Commentary: Setting Aside Tradition When Dealing with Endocrine Disruptors

Theo Colborn

Abstract

In 1996, the US Congress directed the Environmental Protection Agency to produce screens and assays to detect estrogenic and other endocrine-disrupting chemicals in food and water. To date, there are none. Years have been wasted in attempts to utilize traditional toxicological approaches to solve the problem, when in retrospect, it is now apparent that the delay in part stems from the reluctance to attack the problem with entirely new approaches. To develop new testing protocols, it is necessary to set aside much of the dogma of toxicology and to begin again with open minds. A few pertinent examples are provided concerning what has been overlooked and what needs to be done. In particular, it is necessary to give close attention to the selection of animal strain and diet, factors that were only loosely controlled historically when one takes into consideration what has been learned in the last decade. Vast numbers of animals have been sacrificed, and more will be sacrificed, in futile attempts to validate assays and to develop safety standards unless knowledge gained over the past decade concerning the sensitivity and complexity of the endocrine system is taken into consideration.

Key Words: animal strain; bisphenol A; diet; dioxin; endocrine disruptors; low-dose phytoestrogens; testing protocols

Background

It has been nearly 8 yr since the US Congress directed the Environmental Protection Agency (EPA[1]) to produce screens and assays to detect estrogenic and other endocrine-disrupting chemicals in food and water (USC 1996a,b). It was acknowledged by the scientific and regulatory communities that traditional toxicological testing had overlooked chemicals that could penetrate the womb environment and interfere with the development of the embryo and fetus. At that time, some developmental assays were in place, but they did not focus on endocrine-disrupting mechanisms or their impacts on function. Yet to date, not one screen or assay has been validated or standardized to meet this mandate. Years have been wasted in attempts to utilize traditional toxicological approaches to solve the problem, when in retrospect, it is now apparent that the delay in part stems from the reluctance to attack the problem with novel approaches. Instead of utilizing what is already known about the role of the endocrine system in development and integrating this knowledge with directed science from a broad scope of disciplines, there has been a tendency to hang onto the very protocols that missed endocrine disruption in the first place. It is now apparent that many traditional toxicological and statistical approaches must be set aside for EPA to implement its mandate successfully.

In this commentary, I review some of the knowledge gained in laboratories around the world since the early 1990s that has revealed the depth of the complexity and sensitivity of the endocrine system and why rigid control of the conditions under which assays are carried out is imperative when testing for endocrine disturbances. I focus on two examples comprising evidence that to develop, validate, and standardize tests for detecting endocrine disruption, it is critical to give extremely close attention to the selection of animal strain and diet and to keep endocrinological considerations in the forefront of the design.

Letting Go of Tradition

The development of new testing protocols will continue to be stifled by the dogma of toxicology unless the task is tackled with open minds willing to incorporate what has been discovered about endocrine-disrupting chemicals over the past decade. A great deal of research has taken place since 1996 as the result of "thinking outside the box." New information ranges from the molecular to the whole animal level—even the population level—and focuses on the effects of high-profile chemicals for which only equivocal results concerning their toxicity previously existed. These new studies have provided not only new knowledge regarding embryonic and fetal development, but also unique insight regarding the necessity to control the conditions under which assays must be designed to detect endocrine disruptors. In some studies, old toxicological approaches were greatly modified and entirely new, exquisitely delicate laboratory techniques were developed to detect impaired development and function. As these new-design studies

Theo Colborn, Ph.D., is a Professor of Zoology at the University of Florida, Gainesville, Florida.

[1]Abbreviations used in this article: BPA, bisphenol A; DES, diethylstilbestrol; EPA, US Environmental Protection Agency; LH, luteinizing hormone; ME, metabolizable energy; VO, vaginal opening.

continued to probe deeply into the mystery of animal development, more and more was discovered about the processes that lead to normal development. And in synchrony with this new knowledge, the new protocols are also detecting the slightest yet significant developmental changes expressed in response to specific chemicals—changes that can take place very early in development and that have long-term implications for the phenotype and health of the animals. From these studies, new enlightenment has emerged about how testing protocols should be designed and how screens and assays should be validated and standardized.

Paradigm-breaking Approaches

The first breaks in tradition came when study designs took into consideration the exquisitely low concentrations at which signaling chemicals control how the endocrine system functions. Better methods of quantification revealed that only the free hormones and free chemicals in the body initiate developmental and functional responses. This revelation led to the need to determine the amount of free (active) hormone or test chemicals in the study system in an effort to distinguish them from the total amount that is present but "bound to" internal tissue or protein delivery systems.

The new studies also took into consideration the negative feedback activity of the endocrine system that shuts down hormonal responses to maintain homeostasis. Many studies confirmed the inverted U response that classical endocrinology acknowledges (Duft et al. 2003; Gupta 2000; Jobling et al. 2003; Markey 2001a; Takai 2000; Talsness et al. 2000). These new studies demonstrated that exposure to a biologically active chemical within the range in which free hormones operate can have an entirely different suite of effects that change during progressive stages of development than when the same chemical is administered in high doses after an individual has fully developed. Thus, setting standards using traditional high-dose testing with adult animals and extrapolating down to a no-effect level does not adequately protect future progeny (see Sheehan [2000] for an in-depth discussion of why there cannot be a no-effect level when measuring an estrogen response). Some of the new studies tested a broad range of doses to avoid missing the effects of low-dose exposure and to avoid Type II errors. The studies confirmed that endocrine effects are time specific, chemical and/or hormone specific, and dose related.

