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A Cross-Species Mode of Action Information Assessment: A Case Study of Bisphenol A

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ABSTRACT

An approach to using mode-of-action (MOA) information across animal species has been developed to support both integrated ecological and human health assessment methods development and cross-species extrapolation for human health assessments. By assessing the effects and MOA data for a given toxic agent, the relationship between MOA and species relatedness (i.e., evolutionary relationships) can be determined. A case study assessing the utility of this approach was performed for bisphenol A (BPA). BPA, a component of polycarbonate plastics, epoxy resins, and polyester resins, was selected because it is a high production volume chemical; data have been identified for both vertebrate and invertebrate species; and the estrogen agonist MOA (i.e., binding and activating the estrogen receptor to transcribe estrogen-responsive genes) has been well described for a number of vertebrate species. Cross-species MOA information for developmental and reproductive effects of BPA, limited to the animal kingdom, was reviewed from the literature, and the relationship between species relatedness and MOA was assessed. MOA was defined as the key step in the toxic response after chemical interaction at the target site that is responsible for the physiological outcome or pathology. Reproductive and/or developmental in vivo effects data for BPA were identified for 16 species representing seven animal classes (gastropods, crustaceans, insects, amphibians, fish, birds, and mammals) in three phyla (mollusks, arthopods, and chordates). For the tested invertebrate species, the data were insufficient to determine the MOA among mollusks and arthropods. For the tested vertebrate species, the data support a relationship between species relatedness and the estrogen agonist MOA. However, while the data strongly support the estrogen agonist MOA for fish and mammals, the data set was less robust for birds and amphibians. Thus, the cross-species MOA approach holds promise for predicting the MOA among untested species for toxic agents. Such predictions could be useful for applying MOA information in an integrated ecological and human health risk assessment as well as for screening and toxicity testing prioritization of chemicals. For example, cross-species MOA data may provide useful information for chemical prioritization in the Office of Prevention, Pesticides and Toxic Substances' (OPPTS) Endocrine Disruptor Screening Program (EDSP) since the program is concerned with protecting human and wildlife health. This report was developed in support of EPA's Office of Research and Development's Multi-Year Plan for Endocrine Disruptors (2003).

CONTENTS

CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

PREFACE

This report addresses two recommendations of the U.S. Environmental Protection Agency (EPA). First, the Agency has been developing methods that broaden the scope of human health risk assessments from a single-chemical assessment approach that considers risk to one species to an approach that considers risk from multiple sources and chemicals to both wildlife and human health (U.S. EPA, 1997). Risk to wildlife as well as human health is of particular interest for EPA programs that aim to protect wildlife and human health. The Office of Prevention, Pesticides, and Toxic Substances' (OPPTS) Endocrine Disruptor Screening and Testing Program (EDSTP; U.S. EPA, 2005; http://www.epa.gov/oscpmont/oscpendo/index.htm) is a program that considers both wildlife and human health effects from exposure to endocrine disrupting chemicals (EDCs), based on recommendations from the Endocrine Disruptor Screening and Testing Advisory Committee's (EDSTAC, a former Federal Advisory Committee) Report (U.S. EPA, 1998a). Furthermore, one of the research areas identified in the Office of Research and Development's (ORD) Multi-Year Plan for Endocrine Disruptors (U.S. EPA, 2003) is "the development of integrated risk assessment approaches for human and wildlife populations..."

Second, the Agency has been involved in developing approaches to incorporate mode-of-action (MOA) information into risk assessment as a way to better characterize uncertainties in risk assessment. Specifically for EDCs, the ORD's Research Plan for Endocrine Disruptors (U.S. EPA, 1998b) identified the need to understand the relationship between MOA and effects.

A Case Study of Bisphenol A explores a qualitative approach for cross-species extrapolation of chemical-specific effects based on known MOA information. A case study of the EDC bisphenol A (BPA) is presented to demonstrate the approach. This report is neither a health assessment nor an integrated risk assessment of BPA. A phylogenic (evolutionary) relationship, for both invertebrate and vertebrate animals, between reproductive and developmental effects and the MOA of BPA is examined, based on available published literature. This report emphasizes studies that are relevant to the MOA for BPA. Data gaps and research recommendations, including recommendations for future case studies, are included. Future efforts for developing methods, for 1) integrated ecological and human health risk assessment and 2) using mode of action information in human health assessments, may build upon the results of this case study.

LIST OF ABBREVIATIONS AND ACRONYMS

ADME	Absorption, distribution, metabolism, and excretion
AGD	Anogenital distance
AhR	Aryl hydrocarbon receptor
BPA	Bisphenol A
CYP	Cytochrome P450
DES	Diethylstilbestrol
E	Estrogen
EDC	Endocrine disrupting chemical
ER	Estrogen receptor
ERE	Estrogen response elements
FSH	Follicle stimulating hormone
HPG	Hypothalamic-pituitary-gonadal
hSHBG	Human sex-hormone binding globulin
i.p.	Intraperitoneal
i.v.	Intravenous
LH	Luteinizing hormone
LOD	Limit of detection
MOA	Mode of action
ND	Not detected
NP	Not provided
NR	Not reported
NTP	National Toxicology Program
OPPTS	Office of Prevention, Pesticides, and Toxic Substances
p,p'-DDE	p,p'-Dichlorodiphenyldichloroethylene
P450arom	P450 aromatase
ppm	Parts per million
s.c.	Subcutaneous
STW	Sewage treatment works
SXR/PXR	Steroid and Xenobiotic Receptor/Pregnenolone X Receptor
T3	Triiodothyronine
T4	Thyroxine

TCDD	Tetrachlorodibenzo-*p*-dioxin
TRI	Toxic Release Inventory
TSCA	Toxic Substances Control Act
UDPGA	Uridine diphosphate glucuronic acid
U.S. EPA	U.S. Environmental Protection Agency

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1. EXECUTIVE SUMMARY

A case study assessing the relationship between mode-of-action (MOA) and species relatedness was performed for the chemical bisphenol A (BPA). Sources of BPA are the production and processing of polycarbonate plastics and epoxy and polyester resins as well as leaching and degradation of BPA from the products. Studies suggest that BPA exposure may be relatively high for occupationally exposed humans during plastic production and processing and for aquatic species downstream of sewage treatment plants. Nonoccupational human exposure may occur from contact with BPA-containing products, such as oral exposure to BPA leaching and degradation from certain dental sealants. BPA has been detected in the tissue of a number of wildlife species and in newborn human serum.

Reproductive and/or developmental in vivo effects data after BPA treatment were identified for 16 animal species of the total 9 to 10 million. In many invertebrate species, the role(s) of steroid hormones or other signaling molecules for development and reproduction have not been elucidated. Despite this limitation, the comparison of response after BPA vs. estrogen treatment was sometimes used as a method for assessing whether BPA effects were consistent with estrogen agonism, defined herein as binding to and activating the ER. In gastropods, BPA treatment led to sexual differentiation effects consistent with feminization, but data to establish an MOA were lacking. For BPA treatment of crustaceans, effects in daphnids were inconsistent; effects in copepods were consistent with an increase in estrogen activity; and effects in insects were dissimilar from estrogen exposure effects, and some data suggested an ecdysone antagonist MOA. Owing to an insufficient data set and lack of knowledge about the role(s) of steroid hormones in development and reproduction, the MOA for BPA could not be determined for any invertebrate species.

Within the vertebrate species, in vitro estrogen receptor (ER) binding studies indicate that BPA competitively binds to the ER of reptiles, amphibians, fish, birds, and mammals at a much lower affinity (250–12,500 times lower) than ethinylestradiol, a synthetic estrogen that is a relatively potent ER agonist. Some amphibian developmental effects data were consistent with BPA acting as an estrogen agonist, and some in vitro data suggest effects on thyroid hormone bioavailability. In birds, one study observed significant effects after in ovo BPA treatment of two species, and these effects were consistent with an estrogen agonist MOA. In fish, BPA in vivo study findings were consistent with an estrogen agonist, an androgen antagonist, an androgen agonist, steroid hormone bioavailability, and/or a nonendocrine MOA. Some affected endpoints could be explained by different MOAs, and other endpoints were consistent with only one MOA. Fish mechanistic study findings support an MOA leading to increased estrogen activity, including estrogen agonism. Mammalian in vivo effects data, limited to rats and mice, are consistent with an estrogen agonist, an androgen antagonist, an androgen agonist, altered

steroid hormone bioavailability, and/or altered prolactin hormone MOA. Most mammalian mechanistic data support an estrogen agonist MOA, although one study suggests an MOA that decreases androgen bioavailability. Human and rodent in vitro data indicate that BPA can act as an estrogen agonist, binding to the ER and activating estrogen-responsive genes.

Using a weight-of-evidence approach for the BPA MOA within vertebrate species, the estrogen agonist MOA explains the majority of responses. However, some effects were not necessarily the result of estrogen agonism. For example, some male rodent reproductive effects observed after BPA treatment cannot be linked definitively to estrogen agonism; the effects of exogenous estrogens on male development are not well understood. Some of these BPA effects are consistent with androgen antagonism, for example. It is possible that multiple MOAs are affected by BPA. Alternative MOAs include androgen agonist, androgen antagonist, thyroxine agonist, steroid hormone bioavailability (covering a number of specific MOAs), and nonendocrine MOAs.

Although a number of invertebrate species have been shown to be sensitive to either estrogen or BPA exposure, the significance of the findings is unclear because the role of estrogen (or estrogen analogs) in normal invertebrate development is not well understood. Among the tested invertebrates, the freshwater nonbiting midge and two snails (Giant ramshorn and dog whelk) were found to be the most sensitive (i.e., effects at lowest BPA doses) to BPA treatment. Invertebrate sexual differentiation, maturation, and gametogenesis were some of the most sensitive endpoints. Studies to determine a most sensitive life stage for invertebrates are needed. Among the vertebrate species, current data suggest that amphibians and fish are the most sensitive to BPA exposure. Among the tested nonmammalian species, the aquatic invertebrates and vertebrates may be more susceptible to BPA effects because BPA measurements in water and sediment environmental samples were relatively high (among the available studies), and reproductive effects have the potential to lead to population-level effects. Some of the most sensitive endpoints for mammals were anogenital distance, puberty timing and estrus cycling, and mammary gland development, although study findings vary. Among the tested vertebrates, sensitive life stages included the posthatchling stage for fish and the prenatal and peripubertal stages for rodents.

Data indicate that BPA exposure can elicit effects in vertebrate and invertebrate species at concentrations below those that cause morbidity and mortality. For determination of the MOA for BPA in invertebrates, one data gap is an understanding of the signaling pathways (e.g., whether there are analogs to vertebrate steroid hormones) controlling development and reproduction. It is striking that BPA-exposed snails, species with no identified role for estrogens, had feminized reproductive organs that may indicate an effect on the activity of estrogen or an estrogen-like molecule. An elucidation of the basis for the effects of vertebrate steroid hormone treatment on invertebrate development is needed. Mammalian mechanistic

studies to investigate whether BPA affects additional MOAs, at different doses and under different conditions, are needed. Interpretation of the low-dose BPA rodent effects data is complicated by differences in experimental results, inconsistencies in positive control outcomes, and a bias toward investigating the estrogen agonist MOA. More BPA low-dose studies (designed to address concerns, including strain and animal feed differences) examining sensitive developmental and reproductive endpoints are needed.

Some limitations to the case study were noted. First, MOA information for BPA across species, both in the total number of species and the coverage across phyla, were limited. BPA effects data are needed, especially across additional invertebrate species but also for additional vertebrate species. Second, the focus of most mechanistic studies was on the estrogen agonist MOA, and thus studies to assess whether other or alternative MOAs under various conditions (such as high dose vs. low dose and exposure during critical windows of development) are operative for BPA are needed. Third, studies to advance the understanding of the role(s) of hormones in invertebrate sexual differentiation and of estrogens in mammalian male development are needed.

The cross-species MOA approach holds promise for predicting the MOA among untested species for toxic agents. Such predictions could be useful for applying MOA information in an integrated ecological and human health risk assessment as well as for screening and toxicity testing prioritization of chemicals. The ability to predict the MOA for an untested species is more reliable within the chordate phylum because there are the most data for species after BPA exposure. Within the tested chordates, data are consistent with the estrogen agonist MOA. Thus, for an untested vertebrate species, the estrogen agonist MOA could be predicted with varying degrees of confidence depending on the vertebrate class. The data for the fish and mammal classes strongly support the estrogen agonist MOA. The data sets are much smaller for the bird and amphibian classes but are also consistent with an estrogen agonist MOA. However, for the mollusk and arthropod species, not enough data exist for each species, data have not been identified for enough species, and the basic mechanistic understanding of the affected endpoints is inadequate to make any prediction about MOA for an untested species. In the future, MOA information and evolutionary relationships among species may be useful for making predictions about the MOA of an untested species. Based on the experience gained in this case study, a cross-species MOA assessment is a promising approach for use in integrated risk assessment and chemical prioritization.

2. INTRODUCTION

2.1. MOTIVATION FOR CASE STUDY

There is interest in developing approaches for integrating ecological and human health risk assessments. Integrated risk assessment is defined as "a science-based approach that combines the processes of risk estimation for humans, biota, and natural resources in one assessment" in the *Framework for the Integration of Health and Ecological Risk Assessment* (Suter et al., 2001; WHO/IPCS/IRA, 2001). This holistic approach considers humans as part of the ecosystem and combines two risk assessments into one. In the U.S. Environmental Protection Agency's (EPA's) cumulative risk assessment guidance, the term "integration" refers to a cumulative approach, assessing all sources, effects, pathways, stressors, and populations (U.S. EPA, 1997; http://www.epa.gov/osp/spc/2cumrisk.htm).

Historically, human health risk assessment and ecological risk assessment methods developed separately. The recommendations for integrated risk assessment (Suter et al., 2001) are based on the U.S. EPA's *Guidelines for Ecological Risk Assessment* (U.S. EPA, 1998c) and include the same three phases of assessment. Five reasons for performing an integrated risk assessment were provided by Suter et al. (2001). First, coherent expression of assessment results will provide a stronger assessment result (i.e., one assessment that is based on the same assumptions—data based on similar temporal and spatial scales—is more valuable). Second, it is more efficient to perform one assessment instead of two to avoid redundancies in the process. Third, chemical effects across species are often interdependent (e.g., chemical contamination of water may affect wildlife as well as humans), and the loss of a species can affect populations of other species, including humans. Fourth, an integrated assessment may identify sentinel organisms (e.g., nonhuman organisms may have greater exposure to environmental contaminants or may be more sensitive) (NRC, 1991). In fact, a sentinel for a particular MOA may be identified. Fifth, the quality of the assessment should be improved because it is based on more information (effects and exposure to multiple species) and collaborations between experts of different disciplines.

2.2. GOALS, APPROACH, AND SCOPE

The project goals were (1) to develop an approach to use MOA information across species to support integrated ecological and human health assessment that can serve as a template for future cross-species MOA assessments, and (2) to perform a case study assessing the utility of the approach by reviewing and analyzing the MOA data for one chemical across species. Three subobjectives were identified: to determine whether the MOA data could be used to predict effects in untested species, to determine the extent to which MOA data across

phylogenetic relationships can support cross-species extrapolation of risk, and to use MOA data to identify the most sensitive life stages and species (for application to site-specific or media-specific risk assessments) as a method to protect all potentially exposed species. Additional outcomes include a benefit to the risk assessment process. For example, describing and understanding the relationship between species relatedness and MOA may reduce uncertainty in the interspecies default value used in human health assessments.

For purposes of this report, MOA is defined as the key step in the toxic response after chemical interaction at the target site that is responsible for the physiological outcome or pathology (IPCS, 1999). MOA determination requires a link between the MOA and the response (i.e., an alteration of the key step has been shown to be necessary and sufficient to induce the pathology or endpoint of interest). For most toxic chemicals, the MOA is suspected and, for some, it is relatively well defined. An example of a chemical with a well-defined MOA is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); TCDD-associated effects depend on binding to the aryl hydrocarbon receptor (AhR).

The approach was to identify, analyze, and integrate the developmental and reproductive effects and mechanistic information from the published literature for a single chemical that informs the MOA for each species with data. Weight-of-evidence (WOE) criteria would be developed to draw conclusions about MOA from the available data for a given species. By comparing evolutionary relatedness to MOA, patterns may emerge. If enough data on MOA exist for a phylum or class of species, a relationship between species relatedness and MOA may be established. This relationship may be used to predict the MOA of untested species (defined as species without data for the given chemical) that are related to the tested species(defined as species with data for the given chemical).

2.2.1. Bisphenol A Selection for Case Study

The case study was performed to illustrate the use of an approach for conducting cross-species MOA assessment to support integrated risk assessment. Thus, the specific chemical selected is not the focus of the project. A priori criteria for chemical selection were simply that the chemical be of interest to EPA and that some MOA information and effects data across the animal kingdom (i.e., effects data not limited to vertebrates) were available in the published literature.

BPA (2,2-bis(4-hydroxyphenyl)propane; 4,4′-isopropylidenediphenol; CAS no. 80-05-7) was selected because it is a high production volume chemical (Staples et al. 1998), it is an EDC with a well-described estrogen agonist MOA for a number of vertebrate species, and developmental and/or reproductive effects data were identified for some nonmammalian vertebrate and invertebrate species.

BPA is a component of polycarbonate plastics, epoxy resins, and polyester resins (Staples et al., 1998). BPA production has increased during the past decade and is predicted to increase further (Kirschner, 1996). Sources of exposure to BPA include oil, gas, chemicals, plastics, and electronics production (Staples et al., 1998). BPA has also been detected in the tissue of some wildlife species (Tsuda et al., 2000; Larsson et al., 1999) and human newborn serum (Schonfelder et al., 2002).

Effects data for BPA have been identified in some nonmammalian species. In addition, data suggest that the MOA of BPA in many mammalian species is an estrogen agonist, providing a hypothesis to be tested in other species. The first in vivo study indicating that BPA possessed estrogen agonist activity found estrus-related changes in BPA-treated adult ovariectomized rats (Dodds and Lawson, 1936). BPA behaved as an estrogen agonist, binding to the ER in vitro as well (Olea et al., 1996; Brotons et al., 1995; Krishnan et al., 1993). Recently, a number of studies have addressed the reproduction and developmental effects of BPA in a number of diverse animal species.

A highly coordinated, tightly regulated signaling network governs the physiology of all organisms, ranging from bacteria to humans. At the molecular level, receptor-mediated and non-receptor-mediated signaling affect the transcription of target genes, which in turn can cause developmental changes in developing organisms and affect the maintenance of homeostasis in adults. Steroid hormone-mediated signal transduction is an example of receptor-mediated signaling. Steroid hormones bind to their steroid receptor, the hormone-receptor complex undergoes a conformational change leading to activation of the complex, and binding of the hormone-receptor complex to the regulatory region of specific genes leads to activation or repression of transcription of these genes (Figure 1).

A number of environmental chemicals, including BPA, have been shown to lead to physiological changes via an estrogen agonist MOA. An estrogen agonist acts as an estrogen mimic; it binds to the ER and activates transcription of estrogen-dependent genes (Figure 1). Conversely, an estrogen antagonist can bind to the ER but is unable to activate transcription, thus decreasing the response to the endogenous estrogen by blocking its binding to the ER. These two MOAs represent a subset of the MOAs identified for EDCs to date. For a review of the developmental effects of EDCs, see Colborn et al. (1993). The estrogen agonist activity of BPA was identified in the 1930s in rodents (Dodds and Lawson, 1938, 1936). Thus, the estrogen agonist (E) MOA for BPA was likely in other mammalian species, and, as a consequence of the historical focus on the estrogen agonist MOA for BPA, the majority of the mechanistic studies identified in the literature focus on assessing the estrogen agonist MOA. However, it was not clear whether BPA affected additional and/or different MOAs in other mammalian species or whether evidence demonstrated that BPA had an estrogen agonist MOA in nonmammalian species. McLachlan (2001) reviewed the literature for steroid receptors across species with

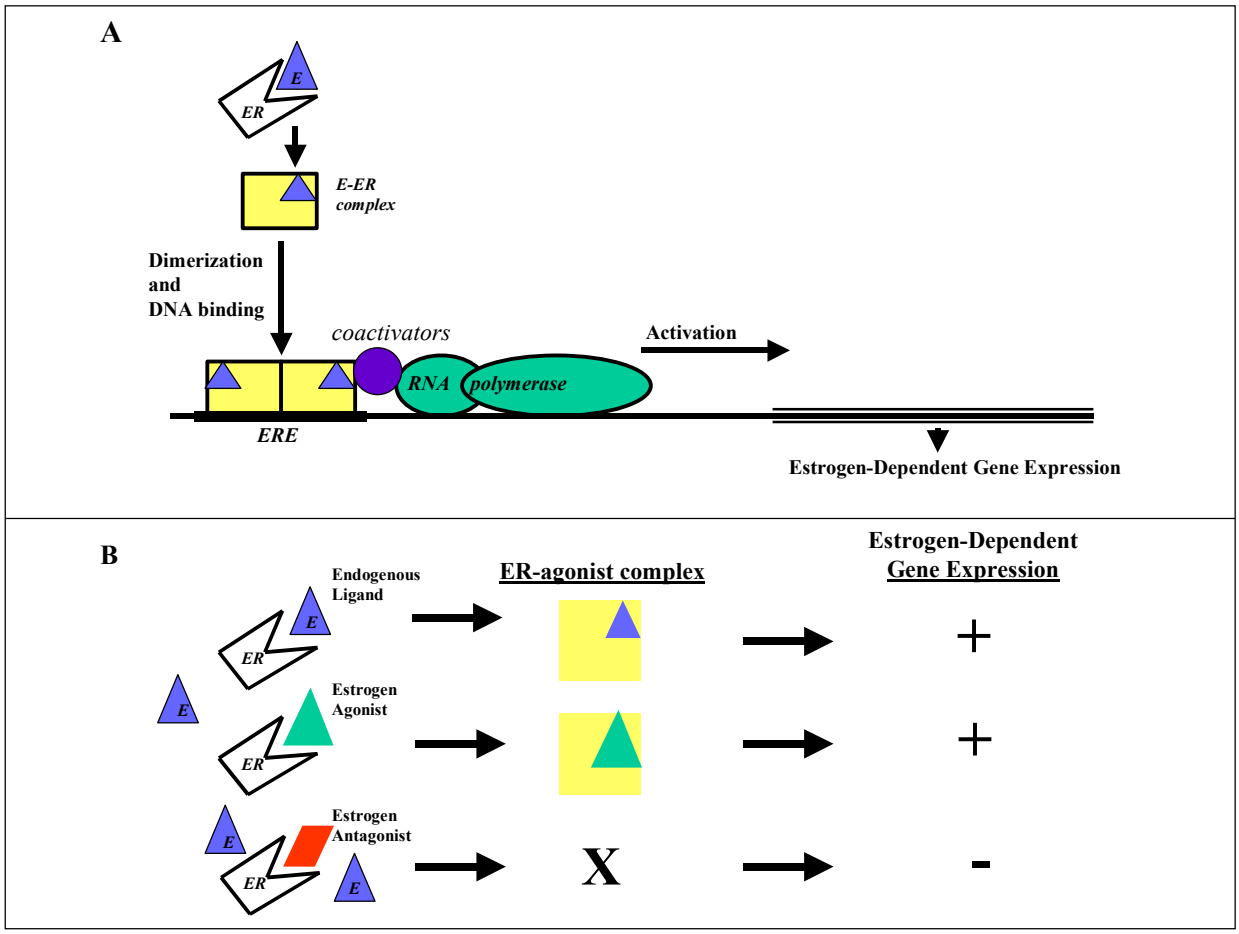

Figure 1. Estrogen mode of action and the effect of exposure to an estrogen agonist or antagonist. *A: Estrogen action of 17 β-estradiol.* Estradiol (E) binds to the estrogen receptor (ER), the E-ER complex undergoes a conformational change and is translocated to the nucleus, and the E-ER complex binds as a dimer to the estrogen response element (ERE) in the regulatory region of genes whose expression is estrogen-dependent. Following binding to the ERE, the E-ER complex activates transcription for the gene shown. (The transcription of some estrogen-dependent genes is inhibited.) *B: A depiction of the effect of exposure to an estrogen agonist or an estrogen antagonist on estrogen-dependent gene expression.* An estrogen agonist mimics the effect of endogenous estradiol but may differ in potency. An E agonist binds to the ER and activates estrogen-dependent gene transcription. An estrogen antagonist competes with endogenous estradiol for binding to the ER, thus blocking formation of the E-ER complex. The E antagonist-ER complex cannot activate transcription of estrogen-dependent gene expression. As a result, estrogen-dependent gene expression is decreased or completely blocked (depending on the potency and dose of the E antagonist).

particular emphasis on the estrogen receptor. For some organisms, estrogen or other steroid hormones and their receptors have not been identified. It is postulated that some organisms without estrogen receptors have estrogen analogous signaling systems that estrogen agonist exposure could affect and, in some cases, to interact with. For example, signaling between a plant and the bacterium *Rhizobium* is analogous to ER signaling. The plant produces the hormone luteolin, which is recognized by the luteolin receptor in the bacteria. Environmental estrogen and estrogen agonists, including BPA, have been shown to bind to and activate the bacterial luteolin receptor (Fox et al., 2001). Study of the action of endogenous and synthetic compounds in the context of the endocrine system led to an appreciation of the conserved aspects of estrogenic systems across many species (for a review, see McLachlan, 2001). The strength of a MOA assessment is that it may aid in cross-species extrapolation and integration of ecological and human health risk evaluations because MOA is most likely a function of evolutionary relationships. This report highlights the MOA and effects data from EPA's internal 2001 draft document entitled *Background Document for an Integrated Human Health and Ecological Species Assessment: A Case Study of Bisphenol A* (McLachlan et al., 2001).

