE supports the conclusion that NMDRs can occur and, in many instances, are explainable in terms of basic biological

processes. NMDR are more likely to be identified after short-term exposures than in longer-term or chronic studies. One reason for this observation is that in short term studies higher doses may be administered without causing overt toxicity than is possible in longer term or chronic studies. The longer term study may achieve an adverse effect at a lower exposure level as the duration of exposure is increased; thus, administration of the higher dose producing an NMDR in a short-term study is precluded. Another possible reason that NMDRs may be more commonly observed in short-term studies is that the animals are exposed during one, or part of one, stage of development, whereas long-term multigenerational studies include exposures during the full life cycle of all of the reproductive events sensitive to estrogen pathway disruption. Therefore, any effects observed in the latter would be a result of the cumulative exposure over multiple life stages. Furthermore the shape of the dose response curve can change over time in studies with repeated samples taken at multiple time points as compensatory mechanisms alter pharmacokinetics and toxicokinetics in a tissue-specific manner.

NMDR occur *in vivo* but are not common and tend to be seen at high doses. Evaluation of NMDR across multiple studies for the same chemical often shows a lack of reproducibility. This lack of reproducibility may be due to variations in sample size, experimental design issues, inappropriate statistical analysis, or lack of a true NMDR. When NMDRs are present and reproducible in *in vivo* studies, they are typically associated with apical adverse effects after a high dose exposure.

The mode of action/adverse outcome pathway concepts should be used to inform the evaluation of data to determine if an NMDR is described and is biologically plausible. Evaluating the key events in the pathway in the context of dose response enables sound conclusions to be drawn as to the influence of an NMDR on characterization of the adverse outcome and its relevance for risk characterization. Minimum conditions are necessary for an NMDR to be present. NMDR can occur when at least two opposing influences shape the curve, with the effect of each influence dominant over a different range of doses (WHO, 2012). For example, disruption of estrogen- and androgen-mediated endpoints can occur when xenobiotics act as ligands for ER and AR or change the activities of enzymes involved in steroid hormone biosynthesis or metabolic clearance. When NMDRs are present they tend to arise from the summation or interaction of multiple processes happening to different degrees at different points along the dose-response spectrum. Potency of the agent as well as its ability to induce repair processes can also impact the description of dose-response and likelihood of an NMDR.

The relevance of an NMDR to human or ecological health should be evaluated for the range of doses or exposures over which the nonmonotonic range occurs. When some or all of the doses

or exposures associated with nonmonotonicity significantly exceed those known or expected to occur at environmental levels of exposure, then the nonmonotonic relationship may not be relevant to the evaluation of health effects.

Nonmonotonicity can also arise by chance. Statistical power and methods, including addressing multiple comparisons, should be carefully evaluated. In addition, important studies should be repeated whenever feasible, as independent replication of NMDR is considered to provide the most scientific support. When replication is not possible, ancillary studies that provide data on pharmacokinetics and the MoA can provide insight into expected dose-response behaviors.

One biological process that theoretically can give rise to NMDR involves compensatory (or adaptive) responses. Organisms have evolved a wide range of mechanisms to maintain homeostatic conditions conducive to normal function(s) when impacted by stressors. The vertebrate endocrine system has been comparatively well-studied in terms of homeostasis Nichols et al. (2011). Various feedback mechanisms operate at different biological levels of organization to maintain a dynamic homeostasis supporting normal reproduction and development. Given the very active nature of feedback signaling in biological systems, maintenance of homeostatic conditions through compensatory responses could be the basis for nonmonotonic features of dose-response relationships in some types of studies.

Fish exposed to exogenous chemicals exhibited several instances wherein changes in gene expression were nonmonotonic. There also were examples of either plasma steroid concentrations or *ex vivo* steroid production exhibiting nonmonotonic relationships with dose. However, in time-course studies, there were no examples of NMDR for endpoints closer in biological level of organization to the apical endpoints examined. These data suggest that responses at molecular and biochemical levels, such as those involved in feedback regulation of homeostasis (including compensation), may more commonly exhibit nonmonotonic characteristics than more integrated, downstream, apical, endpoints.