2.2.2. Scope of the Project

A number of different approaches to integration may be implemented. Because an objective of this case study was to determine whether the relationship between MOA information and species relatedness (i.e., evolutionary relationships) could be used to predict the MOA of a chemical for a species without data, the selected integration approach involved comparing MOA information for BPA across species. By looking across species, species relatedness-MOA pattern(s) may emerge. For example, if BPA is found to act as an estrogen agonist in a group of closely related species, it suggests that BPA may similarly affect other untested closely related species because it is predicted that analogous estrogen signaling mechanisms exist across related species. Furthermore, if BPA has a different MOA in nonmammalian species than in mammalian species, this would indicate that both MOAs need to be considered for an integrated ecological and human health risk assessment. In addition to comparing MOAs across species, the results may identify sensitive or susceptible species, possibly environmental indicator species, and subpopulations and life cycle phases. The scope was limited to the animal kingdom because a small number of published studies of BPA effects for nonanimal species were identified. BPA effects studies in nonanimal species include a bacterium-plant interaction system (Fox et al., 2001) and acute and chronic cell growth studies in green algae and pseudomonas (reviewed in Staples et al., 1998).

The scope of this case study was limited to species of the animal kingdom, the effects part of the assessment, MOA information obtained from the published literature, and focuses on the hazard characterization part of risk assessment (i.e., it is not intended as a complete

integrated risk assessment). Although this project does not develop a risk assessment for BPA, the results of this effort may aid in the development of an integrated evaluation. The literature included peer-reviewed journal articles and publicly available documents, such as the Toxic Release Inventory (TRI) database, published before the end of 2001, with the exception of a large three-generation toxicity study on BPA effects, considered a critical study, by Tyl et al. (2002). Since the literature review was limited to those published by 2001, more recent BPA study findings describing a genotoxic MOA, leading to aneuploidy in mouse oocytes, are not covered in this case study (Hunt et al., 2003; Can et al., 2005).

The audience for this report includes scientists, risk assessors, and policymakers. The report includes information on reproductive and developmental effects and underlying endocrine mechanisms in vertebrate and invertebrate species. Due to this broad scope, the reader is also directed to background materials.

The following questions are addressed in the case study:

- What is the representation of species with effects data after BPA exposure across the animal kingdom?
- What criteria will be used to determine MOA for a species?
- What are the MOA data for BPA for species with data? How well do the data support the proposed MOA for each of the species with data? Are there data suggesting more than one MOA for BPA in any species?
- Are there enough MOA data to establish relationships between MOA and evolutionary relatedness? What is the relationship between BPA MOA and species relatedness?
- What are the data gaps and research needs for an integrated MOA assessment of BPA?
- How applicable is this approach to assessing MOA across species for other chemicals?
- How useful is a cross-species MOA approach for predictive purposes, such as chemical prioritization programs and risk evaluations?

2.3. POTENTIAL FOR EXPOSURE TO BPA

BPA is a component in the manufacturing of polycarbonate plastics, epoxy resins, and polyester resins (Ben-Jonathan and Steinmetz, 1998; Staples et al., 1998), used to make such

products as baby bottles and food containers, epoxy resin food can linings, and dental materials. BPA is a high production volume chemical. Use of BPA in the plastics industry has increased since the 1940s, and its use in epoxy resins has only recently decreased (Bray, 1999). Annual U.S. production of BPA rose from a range of 0.93–1.1 billion pounds in the late 1980s (Alexander et al., 1988) (Figure 2) to 1.6 billion pounds (727 million kg) in 1995 (Kirschner, 1996), with concomitant increases in other industrialized nations. A conceptual model for BPA release into the environment and exposure to animals is shown in Figure 3. Humans and wildlife are exposed to BPA via environmental releases from a variety of industrial sectors, including production of oil and gas, chemicals, plastics, and electronics (Staples et al., 1998); directly from BPA manufacture, processing, or use; and indirectly through disposal BPA-containing products.

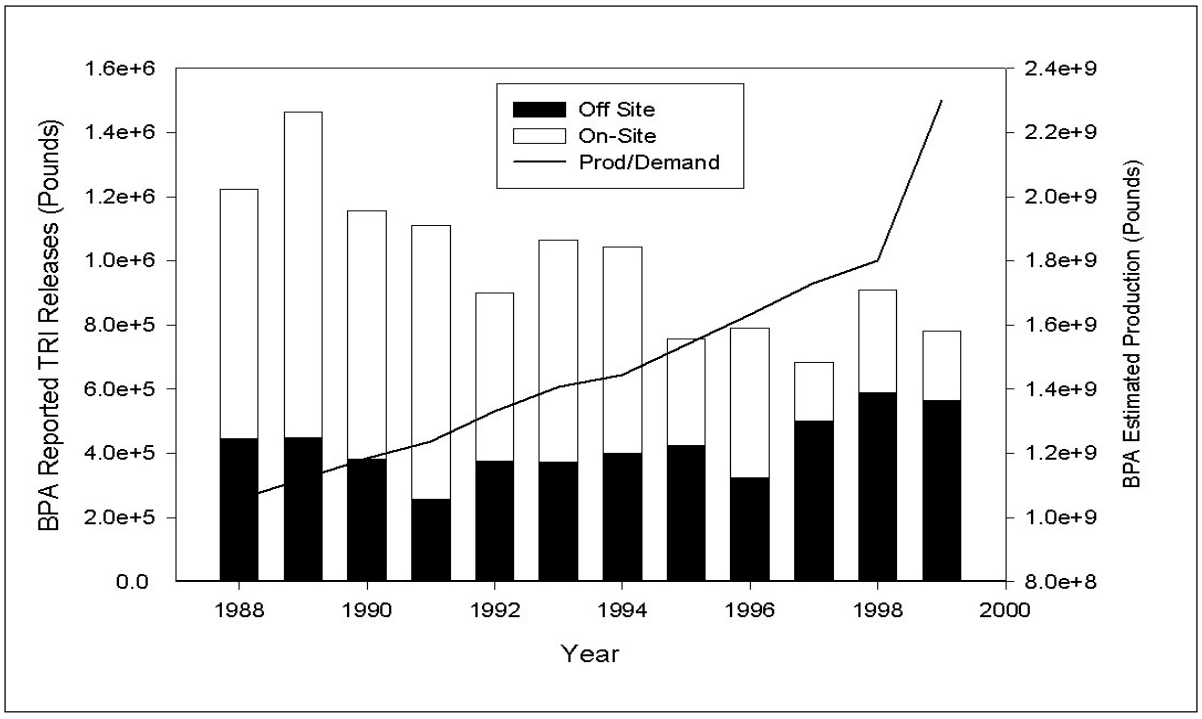

Figure 2. U.S. BPA production and reported releases from 1988 to 1999.
Sources: U.S. BPA reported releases are from U.S. EPA Toxic Release Inventory database, and total U.S. production data are from ChemExpo BPA chemical profile archives.

The number of U.S. counties reporting releases of BPA into the environment to EPA's TRI database increased from 56 to 64 between 1988 and 1999 (U.S. EPA, 1999). However, the number of counties reporting releases in 1995 declined from 83 to 67. At this time, there was a large increase in the number of counties reporting releases of 0 pounds per year. Since the

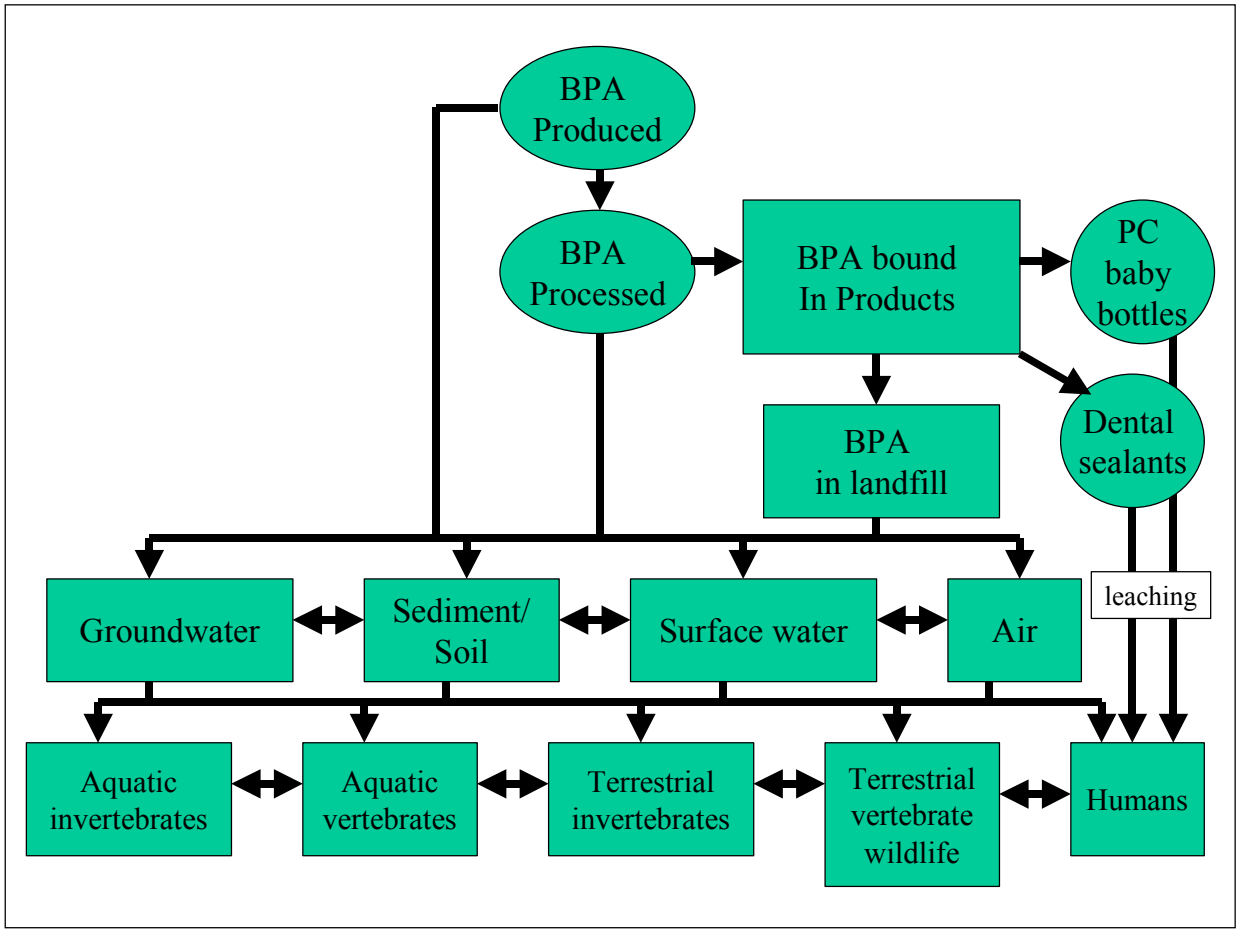

Figure 3. Conceptual model for BPA release into the environment and exposure to animals. Baby bottles and dental sealants are shown as two examples of BPA products with human exposure.

United States annually exports less than 0.1% of its domestic BPA production (approximately 132 million pounds of BPA) (ChemExpo), other industrialized countries represent significant worldwide production and consumption of BPA. Since 99.99% of BPA is destroyed in the manufacturing process or bound in product, the transport of BPA into the environment through leachates in landfills is a potential exposure pathway, particularly given the low volatility of BPA. Leaching from plastics at waste disposal sites has been studied (Yamamoto et al., 2001; Yasuhara et al., 1999; Rudel et al., 1998). The results of one study suggest that softer, more pliable products seem to have greater potential to leach than more rigid ones (Yamamoto and Yasuhara, 1999). After BPA release into the air, water, and land, numerous species can be exposed to BPA. BPA fate and transport in various environmental media are discussed more fully in EPA's draft document, *Background Document for An Integrated Human Health and Ecological Species Assessment: A Case Study of Bisphenol A* (McLachlan et al., 2001).

Human exposure can occur from occupational exposures in the plastics and electronics industries (Angelini et al., 1996). The number of humans occupationally exposed to BPA has increased as the number of workers in plastics industry has increased an average of 3% per year over approximately the past 25 years to more than 1.3 million workers in 1996 (Lewis, 1999). Research on exposure to BPA used for plastics production and processing (e.g., during monomer, polymer, and resin production) and in trade and household use of unpolymerized epoxy resins (Angelini et al., 1996; Maguire, 1988) is very limited. Workers in the BPA-based polycarbonate plastics and epoxy resins industries are potentially exposed to BPA via the inhalation and dermal routes (Bray, 1999; Lewis, 1999; Maguire, 1988). Although exposure to plastic monomers during production has considerably decreased since the 1970s, situations still exist where workers can contact plastic monomers (Lewis, 1999), demonstrated in an epidemiological study of women workers (Aschengrau et al., 1998). Air sampled at a plastics processing workplace had a BPA concentration of 0.208 $\mu g/m^3$, providing evidence for dermal and inhalation BPA exposure to plastics industry workers (Rudel et al., 2001). Workers involved with BPA production as well as production and processing of plastics and resins may be a highly exposed population.

Nonoccupational human exposure to BPA from consumer products can occur from contact or ingestion of water and foods that were manufactured or packaged in BPA-based plastics and certain dental sealants (Munguia-Lopez and Soto-Valdez, 2001; Yoshida et al., 2001; Fung et al., 2000; Arenholt-Blindslev et al., 1999; Aschengrau et al., 1998; Wingender and Niketas, 1998; Howe et al., 1998; Mountfort et al., 1997; Biles et al., 1997a, b; Olea et al., 1996; Brotons et al., 1995). Sources found to leach BPA were dental sealants (Pulgar et al., 2000; Olea et al., 1996), polycarbonate plastic containers (Biles et al., 1997a; Krishnan et al., 1993), and epoxy resin-lined products (Biles et al., 1997b; Brotons et al., 1995). There is controversy about whether BPA leaches from plastics because other studies have found no detectable leaching of BPA from products including polycarbonate plastics and industrial sealants (Geurtsen et al., 1999; Biles et al., 1997a). For the general public, exposure through BPA-contaminated drinking water ingestion is considered to be the primary route of BPA exposure.

A few studies have examined the BPA concentration in human serum (Fung et al., 2000; Inoue et al., 2000; Sajiki et al., 1999). In a study on leaching and absorption of BPA after application of dental sealants to adult participants, 5.8–105 ppb (ng/mL) BPA was detected in saliva, but no BPA was detected in serum samples (5 ng/mL limit of detection [LOD]) collected 1 hour to 5 days postadministration (Fung et al., 2000). Another study examined BPA in blood samples from adult Japanese volunteers and found serum BPA concentrations ranging from not detected (ND) (<0.1 ng/mL) to 1.6 ng/mL in the women and 0.38–1 ng/mL in the men (Sajiki et al., 1999). After storage in blood bags, serum BPA concentrations of 0.32 ng/mL were detected in samples from five healthy volunteers (Inoue et al., 2000). A recent study detected 0.3–18.9

ng/mL BPA in serum of pregnant women (n = 37), 1–104.9 ng/g BPA in placental tissue at birth, and 0.2–9.2 ng/mL BPA in umbilical cord blood of the newborn (Schonfelder et al., 2002).

A study of BPA in human urine examined both free BPA and BPA-glucuronide (Brock et al., 2001). BPA concentrations in urine samples from adults were compared with plasma levels. Prior to deglucuronidation, no free BPA was detected (0.12 ng/mL LOD). After deglucuronidation, BPA was measured at concentrations ranging from 0.11 to 0.51 ng/mL (average 0.28 ng/mL). Interpretation of these results is limited owing to a lack of knowledge about BPA's excretion profile in humans.

2.3.1. Environmental Fate, Transport, and Distribution Pathways

According to EPA's 1999 TRI, 28% of BPA (out of a 779,544-pound total) was released on-site. With regard to reported on-site releases, approximately 79% of BPA was released to the air with lower emissions to land (19%), surface water (<1%), and underground injection (<1%). By comparison, 1989 releases suggest that only 64% of reported BPA releases (out of a 1,223,996-pound total) were released on-site, with emissions to air, land, surface water, and underground water of 29, 55, 16, and <1%, respectively. The most significant changes between the two decades were the decreased on-site releases to land and surface water; most other values remained similar (Figure 2).

BPA's physicochemical characteristics include moderate hydrophobicity, suggesting that is has a moderately low affinity in the aqueous phase; low volatility, low vapor pressure, and rapid air biodegradation, suggesting that it is short lived in air; and a low bioconcentration factor, suggesting that sediments and soils are the most significant sinks for BPA. These characteristics affect subsequent environmental sinks and compartments where humans and wildlife may be exposed to BPA.

Exposure of humans and wildlife to BPA may occur via the water (Table 1). Given the diversity of water-sampling locations, techniques, and study objectives throughout the world during the past several decades, it is difficult to conclusively determine specific regions or industry types that release higher levels of BPA into natural waters. Waters that are affected by industrial or sewage effluent, particularly by paper mills, have found higher BPA concentrations (either in the effluent itself or in receiving waters) than do pristine waters. Based on data submitted by manufacturers required under the Toxic Substances Control Act (TSCA) section 8(e), environmental concentrations resulting from treated process effluent discharge should not exceed 100 µg/L BPA (Dorn et al., 1987).

Staples et al. (2000) analyzed receiving waters upstream and downstream of five manufacturers in 1996 and 1997 and two processors in 1997 during seasonal low-flow periods. BPA was not detectable (<1 µg/L) in any of the surface water samples in 1996 and at only one of seven sites in 1997, where BPA ranged between 2 and 8 µg/L with average concentrations

Table 1. BPA concentrations at aquatic sites worldwide

Location	Site characteristics	Sample Collection Date	Mean/average, µg/L (date)	Range, µg/L (date)	Reference
Tama and Sumida Rivers, Tokyo, Japan	Sewage (majority) and industrial pollution mixture	September and November 1973 (Tama); December 1973 (Sumida); 1974–78 (Sumida and Tama)	ND (September) NR (November) ND (December) 0.12 ± 0.04 (1974–78)	ND (September) 0.01–0.09 (November) 0.06–0.11 (n = 4, Tama, 1974–78) 1.9 µg/L (n = 1, Sumida, 1974–78)	Matsumoto et al. (1977); Matsumoto, (1982)
Ogasawara Islands, Japan	Pristine inland waters	1974–78	ND	ND (n = 35)	Matsumoto (1982)
Five BPA-manufacturing and two BPA-processing facilities in the United States	Seven sites upstream and downstream of facility; effluent during low flow	1996 and 1997	<1 (all sites 1996) <1 (six sites 1996) 4 (upstream one site, 1997) 8 (downstream one site, 1997)	2–8 (one site, 1997)	Staples et al. (2000)
U.S. BPA producers' receiving waters	15 upstream and 15 downstream sites at edge of mixing zone	1996	<1 (all sites)	<1 (all sites)	Staples et al. (1998)
Cape Cod, Massachusetts, United States	Untreated septage	summer 1996 and early 1997	0.82	0.11–1.7	Rudel et al. (1998)
Cape Cod, Massachusetts, United States	Untreated wastewater	summer 1996 and early 1997	0.11	0.094–0.15	Rudel et al. (1998)
Cape Cod, Massachusetts, United States	Treated wastewater	summer 1996 and early 1997	0.038	0.02–0.055	Rudel et al. (1998)
Cape Cod, Massachusetts, United States	Groundwater downgradient; treated wastewater plume	summer 1996 and early 1997	0.016	0.003–0.029	Rudel et al. (1998)
Cape Cod, Massachusetts, United States	Groundwater, downgradient from landfill/septage lagoon	summer 1996 and early 1997	0.32	0.004–1.41	Rudel et al. (1998)
18 sites in Germany	Sewage treatment plant effluents	August 1998–May 1999	0.083	<0.01–1	Spengler et al. (2001)
Town in Lower Austria	Wood, paper, metal, and chemical industries and household effluent	NP	NP	1–72	Fürhacker et al. (2000)

ND = not detected; NR = not reported; NP = not provided.

of 4 and 8 μg/L upstream and downstream, respectively (Staples et al., 2000). In Cape Cod, Massachusetts, Rudel et al. (1998) sampled untreated septage and wastewater, treated septage and wastewater, and groundwater from an effluent downgradient location to assess the potential effects of contamination and treatment on drinking water and found that untreated septage had 0.11–1.7 μg/L (0.82 average) BPA; untreated wastewater had 0.094–0.15 μg/L (0.11 average) BPA; and treated wastewater had 0.02–0.055 μg/L (average 0.038) BPA. Down the hydrologic gradient, groundwater sample concentrations ranged from 0.003 to 0.029 μg/L (average 0.016) in a plume from the wastewater treatment plant and from 0.004 to 1.41 μg/L (average 0.32 μg/L) in a plume from a nearby landfill/septage lagoon. Therefore, septic systems and landfills may represent greater sources of BPA release into the environment than effluent from wastewater treatment methods.

Fürhacker et al. (2000) monitored effluents of industrial emitters and communal wastewaters at 10 sites over a 5-month period and found that industrial point sources showed a maximum BPA concentration of 72 μg/L in wastewater samples. Wastewater treatment plant influent of paper, wood, metal, and chemical companies reached the upper BPA concentrations in a range of 35–50 μg/L BPA (Fürhacker et al., 2000; Table 1). Household area contributions are much lower, as ~90% of the total BPA load was removed during wastewater treatment.

BPA degradation rates in water have wide ranges and depend on site-specific conditions. BPA is most resistant to environmental degradation in groundwater (half-life = 2 days to 12 months), soil (half-life = 1 day to 6 months), and surface water (half-life = 1 day to 5.3 months) (Howard et al., 1991). Studies generally suggest that BPA-degrading bacteria exist throughout aquatic environments but that they cannot completely degrade BPA, leading to the accumulation of some recalcitrant metabolites, such as BPA-glucuronides. When examining microbial interactions with BPA, both the ability of these organisms to biotransform BPA (that reduces impacts on other biota) and the potential for BPA to impair the ecological functioning of these same microorganisms are important to consider. For example, if certain bacteria essential for ecological and anthropocentric functions like nitrification of ammonia are inhibited by the presence of BPA and other compounds, these exposures may have a large impact on the ecosystem.

Studies measuring BPA in biota are limited. Bile samples from juvenile rainbow trout (*Oncorhynchus mykiss*) caged in a stream upstream and downstream of a sewage treatment works (STW), where the sewage is primarily domestic waste from a small city in Sweden, had detectable concentrations of BPA (Larsson et al., 1999). The effluent water detected 490 ng/L BPA. Approximately 5 μg/g BPA was found in fish bile after a 2-week exposure period, and >10 μg/g BPA was detected after 4 weeks in fish caged upstream. The presence of BPA was analyzed in a few fish and shellfish samples caught from Japanese rivers, lakes, and inland seas (Tsuda et al., 2000). Meat and viscera taken from river snails, Melanian snails, Corbicula, and

oyster did not measure BPA above the limit of detection (1 ng/g). Only whole-body analysis of Japanese smelt resulted in detectable BPA at 8 ng/g. No information was reported on ambient BPA concentrations at these locations.

2.3.2. Toxicokinetic Analysis

Information on the toxicokinetics and toxicodynamics of BPA in the animal following external exposure, including absorption, distribution, metabolism, and excretion (ADME) and their kinetic parameters, is important for determining BPA target dose and, thus, susceptibility to BPA exposure. Knowledge of the physiological disposition of BPA comes mainly from rodent ADME studies. Studies of BPA disposition, after oral or intravenous (i.v.) dosing, found that BPA was rapidly absorbed, distributed, and excreted by rat strains (Long et al., 2000; Pottenger et al., 2000; Takahashi and Oishi, 2000; Upmeier et al., 2000; Yoo et al., 2000). A route dependency in the BPA half-life was noted in rats; the i.v. route of exposure had a longer half-life than the oral route (Kurebayashi et al., 2003). The half-life of BPA after a bolus oral gavage route may not necessarily reflect the half-life of BPA for oral human exposure, where one would expect smaller and more frequent dosing. For human oral exposure, the BPA plasma concentration, although not known, may be expected to be lower because there would be smaller and more frequent doses allowing more time for metabolism and excretion. The rat excretion profile indicated enterohepatic recirculation of BPA (Takahashi and Oishi, 2000; Upmeier et al., 2000; Yoo et al., 2000), resulting in a longer terminal excretion half-life. Kurebayashi et al. (2003) found that even a low dose of ^{14}C-BPA leads to a long half-life owing to repeated enterohepatic recirculation. Glucuronidation was the principal pathway of biotransformation (Pottenger et al., 2000; Snyder et al., 2000; Knaak and Sullivan, 1966). BPA-glucuronide was characterized as the primary form of BPA in circulation after oral exposure and in urine regardless of administration route (Pottenger et al., 2000; Snyder et al., 2000; Knaak and Sullivan, 1966). There may be some significant species differences in effective dose of BPA between rodents and primates. Following oral dosing of 0.10 mg/kg ^{14}C-BPA, the plasma concentration was much higher in monkeys than in rats owing to differences in absorption (Kurebayashi et al., 2002). Therefore, primates may be more susceptible than rodents to the same BPA exposure.

Because glucuronidation of BPA is likely to be a means of detoxifying and decreasing the estrogen agonist activity associated with BPA (Elsby et al., 2001; Matthews et al., 2001; Snyder et al., 2000), differences in glucuronidation potential among organisms may affect the observed BPA-related effects among species. In vitro screens demonstrated BPA-glucuronide lacked estrogenic and some other toxic responses. Glucuronidation may also provide a storage depot for BPA (Pottenger et al., 2000; Snyder et al., 2000; Takahashi and Oishi, 2000; Upmeier et al., 2000). Consequently, evaluation of the form of BPA present in an organism is critical to

predicting potential risks associated with exposure. Biotransformation in human liver cells was also examined (Elsby et al., 2001; Suiko et al., 2000; Snyder et al., 2000), and one study found that BPA glucuronidation activity was lower in human liver microsomes than in female rat liver microsomes (Elsby et al., 2001), indicating species differences.

A small number of studies examined BPA distribution in fish (Lindholst et al., 2000; Pedersen and Lindholst, 1999). Absorption of BPA from water into aquatic biota has been demonstrated (Honkanen et al., 2001; Lindholst et al., 2001; Larsson et al., 1999). Physiological disposition studies in fish demonstrated that BPA is absorbed from water in teleost fish eggs and mature animals (Honkanen et al., 2001; Lindholst et al., 2001; Lindholst et al., 2000; Larsson et al., 1999). Japanese quail (*Coturnix japonica*) absorbed BPA after oral exposure and maternally transferred it to developing oocytes (Halldin et al., 2001). BPA is glucuronidated in rainbow trout (*O. mykiss*) (Lindholst et al., 2001), but it is not known whether glucuronidation occurs in other fish. BPA is eliminated in Atlantic salmon (*Salmo salar*) and Japanese quail embryos by unknown conjugation mechanisms (Halldin et al., 2001; Honkanen et al., 2001). No studies investigated BPA-specific biotransformation in wildlife species. BPA is distributed to the liver and muscle of rainbow trout exposed to BPA in ambient water (Lindholst et al., 2001; Lindholst et al., 2000) and was detected in fish eggs and juvenile animals exposed to BPA in water (Honkanen et al., 2001; Lindholst et al., 2001; Larsson et al., 1999). BPA was measured directly in bony fish (Tsuda et al., 2000; Larsson et al., 1999) and also detected in tissues of birds exposed orally to BPA (Halldin et al., 2001).

2.3.3. Exposure During Development

The potential for BPA exposure during animal development and the identification of sensitive developmental periods or critical windows of exposure have been explored in a few studies for a limited number of species. In rats, maternal transfer of BPA to offspring, in utero and via lactation, was observed (Snyder et al., 2000; Takahashi and Oishi, 2000). In birds, deposition of maternal BPA into bird eggs has been demonstrated (Halldin et al., 2001). A recent study assessed BPA exposure to the human fetus and detected BPA in the blood of pregnant women, in the newborn's umbilical cord, and in the placenta (Schonfelder et al., 2002). Potential sources of BPA exposure to human infants and children include milk stored in BPA-based plastic baby bottles, infant formula stored in epoxy-resin lined cans, foods contacting BPA-based plastic products, and dental sealant applications (Yoshida et al., 2001; Fung et al., 2000; Arenholt-Blindslev et al., 1999; Wingender and Niketas, 1998; Howe et al., 1998; Mountfort et al., 1997; Biles et al., 1997a, b; Olea et al., 1996; Brotons et al., 1995).

3. CROSS-SPECIES DEVELOPMENTAL AND REPRODUCTIVE EFFECTS

Several investigators have assessed BPA for teratogenicity and developmental/ reproductive effects, including endocrine-disrupting effects, in a variety of organisms, including mice, rats, copepods, carp, trout, salmon, daphnids, and amphibians. BPA in vivo effects data were identified in only 16 of the total 9 or 10 million animal species. The 16 species fall into three phyla in the animal kingdom (Figure 4). Among the three phyla, only seven classes are represented: gastropods, crustaceans, insects, amphibians, fish, birds, and mammals. Cellular and molecular studies (e.g., using MCF-7 cell lines) have also been conducted. For in vitro estrogen receptor binding data, only five classes (mammals, birds, fish, amphibians, and reptiles) are represented (Table 2). Therefore, the data are limited for making evolutionary comparisons for BPA MOAs. Additionally, among the 16 species with in vivo data, the majority of the studies assessed the mouse and rat, two species within one order (rodentia) within the class mammalia. Thus, even within the small number of species represented, the amount of information is patchy; invertebrate studies are few and rodent studies are many. Therefore, there is incomplete coverage of taxa with BPA effects data (Figure 4).

The following series of tables in this section present the developmental and reproductive effects data, including some mechanistic findings in animal species. The effects studies presented in the tables are those that observed positive outcomes. In addition, a limited number of negative outcome studies were included only if the study had some similarities (e.g., similar study design, strain) to a positive outcome study. Studies are described below, divided into animal classes (class names are in bold italics). In vitro study findings are not included in the tables but are presented in the text.

Developmental and reproductive effects of interest included morphological, cellular, and gene expression changes. The responses affected provided information for determining the potential MOAs but are in many cases influenced by multiple possible hormonal pathways or the MOA is uncertain, especially with regard to male reproductive organ effects (Atanassova et al., 2000; Fisher et al., 1999; Gray et al., 1997). For example, p,p'-dichlorodiphenyl-dichloroethylene (p,p'-DDE) in vivo effects data in rodents are consistent with estrogen agonist or androgen antagonist MOA. Mechanistic studies have indicated that p,p'-DDE acts as an androgen antagonist. The tables include information about possible hormone activities that could be affected by BPA that are consistent with the effects. For estrogen, androgen, and thyroid activity effects, effects on bioavailability of the hormone and/or receptor are additional possible MOAs. For example, increased anogenital distance in rodents is consistent with MOAs that lead to a decrease in androgen activity, including androgen antagonism (at the receptor level) and effects on androgen synthesis. An effect consistent with estrogen agonism could also

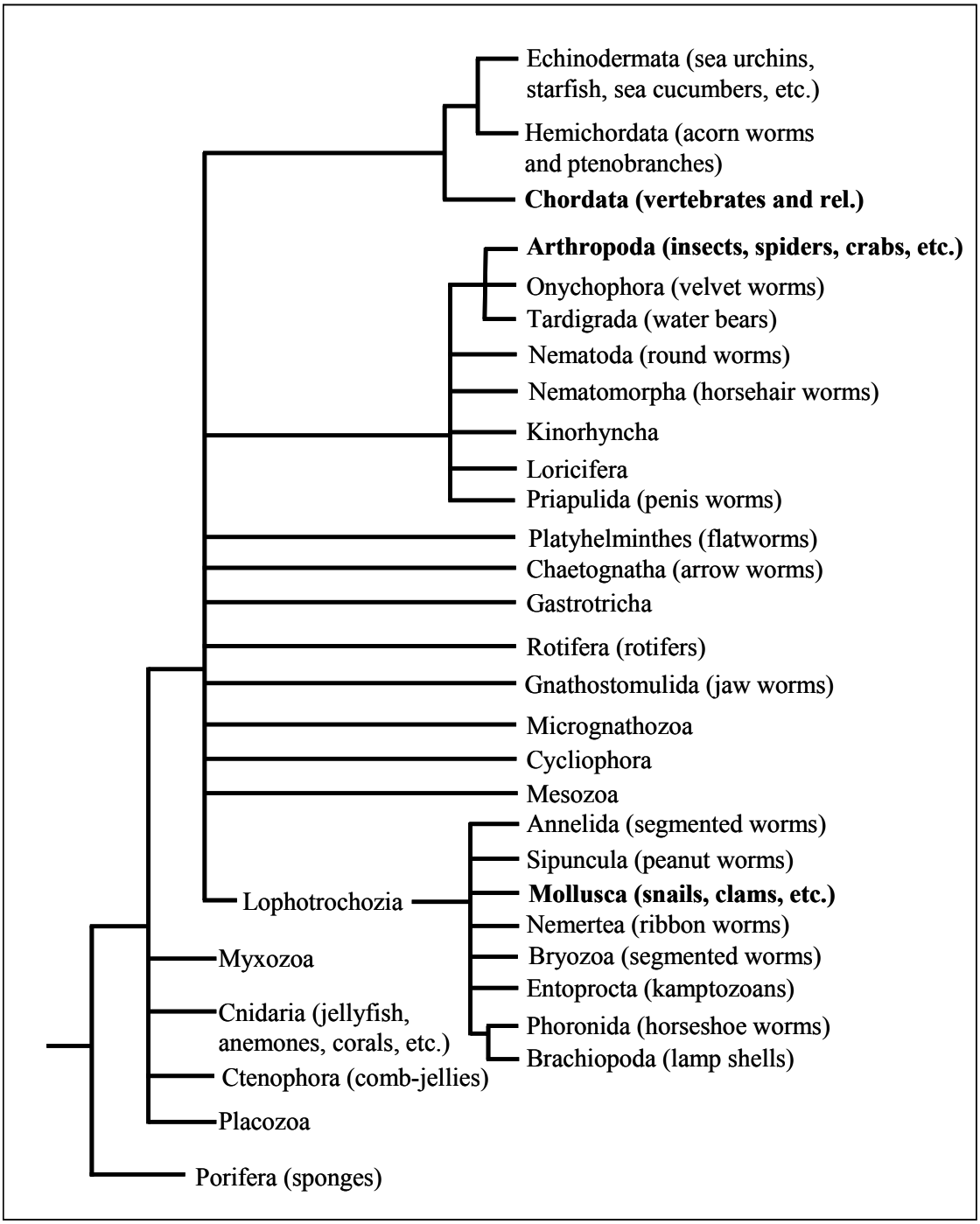

Figure 4. Distribution of BPA effects data across phyla within the animal kingdom. Evolutionary relatedness is shown with the evolutionary diagram (adapted from The Tree of Life Project; www.tolweb.org; http://tolweb.org/tree?group=Bilateria&contgroup=Animals). This project uses molecular studies as the basis for the evolutionary relationships. [Bold type denotes phylum for which BPA effects data have been identified in the literature.]

Table 2. BPA relative estrogen receptor binding affinity across species from competitive binding assays

Species and/or class	Relative ER binding affinity[a]
Human Mammalia	2,000–10,000 (ERα binding) 250–10,000 (ERβ binding)
Rodent Mammalia	12,500 (mouse ERα binding) 300 (rat ERβ binding) <10,000–12,500 (rodent uterine cytosol ER binding)
Gallus gallus Aves	2,273 (ER binding)
Anolis carolinensis Reptilia	770 (ER binding)
Xenopus laevis Amphibia	714 (liver cytosol ER binding)
Piscine	476 (*Oncorhynchus mykiss* ER binding) 833 (*Cyprinus carpio* liver cytosol ER binding)

[a] Fold less than ethinylestradiol binding affinity to the ER.
ER = estrogen receptor.

be explained by an MOA that leads to an increase in the concentration of available ER or estrogen, such as steroidogenesis.

Determining the hormone activities that could be affected was a first step in determining potential MOAs of BPA for each species. Then, the in vitro mechanistic studies were considered along with the in vivo effects data to determine the possible MOAs by a weight-of-evidence approach.

The majority of BPA studies of wildlife species have been conducted through laboratory analyses, either in vitro or in vivo. A number of the laboratory wildlife studies assessed acute and chronic toxicity endpoints such as mortality with no assessment of development and reproduction endpoints and, thus, they are not described in this report (some are reviewed in Staples et al., 1998). A limited number of field studies have examined wildlife in the context of BPA as part of an environmental mixture (Fort et al., 1999). In summary, the studies of nonmammalian wildlife and laboratory species provide evidence that BPA exposure can lead to developmental and reproductive effects in animal species beyond mammals.

3.1. INVERTEBRATE SPECIES

Steroid hormones and receptors have been identified and isolated in some invertebrate species, notably insects and crustaceans. For reviews of invertebrate endocrinology, see Pinder and Pottinger (1998) and the chapter by LeBlanc et al. (1999) in deFur et al. (1999).

3.1.1. Phylum Mollusca

Studies within the mollusks are limited to two species of snails (Table 3).

3.1.1.1. *Class Gastropoda*

BPA treatment of gastropods was reported in one study (Oehlmann et al., 2000) assessing two different species of snails.

3.1.1.1.1. ***Effects after life cycle exposure.*** Effects in the freshwater prosobranch snail, *Marisa cornuarietis*, after 1 or 100 µg/L BPA long-term exposure to egg masses were studied in a life cycle test (Table 3; Oehlmann et al., 2000). Females with genital tract abnormalities, including enlarged albumen and capsule glands and duplicated sex organs, termed "superfemales," were observed in both BPA dose groups. These abnormalities had not been previously observed in controls in this experimental protocol, but the number of snails with genital tract abnormalities after BPA treatment was not statistically significant. Other effects included females sterilized as a result of ruptured oviducts at 1 and 100 µg/L BPA. Effects were observed only in the 100-µg/L BPA dose group; increased spawning egg mass and egg production, and females with male sex characteristics or increased vas deferens sequence index, termed "imposex," were found in statistically significant numbers. An up-regulation in androgen receptors from estrogen agonist exposure was suggested as a possible mechanism behind these observed phenotypes, although the role of vertebrate sex-steroid hormones in gastropod normal sex determination is unknown. Increased mortality occurred in both dose groups after 6 months of exposure and could be explained by oviduct rupture.

Table 3. Phylum Mollusca: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration	Positive control/ effect	Developmental or reproductive effect(s)	BPA doses with observed effect	Statistical significance	Possible hormone activity affected	Reference
Marisa cornuarietis (Giant ramshorn snail) Gastropoda	Parental and life cycle/ 12 m (365 d)	NA	Increased mortality	1, 100 µg/L at 6 m to 12 m of age	<0.05	U	Oehlmann et al. (2000)
Marisa cornuarietis (Giant ramshorn snail) Gastropoda	Adult/ 5 m	NA	Increased mortality (consistent w/oviduct rupture from increased egg production)	1, 5, 25, 100 µg/L	<0.05	U	Oehlmann et al. (2000)
Marisa cornuarietis (Giant ramshorn snail) Gastropoda	Parental and life cycle/ 12 m (365 d)	NA	F: Genital tract malformations	1, 100 µg/L	Not statistically significant (>0.05)	U	Oehlmann et al. (2000)
Marisa cornuarietis (Giant ramshorn snail) Gastropoda	Adult/ 5 m	NA	F: Genital tract malformations, oviduct rupture	1, 5, 25, 100 µg/L	Not statistically significant at <0.05	U	Oehlmann et al. (2000)
Marisa cornuarietis (Giant ramshorn snail) Gastropoda	Adult/ 5 m	NA	F: Enlarged albumen and capsule glands	1, 5, 25, 100 µg/L	NR	U	Oehlmann et al. (2000)
Marisa cornuarietis (Giant ramshorn snail) Gastropoda	Parental and life cycle/ 12 m (365 d)	NA	F: Increased spawning egg mass and production	100 µg/L	<0.05	U	Oehlmann et al. (2000)
Marisa cornuarietis (Giant ramshorn snail) Gastropoda	Adult/ 5 m	NA	F: Increased spawning egg mass and production	1, 5, 25, 100 µg/L	<0.01	U	Oehlmann et al. (2000)
Marisa cornuarietis (Giant ramshorn snail) Gastropoda	Parental and life cycle/ 12 m (365 d)	NA	M: Increased vas deferens sequence index	100 µg/L	<0.001	U	Oehlmann et al. (2000)
Nucella lapillus (dog whelk) Gastropoda	Adult/ 3 m	NA	F: Increased capsule gland length and pallial gland weight	1, 25, 100 µg/L	<0.001	U	Oehlmann et al. (2000)
Nucella lapillus (dog whelk) Gastropoda	Adult/ 3 m	NA	F: Advanced timing of oogenesis	1, 25, 100 µg/L	<0.01	U	Oehlmann et al. (2000)
Nucella lapillus (dog whelk) Gastropoda	Adult/ 3 m	NA	M: Decreased penis and prostate gland length	1, 25, 100 µg/L	<0.05	U	Oehlmann et al. (2000)
Nucella lapillus (dog whelk) Gastropoda	Adult/ 3 m	NA	M: Decreased number with sperm stored	1, 25, 100 µg/L	<0.05	U	Oehlmann et al. (2000)

m = months; d = days; NA = not applicable (no positive control used); F = female; M = male; U = uncertain; NR = not reported.

3.1.1.1.2. ***Effects after adult exposure.*** Similar to the results of the life cycle stage exposure studies with *M. cornuarietis*, adult BPA-exposed prosobranch snails, *M. cornuarietis* and *Nucella lapillus*, developed genital tract and reproductive abnormalities (Oehlmann et al., 2000). BPA concentrations as low as 1 µg/L induced "superfemale" characteristics or genital tract abnormalities in both species. Female Nucella exhibited increased capsule gland length and pallial gland weight and advanced oocyte maturation after 3 months at 1 to 100 µg/L BPA. Species differences in their reproductive tract structure were suggested as the basis for the lack of sterilization and mortality effects observed in *N. lapillus* but not in *M. cornuarietis*. Male Nucella had decreased penis length, prostate length, and number of males with sperm stored at all tested concentrations (1, 25, and 100 µg/L). After exposure to 1 or 100 µg/L BPA, *M. cornuarietis* exhibited the most severe reproductive tract and organ malformations, including enlarged pallial, albumen, and capsule glands in all females and ruptured pallial oviducts in 3.73% of females. Females with a second vagina and opening to the mantle cavity were also described, but their numbers were not statistically significant. Increased spawning egg mass and egg production were noted after exposure to these same BPA concentrations. Female oviduct rupture is one possible explanation for the increased mortality and the sex-ratio trend toward males. BPA-exposed males did not exhibit gametogenesis effects.

3.1.2. Phylum Arthropoda

Table 4 shows the studies identified for arthropod species.

Table 4. Phylum Arthropoda: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration	Positive control/ effect	Developmental or reproductive effect(s)	BPA doses with observed effect	Statistical significance	Possible hormone activity affected	Reference
Acartia tonsa (copepod) Crustacea	ELS (egg)–sexual maturity/ 14 d	23 µg/L E2/increased egg production on d 10	Increased egg production on d 10	20 µg/L	≤0.05	U (E)	Andersen et al. (1999b)
Chironomus riparius (freshwater midge) Insecta	Juvenile and ELS–juvenile/ until adult emergence	1-100 ng/L EE/ inconsistent adult emergence # and timing (some earlier, some later); sex-ratio toward M	Delayed adult emergence or blocked ermergence	1st gen: F: 0.01, 0.55, 77 µg/L 2nd gen: F/M: 0.078 µg/L – 0.75 mg/L; no emergence at 10.4 mg/L	≤0.05	U (AEc)	Watts et al. (2001)

ELS = early life stage; d = days; E2 = 17β-estradiol; EE = 17α-ethinylestradiol; F = female; M = male; NR = not reported; U = uncertain; AEc = antagonizing ecdysteroid activity.

3.1.2.1. *Class Crustacea*

3.1.2.1.1. ***Effects after juvenile exposure.*** Juvenile freshwater daphnids, *Daphnia magna*, were exposed to 316 and 3,160 μg/L BPA for 21 days and examined for changes in pre-adult and adult molting (Caspers, 1998). No changes were observed in pre-adult or total molting frequency.

Exposure to 20 μg/L BPA or 23 μg/L 17 β-estradiol resulted in changes in reproductive parameters in the copepod, *Acartia tonsa* (Andersen et al., 1999b). In both BPA and 17 β-estradiol-treated animals, egg production was significantly increased at 10 days of exposure, suggesting that ovarian maturation was precocious (Andersen et al., 1999b). Egg production was maximal and consistently observed at concentrations of ~20 μg/L for both BPA and estradiol. These effects occurred at concentrations lower than the 72-hour immobility EC_{10} of 0.29 mg/L determined for this species. The similar effects of BPA and estradiol suggest that BPA is acting to increase an estrogen or estrogen-like signal activity. The similar potencies of BPA and estradiol observed in this study were unexpected considering relative potencies determined in vitro and some in vivo studies (in other organisms), which found BPA to be of a much lower potency than estradiol. Since egg production in the copepod was affected by 17 β-estradiol treatment, as has been shown in a number of other crustaceans, it is possible that estrogen receptor-like molecules are present in some crustacean species. However, the mechanism by which 17 β-estradiol and BPA treatment affects oogenesis in copepods, and invertebrates in general, is not clear.

3.1.2.2. *Class Insecta*

3.1.2.2.1. ***Effects after developmental exposure.*** To investigate the effects of BPA exposure on an insect species and examine the estrogenic MOA, a species of nonbiting midge (*Chironomus riparius*) was exposed to BPA or 17α-ethinylestradiol for 2 generations (Watts et al., 2001). Sediments were spiked with 0.01 μg/L to 10.4 mg/L BPA, as larval midges are benthic freshwater insects exposed through contact with sediment contaminants. Larvae were assessed for timing of adult metamorphosis and emergence out of the water, survival, and sex ratio. Delayed emergence of first generation females was observed at 0.01, 0.55, and 77 μg/L BPA. In the second generation, the timing of both male and female adult emergence was delayed n the 0.078-, 0.55-, 77-, and 750-μg/L BPA dose groups and emergence was completely blocked in the 10.4-mg/L exposure group. The greater sensitivity of the second generation to BPA exposure is presumably due to early life stage exposure (beginning in the egg) and/or a longer exposure to BPA. Although no eggs were produced from the 10.4-mg/L exposure group, no dose-response egg production changes were observed. After ethinylestradiol exposure, inconsistent changes in number of adults that emerged, emergence timing, and altered sex ratio toward males were

observed. Since the BPA effects differed from those observed with ethinylestradiol, a nonestrogenic MOA in this midge was suggested (Watts et al., 2001).

Ecdysteroids are the principal steroid hormones in arthropod invertebrates (reviewed in LeBlanc et al., 1999). Ecdysone and 20-hydroxyecdysone (20E), produced by conversion of ecdysone, initiate insect molting; ecdysone affects cell proliferation (Champlin and Truman, 1998), and 20E affects cell differentiation events (see LeBlanc et al., 1999). Ecdysone receptors have been identified in species of the class Insecta and the class Crustacea (reviewed in McLachlan, 2001). Molting in crustaceans has been inhibited by exposure to vertebrate estrogens (Zou and Fingerman, 1997a, b; Baldwin et al., 1995). In insects, BPA demonstrated ecdysone receptor antagonism activity in a *Drosophila melanogaster* B_{II} cell line (Dinan et al., 2001). The delayed emergence observed after BPA exposure to the insect *C. riparius* is also consistent with an ecdysone antagonist MOA (Watts et al., 2001).

3.2. VERTEBRATE SPECIES

For reviews of the endocrinology of nonmammalian vertebrates, see Sparling et al. (2000), DiGiulio et al. (1999), Rolland et al. (1997), and Kendall et al. (1998).

3.2.1. Phylum Chordata

3.2.1.1. *Class Amphibia*

For classes Amphibia and Aves, a limited number of in vivo studies were identified (Table 5).

Table 5. Phylum Chordata, classes Amphibia and Aves: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration	Positive control/ effect	Developmental or reproductive effect(s)	BPA doses with observed effect	Statistical significance	Possible hormone activity affected	Reference
Xenopus laevis (African clawed frog) Amphibia	Posthatch (2–3 d) to metamorphosis/ ~3 m	0.01 and 0.1 µM E2/sex-ratio toward F	Sex-ratio toward F based on gonadal examination	0.1 µM (22.8 µg/L)	<0.05	E	Kloas et al. (1999)
Coturnix japonica (Japanese quail) Aves	Embryo/d 3 of incubation	0.002, 0.02 µg/g egg DES; $p<0.05$ 0.002 µg/g DES; $p<0.001$ 0.02 µg/g DES	F w/abnormal Mullerian ducts	67, 200 µg/g egg	<0.05 for only 200 µg/g BPA	E	Berg et al. (2001)
Gallus domesticus (domestic chicken) Aves	Embryo/d 4 of incubation	0.002, 0.02, 0.2 µg/g egg DES; $p<0.001$ for only 0.02 µg/g DES	M w/ovatestis	67, 200 µg/g egg	<0.001 for only 200 µg/g BPA	E	Berg et al. (2001)

d = days; m = months; E2 = 17β-estradiol; DES = diethylstilbestrol; F = female; M = male; E = increasing estrogen activity.

3.2.1.1.1. ***Effects after early life stage (egg/embryo) exposure.*** A frog embryo teratogenesis assay in Xenopus (FETAX) was used to study the effects of BPA on *Xenopus laevis* development (Fort et al., 1999). Embryos exposed to BPA for 4 days in FETAX solution had maldeveloped guts and craniofacial defects at an EC_{50} value of 35.1 mg/L BPA. After a 30-day exposure, no limb teratogenesis effects were observed. Water and sediment samples from a Minnesota pond site, where deformed frogs had been found, had BPA concentrations of 16.3 and 23.5 μg/L, respectively, but also detected pesticides, including diphenylamine, atrazine, maneb, and permethrin. Embryos exposed to these media also developed craniofacial and eye defects similar to those observed in the field.