This review did not include a systematic evaluation of the epidemiologic literature for NMDRs in human estrogen, androgen, or thyroid systems. However, data from epidemiology and human studies may provide a perspective not captured by traditional toxicologic testing. Thus, there is a need within the community of environmental health scientists to develop guidance for synthesizing evidence from various study types and to strengthen the design of future human studies and next generation toxicity tests EFSA (2013; WHO (2012). Ideally, epidemiologic studies of high quality and adequate statistical power should be integrated with the *in vitro* and *in vivo* aquatic and mammalian testing data for common MoA for chemicals across different levels of biological organization.

Conclusions:

1. NMDRs do occur in estrogen, androgen, and thyroid systems as evidenced in ecological and mammalian studies.
2. NMDRs are not unexpected *in vitro* particularly when evaluating high dose levels and/or lower-order biological endpoints in estrogen androgen or thyroid systems.
3. NMDRs 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.
4. The nature of a dose response will vary over time, and nonmonotonicity due to compensation may be observed.
5. NMDRs observed in endocrine endpoints may be biologically relevant and should be evaluated in context with the totality of the available scientific data, including epidemiologic and human studies.
6. There is currently no reproducible evidence that the early key events involved in the expression of NMDRs that are identified at low dose are predictive of adverse outcomes that may be seen in humans or wildlife populations for estrogen, androgen or thyroid endpoints.
7. Therefore, current testing strategies are unlikely to mischaracterize, as a consequence of NMDR, a chemical that has the potential for adverse perturbations of the estrogen, androgen or thyroid pathways.

5.1.1 Overall Conclusions: Estrogen, Androgen, and Thyroid

Effects with NMDRs were frequently observed in studies evaluating estrogenic or androgenic activity of chemicals *in vitro*. However, they tend to be indentified when high concentrations are used in the medium; these concentrations projected to *in vivo* scenarios would be too high to be environmentally relevant or attainable. Therefore, while the data from *in vitro* studies provide valuable insights into the endocrine activity and potential mechanisms of action of chemicals, the relevance of the dose response curves from these studies to understanding dose response relationships *in vivo* is highly questionable.

When NMDRs were seen i*n vivo* for estrogen or androgen signaling pathway effects, the observations were often from large multigenerational studies wherein numerous measurements were taken and a great many comparisons were made across control and exposed groups of animals over several generations. Given the large number of measurements and comparisons made in these types of studies, one would expect to see some NMDRs by

chance. To determine biological relevance one would generally weight more heavily results from experiments that have been replicated or explained mechanistically.

In some cases, NMDR were observed in non-multigeneration studies, but they were not replicated in other studies using similar exposure scenarios. This was often attributed to small sample sizes, questionable experimental designs (*e.g.*, route of exposure not relevant to environmental scenarios), and/or inappropriate statistical analyses (*e.g.*, not accounting for litter effects). Reproducible effects following an NMDR were seen for several *in vivo* studies for adverse, downstream phenotypic effects. In these cases, the effects were usually at high and not low doses.

In general, NMDR for the estrogen and androgen signaling pathways, while identified for some apical endpoints, are more likely with genomic and hormonal measures than with phenotypic effects and endpoints such as fertility, histopathology or malformations. These types of measures tend to be more variable and less reproducible, which could explain why similar findings often were not observed in studies with comparable exposures.

For a specific endocrine mechanism of toxicity, the shapes of the dose response curves will be tissue- and cell-type specific due to the complexity involved in the tissue-specific molecular events modulating the different responses to a chemical that disrupts estrogen or androgen signaling. In the case for estrogens, responses are frequently tissue specific due to tissue specific ratio of ERα and ERβ, which may antagonize one another when activated. Likewise, there may be different levels of the two estrogen receptors, corepressors and coactivators that regulate effects on gene expression, metabolism, distribution and elimination. In addition, the set of compounds for which NMDR for the estrogen hormone system were identified were most frequently synthetic hormones that were designed to have endocrine activity. Some of the reasons for tissue specificity for estrogens are also applicable for the androgen signaling pathway. Different androgen sensitive tissues have different corepressors and coactivators that regulate the effects of the chemical's ability to alter gene expression and metabolism, distribution and elimination of the chemical can vary from tissue to tissue.