3.2.1.1.2. ***Effects after immediate posthatch (neonatal) exposure.*** One study investigated the outcome of posthatch exposure to low doses of BPA (Kloas et al., 1999). Exposure of *X. laevis* tadpoles from 2 to 3 days posthatch to metamorphosis to 100 nM (22.8 μg/L) BPA, or 10 or 100 nM estradiol resulted in significant increases in female phenotypic frogs relative to controls based on gonadal examination (Kloas et al., 1999). Approximately 65–70% of the 100 nM BPA-exposed or the 10 nM estradiol-exposed frogs developed as female. The 100 nM estradiol group was almost entirely female and had increased mortality rates. No changes in growth or survival rates were noted in any other treatment groups. In this assay, BPA was only 10 times less potent than estradiol.

3.2.1.2. ***Class Aves***

3.2.1.2.1. ***Effects after early life stage (egg/embryo) exposure.*** Different results from studies assessing effects in Japanese quail (*C. japonica*) were observed after in ovo BPA exposure at 67 or 200 μg/g of egg (Berg et al., 2001; Halldin et al., 2001). In a study assessing the embryo stage for morphological effects, Berg et al. (2001) observed statistically significant oviduct effects in females after in ovo BPA exposure at 67 and 200 μg/g egg (Table 5). However, in a study assessing adults after a similar in ovo BPA exposure regime, no significant effects on adult male reproductive behavior, testosterone levels, or testis weight and no changes in female egg laying were observed, while a trend increase in the fraction of females with right oviduct retention, an effect seen after exposure to estrogens, was observed (Halldin et al., 2001). These results may indicate that the reproductive behavior of the quails was not affected by the reproductive tract structural abnormalities that resulted in the embryos and some of the adults after in ovo BPA exposure. In the chicken (*Gallus domesticus)*, statistically significant numbers of males with ovatestis were observed after 200 μg/g egg in ovo BPA exposure (Berg et al., 2001). Since DES in ovo treatment in chicken leads to male ovatestis and diethylstilbestrol (DES) in ovo treatment

in quail leads to females with abnormal Mullerian ducts, the MOA for BPA is consistent with an estrogen agonist.

3.2.1.3. *Class Pisces*

A relatively large number of developmental/reproductive studies with BPA exposure in fish were identified (Table 6).

3.2.1.3.1. ***Effects after early life stage (egg/embryo) exposure.*** Fertilized Japanese medaka (*Oryzias latipes)* eggs were exposed to BPA ranging from 2.28 to 1,820 μg/L until 60 days posthatch (Yokota et al., 2000). Morphological development, total length, and body weight were significantly suppressed only at 1,820 μg/L and an inverse relationship between an increasing BPA concentration and the effects were observed, suggesting general growth suppression. However, hatching rates, time to hatch, and cumulative mortality were not affected at any tested BPA concentration. A trend toward a decreasing number of males at 355 μg/L and at 1,822 μg/L BPA was observed. Examination of their secondary sex characteristics found no male phenotypes and 32% of the female fish had ovatestis, tissue composed of both testicular germ cells and oocytes, indicating a masculinization of females. Decreased spermatozoa numbers were found, but statistical significance was not tested. No behavioral abnormalities were noted.

P450 aromatase (P450arom) isoform B is primarily localized in the brain of zebrafish and is induced by estradiol at concentrations as low as 0.01 μM. Zebrafish (*Danio rerio*) embryos were exposed to 0.01 to 10 μM (2.28–2,280 μg/L) BPA from 2 to 48 hours postfertilization and examined for changes in P450arom expression (Kishida et al., 2001). 10 μM BPA or 0.01 μM DES exposure increased P450aromB expression. P450aromA, an isoform primarily found in the zebrafish ovary, exhibited no changes in expression after treatment with BPA, estradiol (0.01–10 μM), or DES (0.01–2 μM). Changes in P450arom expression could affect the androgen-to-estrogen ratio by influencing the conversion of testosterone to estrogen. The effects found in zebrafish exposed to BPA are consistent with an estrogen agonist MOA.

Table 6. Phylum Chordata, class Pisces: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,b]	Statistical significance[b]	Possible hormone activity affected[d]	Reference
Oryzias latipes (Japanese medaka) Piscine/ Osteichthyes (Subclass: Actinopterygii)	ELS (egg)/ to 60 d posthatch (~71 d)	NA	M and F: DR decrease in mean total length and BW	2.28–1,820 µg/L stat. sig. at 1,820 µg/L	≤0.017	U (NE, E)	Yokota et al. (2000)
Oryzias latipes (Japanese medaka) Piscine (Subclass: Actinopterygii)	ELS (egg)/ to 60 d posthatch (~71 d)	NA	Ovatestis	1,820 µg/L	<0.001	E	Yokota et al. (2000)
Oryzias latipes (Japanese medaka) Piscine (Subclass: Actinopterygii)	Posthatch/ to a length of 1.5 cm (84–109 d)	0.01 µg/L E2/ovatestis (EE, Estriol, Estrone)	Phenotypic M Ovatestis	~10 µg/L	NR	E	Metcalfe et al. (2001)
Oryzias latipes (Japanese medaka) Piscine (Subclass: Actinopterygii)	ELS (egg)/ to 60 d posthatch (~71 d)	NA	Sex-ratio toward F based on secondary sex characteristics/ gonadal histology	1,820 µg/L	<0.001	E	Yokota et al. (2000)
Oryzias latipes (Japanese medaka) Piscine (Subclass: Actinopterygii)	Posthatch/ to a length of 1.5 cm (84–109 d)	E2 (dose NR)/ testicular fibrosis	M: Testicular fibrosis	50, 100, 200 µg/L	NR	U (E)	Metcalfe et al. (2001)
Oryzias latipes (Japanese medaka) Piscine (Subclass: Actinopterygii)	Posthatch/ to a length of 1.5 cm (84–109 d)	0.001–1 µg/L E2, 0.0001–1 µg/L EE, 0.01–10 µg/L Estriol, 0.01–10 µg/L Estrone	F: Advanced oogenesis	200 µg/L	NR	E	Metcalfe et al. (2001)
Oryzias latipes (Japanese medaka) Piscine (Subclass: Actinopterygii)	Posthatch/ to a length of 1.5 cm (84–109 d)	E2, EE, Estriol, Estrone: spermatozoa changes NR	M: Decreased number of spermatozoa	50, 100, 200 µg/L	NR	U (E)	Metcalfe et al. (2001)
Oryzias latipes (Japanese medaka) Piscine (Subclass: Actinopterygii)	Adult/14 d	3 nM E2/ sig. decrease in same effects	Exposed M and control F: Decreased number of spawned and hatched eggs	10 µM (2,280 µg/L)	0.01	E	Shioda and Wakabayashi (2000a); Shioda and Wakabayashi (2000b)
Danio rerio (zebrafish) Piscine (Subclass: Actinopterygii)	ELS (egg)/ 2 d	0.01 µM E2 and DES/ increased P450 aromB	Increased p450 aromB expression threefold above neg. controls	10 µM (2,280 µg/L)	NR	E	Kishida et al. (2001)

Table 6. Phylum Chordata, class Pisces: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints (continued)

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,b]	Statistical significance[b]	Possible hormone activity affected[d]	Reference
Oncorhynchus mykiss (rainbow trout) Piscine (Subclass: Actinopterygii)	Juvenile	NA	Increased VTG F: d 5 M: d 7	35,000 µg/kg-d	<0.05	E	Lindholst et al. (2000)
Oncorhynchus mykiss (rainbow trout) Piscine (Subclass: Actinopterygii)	Juvenile/ 12 d	1 µg/L E2/increased VTG	DR induction of plasma VTG	70, 100, 500 µg/L (stat. sig. at 500 µg/L)	<0.05	E	Lindholst et al. (2000)
Salmo salar (Atlantic salmon) Piscine (Subclass: Actinopterygii)	Juvenile	5 mg/kg-d E2/increased VTG and zr proteins	DR induction of VTG and zr proteins measured 2 w later	5, 25, 125 mg/kg-d	<0.01 at 25 mg/kg	E	Arukwe et al. (2000)
Xiphophorus helleri (green swordtail) Piscine (Subclass: Actinopterygii)	Juvenile/ 60 d	NA	Decreased sword length (2^o sex characteristic)	2, 20 µg/L	<0.05	U (E, AA)	Kwak et al. (2001)
Xiphophorus helleri (green swordtail) Piscine (Subclass: Actinopterygii)	Adult/ 3 d	NA	Testis cellular necrosis; apoptosis at 10,000 µg/L	400–10,000 µg/L	NR	U	Kwak et al. (2001)
Pimephales promelas (fathead minnow) Piscine (Subclass: Actinopterygii)	Adult/ 43–164 d	NA	M: DR increased plasma VTG (at 164 d levels > 3,300× above controls)	640, 1,280 µg/L at 43 d and 160 µg/L at 71 d	≤0.027	E	Sohoni et al. (2001)
Pimephales promelas (fathead minnow) Piscine (Subclass: Actinopterygii)	Adult/ 43–164 d	NA	F: DR increased plasma VTG	640 µg/L at 164 d	<0.05	E	Sohoni et al. (2001)
Pimephales promelas (fathead minnow) Piscine (Subclass: Actinopterygii)	Adult/ 43–164 d	NA	M+ F: inhibited gonadal growth (GSI)	640, 1,280 µg/L at 164 d	<0.05	E	Sohoni et al. (2001)
Pimephales promelas (fathead minnow) Piscine (Subclass: Actinopterygii)	Adult/ 43–164 d	NA	M: altered sex cell type proportion in testes	16 µg/L	<0.05	NE	Sohoni et al. (2001)
Pimephales promelas (fathead minnow) Piscine (Subclass: Actinopterygii)	Adult/ 43–164 d	NA	M: Negative DR relationship w/somatic growth (length and weight)	640, 1,280 µg/L at 43, 51, 164 d of exposure	≤0.017	U	Sohoni et al. (2001)

Table 6. Phylum Chordata, class Pisces: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints (continued)

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,b]	Statistical significance[b]	Possible hormone activity affected[d]	Reference
Pimephales promelas (fathead minnow) Piscine (Subclass: Actinopterygii)	Adult/ 43–164 d	NA	F: decreased egg production	1,280 µg/L	<0.05	U	Sohoni et al. (2001)
Pimephales promelas (fathead minnow) Piscine (Subclass: Actinopterygii)	Adult/ 43–164 d	NA	Increased proportion spermatogonia (immature germ cells)/decreased proportion spermatozoa (mature germ cells)	640, 1,280 µg/L at 164 d	0.001	U (E)	Sohoni et al. (2001)
Poecilia reticulata (guppy) Piscine (subclass: Actinopterygii)	Adult/ 21 d	NA	DR decrease in sperm count (40–75%)	274, 549 µg/L	0.003	U (E)	Haubruge et al. (2000)

[a] ELS = early life stage; cm = centimeter; d = days.
[b] NA = not applicable (no positive control used); E2 = 17β-estradiol; EE = 17α-ethinylestradiol; stat. sig. = statistically significant; VTG = vitellogenin; aromB = aromatase B; zr = zona radiata; DES = diethylstilbestrol; NR = not reported.
[c] M = male; F = female; BW = body weight; GSI = gonadosomatic index; DR = dose-response; w = weeks.
[d] Observed effects consistent with an affect on one or more of the following hormone activities: AA = antagonizing androgen activity; E = increasing estrogen activity; NE = nonendocrine/general toxic MOAs; U = uncertain.

3.2.1.3.2. ***Effects after immediate posthatch (neonatal) exposure.*** Responses in Japanese medaka exposed to BPA (10–200 µg/L) or various estrogens (0.001–1 µg/L) for 100 days from 1-day posthatch included growth changes, feminization, and morphological abnormalities (Metcalfe et al., 2001). Increased total body weight and length occurred in fish exposed to 0.1 and 1 µg/L of estradiol, respectively. Fish exposed to 100 or 200 µg/L BPA had an increased weight-to-body length ratio. Ovatestis histology was observed after exposure to 0.01 µg/L of estradiol, whereas it was observed in fish exposed to a mean BPA concentration of ~10 µg/L. However, testicular fibrosis observed at the higher BPA concentrations may have precluded ovatestis discrimination (Metcalfe et al., 2001). Significantly more females, based on gonad histology, developed after exposure to 0.1 µg/L estradiol and no males were found at 1 µg/L estradiol. However, no sex-ratio shift was observed at any of the tested BPA doses. Ovaries from females in the 200-µg/L BPA exposure group had eosinophilic fluid and advanced oogenesis consistent with changes observed in estradiol-exposed females. Males exposed to BPA at 50 µg/L and greater had testicular morphological abnormalities, fibrosis, and decreased number of sperm cells. BPA effects were consistent with an estrogen agonist MOA. Based on

the concentrations of BPA and estradiol affecting normal gonadal differentiation, BPA was approximately 3,000 times less potent than estradiol.

3.2.1.3.3. ***Effects after juvenile exposure.***

3.2.1.3.3.1. ***Vitellogenin and zona radiata protein expression*****.** Vitellogenin is a precursor egg yolk protein found normally in oviparous vertebrates (Palmer et al., 1998). Vitellogenin expression is responsive to estradiol and normally only present in adult, egg-laying females. The presence of vitellogenin in some juvenile and male oviparous species has become an accepted biomarker of exogenous estrogen exposure (NRC, 1999). In vivo induction of the estrogen-responsive gene vitellogenin was examined in rainbow trout (*O. mykiss*) (Lindholst et al., 2001, 2000; Christiansen et al., 2000, 1998) and Atlantic salmon (*S. salar*) (Arukwe et al., 2000). In addition, concentrations of zona radiata protein, another protein whose expression is estrogen-regulated, were examined in Atlantic salmon (Arukwe et al., 2000). However, the sex of juvenile trout and salmon was indistinguishable in most studies. This is a problem especially because the mixed population can influence vitellogenin expression and females can be less sensitive to increases in vitellogenin expression as demonstrated in BPA exposure studies (Sohoni et al., 2001).

Juvenile rainbow trout given three 50-mg/kg intraperitoneal (i.p.) doses of BPA exhibited increased plasma vitellogenin concentrations 6 days after the final dose (Christiansen et al., 2000). Juvenile rainbow trout of both sexes administered a single 50-mg/kg i.p. injection of BPA had significantly increased plasma vitellogenin concentrations 9 days later, although wide interindividual differences were noted. These fish also had an increased hepatosomatic index (liver weight relative to body weight). BPA-induced vitellogenin concentrations were 27 times lower than those seen in fish exposed to 1 mg/kg estradiol.

Vitellogenin induction was examined in males versus females in one juvenile exposure assay (Lindholst et al., 2001). Vitellogenin induction was observed in rainbow trout given a single i.p. injection of 35 mg/kg BPA with differences found based on the sex of the fish (Lindholst et al., 2001). Significant increases in plasma vitellogenin concentrations first occurred at 120 hours of treatment for females and at 168 hours for males. Maximum levels of vitellogenin were higher in females than males after BPA treatment. Liver measurements of BPA demonstrated a lag-time from time of BPA exposure to time of vitellogenin response detection because a very low concentration of BPA was measured at the time of maximum vitellogenin induction.

Vitellogenin induction also occurred in juvenile rainbow trout exposed to BPA through ambient water (Lindholst et al., 2000). Vitellogenin concentration increased in trout exposed at greater than 70 µg/L BPA and the increase was statistically significant at 500 µg/L BPA. The vitellogenin induction at 500 µg/L BPA was similar to that observed in 1 µg/L estradiol-exposed

fish. As only one dose of estradiol was examined, a relative potency difference could not be calculated. Liver and muscle samples were examined for BPA. Higher BPA liver concentration was associated with vitellogenin levels in a dose-dependent manner.

Juvenile Atlantic salmon had increased vitellogenin and zona radiata protein concentrations 2 weeks following BPA exposure at 25 mg/kg i.p. (Arukwe et al., 2000). Both vitellogenin and zona radiata protein, an eggshell protein that is a sensitive marker for estrogen exposure, increased as a function of increasing BPA exposure concentrations. Cytochrome P450 1A (CYP1A) monooxygenase activity was slightly decreased in hepatic samples from groups of fish exposed to 1 mg/kg BPA.

3.2.1.3.3.2. ***Secondary sex characteristics*.** Male sword length/growth was examined in juvenile swordtail fish (*Xiphophorus helleri*) after exposure to 0.2, 2, or 20 ppb (μg/L) BPA for 60 days (Kwak et al., 2001). In the swordtail, the sword is a male-specific secondary sexual characteristic and a longer swordtail length is preferred by females. Sword length was decreased by BPA exposure in a dose-dependent relationship that was statistically significant ($p<0.05$) at 2 and 20 μg/L compared with controls. The decrease in sword length was in the absence of significant effects on body length relative to controls. Adult males exposed to 400 μg/L BPA did not exhibit a significant increase in liver vitellogenin expression, demonstrating that sex-specific effects can occur after exposure to BPA at concentrations below those that induce vitellogenin in this species. This suggested that male sword length is a sensitive endpoint for BPA exposure.

3.2.1.3.4. ***Effects after adult exposure*.** Reproductive effects were examined in adult male Japanese medaka in a dose-response study with 0.3–10 μM (68.4–2,280 μg/L) BPA or 1–100 nM estradiol (Shioda and Wakabayashi, 2000a, b). Exposed males were paired with untreated females to examine egg spawning and hatching success. Both of these parameters were impaired at 10 μM (2,280 μg/L) BPA or 3 nM (1.68 μg/L) estradiol.

Growth parameters were assessed in sexually mature fathead minnows (*Pimephales promelas*) exposed for up to 164 days to 1, 16, 160, 640, or 1,280 μg/L BPA (Sohoni et al., 2001). Fish were also placed in breeding pairs after 42 days of exposure, and a subset of fertilized eggs were continually exposed. Growth inhibition was observed in females only at 71 days of exposure at 640 and 1,280 μg/L BPA and in males at 71 and 164 days of exposure at the same doses. Plasma vitellogenin was increased in females on day 164 at 640 μg/L BPA and in males at days 43, 71, and 164 at 160, 640, and 1,280 μg/L BPA. After 164 days of exposure, decreased gonadal growth was observed in both females and males at 640 and 1,280 μg/L. Females exhibited no changes in oocyte development. Males examined after 164 days of exposure had a decreased proportion of mature sperm and increased immature sperm cells at 640 and 1,280 μg/L BPA. Increased egg production at 1 to 640 μg/L BPA was not significant and

may be the result of low egg production in controls. The highest BPA dose group exhibited a significant decrease in egg production. Hatching success was decreased in the 640-µg/L BPA group, and no hatching was observed in the 1,280-µg/L dose group. At 16 µg/L BPA, a change in the proportion of sex cell types in the testes consistent with inhibition of spermatogenesis. These BPA effects are consistent with an estrogenic MOA.

Male reproductive parameters were assessed in adult male guppies (*Poecilia reticulata*) exposed to 274 or 549 µg/L BPA for 21 days. Effects on testicular function at 274 µg/L BPA were observed (Haubruge et al., 2000). Sperm counts were significantly lower in both dose groups in a dose-dependent manner. Neither sperm length nor testis weight was affected at either dose. The spectrum of effects suggests a non-endocrine-mediated MOA affecting sperm production (Haubruge et al., 2000).

3.2.1.4. *Mechanistic Studies for Nonmammalian Vertebrates*

3.2.1.4.1. ***Estrogen receptor binding studies.*** BPA directly interacts with the ERs of nonmammalian species. BPA competitively bound to ERs in species representing all classes of vertebrates (Table 2; Kloas et al., 2000; Matthews et al., 2000; Lutz and Kloas, 1999). ERs alpha (α) and beta (β)have been isolated in nonmammalian species (Foidart et al., 1999; Lutz and Kloas, 1999; Tchoudakova, 1999; Xiaotian et al., 1999; Andersen et al., 1999a), and the presence of a third ER subtype, gamma (γ), was recently described in the teleost fish, Atlantic croaker (Hawkins et al., 2000). BPA exhibited potencies ~700 and ~800 times less than estradiol in liver cytosolic assays from the frog *X. laevis* and the common carp, respectively (Table 2; Kloas et al., 2000; Lutz and Kloas, 1999).

ER agonist activity of BPA was demonstrated in reporter gene and vitellogenin induction assays. In vitro assays for ER binding revealed BPA was able to competitively bind ER in *Gallus gallus* (domestic chicken), *Anolis carolinensis* (green anole lizard), and *X. laevis* at relative potencies of 2,300, 770, and 714 times less than estradiol, respectively (Matthews et al., 2000; Lutz and Kloas, 1999). BPA was also found to competitively bind to ERs from piscine species *Cyprinus carpio* (common carp) and *O. mykiss* (rainbow trout) with potencies approximately 800 and 500 times less than estradiol, respectively (Kloas et al., 2000; Matthews et al., 2000).

3.2.1.4.2. ***Gene expression studies.*** Estrogen-response elements (EREs) in the vitellogenin gene promoter region are bound and activated by E-ER complex binding (Hiroi et al., 1999). In vitro, BPA treatment induced vitellogenin expression in *X. laevis, O. mykiss*, *C. carpio*, and the expression of vitellogenin and zona radiata proteins in *S. salar* (Pawlowski et al., 2000; Shilling and Williams, 2000; Celius et al., 1999; Islinger et al., 1999; Kloas et al., 1999; Smeets et al., 1999a, b; Hansen et al., 1998; Schrag et al., 1998). Vitellogenin induction was observed in liver

cultures from juveniles and adults of both sexes. An additive vitellogenin response was observed after carp hepatocytes were exposed to BPA or estradiol (Smeets et al., 1999b). Additionally, BPA induced ERE reporter gene expression in a cell culture assay co-transfected with an *O. mykiss* ER expression vector (Andersen et al., 1999a).

Determining the precise potency differences between BPA and estradiol is complicated by the lack of standardized assays, differences in experimental protocol, and assay specificity for estrogenic activity. However, BPA consistently ranged from 1,000 to 10,000 times less potent than estradiol in most of the in vitro assays examined (Matthews et al., 2001; Chun and Gorski, 2000; Jorgensen et al., 2000; Sheeler et al., 2000; Inadera et al., 2000a; Andersen et al., 1999a; Bolger et al., 1998; Kuiper et al., 1998; Perez et al., 1998; Sohoni and Sumpter, 1998; Gaido et al., 1997; Kuiper et al., 1997; Steinmetz et al., 1997; Olea et al., 1996; Brotons et al., 1995). The variability observed for estradiol in MCF-7 endogenous gene expression assays (Leffers et al., 2001; Jorgensen et al., 2000) could reflect differences in effects on ER-mediated transcriptional events among estrogen-regulated genes. Therefore, BPA may exhibit different estrogenic potencies depending on the specific estrogen-responsive target genes assessed.

The results observed for BPA in competitive binding assays suggest that certain nonmammalian species may be more sensitive than mammals to the estrogenic activity of BPA; BPA is less than 1,000 times as potent as estradiol for all nonmammalian species examined with the exception of the domestic chicken (2,300 times less than estradiol) (Kloas et al., 2000; Matthews et al., 2000; Lutz and Kloas, 1999). BPA was found to be 100 times less potent than estradiol in an amphibian vitellogenin screen (Kloas et al., 1999). In contrast, piscine in vitro assays found lower potencies of BPA (1,000–20,000 times less than estradiol) with a focus on vitellogenin expression in liver cultures (Pawlowski et al., 2000; Shilling and Williams, 2000; Islinger et al., 1999; Smeets et al., 1999b). Overall, the small number and different assays used in nonmammalian in vitro studies precluded a rigorous determination of relative potency for BPA across species.

3.2.1.4.3. ***Other hormone receptor binding studies.*** An in vitro assay demonstrated BPA was as potent as thyroxine (T4) in displacing triiodothyronine (T3) in plasma assays with two amphibians, the bullfrog (*Rana catesbeiana*) and the African clawed frog (*X. laevis*) (Yamauchi et al., 2000). This result suggests that BPA may affect T3 bioavailability, possibly as a thyroxine agonist. Thyroid hormone activity plays an important role in amphibian metamorphosis, and the results suggest the potential for BPA to interfere with hormonal signaling pathways other than estrogens.

3.2.1.5. *Class Mammalia*

For mammalian species, all in vivo BPA studies identified were conducted with the rat and mouse (Table 7). At high doses, BPA administration resulted in maternal and reproductive toxicity to both male and female rodents, but not developmental toxicity in the prenatally exposed offspring (Morrissey et al., 1989, 1987). Lower doses of BPA exposure can lead to effects on adult male and female rodent reproductive organ weights, cell height and differentiation, and prolactin concentration (Ashby et al., 2000; Stoker et al., 1999; Steinmetz et al., 1997; Dodds and Lawson, 1936). Studies exposing pregnant mice to BPA at the ng/g (parts per billion) dose range found different results; some studies observed effects on reproductive endpoints in male and female offspring (Gupta, 2000; Howdeshell et al., 1999; Cagen et al., 1999a; Vom Saal et al., 1998; Nagel et al., 1997).

3.2.1.5.1. ***Effects after prenatal exposure.*** Differences in experimental findings after low-dose prenatal BPA exposure have been observed. The National Toxicology Program (NTP) Workshop on the Endocrine Disruptors Low Dose Peer Review was convened to evaluate whether low-dose effects occurred for a number of different classes of EDCs, including environmental estrogens and BPA. The BPA Subpanel was instructed to define low dose as <5 mg/kg/d. The BPA Subpanel (NTP, 2001) found that some studies provided credible evidence of low-dose effects but that other, also credible, studies with low-dose treatment found either no effect or conflicting effects (i.e., different direction of effect). Their consensus statement was as follows:

> There is credible evidence that low doses of BPA can cause effects on specific endpoints. However, due to the inability of other credible studies in several different laboratories to observe low dose effects of BPA, and the consistency of these negative studies, the Subpanel is not persuaded that a low dose effect of BPA has been conclusively established as a general or reproducible finding. In addition, for those studies in which low dose effects have been observed, the mechanism(s) is uncertain (i.e., hormone related or otherwise) and the biological relevance is unclear (NTP, 2001).

The NTP BPA Subpanel suggested that substrain sensitivity differences may be a critical factor explaining the conflicting findings for body and prostate weight endpoints after low-dose BPA exposure. Differences in mouse strain sensitivity to male reproductive organ effects after estradiol exposure have been observed (Spearow et al., 1999). The Subpanel also discussed other differences that could contribute to different responses to BPA, including food phytoestrogen content, uterine horn position, housing protocol, and continuous versus short-term exposure, each of which could alter the

Table 7. Phylum Chordata, class Mammalia: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints [Note: Not all negative outcome studies after BPA treatment are included in this table; negative outcome studies are included only if they use the same species, measure the same endpoint, and have study designs similar to those of a positive outcome study.]

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,c]	Statistical significance[b]	Possible hormone activity affected[a,d]	Reference
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0–F3 adult)	NA	F: Decreased BW	750 ppm in F1 only; 7,500 ppm for F0–F3	<0.05 for 750 ppm effects; <0.001 for 7,500 ppm effects	NE	Tyl et al. (2002)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Prenatal/ GD 6 through lactation at ~21 d of age	NA	F: Increased BW 4–11 d of age[e]	100, 1,200 µg/kg-d	<0.0001	E	Rubin et al. (2001)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Prenatal/ GD 6 through lactation at ~21 d of age	NA	F: Increased BW 22–110 d of age[e]	100 µg/kg-d	<0.05	E	Rubin et al. (2001)
Rattus norvegicus (Crl:CD BR SD rat) Mammalia	Prenatal/ GD 11 through 20 d old	15 µg/kg-d DES/no change in BW	F: No change in BW at 1, 7 d old[e] M: No change in BW at 1, 7 d, and 6 m old	3,200–320,000 µg/kg-d[e]	<0.05	NA	Kwon et al. (2000)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Prenatal/ GD 6 through lactation at ~21 d of age	NA	M: Increased BW 4–11 d of age[e]	100, 1,200 µg/kg-d	<0.0001	A	Rubin et al. (2001)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0–F3 adult)	NA	M: Decreased BW	750 ppm in F1and F2 only; 7,500 ppm for F0–F3	<0.001 to <0.05	NE	Tyl et al. (2002)
Rattus norvegicus (Han-Wistar Albino rat) Mammalia	Prenatal/ GD 0 through weaning at 22 d old	6–36 µg/kg-d DES/no change in BW	M: No changes in BW at 90 d old[e]	10–10,000 µg/L (1–4 and 775–4,022 µg/kg-d)[e]	<0.01 Levene's test	NA	Cagen et al. (1999b)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Adult/ 15 d	NA	F ovx: Decreased BW gain and ratio of BW gain to food intake	4,000 µg/d (18,000 µg/kg-d)	<0.05	U (E)	Nunez et al. (2001)
Rattus norvegicus (Alpk:ApfSD Wistar rat) Mammalia	Adult/ 3 d	11.2 µg/d (38.5 µg/kg-d)/no change in BW	F ovx: No change in BW	33,400 µg/d (114,600–118,500 µg/kg-d)	<0.05	NA	Laws et al. (2000)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0–F3 adult)	NA	Decreased testes wt (g)	0.015 ppm for F3 only; 0.3 ppm and 750 ppm for F2 and F3; 7,500 ppm for F1–F3	<0.001 to 0.05	U	Tyl et al. (2002)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0-F3 adult)	NA	Increased testes wt (% sacrifice wt)	7,500 ppm for F0–F3	<0.001	U	Tyl et al. (2002)

Table 7. Phylum Chordata, class Mammalia: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints (continued)

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,c]	Statistical significance[b]	Possible hormone activity affected[d]	Reference
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0–F3 adult)	NA	Decreased epididymides, preputial gland, seminal vesicles w/coagulating glands, and prostate wt (g) [Note: No change when assessed % sacrifice wt for preputial gland and prostate]	Epididymides: 750 ppm for F3 only; 7,500 ppm for F1–F3 Preputial gland: 7,500 ppm for F1 only Seminal vesicles: 4.5 ppm for F0 and 7,500 ppm for F0–F3 Prostate: 7,500 ppm for F0–F3	<0.001 to <0.05	U	Tyl et al. (2002)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0–F3 adult)	NA	Increased epididymides wt (% sacrifice wt)	750 ppm for F2 only; 7,500 ppm for F0–F3	<0.001 to <0.05	U	Tyl et al. (2002)
Rattus norvegicus (Wistar rat) Mammalia	Neonatal/ 11 d	3.7, 37, and 370 µg/kg-d DES/ decreased testis wt	Increased testis wt at 80–90 d of age[n]; (conflicting results of testis wt at 18–75 d old)	37,000 µg/kg-d	<0.01	U (AT)	Fisher et al. (1999); Atanassova et al. (2000)
Rattus norvegicus (Han-Wistar Albino rat) Mammalia	Prenatal/ GD 0 through lactation at 22 d old (Mother: prior to mating–lactation, 10 w)	100 µg/L (6–36 µg/kg-d) DES/no changes	M: No changes in wts of accessory sex organs or testis at 90 d old[m]	10–10,000 µg/L (1–4 and 775–4,022 µg/kg-d)	<0.01 Levene's test	NA	Cagen et al. (1999b)
Rattus norvegicus (Crl:CD BR Sprague-Dawley rat) Mammalia	Prenatal/ GD 11 through 20 d old	15 µg/kg-d DES/no changes	M: No change in wts of accessory sex organs or testis at 6 m old[m]	3,200–320,000 µg/kg-d	<0.05	NA	Kwon et al. (2000)
Rattus norvegicus (Han-Wistar Albino rat) Mammalia	Prenatal/ GD 0 through weaning at 22 d old	100 µg/L (6–36 µg/kg-d) DES/no changes	M: No changes in sperm conc., daily sperm production, or sperm production/g testis at 90 d old	10–10,000 µg/L (1–4 and 775–4,022 µg/kg-d)	<0.01 Levene's test	NA	Cagen et al. (1999b)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0-F3 adult)	NA	Decreased paired ovaries (g and % sacrifice wt) and uterus wt (g) [Note: No change when assessed % sacrifice wt for uteri]	Ovaries: 0.015, 4.5, and 75 ppm for F2 only; 750 ppm for F1 only; 7,500 ppm for F0–F3 Uterus: 0.015 ppm for F0 only; 7,500 ppm for F0, F1, and F2	<0.001 to <0.05	U (AE)	Tyl et al. (2002)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0–F3 adult)	NA	F: increased AGD[h,i]	0.015, 0.3, 4.5, and 750 ppm in F2s only	<0.01 to <0.05	U (A)	Tyl et al. (2002)

Table 7. Phylum Chordata, class Mammalia: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints (continued)

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,c]	Statistical significance[b]	Possible hormone activity affected[d]	Reference
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0–F3 adult)	NA	M: No change in AGD	0.015, 0.3, 4.5, 75, 750, and 7,500 ppm	No statistical significance	NA	Tyl et al. (2002)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Prenatal/ GD 6 through lactation at ~21 d old	NA	M and F: No change in AGD	100, 1,200 µg/kg-d	Statistical significance but no level given	NA	Rubin et al. (2001)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0–F3 adult)	NA	M: Delayed age at preputial separation adjusted for BW	750 ppm for F1 only; 7,500 ppm	<0.01 to <0.001	U (AA)	Tyl et al. (2002)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Prenatal/ GD 6 through lactation at ~21 d of age	NA	Irregular estrus cycling (4 and 6 m old)[j]	1,200 µg/kg-d	<0.005	E; AT	Rubin et al. (2001)
Rattus norvegicus (Crl:CD BR SD rat) Mammalia	Prenatal/ GD 11 through 20 d old	15 µg/kg-d DES/ Decreased estrus length	F: No change in timing of 1st estrus or in estrus cycling at 4 m old[j]	3,200–320,000 µg/kg-d	<0.05	NA	Kwon et al. (2000)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Continuously, three-generation diet (prebreed F0–F3 adult)	NA	F: Delayed age at vaginal patency adjusted for BW	7,500 ppm	<0.001	U (AE)	Tyl et al. (2002)
Rattus norvegicus (Long-Evans rat) Mammalia	Adult/ 25 d	5 µg/kg-d E2; 10 and 100 µg/kg-d EE/ (decreased estrus cycles but different patterns from BPA exposure)	F: Decreased estrus cycling	100,000 µg/kg-d	<0.05	E; AT	Laws et al. (2000)
Rattus norvegicus (Nobel rat) Mammalia	Peripubertal/ 11 d	NA	Increased proliferation and differentiation of mammary epithelial cell structures and cell cycle kinetics (lobules, terminal ducts, and end buds)	100, 54,000 µg/kg-d	<0.05	E	Colerangle and Roy (1997)
Rattus norvegicus (F344 rat) Mammalia	Adult/ 3 d	1 µg/d E2/increased uterine epithelial ht 3.5- fold	F ovx: Increased uterine epithelial ht 2.5-fold	50 µg/d (300 µg/kg-d)	<0.001	E	Steinmetz et al. (1998)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Adult/ 3 d	1 µg/d E2/increased vaginal epithelial ht and proliferation	F ovx: No change uterine epithelial ht and differentiation	50 µg/d (300 µg/kg-d)	NR	NA	Steinmetz et al. (1998)
Rattus norvegicus (F344 rat) Mammalia	Adult/ 3 d	1 µg/d E2/increased vaginal epithelial ht and proliferation	F ovx: Increased vaginal epithelial ht and differentiation	50 µg/d (300 µg/kg-d)	NR	E	Steinmetz et al. (1998)

Table 7. Phylum Chordata, class Mammalia: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints (continued)

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,c]	Statistical significance[b]	Possible hormone activity affected[d]	Reference
Rattus norvegicus (F344 rat) Mammalia	Neonatal/ 5 d	5 µg/d DES/ increased PRL @ 25 d old	F: Increased serum PRL concentrations at 20 and 30 d old M: Increased serum PRL concentrations at 25 d old	100, 500 µg/d	<0.05	E	Khurana et al. (2000)
Rattus norvegicus (Wistar rat) Mammalia	Peripubertal/ 11 d	4 mg/mL E2/increased PRL @ 29 d old	M: Increased serum PRL concentrations at 29 d old	50,000 µg/kg-d	<0.05	E	Stoker et al. (1999)
Rattus norvegicus (F344 rat) Mammalia	Adult/ 3 d	1.2–1.5 µg/d E2/increased PRL	F ovx: Increased serum PRL concentrations[k]	40–45 µg/d	<0.05	E	Steinmetz et al. (1997)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Adult/ 3 d	1.2–1.5 µg/d E2/increased PRL	F ovx: No change serum PRL concentrations[k]	40–45 µg/d	<0.05	NA	Steinmetz et al. (1997)
Rattus norvegicus (Wistar rat) Mammalia	Adult/ 7 d	NA	F ovx: DR increase of serum PRL concentrations	78,000–250,000 µg/kg-d	<0.05 at 128,000 µg/kg-d	E	Goloubkova et al. (2000)
Rattus norvegicus (Wistar rat) Mammalia	Adult/ 14 d	NA	M: Increased plasma PRL concentrations	1,000 µg/d (~300 µg/kg-d)	<0.05	E	Tohei et al. (2001)
Rattus norvegicus (Wistar rat) Mammalia	Adult/14 d	NA	M: Decreased plasma testosterone concentrations, but not those of the testes	1,000 µg/d (~300 µg/kg-d)	<0.05	U	Tohei et al. (2001)
Rattus norvegicus (Wistar rat) Mammalia	Neonatal/ 11 d	DES (dosed every other d) for 10 d: 370 µg/kg-d/decreased T 37 µg/kg-d/no change in T	M: Increased plasma T @ 18 d old	37,000 µg/kg-d	<0.001	U	Williams et al. (2001)
Rattus norvegicus (Sprague-Dawley rat) Mammalia	Prenatal/ GD 6 through lactation at ~21 d of age	NA	F ovx: Decreased LH concentrations	1,200 µg/kg-d	<0.001	U (E)	Rubin et al. (2001)
Rattus norvegicus (Wistar rat) Mammalia	Adult/ 14 d	NA	M: Increased plasma LH concentrations	1,000 µg/d (~300 µg/kg-d)	<0.05	U	Tohei et al. (2001)
Rattus norvegicus (Wistar rat) Mammalia	Neonatal/ 11 d	3.7–370 µg/kg-d (5 doses) DES/ increased FSH and decreased FSH	M: Increased plasma FSH 18 and 25 d old (No changes in inhibin β concentrations)	37,000 µg/kg-d	<0.05	U	Atanassova et al. (2000)

Table 7. Phylum Chordata, class Mammalia: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints (continued)

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,c]	Statistical significance[b]	Possible hormone activity affected[d]	Reference
Mus musculus (Swiss CD-1 mouse) Mammalia	Adult/ ~100 d	NA	Mated exposed M and F: Decreased # litters and live pups/litter	875,000, 1,750,000 µg/kg-d	<0.05	NE	Morrisey et al. (1989); Reel et al. (1997)
Mus musculus (Swiss CD-1 mouse) Mammalia	Adult/ ~100 d	NA	Mated exposed M and control F: Decreased # pups/litter	1,750,000 µg/kg-d	<0.05	NE	Morrisey et al. (1989); Reel et al. (1997)
Mus musculus (Swiss CD-1 mouse) Mammalia	Adult/ ~100 d	NA	Mated exposed F and control M: Decreased live pups/litter	1,750,000 µg/kg-d	<0.05	NE	Morrisey et al. (1989); Reel et al. (1997)
Mus musculus (Crl:CD-1 ICR BR mouse) Mammalia	Adult/ 10 d	NA	Mated exposed F and control M: Increased fetal resorptions	1,250,000 µg/kg-d	<0.05	NE	Morrisey et al. (1987)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17 7 d	NA	F (uterine position 0M or 1M): Increased BW at 22 d old[f]	2.4 µg/kg-d	<0.001	E	Howdeshell et al. (1999)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17	0.2 µg/kg-d DES/ decreased BW at 23–37 d old	F: No changes in BW at 23–44 days old, 155 d (5 m) and 310 d old (11 m)[f]	2, 20 µg/kg-d	<0.05 (litter based)	NA	Ashby et al. (1999)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17	0.2 µg/kg-d DES/no change in BW	M: Increased BW 36–90 d old (20 µg/kg-d); 90 d old only (200 µg/kg-d)[f]	20, 200 µg/kg-d	<0.01 Levene's test	A	Cagen et al. (1999a)
Mus musculus (CD-1 mouse) Mammalia	Prenatal/ GD 16–18 3 d	200 µg/kg-d DES/ decreased BW at 3 and 60 d old	M: No change in BW at 3, 21, and 60 d old[f]	50 µg/kg-d	<0.05	NA	Gupta (2000)
Mus musculus (CD-1 mouse) Mammalia	Peripubertal/ 3 d	5 µg/kg-d E2/no BW changes	F: Increased and decreased BW[g]	500 µg/kg-d (increased); 100,000 µg/kg-d (decreased)	<0.05	Increased BW: E Decreased BW: NE	Markey et al. (2001a)
Mus musculus (AP mouse) Mammalia	Peripubertal/ 3 d	10 µg/kg-d DES/no change BW	F: No change in BW[g]	200–300,000 µg/kg-d	<0.01	NA	Tinwell et al. (2000)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17 7 d	NA	M: Decreased BW 6 m old[f]	2 µg/kg-d	<0.05	E; NE	Nagel et al. (1997)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17 7 d	0.2 µg/kg-d DES/no change	M: Increased BW 6 m old[f]	2 µg/kg-d	<0.05 (litter based)	A	Ashby et al. (1999)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17 7 d	NA	M: Increased prostate wt 6 m old[l]	2, 20 µg/kg-d	<0.01	A; E	Nagel et al. (1997)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17	0.2 µg/kg-d DES/no change	M: No change in prostate wt 6 m old[l]	2, 20 µg/kg-d	<0.05	NA	Ashby et al. (1999)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17 7 d	0.2 µg/kg-d DES/no change	M: Increased testis wt at 6 m old[o]	2, 20 µg/kg-d	<0.05	U (AT)	Ashby et al. (1999)

Table 7. Phylum Chordata, class Mammalia: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints (continued)

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,c]	Statistical significance[b]	Possible hormone activity affected[d]	Reference
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17 7 d	0.2 µg/kg-d DES/no change	M: Increased epididymal wt at 6 m old[p]	2, 20 µg/kg-d	<0.05 (litter based)	U	Ashby et al. (1999)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17	0.2 µg/kg-d DES/no change	M: No changes in wts of accessory sex organs or testis at 90 d old[o,p]	0.2–200 µg/kg-d	<0.01 Levene's test	NA	Cagen et al. (1999a)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17 7 d	NA	Decreased sperm production/g testis[m]	20 µg/kg-d	<0.05	U (E)	Vom Saal et al. (1998)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17 7 d	0.2 µg/kg-d DES/no change	M: Increased daily sperm production at 6 m old[m]	2, 20 µg/kg-d	<0.05	U (A; AT)	Ashby et al. (1999)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17	0.2 µg/kg-d DES/no changes	M: No changes in sperm conc., daily sperm production, or sperm production/g testis at 90 d old[m]	0.2–200 µg/kg-d	<0.01 Levene's test	NA	Cagen et al. (1999a)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17	0.2 µg/kg-d DES/no changes	M: No change sperm conc., sperm production/g testis at 6 m old[m]	2, 20 µg/kg-d	<0.05	NA	Ashby et al. (1999)
Mus musculus (CD-1 mouse) Mammalia	Prenatal/ GD 16–18 3 d	0.1 µg/kg-d DES/ increased AGD	M: Increased AGD at 3–60 d old[h]	50 µg/kg-d	<0.05	A	Gupta (2000)
Mus musculus (CF-1 mouse) Mammalia	Prenatal/ GD 11–17 7 d	NA	F (uterine position 0 M): Decreased interval (d) from VO to first estrus	2.4 µg/kg-d	<0.01	E	Howdeshell et al. (1999)
Mus musculus (B6C3F1 mouse) Mammalia	Adult/ 4 d	0.4 µg/d E2/increased uterine luminal epithelial ht and effect reversed w/ICI Increased uterine stromal/ myometrial ht and effect not changed w/ICI	Increased uterine luminal epithelial ht: Decreased to control levels with ICI (200 µg/d) Increased uterine stromal and myometrial ht: Not decreased to control levels with ICI (200 µg/d)	2,000 µg/d	<0.05	E	Papaconstantinou et al. (2000)
Mus musculus (AP mouse) Mammalia	Peripubertal/ 3 d	10 µg/kg-d DES/ increased endometrial ht	F: No change in uterine endometrial ht	200–300,000 µg/kg-d	<0.01	NA	Tinwell et al. (2000)
Mus musculus (CD-1 mouse) Mammalia	Peripubertal/ 3 d	5 µg/kg-d E2/increased uterine epithelial ht	Increased uterine epithelial ht	75,000, 100,000 µg/kg-d	<0.05	E	Markey et al. (2001a)
Mus musculus (AP mouse) Mammalia	Peripubertal/ 3 d	10 µg/kg-d DES/ increased epithelial ht	Increased uterine epithelial ht in one of two trials	200,000 µg/kg-d (s.c.)	<0.05	E	Tinwell et al. (2000)

Table 7. Phylum Chordata, class Mammalia: Bisphenol A (BPA) in vivo response data highlighting developmental and reproductive endpoints (continued)

Species (strain and/or common name) and class	Life stage of exposure/ exposure duration[a]	Positive control/ effect[a,b]	Developmental or reproductive effect(s)[a,b,c]	BPA doses with observed effect[a,c]	Statistical significance[b]	Possible hormone activity affected[d]	Reference
Mus musculus (CD-1 mouse) Mammalia	Prenatal/ GD 9 to parturition 12 d	NA	Extensively increased area of mammary terminal ducts and end bud and alveolar buds at 6 m old	25, 250 µg/kg-d	<0.05	E	Markey et al. (2001b)
Mus musculus (CD-1 mouse) Mammalia	Prenatal/ GD 9 to parturition 12 d	NA	Increased percentage of mammary alveoli with secretory product	25 µg/kg-d	<0.05	U	Markey et al. (2001b)
Mus musculus (CD-1 mouse) Mammalia	Prenatal/ GD 9 to parturition 12 d	NA	F: Increased DNA synthesis in stromal layer of mammary gland	25, 250 µg/kg-d	<0.05	E	Markey et al. (2001b)
Mus musculus (C57BL/6 mouse) Mammalia	Peripubertal/ 28 or 56 d	NA	M: Decreased plasma T concentrations at 13 w old	50,000 µg/L (10,400 µg/kg-d) at 56 d	<0.02	U	Takao et al. (1999)
Mus musculus (CD-1 mouse) Mammalia	Peripubertal/ 3 d	5 µg/kg-d E2/increased uterine lactoferrin	Increased uterine lactoferrin expression	75,000, 100,000 µg/kg-d	<0.01	E	Markey et al. (2001a)
Mus musculus (C57BL/6-SJL ERIN Transgenic mouse) Mammalia	Adult/ 1 d	5 µg/kg-d DES/ increased reporter gene expression	F ovx: Increased ERE transfected reporter gene expression in uterus	25,000 µg/kg-d	<0.01	E	Nagel et al. (2001)

[a] GD = gestation day; w = weeks; d = days; gen. = generation; F# = generation number.

[b] NA = not applicable (no positive control used); BW = body weight; AGD = anogenital distance; T = testosterone; E2 = 17β-estradiol; EE = 17α-ethinylestradiol; FSH = follicle stimulating hormone; ICI = ICI 182,780; PRL = prolactin; ht = height; wt = weight; DES = diethylstilbestrol.

[c] M = male; F = female; LH = luteinizing hormone; s.c. = subcutaneous injection; VO = vaginal opening; 0M = fetus positioned between 0 males in uterine horn; 1M = fetus positioned next to one female and one male in uterine horn; ovx = ovariectomized; ERE = estrogen response element; m = months.

[d] NA = not applicable (no change observed); NE = nonendocrine/general toxic MOAs; U = uncertain; observed effects consistent with an affect on one or more of the following hormone activities: A = increased androgen activity; AA = antagonizing androgen activity; AE = antagonizing estrogen activity; E = increasing estrogen activity; AT = antagonizing thyroid hormone activity.

[e] Studies identified with this footnote are rat prenatal studies assessing BW with similar protocols that found different results; some observed an increase and others observed no change in BW.

[f] Studies identified with this footnote are mouse prenatal exposure studies assessing BW with similar protocols that found different results; studies observed an increase, a decrease, or no change in BW was observed.

[g] Studies identified with this footnote are mouse peripubertal exposure studies assessing BW that found different results; studies observed an increase, a decrease, or no change in BW.

[h] Studies identified with this footnote are rodent studies assessing AGD that found different results; studies observed either an increase or no change in AGD.

[i] Tyl et al. (2002) authors concluded that the AGD effects were not biologically plausible and that the change was too small to be meaningful.

[j] Studies identified with this footnote are rat studies assessing estrus cyclicity that found different; studies observed either irregular cycling or no change.

[k] Studies identified with this footnote are rat studies assessing serum PRL that observed an increase or no change in serum PRL concentrations depending on the rat strain.

[l] Studies identified with this footnote are mouse studies of similar protocol assessing prostate weight that found different results; studies observed either an increase or no change in prostate weight.
[m] Studies identified with this footnote are mouse studies of similar protocol assessing sperm production that found different results; studies observed a decrease, an increase, or no change in sperm production.
[n] Studies identified with this footnote are rat studies of similar protocol assessing testis weight that found different results; studies observed either an increase or no change in testis weight.
[o] Studies identified with this footnote are mouse studies of similar protocol assessing testis weight that found different results; studies observed either an increase or no change in testis weight.
[p] Studies identified with this footnote are mouse studies of similar protocol assessing epididymal weight that found different results; studies observed an increase or no change in epididymal weight.

effective estrogen concentration of the animals (NTP, 2001). For example, continuous BPA exposure, which is relevant to some individual human exposures, could lead to a reduction in response (i.e., desensitization). They also pointed out that a number of the examined endpoints are sensitive and variable to hormonal changes during development and are affected by genetic and environmental factors. Additionally, although some of the studies were performed in a similar manner, no two studies were performed with identical strain and colony, methods (e.g., feed), and study design (e.g., number). For a review of the NTP workshop, see Melnick et al. (2002); for other analyses of the BPA low-dose studies, see reviews by Witorsch (2002) and Milman et al. (2002); and for analysis of low-dose effects of EDCs in general, see Welshons et al. (2003).