Studies conducted in mammalian models *in vivo* rarely resulted in estrogen or thyroid signaling pathway effects that were NMDR. Studies conducted in mammalian models *in vivo* resulted in androgen signaling pathway effects that were nonmonotonic more often than reported for the estrogen or thyroid signaling pathways. Furthermore, when NMDRs occurred in these hormone systems they often were not reproducible within or across studies.

In the review of the mammalian, fish, and amphibian literature for xenobiotics that have been shown to perturb the thyroid hormone system, evidence of NMDR for thyroid disruption was

found in a small number of instances. In fish and amphibians, the appearance of NMDR was often confounded by toxicity induced at high doses, inadequate temporal sampling, or from variable and inconsistent measurements. In mammals, when nomonotonicity was detected, it was often not the critical effect or sole determinant that would determine the NOAEL or LOAEL. In the instances wherein the NMDR was at the lowest dose levels, the reported effects were on serum T3 or T4 and were inconsistent with the proposed MoA identified for those chemicals. In addition, the declines in serum TH observed at low doses in some studies were inconsistent with other thyroid related measures, and suggest that there are observations that require replication before being given further consideration. Overall, a more complete understanding of the temporal dynamics of compensatory processes in thyroid hormone regulation and activity would assist interpretation of the biological relevance of NMDR when they are identified.

5.2 Central Scientific Questions and Answers

We identified three central scientific questions to be addressed in the current state of the science assessment. The central scientific questions, and summarized responses are these.

5.2.1 Do nonmonotonic dose responses (NMDRs) exist for chemicals and if so under what conditions do they occur?

Yes, we concluded that exposures to chemicals can result in NMDRs for specific endpoints. NMDRs arise from complex relationships between the dose of toxicant at its target site and the effect of interest WHO (2012). NMDRs are biologically plausible and can arise when the biological system that is activated in response to toxicant exposure consists of at least two activities that can act in opposition to each other Conolly and Lutz (2004), WHO (2012). We determined that NMDRs are more frequently identified in *in vitro* studies, high dose range studies, or short-term studies. Assays that provide data at a lower level of biological organization (such as proteomics or transcriptomics) are more likely to identify NMDRs than studies that provide data on apical adverse events further downstream from the molecular initiating event. Reproducibility of NMDRs is important in establishing plausibility of a response and its potential applicability as part of the hazard characterization. Factors that influence reproducibility include:

- Study design – dose selection, sample size, organism strain, diet, housing environment, statistical methods;
- Robustness of physiology – physiologic compensation producing changes in slope; and
- Competing processes– induction of metabolism, repair, or independent mechanisms.

5.2.2 Do NMDRs capture adverse effects that are not captured using our current chemical testing strategies (*i.e.*, false negatives)?

There are certainly adverse biological changes that may occur in a nonmonotonic manner that would not be captured using current testing strategies. No testing strategy is able to assess all potential adverse effects, for all biological systems, in all tissues, for all species, in all developmental time points.

As the work group progressed with its review and discussions of the science, it became clear that our second question needed to be further defined so that it could be more accurately and fully answered in the context of the science as evaluated. Thus, question 2 was further expanded for clarification.

- Are there adverse effects with NMDRs that are not being identified using the current chemical testing strategies?
- Are there NMDRs for adverse effects below the no observed adverse effect levels (NOAELS) or benchmark doses (BMD) derived from the current testing strategies?
- Do EPA chemical testing strategies detect relevant adverse effects for chemicals which produce NMDR for specific endpoints?