Alterations in mouse mammary gland differentiation (Markey et al., 2001b) occurred after maternal subcutaneous (delivered by osmotic pumps) exposure to 25 and 250 μg/kg/day BPA. Alterations in the epithelium and stroma and an increase in terminal ducts and terminal end buds were observed in both dose groups, providing evidence of developmental effects in the low-dose range. This study was not analyzed by the NTP low-dose workshop.

In a three-generation reproductive toxicity study in rats, in which animals were continuously fed 0.015, 0.3, 4.5, 75, 750, or 7,500 parts per million (ppm) (equivalent to ~0.001, 0.02, 0.3, 5, 50, or 500 mg/kg/day) BPA, reproductive organ histopathology and function were not affected (Tyl et al., 2002). Only at 7,500 ppm (~500 mg/kg/day) was the timing of vaginal patency (in females) and preputial separation (in males) delayed in offspring, and these changes were associated with a reduction in body weight. At 0.015, 0.3, 4.5, and 750 ppm (~0.001, 0.02, 0.3, and 50 mg/kg/day), the anogenital distance (AGD), a sensitive endpoint, of F2 females showed a statistically significant increase. However, the authors argue that this observation was not biologically meaningful because AGD has been shown to be regulated by androgens during development (Gray et al., 1998; Gray and Ostby, 1998). However, this reasoning presupposes that BPA has no effect on androgen activity and that exogenous estrogen exposure cannot affect AGD. Tyl et al. (2002) also observed some statistically significant increases in ovarian weight in the F2 generation in the low-dose range. For the ovarian weight and AGD changes, the NTP

BPA Subpanel found that the dose-response relationships were not consistent throughout the range of doses with observed changes, and some changes lost statistical significance after correcting for body weight differences. As previously mentioned, one possible reason for the discrepancies in findings, particularly in the low-dose range, is that continuous BPA treatment of the animals, as was done in the Tyl study, may lead to desensitization of response compared with BPA exposures of shorter duration. For the purposes of this case study, all published studies with statistically significant effects were included and additional details (e.g., positive controls, strain differences) are presented in Table 7. Resolution of the low-dose effects issue was not a goal of this report and is discussed herein to raise one of the issues surrounding the BPA developmental and reproductive effects literature.

Although exposure duration and route differed among studies and there is uncertainty about the effects of BPA on body weight, male accessory sex organ and gland weights, and estrus timing in CF-1 mice, some developmental/reproductive effects were consistently observed after prenatal exposure to BPA. Behavioral changes in SD rats occurred after prenatal through postnatal exposure to BPA at a dose of 40 μg/kg (Farabollini et al., 1999).

Increased expression of ERα and ERβ in the anterior pituitary of males and ERα in the hypothalamus of female F344 rats exposed neonatally to BPA (Khurana et al., 2000) demonstrate that BPA can affect gene expression in the main neuroendocrine brain centers in both sexes. The increase in prolactin and follicle stimulating hormone (FSH) levels observed in males also shows an influence on this important regulatory system (Williams et al., 2001; Atanassova et al., 2000; Khurana et al., 2000). Alterations in the hypothalamic-pituitary-gonadal (HPG) axis can lead to puberty effects (Hadley, 2000; Gray et al., 1997). Therefore, the HPG changes observed in ER expression and gonadotropin levels provide a mechanistic explanation for the shortened interval of puberty in females exposed prenatally to BPA (Howdeshell et al., 1999) and effects in males exposed neonatally (Williams et al., 2001; Atanassova et al., 2000). The male and female puberty effects are consistent with an estrogen agonist MOA. Because estradiol benzoate exposure at low doses has demonstrated an ability to advance puberty in male Wistar rats exposed neonatally, effects on male puberty may be consistent with the estrogen agonist MOA (Putz et al., 2001b). The HPG effects in female and male F344 rats exposed neonatally to BPA at 100 μg/kg (Khurana et al., 2000), together with the effects on vaginal and uterine epithelium after BPA exposure of adult F344 rats (Steinmetz et al., 1998, 1997), were considered credible evidence for low-dose effects of BPA by the NTP BPA Subpanel (NTP, 2001).

Mammary gland development was also affected in CD-1 mice after prenatal exposure to BPA at doses of 25 and 250 μg/kg (Markey et al., 2001b), and a trend of increasing mammary tumors in SD rats exposed prenatally and neonatally to BPA at 0.1 and 1.2 mg/kg was also observed (Rubin et al., 2001). Earlier onset of vaginal opening was observed in CD-1 mice after

prenatal exposure at 0.1 and 100 mg/kg subcutaneous (s.c.) BPA (Markey et al., 2001a). Body weight increases at 5 mg/kg and decreases at 100 mg/kg suggested a nonmonotonic dose-response curve that was not found for uterine wet weight (increased only at 100 mg/kg).

3.2.1.5.2. ***Effects after peripubertal exposure.*** A limited number of studies examined reproductive and developmental effects after peripubertal BPA exposure. In male rodents, effects on body weight and lateral prostate weight after BPA peripubertal exposure were observed (Stoker et al., 1999; Hanioka et al., 1998). No weight changes were observed in the ventral prostate, and a histological examination was not included in this study to compare effects with stromal differentiation observed after prenatal BPA exposure (Ramos et al., 2001). Testosterone levels after BPA administration were decreased in C57BL/6 mice (Takao et al., 1999). For peripubertal BPA exposure in females, the lowest concentration at which effects on mammary cell differentiation, maturation, and proliferation were observed was 0.1 mg/kg (s.c.) (Colerangle and Roy, 1997).

3.2.1.5.3. ***Effects after adult exposure.*** Studies on F344 adult ovariectomized rats demonstrated that BPA exposure increased uterine and vaginal *c-fos* gene expression, epithelial height, and differentiation after a single dose in the absence of endogenous estrogen production (Long et al., 2000; Steinmetz et al., 1998). These changes, along with increased uterine wet weight, were also observed in F344 rats after three daily administrations of BPA at 50 μg/day (0.3 mg/kg). BPA at 40–45 μg/day (<0.3 mg/kg) also affected the neuroendocrine system by increasing prolactin concentrations and anterior pituitary weight in this rat strain (Steinmetz et al., 1998). These responses paralleled those found with estradiol exposure, suggesting an estrogenic MOA.

BPA also affected endpoints in intact female and male rats (Tohei et al., 2001; Laws et al., 2000). Estrus cycling was altered in Long-Evans rats after daily exposure to BPA at 100 mg/kg for 25 days (Laws et al., 2000). At this dose, uterine weight was not increased in ovariectomized female Long-Evans rats in the standard 3-day uterotrophic assay. Similarly, prenatally exposed SD rats also exhibited changes to estrus cycling at doses below those that resulted in increased uterine wet weight in adult ovariectomized females (Rubin et al., 2001), demonstrating the importance of including multiple endpoints, exposure intervals, and doses for determining the effects after BPA exposure. Male Wistar rats exposed to BPA at 1 mg/day for 14 days exhibited changes in prolactin, luteinizing hormone (LH) and testosterone levels.

3.2.1.6. *Mechanistic Studies of Mammalian Vertebrates*

Early assays of the toxicological activity of BPA focused on cornification of vaginal epithelium in ovariectomized rats (Dodds and Lawson, 1936). This assay defined a physiological response that was consistent with estrogen action. Recognition that endogenous

estrogen acted via an intracellular receptor (Jensen et al., 1968; Toft and Gorski, 1966; Jensen and Jacobson, 1962) led to the development of techniques to examine endocrine processes from a molecular as well as physiological level. In vitro assays examined BPA responses in screens designed to assess hormonal activity. The assays primarily focused on human ER-mediated activity and demonstrate the ability of BPA to competitively bind and activate human ERα and ERβ (Matthews et al., 2001; Sheeler et al., 2000; Andersen et al., 1999a; Bolger et al., 1998; Kuiper et al., 1998, 1997; Sohoni and Sumpter, 1998; Gaido et al., 1997).

3.2.1.6.1. ***Estrogen receptor binding studies.*** The studies on ER binding provided important information on the potential for BPA to elicit estrogen agonist activity. BPA was only 250 times less potent than estradiol in one study (Matthews et al., 2001). But another study found the affinity of BPA for ERβ was 10,000 times lower (Kuiper et al., 1998). In rodent uterine cytosolic assays, BPA was 10,000 times less potent than estradiol (Matthews et al., 2001; Blair et al., 2000; Yoon et al., 2000).

The range of relative potency values determined for BPA in ER competitive binding assays could be explained by the low sensitivity of these assays compared to other in vitro assays used to assess hormonal activity (Gutendorf and Westendorf, 2001; NRC, 1999; Andersen et al., 1999a). Binding assays using tissue extracts measure binding based on both ER subtypes, which can lead to different binding affinity values from those observed in recombinant assays (Andersen et al., 1999a). Evaluation of BPA in more biologically complex assays that are more sensitive and can distinguish agonist and antagonist activity will improve our understanding of interactions between BPA and hormonal activities, including receptor interactions.

In a reporter gene assay, BPA was found to have a different response than endogenous estrogens in a human ERα with a mutated activation function region (Yoon et al., 2000; Gould et al., 1998); BPA behaved as a weak estrogen and exhibited possible anti-estrogenic activity in vitro.

3.2.1.6.2. ***Gene expression studies.*** MCF-7 cellular proliferation in response to BPA and estradiol is one of the most sensitive in vitro assays to detect hormonal activity effects (Andersen et al., 1999a; Perez et al., 1998; Gray et al., 1997; Olea et al., 1996). BPA treatment elicited gene expression changes in the human cell line MCF-7 that were similar to changes after estradiol treatment, many of which were blocked by the ER antagonist, ICI 182,780 (Inadera et al., 2000a, b; Jorgensen et al., 2000; Perez et al., 1998; Krishnan et al., 1993). BPA was found to be approximately 10,000 times less potent than estradiol in this screen (Andersen et al., 1999a; Perez et al., 1998; Olea et al., 1996). Although this assay is highly sensitive, it is not highly specific for characterizing estrogen agonist activity (Gray et al., 1997), and under some conditions BPA exhibited estrogen antagonistic activity (Yoon et al., 2000; Hiroi et al., 1999).

The weight of evidence for the in vitro studies demonstrates that BPA acts as an ER agonist in mammals.

3.2.1.6.3. ***Other hormone receptor binding studies.*** Assays investigating direct receptor-mediated responses for other hormones were limited to competitive binding and recombinant receptor-reporter gene constructs for mammalian receptors. BPA demonstrated a low potential for interacting with rat progesterone receptors but no agonist or antagonist activity for human progesterone receptors (Laws et al., 2000; Gaido et al., 1997; Tran et al., 1996). Studies examining BPA in a human androgen receptor activation system also suggested a low potential for BPA to act directly via this receptor (Gaido et al., 2000, 1997; Sohoni and Sumpter, 1998). BPA treatment did demonstrate agonist activity in a human SXR/PXR recombinant-reporter gene assay (Takeshita et al., 2001) but not in mouse studies (Masuyama et al., 2000). The induction of CYP3A enzymes is species-specific, and the SXR/PXR genes are one factor conferring species-specificity (Xie et al., 2000). This species difference may be explained by the fact that the predicted amino acid sequence of the ligand-binding domains of the human and mouse SXR/PXR are 77% similar, but their DNA-binding domains are 96% similar (Takeshita et al., 2001). These results suggest that BPA may act as a SXR/PXR agonist in humans, but further confirmation is needed.

3.2.1.6.4. ***Estrogenicity of BPA-glucuronide metabolite.*** BPA-glucuronide is unable to bind to either ER subtype or to induce estrogen-responsive gene expression (Matthews et al., 2001; Snyder et al., 2000). Estrogenic activity also decreased in human and rat liver microsomes after incubation with uridine diphosphate glucuronic acid (Elsby et al., 2001). In addition, formation of the BPA-glucuronide in rat hepatocytes decreased BPA-induced cytotoxicity as measured by cell death and adenine nucleotide loss (Nakagawa and Tayama, 2000). These assays provide evidence that BPA glucuronidation decreased the estrogenic and toxic response of BPA.

4. DISCUSSION

4.1. WEIGHT-OF-EVIDENCE MODE-OF-ACTION ASSESSMENT ACROSS SPECIES

Tables 3 to 7 present the possible MOAs to explain the in vivo effects data. Using a WOE approach, that considered the in vivo effects and in vitro mechanistic data, the MOA for BPA for different species with data (Figure 5). The WOE criteria for listing an MOA were: 1) in vivo endpoints consistent with the MOA, and 2) mechanistic studies supporting the MOA. In addition, biological plausibility for the MOA in the species; data set quantity; and data set quality (including consistency of outcomes) were considered.

4.1.1. Invertebrate Species

The affected MOAs underlying the observed effects in BPA-exposed invertebrate species, *M. cornuarietis*, *N. lapillus* (Oehlmann et al., 2000)*, A. tonsa* (Andersen et al., 1999b), and *C. riparius* (Watts et al., 2001) are difficult to characterize. Little is known about timing and hormonal control of sexual differentiation in invertebrates (Oehlmann et al., 2000; Olmstead and LeBlanc, 2000; Ankley et al., 1998; Tyler et al., 1998). Direct BPA estrogenic signaling has not been demonstrated through ER binding and activation in invertebrates (McLachlan, 2001). Sex-steroid hormones may influence gastropod gonadal development (Oehlmann et al., 2000; Tyler et al., 1998). Estradiol has been identified in a few invertebrate species within 4 phyla (cnidarians, arthropods (crustaceans), mollusks, and echinoderms), although estrogen receptors have not been isolated in any invertebrate species (reviewed in McLachlan, 2001). More dose-response data are necessary because the studies assessed few dose groups and observed effects in a subset. Overall, the WOE of the MOA is unknown for tested species within the phylum Mollusca; the data set is small and limited to two species of snails, and there is little known about the signaling molecules and receptors for sexual differentiation in mollusks. However, it is interesting to note that the effects of BPA exposure in snails are consistent with feminization effects and, therefore, could be the result of effects on estrogen or estrogen-analog signaling.

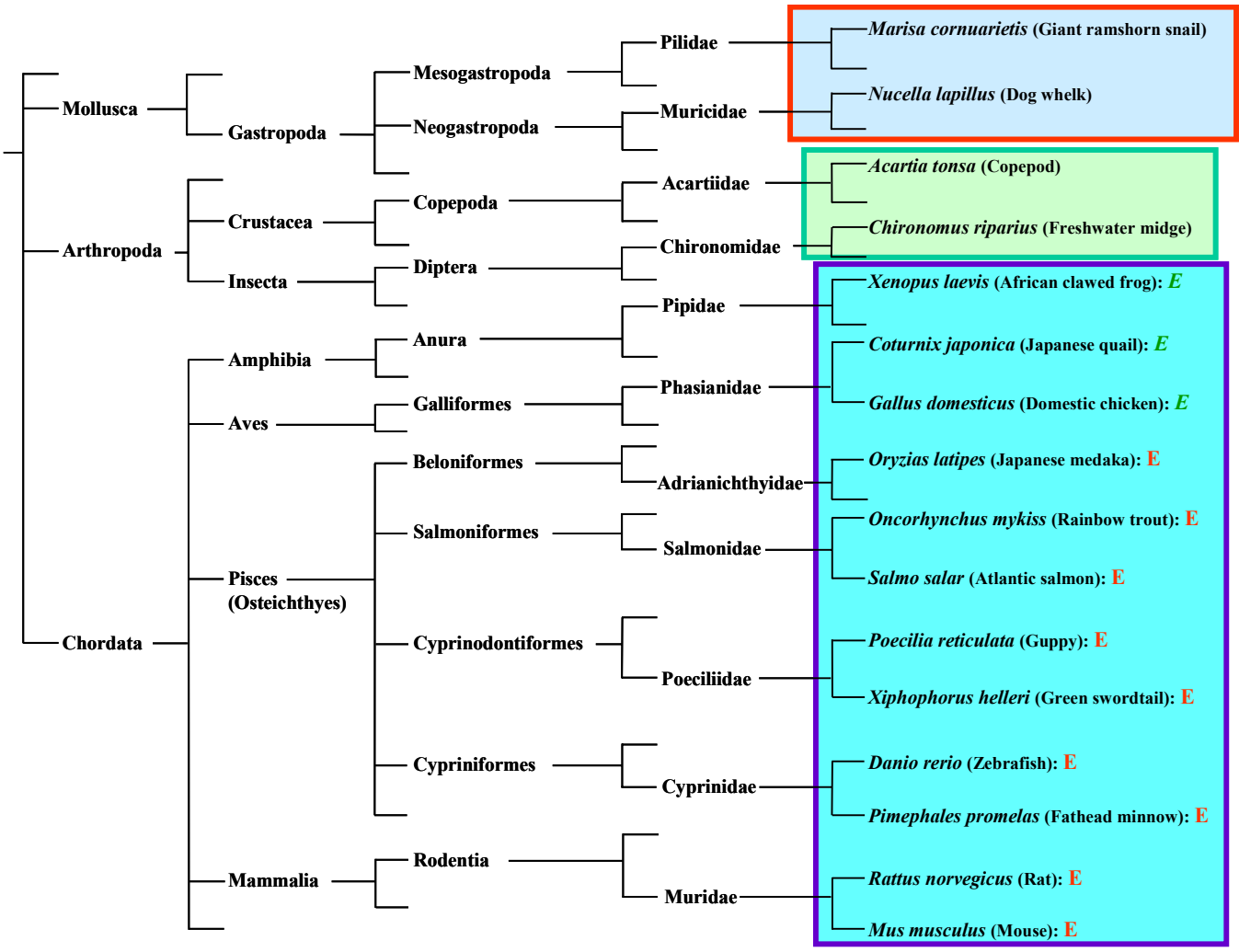

Figure 5. Weight-of-evidence for BPA mode-of-action determination among animal species with data. The weight-of-evidence supports the estrogen agonist (E) mode of action within the chordate species but is unknown for the mollusk and arthropod species. A red E indicates that the dataset is relatively large and consistent with an estrogen agonist MOA; A green italicized E indicates that the dataset, while consistent with an estrogen agonist MOA, is smaller and includes fewer studies to conclusively support this MOA. [Lines indicate evolutionary relationships. Lines without names indicate additional phylogenetic tree branches that are not shown. Classifications are aligned in columns representing phylum, class, order, family, and genus species (common name), from left to right. Species included are those with published BPA in vivo effects data. Boxes indicate the species within the three phyla (Mollusca, Arthropoda, and Chordata) for which there are BPA effects data.]

In some experiments with invertebrates, an estrogen control was performed and the outcome was compared to the outcome after BPA treatment, providing important information about possible MOAs. In the class Gastropoda, data are limited and an estrogen positive control was not performed (Oehlmann et al., 2000). Therefore, assessing whether the observed BPA-associated effects were consistent with an estrogen control was not possible. However, the effects observed are all consistent with a feminization or hyperfeminization of males. For example, enlarged reproductive tracts and glands, increased spawning egg mass and production, and advanced timing of oogenesis in females and decreased penis and prostate gland length and number of sperm stored in males are feminization effects that may be consistent with an estrogen or estrogen-like agonist MOA.

In the phylum Arthropoda, some data indicate an estrogen agonist MOA in the copepod and an ecdysone antagonist MOA in the freshwater midge. Increased egg production was observed in the copepod *A. tonsa* exposed to estradiol or BPA, suggesting that BPA affects estrogen activity in this species as well (Andersen et al., 1999b). Ecdysteroids are known to have a role in development, molting, and reproduction for arthropods (Oberdörster et al., 2001; Tyler et al., 1998). As molting and development are regulated by ecdysteroids in arthropods, the delay in adult insect emergence after BPA treatment (Watts et al., 2001) could suggest an effect on ecdysteroidal activity. BPA did exhibit direct ecdysteroid antagonist activity in an in vitro *D. melanogaster* ecdysone receptor screen, although the EC_{50} value was high at 100 μM (Dinan et al., 2001). However, other studies suggest this is not a signaling pathway affected by BPA (Oberdörster et al., 2001). In addition, BPA did not affect molting behavior in *Daphnia magna* at concentrations of 0.316 and 3.16 ppm (mg/L) BPA (Caspers, 1998). Differences in effects observed between BPA and ethinylestradiol-exposed *C. riparius* suggest different modes or mechanisms of action (Watts et al., 2001). This finding corroborates another study's finding that vertebrate sex-steroid hormones did not disrupt arthropod (Insecta phylum) physiology (Oberdörster et al., 2001).

The WOE indicates that the MOA for BPA is unknown in arthopods. This is based on the relatively small dataset, limited to two in vivo studies and one mechanistic in vitro study in two classes. MOA findings were inconsistent; two were consistent with ecdysone antagonism and one was consistent with estrogen agonism.

Since a number of phyla do not have ERs, it may be more appropriate to consider the MOA as that of a steroid hormone agonist. The BPA MOA to be considered, to be inclusive of vertebrate and invertebrate endocrinology, is that of a steroid hormone agonist and not specifically as an estrogen agonist. From this perspective, data that found effects consistent with ecdysone receptor antagonist activity in insects and crustaceans would unite the MOA across invertebrate and vertebrate species. Beyond the animal kingdom, BPA has been shown to interact with a bacterial receptor, NodD (Nod for nodulation) (Fox et al., 2001). BPA

demonstrated NodD antagonist activity in the presence of one of its natural ligands, luteolin, from a plant. Luteolin shares some characteristics with estrogen and is therefore considered an estrogen-like molecule (McLachlan, 2001). BPA exposure disrupted this plant-bacteria interaction by binding to NodD. This study reveals that BPA, an estrogen agonist at least in vertebrates, has the ability to bind to an ER-like molecule in taxa distantly related from the animal kingdom. However, owing to the data gaps, considering the invertebrate effects data and MOA assessment from this perspective does not allow for a conclusive determination of the BPA MOA in invertebrates to be that of a steroid hormone agonist.

4.1.2. Vertebrate Species

4.1.2.1. *Nonmammalian Vertebrate Species*

Many of the molecular to physiological responses observed across nonmammalian species are consistent with effects on estrogen signaling. In vitro findings of vitellogenin induction were extended to in vivo gene expression in *O. mykiss, S. salar,* and *P. promelas* (fathead minnow) following BPA exposure (Lindholst et al., 2001, 2000; Sohoni et al., 2001; Arukwe et al., 2000; Christiansen et al., 2000, 1998), thus providing evidence for an estrogenic MOA for BPA in these fish species.

While in vitro assays provide evidence that BPA induces vitellogenin through ER binding and activation, indirect estrogen activity effects, such as through changes in estradiol bioavailability, could explain the vitellogenin induction observed in vivo. Estradiol levels were not examined directly in any nonmammalian species assay. BPA did exhibit a low binding affinity for sex- steroid binding proteins and increased aromatase expression, the enzyme responsible for the conversion of testosterone to estradiol in fish, and thus BPA may influence estradiol bioavailability (Kishida et al., 2001; Kloas et al., 2000).

Effects on organism function due to the abnormal presence of vitellogenin have been suggested but not conclusively shown (NRC, 1999; Metcalfe et al., 2001; Ankley et al., 1998). For example, inhibition of testicular development was associated with increases in vitellogenin concentrations in *O. mykiss*. Although vitellogenin concentrations were not examined in this study, excess vitellogenin that is not taken up by maturing ovaries may result in the deposition of eosinophilic fluid in female medaka exposed to 0.1 μg/L estradiol or 200 μg/L BPA (Metcalfe et al., 2001).

Although the mechanism differs among species, sex determination is the genetic and/or environmental (e.g., temperature) mechanism that controls the pathway to testicular versus ovarian development (Clinton and Haines, 1999). Studies on sex-steroid manipulation demonstrate that excess estrogenic exposure can result in ovatestis conditions and even total sex reversal, suggesting effects on sex determination. Sexual differentiation is the series of events that lead to permanent, irreversible sex-specific organization (Ottinger and Abdelnabi, 1997).

Exogenous estradiol or environmental estrogen agonist exposure can result in reproductive tract abnormalities in vertebrates, presumably by affecting sexual differentiation (reviewed in NRC, 1999). The mechanism of sexual determination in anuran (e.g., *X. laevis*) and urodele amphibians is genetic (Schmid and Steinlein, 2001) and likely a combination of genetic and environmental effects in gonochoristic (single sex and sex retained over the organisms lifetime) fish (e.g., *O. latipes, O. mykiss, P. promelas*, and most temperate, freshwater fish) (Tyler and Routledge, 1998). The processes of sexual differentiation in amphibians and fish are not thoroughly characterized but are influenced by sex-steroid hormones (Metcalfe et al., 2001; Scholz and Gutzeit, 2000; Kloas et al., 1999; Ankley et al., 1998; Jobling et al., 1998; Tyler et al., 1998).

Posthatch exposure to a BPA concentration of 100 nM (22.8 μg/L) also resulted in more phenotypic females than males based on gonadal histology in the amphibian *X. laevis* (Kloas et al., 1999). *X. laevis* exposed to estradiol by the same protocol also produced more females than males; at 0.1 μM estradiol almost all animals in that exposure group were female. A sex-ratio change toward females in *X. laevis* exposed to BPA suggests a feminizing influence on sex determination. These alterations in sexual development are consistent with an estrogenic MOA for BPA and the ER binding data further supports the E agonist MOA. However, the data set is small and some effects are consistent with a thyroid activity MOA. Thus, the WOE supports E agonism but not conclusively (Figure 5).

The Berg et al. (2001) study strongly supports the estrogen agonist MOA in birds. ER binding data and a known role for estrogens in sexual differentiation of birds provides further support. However, the data set for birds is relatively small. Thus, similar to the amphibian data set, the WOE supports E agonism but not conclusively.

In the fish medaka, 1,820 μg/L early life stage through posthatch, or ~10 μg/L posthatch only exposure to BPA resulted in formation of ovatestis histology (Metcalfe et al., 2001; Yokota et al., 2000). The observation of ovatestis illustrates atypical estrogenic signaling by BPA at the level of sexual differentiation of the gonad. A shift in the sex ratio toward females was observed in medaka exposed to 1,820 μg/L BPA beginning in the embryonic stage (Yokota et al., 2000) but not in fish exposed to ~10 μg/L BPA beginning in posthatch (Metcalfe et al., 2001). .

Physiological responses reported in bony fish following BPA exposure included alterations in secondary sex characteristics and gametogenesis (Kwak et al., 2001; Metcalfe et al., 2001; Sohoni et al., 2001; Haubruge et al., 2000). Both processes are dependent on sex-steroid hormones and thus can be affected by environmental hormones (Tyler et al., 1998; LeBlanc and Bain, 1997). Distinguishing between the underlying mechanisms is more difficult because multiple factors could result in a similar observation. Effects on a male secondary sex characteristic, such as decreased sword length in *X. helleri,* could occur by antiandrogenic or

estrogenic MOAs (Kwak et al., 2001). And gametogenesis effects can be attributed to changes in more than one hormonal signaling pathway (Ankley et al., 1998).

Together, these studies demonstrate the ability of BPA to elicit reproductive and development effects in nonmammalian vertebrate species (Metcalfe et al., 2001; Yokota et al., 2000; Shioda and Wakabayashi, 2000a, b; Kusk and Wollenberger, 1999; Andersen et al., 1999b), and a number of effects are consistent with exogenous estradiol exposure (Metcalfe et al., 2001; Christiansen et al., 2000, 1998; Lindholst et al., 2000; Yokota et al., 2000; Shioda and Wakabayashi, 2000a, b; Kloas et al., 1999; Andersen et al., 1999b). There is a known role for estrogen and estrogen receptor in fish that further supports the estrogen agonist MOA. Given the relatively large data set which includes both in vivo and mechanistic studies for fish, the WOE supports an estrogen agonist MOA. However, some effects may be explained by alternate MOAs.

4.1.2.2. *Mammalian Vertebrate Species*

The first assays designed to detect estrogenic activity were based on in vivo changes to the reproductive tract of female rodents and are still considered some of the best screens for estrogenicity in mammals (Gray et al., 1997). Proliferation and differentiation of vaginal epithelial cells to a keratinized or cornified state is a highly specific estrogenic response (Dodds and Lawson, 1936; NRC, 1999; Gray et al., 1997). Increased uterine wet weight or the uterotrophic response is another response typically used as an indicator of estrogenic activity but is a less sensitive endpoint when assessing environmental estrogens. These assays have also been expanded to examine ER activation and changes in the expression of estradiol-mediated genes, such as lactoferrin and *c-fos*.

BPA effects are consistent with estrogenic activity in female rodent reproductive tract assays. Vaginal epithelial cell proliferation and differentiation are induced by BPA exposure (Ashby et al., 2000; Steinmetz et al., 1998; Dodds and Lawson, 1936). Increased uterine wet weight was observed in rodents exposed to BPA during peripubertal or adult life stage (Matthews et al., 2001; Markey et al., 2001a; Diel et al., 2000; Goloubkova et al., 2000; Laws et al., 2000; Long et al., 2000; Papaconstantinou et al., 2000; Yamasaki et al., 2000; Steinmetz et al., 1998; Ashby and Tinwell, 1998; Dodge et al., 1996). At a gene expression level, rodents exposed to BPA exhibited increased uterine *c-fos* and lactoferrin induction (Markey et al., 2001a; Steinmetz et al., 1998). Vaginal *c-fos* expression was also increased following BPA exposure (Steinmetz et al., 1998).

Both in vitro and in vivo studies demonstrated that BPA does act as an ER agonist in mammals. In vitro assays characterized BPA as an ER agonist in mammalian recombinant and endogenous gene expression assays (Matthews et al., 2001; Jorgensen et al., 2000; Sheeler et al., 2000; Inadera et al., 2000a, b; Andersen et al., 1999a; Kuiper et al., 1998; Perez et al., 1998;

Sohoni and Sumpter, 1998; Gaido et al., 1997; Krishnan et al., 1993). Direct uterine ER binding and activation was observed in ovariectomized transgenic ERIN mice after BPA exposure (Nagel et al., 2001). Examination of the IGF-I signaling pathway in CD-1 and αERKO mice also found that activation of this signaling pathway was dependent on ERα, suggesting that BPA was an agonist for ERα in vivo (Klotz et al., 2000). In addition, increases in uterine wet weight and epithelial and stromal height were reversed in B6C3F1 mice co-administered BPA and the synthetic ER antagonist ICI 182,780, providing additional evidence that these changes were likely mediated through ERs (Papaconstantinou et al., 2000).

Although studies have demonstrated that BPA is capable of acting as an ER agonist in both in vitro and in vivo assays, the potential for BPA to influence estradiol bioavailability, for example, (thus contributing to its estrogen activity) has not been directly examined. BPA has been shown to influence mammalian cytochrome P450 enzymes involved in the metabolism of estradiol (Takeshita et al., 2001; Niwa et al., 2000). Changes in ER expression following BPA exposure have been observed in vivo (Funabashi et al., 2001; Khurana et al., 2000) and may also represent another indirect mechanism for influencing estrogenic activity.

The binding affinity of BPA for plasma proteins is important when examining the bioavailability of BPA in the plasma, in addition to that of endogenous hormones potentially displaced from carrier proteins. In competitive binding assays with estradiol and testosterone, BPA had a low binding affinity to the human sex-hormone binding globulin (hSHBG) (Dèchaud et al., 1999; Nagel et al., 1997). However, in adult human plasma, BPA increased the concentration of hSHBG-unbound estradiol or testosterone, indicating that BPA is a potent hSHBG ligand (Dèchaud et al., 1999). These findings suggest that BPA may have an additional mammalian MOA, to increase the bioavailability of free estradiol and testosterone in plasma, leading to an increase in estrogen and androgen action. In the assessment of BPA binding affinity to α -fetoprotein, BPA did not displace estradiol in rat amniotic fluid (Milligan et al., 1998).

Mammary tissue is also sensitive to both direct and indirect estradiol signaling via epidermal and transforming growth factors during development and via prolactin and progesterone in pregnancy maturation, effects mediated through ERα (for review, see Couse and Korach, 1999). Mammary gland alterations at the cellular and physiological levels after BPA exposure (Markey et al., 2001b; Colerangle and Roy, 1997) provide additional support for the estrogen agonist MOA of BPA.

Estradiol plays an important role in female puberty onset and progression; exogenous estradiol exposure can lead to an advanced pubertal onset (Andersen et al., 1999a). BPA exposure affected pubertal changes in only a few rodent assays (Markey et al., 2001a; Howdeshell et al., 1999; Ashby and Tinwell, 1998). The timing of vaginal opening, a marker of puberty onset, was advanced only after high subcutaneous doses in one rat strain (Ashby and

Tinwell, 1998). CD-1 mice exposed peripubertally to three treatments of BPA at 0.1 or 0.5 mg/kg exhibited an advance in the timing of vaginal opening (not statistically significant at 0.5 mg/kg) and increased body weight (not statistically significant at 0.1 mg/kg) (Markey et al., 2001a). A subset of female CF-1 mice exposed prenatally to BPA also exhibited increased pubertal body weight and a shorter interval between age at vaginal opening and first estrus for female fetuses positioned between 0 males in the uterine horn (0M) and fetuses positioned next to one female and one male in the uterine horn (1M) (Howdeshell et al., 1999). However, another study did not find any changes in pubertal effects following similar experimental protocol using CF-1 mice, suggesting strain sensitivity differences (Ashby et al., 1999).

Estrus cycling is another physiological process regulated through the HPG axis and influenced by estrogens (Hadley, 2000; Andersen et al., 1999a). Alterations in estrus cycling in rats exposed to BPA suggest the ability of BPA to interfere with this system (Laws et al., 2000; Rubin et al., 2001). Estradiol exposure affects estrus cycling in Long-Evans rats, but the types of changes observed were slightly different than in BPA-exposed females; most of E2-exposed animals had extended diestrus suggesting pseudopregnancy, whereas BPA-exposed rats had either extended diestrus or estrus, suggesting possible subtle mechanistic differences between estradiol versus BPA (Laws et al., 2000). Other effects of BPA on the HPG include increased prolactin (a gene directly responsive to estradiol) levels (Goloubkova et al., 2000; Steinmetz et al., 1997), increased ER expression (Khurana et al., 2000), and decreased LH levels (Rubin et al., 2001) in vivo that are consistent with estradiol-mediated changes. However, the elucidation of the BPA MOA is complicated by the fact that the regulation of the HPG is elaborate and its development and homeostasis are influenced by many neurological and hormonal responses, including feedback activities.

Decreased thyroidal activity can also affect estrus cycling (Gray et al., 1997) and is another hormonal system regulated through the hypothalamus and pituitary system. Mammalian assays designed to assess thyroid activity have not been performed with BPA.

Determining whether the MOA for BPA in males is an estrogen agonist is more difficult owing to a lack of defined phenotypic markers specific for detecting atypical estrogenic activity in males. Body weight is an endpoint that is decreased in males in response to exogenous estrogens and is described as possibly the "most sensitive response of the male rat to an estradiol substance" (Gray et al., 1997). However, male body weight can also be decreased by exposure to antiandrogens, increased by exposure to androgens, and affected by other endocrine and nonendocrine mechanisms, including general organism toxicity (Gray et al., 1997).

Body weight was assessed in rodents exposed to BPA at different life stages. Decreased birth weight was observed only in offspring exposed to high doses of BPA in utero (Kim et al., 2001; Morrissey et al., 1987; Hardin et al., 1981), and in one study a decrease specifically in male offspring was observed (Kim et al., 2001). Body weight decreases were likely the result of

a general toxic effect as animals also exhibited other signs of toxicity. Most studies found male offspring exposed prenatally to low doses of BPA had increased body weight or no change when examined from neonatal to adult ages (Rubin et al., 2001; Gupta, 2000; Ashby et al., 1999; Cagen et al., 1999a, b). Only one prenatal exposure study observed decreased body weight in 6-month-old males (Vom Saal et al., 1998; Nagel et al., 1997). Increased body weight was observed at weaning in offspring exposed to BPA in vitro as embryos prior to implantation, although this finding was not distinguished by sex (Takai et al., 2001). Male Wistar rats exposed to BPA peripubertally exhibited decreased weight in one study (Hanioka et al., 1998) and no change in another (Stoker et al., 1999), although differences in route of exposure (intraperitoneal versus subcutaneous) may explain the different findings. Body weight effects alone, after BPA exposure, do not provide strong evidence for differentiating between an estrogenic, antiandrogenic, or androgenic MOA due to different outcomes across studies, and body weight can be affected by both androgen and estrogen activity effects. However, the antiandrogenic MOA of BPA in the case of body weight was unlikely, as changes in female body weight (Howdeshell et al., 1999) were observed along with decreased male body weight (Vom Saal et al., 1998; Nagel et al., 1997) in studies using similar protocols. Antiandrogenic activity would be expected to affect only male body weight (Gray et al., 1997).

AGD is another endpoint that can be influenced by androgens in rodents; male mammals have a longer AGD than females (Gray et al., 1997). It is not clear whether estrogens can affect AGD. Increased AGD was observed in CD-1 males in one study, suggesting androgenic activity by BPA (Gupta, 2000), although other studies observed no changes in AGD in male or female offspring exposed to low doses of BPA in utero (Ramos et al., 2001; Rubin et al., 2001). Tyl et al. (2002) observed a statistically significant increase in AGD in females and no effect on AGD in males. The significance of the different outcomes is unclear.

Determining the effect of estradiol and environmental chemicals on male reproductive development is an ongoing area of research. Male accessory sex organ and gland development and weights are known to be responsive to androgens (Gray et al., 1997). Exposure to exogenous estradiol and synthetic estrogens during development can result in male reproductive organ structural effects in both humans and laboratory rodents (McLachlan, 2001; NRC, 1999; Gray et al., 1997). Additionally, changes in male accessory sex organ and gland weights were observed in mice after exposure to estradiol concentrations in excess of 23 pg/mL (estradiol concentration increases due to their uterine horn position next to females) (Howdeshell and Vom Saal, 2000; Nagel et al., 1997). Increased prolactin levels have also been associated with prostate inflammation and weight increases (Stoker et al., 1999). Recent research has suggested that accessory sex organ weight changes or an earlier onset of male puberty can be the result of an altered ratio of androgen and estrogen activity (Williams et al., 2001; Putz et al., 2001a; Putz et al., 2001b). The finding that estradiol benzoate exposure could advance the timing of puberty

in male Wistar rats suggested the possibility of an androgen agonist MOA for estradiol. However, this effect may be the result of downstream (indirect) effects of HPG changes. Increased testis weight and spermatogenesis have also been observed in response to hypothyroidism (Creasy, 1999) suggesting the possibility of a thyroid antagonism MOA.

Determining the MOA responsible for the male reproductive organ and gland effects after BPA exposure is complicated by differences in experimental results and uncertainties in the role of estradiol during normal male reproductive system development (Ramos et al., 2001; Williams et al., 2001; Putz et al., 2001a, b; Atanassova et al., 2000; Gupta, 2000; Kwon et al., 2000; Ashby et al., 1999; Fisher et al., 1999; Nagao et al., 1999; Stoker et al., 1999; Cagen et al., 1999a, b; Vom Saal et al., 1998; Nagel et al., 1997). Prenatal BPA exposure increased prostate weight in some rodent studies (Gupta, 2000; Stoker et al., 1999; Vom Saal et al., 1998). However, other studies did not observe significant changes in prostate weight (Kwon et al., 2000; Ashby and Lefevre, 2000; Ashby et al., 1999; Cagen et al., 1999a, b). Study results have also differed on epididymal weights: decreases (Nagel et al., 1997), increases (Ashby et al., 1999), and no changes (Cagen et al., 1999a, b) after prenatal BPA exposure have been reported. No change in epididymal weight was found in neonatal and peripubertal BPA exposure studies (Ashby and Lefevre, 2000; Nagao et al., 1999). Studies have also differed on the effect of BPA on testis weight (Atanassova et al., 2000; Ashby and Lefevre, 2000; Ashby et al., 1999; Fisher et al., 1999; Cagen et al., 1999a, b) and sperm production (Ashby et al., 1999; Cagen et al., 1999a, b; Vom Saal et al., 1998).

Some of the in vivo responses observed after BPA exposure could suggest effects on androgen activity that could be mediated via direct interaction with the androgen receptor or through a number of other effects on androgen activity (e.g., effects on androgen bioavailability). Mechanisms that would affect estradiol bioavailability could also affect androgens. In vivo rodent studies have demonstrated changes (decreases and increases found) in testosterone levels after BPA exposure (Tohei et al., 2001; Williams et al., 2001; Takao et al., 1999). Steroidogenesis effects, particularly via cytochrome P450 inhibition, are a common mechanism of a number of different environmental endocrine disruptors (Gray et al., 1997). BPA can alter the functioning of numerous rodent and human cytochrome P450s, including isoforms involved in testosterone metabolism (Takeshita et al., 2001; Cannon et al., 2000; Hanioka et al., 2000, 1998; Niwa et al., 2000). BPA displacement of testosterone from plasma proteins has also been observed (Dèchaud et al., 1999). Testosterone synthesis could also be inhibited directly in the Leydig cells, as suggested by an in vitro assay that found BPA interfered with the signaling of LH receptors resulting in progesterone release decreases (Nikula et al., 1999). Testosterone is also influenced through this pathway, and decreased testosterone levels were attributed to a direct effect of BPA on the testes in one study (Tohei et al., 2001).

Another possibility is that changes in androgen receptor expression in the prostate could explain the underlying androgen activity effects (Ramos et al., 2001; Gupta, 2000). The androgen activity effects could be related to the estrogenic activity of BPA. For example, androgen receptor expression can be affected by exposure to estrogenic substances (Williams et al., 2001; Gupta, 2000), and testosterone levels are also regulated through the HPG, which is responsive to estrogens. Increased ER expression and prolactin levels were observed in male rodents after BPA exposure (Tohei et al., 2001; Khurana et al., 2000; Stoker et al., 1999), suggesting that BPA can affect the male HPG through estrogenic mechanisms.

Inconsistent findings of opposite responses in male body, testis, and accessory sex organ and gland weights may be the result of variability in the interactions of BPA on one or multiple MOAs that indirectly affect androgenic activity or directly alter estrogenic responses. To elucidate MOAs by which BPA exerts the observed responses in male rodents, molecular endpoints specifically responsive to individual hormonal activities need to be characterized and examined after BPA exposure.

The mammals were the most extensively studied for effects following BPA exposure. These studies encompassed the widest variety of endpoints, including those representing molecular to physiological responses. However, characterizing the MOA responsible for the effects associated with BPA exposure was complicated by the large percentage of studies that examined molecular responses only sensitive to estrogenic activity and in vivo effects at higher levels of biological functioning that are influenced by many hormonal and nonhormonal mechanisms. Studies of reproductive tract changes after BPA exposure in female rodents provide strong evidence that BPA can act as an estrogen agonist in vivo. Mammary gland and estrus cycling changes also support the estrogen agonist MOA for BPA. In male rodent assays, effects were observed that could be mediated through affects on estrogen or androgen activity MOAs. More detailed molecular studies will be needed to conclusively elucidate these MOAs. In vitro studies suggest BPA lacks androgen receptor agonist or antagonist activity, suggesting that if BPA does affects androgen activity, then it does so through an androgen bioavailability MOA. Hypothyroidism has also been shown to affect estrus cycling and male reproductive parameters (Gray et al., 1997) and is a hormonal system not directly examined in the mammalian assays described herein. The data set for mammals, although limited to two rodent species, is relatively large, includes mechanistic and in vivo studies, and the role of estrogens in development and sexual differentiation in females is well-understood. Thus, the WOE supports the estrogen agonist MOA although additional MOAs may be operative in mammals.

4.1.3. Sensitive Species and Endpoints

Based on the physicochemical properties of BPA and its presence in some surface waters and sediment, aquatic organisms are likely to be exposed to BPA (Staples et al., 2000, 1998; Lee

and Peart, 2000; Rudel et al., 1998). The nonbiting midge, *C. riparius*, and prosobranch snails, *M. cornuarietis* and *N. lapillus*, were the most sensitive of the invertebrate species to effects following exposure to low concentrations of BPA (Watts et al., 2001; Oehlmann et al., 2000). *C. riparius* exhibited delays in adult emergence after exposure to BPA at concentrations as low as 0.01 (females) and 0.078 μg/L (males) BPA, suggesting BPA affected developmental processes in this species (Watts et al., 2001). Sexual differentiation and gametogenesis were affected at a BPA concentration of 1 μg/L in *M. cornuarietis* and *N. lapillus* with increased mortality observed at the same concentration in *M. cornuarietis* (Oehlmann et al., 2000). Like prosobranch snails, the copepod *A. tonsa* exhibited increased egg production after exposure to BPA at 20 μg/L but not at lower concentrations (lowest tested, 0.2 μg/L) (Andersen et al., 1999b).

BPA studies in nonmammalian vertebrate species observed effects at relatively low concentrations of 2 to 50 μg/L (Kwak et al., 2001; Metcalfe et al., 2001; Sohoni et al., 2001; Kloas et al., 1999; Andersen et al., 1999b). The amphibian *X. laevis* exhibited an increase in female phenotypes after posthatch to metamorphosis exposure to 0.1 μM (22.8 μg/L) but not at 0.01 μM (2.28 μg/L) BPA (Kloas et al., 1999). In male fish, ovatestis histology was observed after exposure to ~10 μg/L, the minimum concentration tested, and decreased sperm counts and testicular morphological abnormalities, including fibrosis, at 50 μg/L in Japanese medaka (*O. latipes*) (Metcalfe et al., 2001). Male fathead minnows (*P. promelas*) exhibited a change in the proportion of sex cell types in the testes at 16 μg/L BPA (Sohoni et al., 2001). Sword length, a secondary sex characteristic, was decreased in swordtail fish (*X. helleri*) after exposure to 2 μg/L but not at 0.2 μg/L BPA (Kwak et al., 2001).

The effects observed in invertebrate and nonmammalian vertebrate species following BPA exposure suggest that sexual differentiation, maturation, and gametogenesis are some of the endpoints most sensitive to BPA. Sexual differentiation was altered by life cycle exposure to BPA in female *M. cornuarietis* (Oehlmann et al., 2000). In addition, female and male two snail species exposed to BPA as adults exhibited genital tract effects suggestive of alterations in sexual maturation. Gonadal sexual differentiation, and possibly sexual determination, was also affected in response to BPA in *X. laevis* (Kloas et al., 1999) and Japanese medaka (Yokota et al., 2000; Metcalfe et al., 2001), as observed by ovatestis histology and sex ratio shifts toward females. Gametogenesis changes in females were found in advanced timing of oogenesis in Japanese medaka (Metcalfe et al., 2001) and *N. lapillus* (Oehlmann et al., 2000) and in increased egg production in *A. tonsa* (Andersen et al., 1999b) and prosobranch snails (Oehlmann et al., 2000). Decreased sperm counts were also observed in three piscine species (Metcalfe et al., 2001; Sohoni et al., 2001; Haubruge et al., 2000; Yokota et al., 2000), and the number of males with sperm stored decreased in *N. lapillus* (Oehlmann et al., 2000).

As BPA has been measured in environmental water samples at 1 μg/L (Staples et al., 2000; Rudel et al., 1998), there is the potential for effects to aquatic organisms in the wild. Of the invertebrate and nonmammalian vertebrate species evaluated in laboratory studies, the aquatic invertebrates, amphibians, and bony fish were the most sensitive to developmental and reproductive effects from BPA exposure, especially since the observed effects could potentially lead to population-level effects. Although decreased reproductive success was observed only after exposure to high BPA concentrations (minimum of 640 μg/L) (Sohoni et al., 2001; Shioda and Wakabayashi, 2000a, b), few studies evaluated reproduction in piscine species. Altered sex ratio (Kloas et al., 1999) and genital organ differentiation and maturation (Metcalfe et al., 2001; Oehlmann et al., 2000) effects, after exposure to BPA below 22.8 μg/L in amphibian, gastropod, and piscine species, have the potential for population-level effects. The multiple reproductive effects observed in *M. cornuarietis* and *N. lapillus* prosobranch snails after exposure to BPA at 1 μg/L, including sterility and increased mortality (Oehlmann et al., 2000), are likely effects that could lead to population-level changes to gastropod species in the wild. Gastropod species exposed to another environmental contaminant, tributyltin, exhibited alterations in sexual differentiation resulting in imposex genitalia, and imposex led to decreased reproduction and increased mortality in at least 45 gastropod species that subsequently initiated widespread population-level changes (Ankley et al., 1998; Tyler et al., 1998; LeBlanc and Bain, 1997).

Sensitive mammalian endpoints include mammary gland differentiation at 25 μg/kg-d BPA (Markey et al., 2001b), decreased paired ovary weights and increased AGD in F2 females at ~1 μg/kg/day (Tyl et al., 2002). Among these endpoints, mammary gland changes exhibited a consistent dose-response pattern and were consistent with the estrogen agonist MOA.

4.2. DATA GAPS AND RESEARCH NEEDS

4.2.1. Role of Steroid Hormones in the Development of Nonmammalian Vertebrates and Invertebrates

One of the largest data gaps for evaluating the MOA for BPA in nonmammalian species is the lack of knowledge of vertebrate steroid hormones and their role in normal development and reproduction, and determination of whether there are analogous signaling pathways in invertebrate species. Although a number of species have been shown to be sensitive to estrogen exposure, the MOA has not been clearly defined for many cases. Research to further elucidate the roles of steroid hormones in nonmammalian species is needed. The knowledge gained in understanding normal physiological processes and comparative endocrinology will assist in predicting effects and MOA from environmental hormones. Characterizing the role of estrogens in invertebrate development, in particular, is critical to understanding the potential for BPA and other environmental estrogens to affect these processes and to conclusively determine the MOA

for BPA in various species. In addition, studies exploring the potential action of BPA through the ecdysone receptor system are needed.

4.2.2. Developmental Susceptibility to BPA Exposure and Population Effects in Wildlife

Because available studies that examined the same endpoints in the same species after exposure at different life stages are limited, studies to assess life stage sensitivity to BPA are needed. Field studies to examine the long-term species survival effects and associations with gonadal histological changes are important to determine risk to aquatic organisms in the wild. In laboratory studies, the sensitivity of gastropod and copepod species to BPA exposure warrants further investigation into the effects of BPA in those and other species of invertebrates (Oehlmann et al., 2000; Andersen et al., 1999b). The limited number of BPA exposure studies conducted on nonmammalian species precluded any conclusive inference on relative life stage sensitivity.