Chemicals that operate through endocrine modes of action (MoA) have multiple targets across organs, tissues, and cellular systems in various species, and across all life stages. It is not possible or feasible for chemical testing to measure or analyze all possible endpoints for all chemical MoA in all tissues. The objective of USEPA's chemical testing strategy is not to identify all possible adverse effects, but rather, to identify sensitive endpoints relevant to human or ecological health, providing confidence that adverse effects are not being induced at dose levels below what was determined to be a NOAEL.

For estrogen, androgen or thyroid MoA that provide adequate information to make an assessment, our evaluation shows that there is not sufficient evidence of NMDRs for adverse effects below the NOAELS or BMD derived from the current testing strategies. For some MoA, however, the scientific database remains too limited to conclude this with certainty.

While there are biological changes that may occur in a nonmonotonic manner in the low dose region, our review indicates that reproducible NMDRs for adverse effects occur in the high dose region and not the low dose region of the dose response curve. Thus, the current testing approaches do not fail to identify or establish appropriate NOAELS in the low rose range of exposure, even if not all effects for every chemical are identified. The extensive evaluation conducted in the present review, as well as almost two decades of experience with screening

assays for hazard identification, indicate that these assays do not fail to detect chemicals with endocrine activity for the estrogen or androgen hormone systems. Dose response assessment is not an issue for screening assays. NMDRs would be problematic only if a chemical with estrogen, androgen, or thyroid activity produced an effect *in vivo* at a dose below those used in screening, and the chemical had no effect on estrogen, androgen, or thyroid related endpoints at the higher screening dosage levels. Although such NMDRs have been hypothesized, they have not been demonstrated reproducibly, and none were found in the present evaluation.

Our assessment of the adequacy of the current testing assays concludes that a number of standardized short- and long-term assays are sensitive in detecting chemicals that interfere with the estrogen, androgen and thyroid signaling pathways. Specifically, the EDSP screening battery can detect disruption of these pathways using combined *in vitro* and *in vivo* assays in mammalian and aquatic models. Standard multigenerational test guidelines have measures that are sensitive to disruption of the estrogen and androgen signaling pathways. While these studies are considered the current standard for assessing the potential of a chemical to be a reproductive toxicant and for use in setting NOAELS, they are not without limitations. USEPA testing strategies are reviewed periodically to assure they are incorporating the most sensitive and biologically relevant endpoints.

Further, if an objective of testing is to define the shape of the dose response curve and thereby identifying potential NMDRs, then the three treated groups and a control group used in current guideline studies may not be sufficient for this purpose. Modifications that could lead to a more clearly defined dose response characterization and increase the statistical power to detect low dose effects may be appropriate in specific instances.

5.2.3 Do NMDRs provide key information that would alter EPA's current weight of evidence conclusions and risk assessment determinations, either qualitatively or quantitatively?

Data from studies in which NMDRs are identified may be biologically relevant and as such should be evaluated in context with the totality of the available scientific data in weight of evidence (WoE) conclusions and risk assessment determinations. These data should be considered and analyzed, as all data are, and factored into the WoE based on standard criteria including, but not limited to, conduct of the studies, representation of biological processes that are relevant to the evaluation, biological plausibility, and reproducibility. NMDRs can have impact on both qualitative and quantitative risk assessments, but cannot be considered in isolation from other data for the chemical and biological response being considered.

5.3 Summary

NMDR after exposure to xenobiotics occur in estrogen, androgen, and thyroid hormone systems but are generally not common. Where NMDRs were observed, they were often at high dose. Biological endpoints closest to the molecular initiating event were more likely to identify a nonmonotonic point of inflection than those effects further downstream, including the apical adverse outcomes. The goal of chemical testing is to identify the potential for hazard after exposure to the xenobiotic of concern, not to identify and describe 100% of all the possible biological effects. As such, the current testing approaches perform this function successfully and, based on the current evaluation, are unlikely to mischaracterize a chemical that has the potential to adversely perturb the endocrine system due to an NMDR.

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7. Appendices