Amphibians, *X. laevis*, and bony fish, *O. latipes*, exhibited effects on sexual differentiation after exposure to BPA concentrations below 100 μg/L beginning at posthatch (Metcalfe et al., 2001; Kloas et al., 1999). Further low-dose testing in additional vertebrate species is needed. Ovatestis histology, decreased sperm counts, and growth effects occurred in Japanese medaka exposed to BPA during early life and posthatch stages but were observed at lower concentrations in fish exposed during posthatch only (Metcalfe et al., 2001; Yokota et al., 2000). Greater sensitivity to BPA exposure during posthatch in piscine species could be explained by studies in rainbow trout (*O. mykiss*) that demonstrated gonadal differentiation occurs during this life stage (Feist and Schreck, 1996).

4.2.3. Development of New Nonmammalian Endpoints and Biomarkers for Estrogen Agonist Activity

The use of vitellogenin as a biomarker of estrogenic exposure may not be sensitive enough for BPA studies. In *O. mykiss*, vitellogenin was detected after exposure to 500 μg/L (Lindholst et al., 2000), but developmental effects were observed at ~10 μg/L in *O. latipes* (Metcalfe et al., 2001). In vitro molecular expression assessments need to be expanded beyond vitellogenin to understand the estrogenic and other potential MOAs of BPA in nonmammalian species (Ankley et al., 1998). Additional studies assessing the estrogen agonist activity of BPA in fish species should include assays for vitellogenin and zona radiata protein induction as well as histological and morphological examinations to determine whether estrogen-responsive genes are predictive biomarkers for estrogen agonist exposure. Endpoints for MOAs other than estrogen agonism are needed.

4.2.4. Development of New Mammalian Endpoints and Biomarkers for Estrogen Agonist Activity

To resolve whether BPA exposure leads to low-dose effects, it is necessary to determine the most sensitive indicators of estrogen agonism within and across species (and, perhaps, to develop more sensitive predictive endpoints). Whether effects after low dose BPA treatment were observed could either reflect a lack of response, evaluation of less sensitive endpoints, and/or exposure intervals did not correspond to sensitive or critical windows for BPA for a particular species. Mammary gland changes appear to be the most sensitive endpoint for BPA exposure regardless of the development stage of exposure. Mammary gland changes were observed at low BPA doses (at 25 and 250 μg/kg in CD-1 mice and at 0.1 mg/kg in Nobel rats) exposed prepubertally (Markey et al., 2001b; Colerangle and Roy, 1997). There is still the potential that even lower doses of BPA may affect this sensitive tissue and, thus, lower doses need to be tested. Sex-specific changes in other behavioral and neuroendocrine responses have shown that these endpoints are highly sensitive to exogenous estradiol and environmental hormone exposure (Gray et al., 1997). Behavioral and neuroendocrine effects, including pubertal and estrus cycle alterations, occurred in both males and females in response to low-dose exposure to BPA during prenatal (Rubin et al., 2001; Farabollini et al., 1999), neonatal (Atanassova et al., 2000; Khurana et al., 2000), peripubertal (Markey et al., 2001a), and adult (Steinmetz et al., 1997) life stages. However, altered reproductive behavior was not observed after prenatal BPA exposure in females (Kwon et al., 2000) and after neonatal exposure in males (Nagao et al., 1999).

Another data gap is an understanding of the estrogen agonist and other possible alternate MOAs in vertebrates. In part, this gap could be aided by in vivo studies assessing the effect of BPA on estradiol as well as other hormone levels.

4.2.5. Resolving Different Findings Among Mammalian BPA Studies

A large body of data supports an estrogenic MOA for BPA in rodents. However, studies that examine whether BPA is affecting additional MOAs in mammals are needed. In particular, alterations to other hormone levels are other possible MOAs by which EDCs can act. Decreased testosterone levels were observed in the one in vivo study that examined effects to steroid hormone levels following BPA exposure (Takao et al., 1999). Future BPA studies need to include testosterone and other hormones as well as estradiol levels in their evaluations. Potential actions of BPA through androgen, and SXR/PXR receptor systems are still areas in need of further investigation.

Studies are needed to resolve differing findings observed in many of these studies (Tables 7). Prenatal BPA exposure studies could examine dose-response relationships with a broader endpoint evaluation including molecular and mechanistic responses associated with in vivo

endpoint assessment, such as increased prolactin levels observed with prostatitis in adult males exposed to BPA peripubertally (Stoker et al., 1999).

The mechanisms to explain the observed rodent species- and strain-specific effects should be investigated (NTP, 2001), and these differences could provide information about related mechanisms in humans. Previous research findings have also provided direction for the choice of rodent species and strain. Considerable similarities in reproductive tract development exist between mice and humans (Cunha et al., 1999). In particular, CD-1 mice exposed prenatally to DES have exhibited numerous adverse effects that are consistent with those observed in humans exposed to DES in utero (Newbold and McLachlan, 1996; Newbold, 1995). Other rodent species and strains have also shown adverse effects in limited reproductive endpoints similar to those observed in humans following prenatal exposure to DES (Khan et al., 1998; Hendry et al., 1997; Plapinger and Bern, 1979), which may prove useful in elucidating common mechanisms of endocrine disruption. Considerations should also include strain responses to other hormonal mechanisms that can be influenced by synthetic chemicals, such as thyroid functions.

4.2.5.1. *Sensitive Mammalian Developmental Stages and Critical Windows of Exposure*

Examining the BPA concentrations that lead to effects and comparing developmental stages of exposure were complicated by differences in endpoints evaluated, rodent strains, doses and routes of exposure, and the fact that, in similar studies, differing results were sometimes reported (Table 7, see footnotes). Comparable studies at low doses and with exposure at the many possible sensitive developmental times are needed to determine the critical windows of exposure in mammals and other species.

4.2.6. BPA Exposure Studies

Overall, a need exists for BPA exposure studies in wildlife species. Currently, measurements of BPA in the environment are inadequate to determine the geographic scope and relative levels of BPA environmental contamination. The available studies indicate that BPA concentrations measured in water and sediment samples in parts of Canada, Korea, and Austria (Fürhacker et al., 2000; Lee and Peart, 2000; Khim et al., 1999) may be high enough to elicit effects in certain aquatic species.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. BPA CROSS-SPECIES MODE-OF-ACTION CASE STUDY CONCLUSIONS

BPA in vivo developmental and/or reproductive effects data were identified in 16 animal species represented by three phyla of the animal kingdom (Figure 4). Therefore, the total number of species and the coverage across phyla are limited and patchy. Although limited, the effects data indicate that BPA can elicit developmental and reproductive effects in vertebrates and invertebrates.

The estrogen agonist MOA is well characterized for BPA based on mechanistic studies of vertebrate species, and this MOA can explain the majority of the observed effects across vertebrate species. For the invertebrate species with data, some of the sexual differentiation effects were consistent with an estrogen agonist or estrogen-analog MOA. For insects, some data are consistent with an ecdysone antagonist MOA. However, without knowledge of the signaling pathways responsible for the endpoints in invertebrates and additional studies, a definitive MOA determination for BPA cannot be made. The MOA for BPA in mollusks and arthropods is unknown. Therefore, for invertebrate species, there are not enough studies, species with data, or the basic mechanistic understanding of the affected endpoints to draw any conclusions or make predictions about MOA for untested invertebrate species.

Together, these studies demonstrate the ability of BPA to directly bind to the ERs in a number of species (humans, rats, mice, chicken, green anole lizard, rainbow trout, common carp, and African clawed frog) representing five classes of vertebrates (mammal, avian, reptile, bony fish, amphibian). BPA exhibited a higher ER affinity in nonmammalian species than in mammals (Table 2). The binding affinity values suggest that BPA has a higher affinity for the ERα homologous receptor tested in nonmammalian vertebrates, including rainbow trout, green anole, and chicken versus ERα in mammals (Matthews et al., 2000). Adverse effects observed across the vertebrate species reported here are generally consistent with an estrogenic MOA. However, some affected endpoints were not explained by an estrogen agonist MOA. Other possible MOAs include androgen agonist, androgen antagonist, thyroxine agonist, steroid hormone bioavailability (covering a number of specific MOAs), and nonendocrine MOAs. In addition, a number of endpoints, such as male rodent reproductive effects, are not clearly explained by a particular MOA because the effects of exogenous estrogens on male development are not well understood. Since there is some evidence of other MOAs for some vertebrate species, a strong research recommendation is to explore the possibility of multiple BPA MOAs for chordates and whether different or multiple MOAs exist under certain exposure conditions (e.g., high dose vs. low dose; exposure during critical windows of development). Given these caveats, the estrogen agonist MOA would be predicted for untested species within the chordate

phylum. Within the vertebrates, there would be greater confidence for predicting the estrogen agonist MOA for an untested mammal or fish species compared with an untested amphibian or bird species.

5.2. WHAT DID WE LEARN?

The project goals as delineated in Section 2.2 (to develop an approach to use MOA information across species to support integrated ecological and human health assessment and to perform a case study assessing the utility of the approach) were accomplished. The subobjectives (to determine whether the MOA data can be used to predict effects in untested species, to determine the extent to which MOA data across phylogenetic relationships can support cross-species extrapolation of risk, and to use MOA data to identify the most sensitive life stages and species (for application to site-specific or media-specific risk assessments) as a method to protect all potentially exposed species) were accomplished to differing degrees of detail.

This report establishes that the cross-species MOA assessment approach is promising for predicting the MOA for a toxic agent for species without data, when there are data on a number of related species. The basis for predicting the MOA can be an established phylogenetic relationship. However, the strength of evolutionary closeness that would predict the same MOA depends partly on the number of species with MOA information within a given evolutionary grouping. For example, in the case of BPA, there is a strong basis for predicting the estrogen agonist MOA for untested fish species but not necessarily for amphibians. This approach, in turn, could be applied to make MOA and effects predictions for species without data in human health assessments, and chemical prioritization, especially for chemical screening and testing programs that include concern for wildlife, such as EPA's Endocrine Disruptor Testing Program (http://www.epa.gov/scipoly/oscpendo/index.htm).

Based on the experience gained in this case study, a cross-species MOA assessment is a promising method for supporting the goal of integrated risk assessment when there is an appropriate data set across species for a toxic agent. The cross-species effects assessment is an excellent method for assessing relative sensitivities among species with data. For example, identifying the most susceptible species, among tested species, and predicting other possible susceptible species has great value in an integrated or ecological assessment.

An important question was raised by performing this case study: What is an appropriate data set for use of this method? Data set needs are partly determined by the scope of the assessment. For example, if the approach was being applied to a human health assessment, then the species could be limited to mammals. The minimum data set needs include the following: (1) MOA information for more than one species within the phylogenetic grouping (e.g., animal

kingdom, mammals) of interest to the assessment; (2) species relatedness information; and (3) in vivo effects data for more than one species within the phylogenetic grouping of interest.

Beyond the project goals, this report meets an additional objective. In reviewing and analyzing the MOA data for BPA across taxa in the animal kingdom, the report provides a template for an approach for future cross-species MOA comparative evaluations. The hope is that this template will be of value for future MOA human health work and for integrated risk assessment efforts.

5.3. RECOMMENDATIONS FOR FUTURE CASE STUDIES AND DEVELOPMENT OF RISK ASSESSMENT METHODS

5.3.1. Future Case Studies

Recommendations for selection criteria for a developmental and/or reproductive toxic agent for a future case study to explore a cross-species MOA analysis are as follows:

1. Optimize the amount of cross-species data by selecting an agent based on a relatively strong cross-species data set. This could be accomplished by performing literature searches (e.g., BIOSIS and PUBMED) and interviewing experts to identify agents for which effects data exist for the largest number of species.

2. Data for the largest number of species would not be the only criteria. Because the goal would be to have enough data to develop predictions about untested species, the optimum data clustering would be to have data for a number of species within one phylum. Thus, having a large number of species with fairly close evolutionary relatedness would enhance the possibility of using relatedness to predict MOA.

3. An agent that has relatively high potency should be selected. For example, ethinylestradiol may have been a better choice because it is a more potent estrogen agonist than BPA. In addition, there would be less controversy about effects at low doses and reproducibility when assessing a chemical with a relatively high potency.

4. An agent with a well-established MOA, although there may be a different MOA in different species, in invertebrate and vertebrate species should be selected. In addition, this should be an agent for which the effects and MOA data are consistent within a given species. This will minimize uncertainties in the analysis.

5.3.2. Development of Methods for Integrated Risk Assessment and Use of MOA Information in Risk Assessment

The integrated assessment aims to provide more than the sum of its parts, i.e., a more complete picture of risk than either the ecological and human health assessments alone. Future development of methods for integrated ecological and human health risk assessment will need to address issues about critical qualitative and quantitative risk assessment steps. For example, how

will the reference dose be determined when looking across species? Will this be determined by identifying the lowest exposure level eliciting a detectable biological effect in the most sensitive endpoint in the most sensitive species? In order to answer these questions the purpose of the individual assessment, developed in the problem formulation phase, needs to be considered. Depending on the purpose of an assessment, the MOA information could be used to limit the species of concern in the assessment. For example, different MOAs may correspond to different phyla (e.g., ecdysteroid receptor agonists might be expected to be primarily a concern for arthropods).

The cross-species approach presented in this report could potentially be used in human health assessments as well. For example, the cross-species MOA information provides the human health risk assessor with the range of effects observed across species for a chemical. This information may allow the assessor to consider human relevancy of the effects in various species used in testing and this information may, in turn, aid in selecting which effects to evaluate in the assessment. Additionally, if gaps exist in the assessment database, the MOA information may aid in predicting effects. Finally, cross-species MOA information could possibly aid in the context of the dose-response assessment by developing better physiologically-based pharmacokinetic (PBPK) models for considering intra- and interspecies variability and high to low dose extrapolations.

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APPENDIX:
RESPONSE TO EXTERNAL PEER REVIEW OF THE DRAFT REPORT ENTITLED *AN INTEGRATED HUMAN HEALTH AND ECOLOGICAL SPECIES EFFECTS ASSESSMENT: A CASE STUDY OF BISPHENOL A*

Major External Reviewer Comments Summarized
(External Reviewers: George Daston [GD], Peter deFur [PdF], and Frank Welsch [FW])

Charge question	Reviewer's initials	Comment/Suggestion	Response
1	GD	Good template (organization) for the report	*No response or change necessary.*
1	GD	Needs better articulation of project goals in terms of the practical utility of mode-of-action (MOA) cross-species information. How will it be used? Need to know where this effort is going. State objectives more clearly on p. 11, in addition to stating limits of project. "Set up objectives in ascending scale of difficulty: 1. To determine for the species used in human and ecological risk assessment (RA), whether right endpoints have been evaluated for BPA's putative MOA; 2. To determine whether the MOA data are strong enough to predict the nature of effects expected in untested species; 3. To determine the extent to which MOA data can support cross-species extrapolation of risk and how far in the phylogenetic relationships they can predict risk; 4. Use MOA data to do integrated RA for a given medium (e.g., water would be relevant for BPA) or location that could protect all species potentially exposed." At end of report, draw conclusions for each of the four objectives.	We agree with the reviewer's comment with the exception of adding suggested subobjective 1. Broke down the objectives (p. 11) into subobjectives. Moved statement of limitations of project to this section. In the discussion section, addressed the conclusions for each subobjective.

Charge question	Reviewer's initials	Comment/Suggestion	Response
1	PdF	1. Figure 4 is ignored. 2. Who is audience? Report reads as if for only scientist audience. 3. Presentation of conflicting results for mammalian repro effects needs further clarity and education of reader. 4. Not enough explanation of the link between test animals and humans for BPA data.	1. Cited Figure 4 where appropriate. 2. Added statement of the audience early in Intro. The audience includes risk assessors, basic scientists, and policy makers. Added references to review articles where appropriate to educate across scientific disciplines. Reviewer provided a number of suggestions for invertebrate endocrinology reviews and citation of these has been added, where appropriate. 3. Addressed clarity in description of "conflicting results" (similar comment made by GD) and reworded to "differences in experimental results." 4. The meaning of this comment is not clear. However, have added language to note some known endocrinological differences between rodent species and humans.
1	FW	Generally clear presentation; very ambitious and ahead of what is possible due to risk assessment process; calls into questions selection of BPA for case study. Suggests doing a case study with ethinylestradiol.	Cannot address reviewer's comments, as we did already select BPA for reasons cited in the document. Disagree that BPA was a poor choice, given that the purpose of the case study was to illustrate an approach to performing cross-species MOA assessment to support integrated risk assessment. Agree with comment that ethinylestradiol is a promising candidate for a future cross-species MOA case study (this was stated in the Recommendations for Future Case Study section).
2a	GD	Valid approach; "often unacknowledged foundation of current cross-species extrapolation for hazard and RA." BPA good for case study because "estrogen receptors are highly conserved phylogenetically and steroid-mediated signaling is among the archetypal signal transduction pathways." Also, relevant because exposure to humans and wildlife.	*No response or change necessary.*

Charge question	Reviewer's initials	Comment/Suggestion	Response
2a	PdF	Valid approach for vertebrates; not yet for invertebrates.	Not sure what is meant by "not yet valid for invertebrates." Perhaps this comment refers to the fact that for a chemical that has an estrogen agonist MOA in vertebrates, the MOA in invertebrates is not well-defined, although some estrogen-like effects were observed.
2a	FW	Integrated approach seems "far removed from practical realities. We are still struggling with interspecies extrapolations regarding mammalian data of chemical exposure to single agents in risk assessment."	Added the possible benefit of performing the case study to the introduction. A cross-species MOA assessment may be promising for aiding cross-species extrapolation for mammals, which would be useful in human health assessments.
2b	GD	Approach holds promise for use as MOA predictive tool for untested species.	In the discussion, added description of data set needs for this approach to MOA cross-species assessment.
2b	PdF	Approach has promise if have established reference for link between tested and untested animals.	Added this statement to the discussion.
3	GD	Comprehensive, although some issues of accuracy in summary: P. 20, par. 2, lines 4–6: line 4 about LC50 values as less than 1 mg/L BPA, but lines 5–6 characterize the range of toxicity as 1.17–39.5 mg/L which is a range that does not include 1 mg/L; Indicate whether sword length was normalized to overall body length or some other index of animal size; P. 37, lines 2–3: add sentences about relevance (or not) of PXR agonist activity, given that there does not appear to be any cyp3a-mediated metabolism of BPA; Summary of NTP low-dose workshop needs to be that "the effects being measured in the studies in question are sufficiently variable, and the purported magnitude of effects so small, that they may be influenced by variables that are difficult or impossible to control" (pp. 32–33 of NTP report) and change Tyl et al. and NTP report conclusions were not opposite or different; P. 8, last two lines: change sentence to include non-receptor-mediated signaling; find primary sources for Ben-Jonathan and Steinmetz reference in line 5, p. 12, to support statements about commercial applications of BPA.	Addressed the comments by making suggested corrections as well as additional discussion items

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3	PdF	1. For nonmammalian vertebrates, literature is not as well covered as for mammals; for lower vertebrates, see chapters and books, such as Kendall et al., 1996; Mahaich et al., 1999; Roland et al., 1998; Sparling et al., 2000. 2. Invertebrate literature is only cursory. Oberdorster 2001 is not the reference for elucidating the role of ecdysone in molting in arthropods. Find original paper by looking at deFur et al., 1999; also, for other invertebrate endocrinology, see endocrine reviews by ES Chang and M Fingerman, and for endocrine disruptors see deFur et al., 1999; Pinder and Pottinger, 1998; need to include general literature on invertebrate endocrinology and endocrine disruptors in invertebrates.	Where appropriate, added citations to suggested references.
3	FW	Coverage of mammalian literature is reasonable. Referral to NTP 2001 report is not sufficient—see public comments of Final Report. There is not as much harmony in the outcome of the workshop as was stated. Read: Witorsch RJ. Low dose in utero effects of xenoestrogens in mice and their relevance to humans; an analytical review of the literature. Food Chem Toxicol 40; 905–912. Milman HA et al. Evaluation of the adequacy of published studies on the rodent prostate for use in human risk assessment. Regulatory Toxicol Pharmacol 35: 338–346, 2002.	Already addressed similar comment re NTP by GD (#3 above). Included two suggested references in report.
4	GD	Conclusions: Agrees w/conclusions: 1. WOE approach not explicit. 2. Regarding recommendations that multiple mechanisms of action should be explored (see p. 55), needs further comment. This section could be improved by a quantitative assessment of the data and even if don't do a quantitative assessment, state that this could be done. 3. P. 56 recommendations: add that the next case study should be done with one with a known MOA (even if other MOAs are possible) that is known to be present across taxa; example of ethinylestradiol fulfills this additional criterion.	1. Explicitly stated using a WOE approach in the introduction and discussion. 2. Stated that quantitative assessment is the next obvious step. 3. Addressed this comment by including a "criteria" section. 4. Addressed comment by including this data gap.

Charge question	Reviewer's initials	Comment/Suggestion	Response
		4. Data gaps section: add comparative toxicokinetics; toxicokinetics may play a large role in explaining greater or lesser sensitivity of particular species.	
4	PdF	Conclusions were that more research is required and research needs to focus on lower doses of BPA. Need to pull out more conclusions and need to stress that MOA across phyla one must compare BPA as a steroid and not specifically as an estrogen since not all phyla have estrogens but all have steroids; BPA action may be at level of steroidal receptor (e.g., EcR in insects and crustaceans).	Addressed comments (similar to GD 1) and incorporated suggested points.
4	FW	Conclusions flow logically. However, reviewer does not consider the exercise to have been successful.	Disagree with comment that exercise was not successful. Reviewer's reasons for finding the exercise unsuccessful hinge on the selection of BPA as the chemical for the case study. Other two reviewers commented: "valid approach," "approach has promise," and "good start to integrating ecological and human health assessments." See response (above) to FW comment to question 1.
5	GD	Add that this report meets two objectives: reviewing MOA data for BPA across taxa in animal kingdom and creating template for future comparative evaluations of MOA.	Added this suggestion in introduction and discussion. Also, see GD question 1 comment and response (above).
5	PdF	Report made the case for BPA as estrogenic chemical across some vertebrate species but stated goal was to go farther than vertebrates (*was it?)*; goal stated on pages 4 and 8 was never truly met. Discuss links of test animals against untested animals in a chart that lists similarities and differences among animals in endocrinology (i.e., steroid receptors, endpoints). See pages 4 and 8 to see if the goal can be redefined.	Added a comparison of endocrinology for species within the three different phyla discussed in this report. The suggestion of a table to compare tested and untested species is a very large exercise (there are a lot of untested species!), worthy of its own report.
5	FW	Original goal was not met (pp. 55–56). Overall, we have learned that the approach is not feasible (yet). And BPA has too many data gaps for MOA across phyla.	Reviewer's perspective appears to be a human health-centered (i.e., a narrower) viewpoint, whereas this case study is relevant to integrated ecological and human health assessment. Since reviewer suggests working on human health RA issues and not using BPA for the case study (which has already been done), these comments cannot be addressed. It appears that the reviewer missed the intent of the study; having data gaps in MOA information across phyla does not indicate that the analysis should not be conducted.

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6	GD	Approach has real potential; add some issues into discussion: add that the approach "has potential to provide info about nature of effects that the assessor would want to evaluate in order to ensure that database was adequate for RA. . . . If there are gaps in the database, the MOA data would provide the assessor with information that could allow the tailoring of subsequent studies." Would be good to add something about how useful cross-species MOA info could help quantitative RA, in the absence of pharmacokinetic data; for rodent to human use default uncertainty factors but for nonmammalian data would be hard to use. . . discuss this. Could MOA info be used to "limit the species of concern or ID the most susceptible species in an integrated RA?" Different MOAs may match up with different phylum. . . e.g., ecdysteroid receptor agonists might be expected to be primarily a concern for arthropods.	Addressed valuable comments by incorporating suggestions into the discussion.
6	PdF	Good start on integrating ecological and human health risks; 1. Too much science jargon 2. Reviewer is not sure whether or not an integrated RA can be done; integrated RA has to do more than select the most sensitive species of endpoint and more than comparing across phyla—it has to identify that which is not present in either human or ecological RAs alone.	1. Stated the conclusion of each section in less technical language and stated up front that this is a report for the science and policy expert (see PdF 1 comment and response). 2. No suggestion provided. It appears that the reviewer misunderstood the intent of the project. The purpose of the analysis was not to develop a full risk assessment.
6	FW	Premature approach; what is needed is higher degree of certainty that human health assessments for single agents are trustworthy—need better mammalian data avoid reliance on uncertainty factors. Poor choice of BPA; no agreement about how to extrapolate among mammals; no consensus about human BPA exposure levels among population.	Some relevant questions raised for discussion (but not answered) have been included in the discussion: 1. What numbers will be used for arriving at reference doses to be applied? 2. Is it going to be the exposure level eliciting a detectable biological effect on what appears to be the most sensitive endpoint in the most sensitive species of the most sensitive phylum that sets the stage? How do the scientists who are proposing the pursuit of the holistic approach to integrated risk assessments envision those critical steps?

Charge question	Reviewer's initials	Comment/Suggestion	Response
			While these are important questions for integrated risk assessment, they cannot be answered since the development of integrated risk assessment methods is in the early stages. It appears the reviewer may have misunderstood the goal of the case study, which was to develop a method for cross-species MOA assessment and not to perform a full integrated risk assessment. Therefore, the title of the document has been changed to "A cross-species mode of action assessment method: A case study of bisphenol A," and the project description in the introduction has been clarified.