Differences of Low-dose Effects

Dioxin provides an excellent example of the importance of incorporating endocrinological considerations in a study when determining the health impacts of a chemical. In a 1991 study (Mably et al. 1991), the administration of the usual high doses of dioxin were set aside, and instead, pregnant rats were exposed to a single low dose of dioxin consisting of 0.00, 0.064, 0.16, 0.4, or 1.0 μg of 2,3,7,8-tetrachlorodibenzo-p-dioxin/kg on day 15 of gestation. Their offspring were monitored for a nontraditional list of innovative and occult endpoints, which provided a profile about the adverse health effects of dioxin in the absence of clear signs of clinical illness. The results were remarkable and disturbing. Changes in responses were quantified for a total of 21 endpoints, confirming that dioxin had significantly and irreversibly altered the development of the reproductive system, sexual development, and behavior of the in utero-exposed rats (Peterson et al. 1992). Changes included the following: decreased anogenital distance, time to testis descent, plasma luteinizing hormone (LH^1), seminal vesicle weight, ventral prostate weight, testis weight, epididymis weight, sperm cauda epididymis weight, daily sperm production, and seminiferous tubule diameter; increased mount latency; intromission latency; ejaculatory latency, number of mounts, number of intromissions, postejaculatory interval, and decreased copulatory rate; and in females, increased lordosis quotient, lordosis intensity score, and progesterone-induced LH surge. Indeed, if only traditional endpoints such as litter size, live birth index, pup survival, sperm production, and fertility had been used in this study, the insidious endocrine effects would have been missed. The doses used were well below those that would have caused differences in maternal health or reproductive success.

Coincidentally, the list of changes in sexual behavior in adult male rats as the result of exposure to dioxin in utero and during lactation were strikingly similar to those reported in a 1989 review of studies in which the phenotype of male mouse pups was altered because they developed between two females in the uterine horn (vom Saal 1989; also reviewed in Vandenbergh 2004, in this issue). Results in the study indicated that the same hormone-specific system was being affected in each study. Both studies revealed the sensitivity of the developing individual to the slightest chemical perturbation during development. Although the affected animals' phenotypes were altered, from a clinical perspective they would have been considered healthy. In addition, if each individual endpoint was considered alone, the results might have seemed insignificant; but as a constellation or sequelae of effects, they soon begin to resemble disorders or undesirable trends that could eventually affect a population. These results are beginning to generate thinking about how to broaden the scope and efficiency of a single assay or screen to involve more sensitive endpoints. Such improved future designs will better detect the cumulative impact of a chemical on an animal and will provide a more accurate reflection of how endocrine disruptors undermine health. They could continue to uncover various sequelae of change that are beginning to be explored in terms of increases in prevalence of unexplained health disorders in the human population (Colborn 2004; Skakkebaek et al. 2001). Assays that are designed to detect disruption in multiple endocrine-specific systems would considerably re-

duce the number of animals required and may better reflect how humans would be affected.

In subsequent years, another widely dispersed and economically important chemical, bisphenol A (BPA[1]), came under scrutiny in independent laboratories around the world. As with dioxin, very low or ambient exposure concentrations of BPA were administered to pregnant mice producing occult but life-altering changes in both male and female offspring. There are now more than 80 publications that demonstrate endocrine disturbances in 15 species at concentrations of BPA (0.1 ppt to 1000 ppb) as low as 5 to 7 orders of magnitude less than the EPA lowest observed adverse effect level of 50 mg/kg.

Similarly, in studies designed to determine whether fetal position in the uterus could affect the development and behavior of mice, the changes discovered in male mice that developed between two females were almost identical to the endpoints found in the BPA in utero-exposed mice (vom Saal et al. 1998). It was also determined that changes in the males were the result of increased blood levels of less than 1/10th part per trillion per gram (0.1 ppt) of free 17-beta-estradiol released by their sisters (vom Saal et al. 1992). Like the dioxin and BPA studies, the intrauterine position study demonstrates that the gestational timing of exposure can significantly reduce the amount of chemical or hormone needed to elicit an estrogenic response, reflecting the sensitivity of the embryo and fetus (Howdeshell et al. 1999). In addition, a number of the BPA low-dose studies reported the nonmonotonic inverted U dose response in addition to showing effects of BPA at very low doses (Gupta 2000; Jobling et al. 2003; Levy et al. 2004; Schulte-Oehlmann et al. 2001; Takai et al. 2000, 2001; Talsness et al. 2000; Watts et al. 2003; Wetherill et al. 2002).

Dealing with "Chameleon Chemicals"

Complicating the difficulty of designing simple screens to detect endocrine-disrupting chemicals has been the discovery that chemicals do not always fit the traditional dogma that a compound is either a hormone agonist or antagonist. It was not uncommon before 1993 to find the following type of statements in the literature: "all estrogens act through a common mechanism—binding to receptor proteins in cells that are targets for hormones." Unfortunately, it is now recognized that a simple in vitro assay designed to detect an estrogenic or androgenic chemical will miss chemicals that can induce sex hormone-like effects without binding to the estrogen or androgen receptor. For example, as noted above, both dioxin and BPA could be called chameleon chemicals because the result of their activity changes depending on their concentration and/or the specific stage of development of the tissue with which they come into contact (Gould et al. 1998; MacLusky et al. 1998; Nagel et al. 1997). In addition, although dioxin does not bind to the estrogen receptor (Romkes et al. 1987) and BPA is a weak estrogen in vitro (Markey et al. 2001b), they both turn on auxiliary systems in cells that significantly enhance cell function in vivo (Barthold et al. 1999; Olsen et al. 1994).

Under some circumstances, the ultimate effect of dioxin and BPA is that of a powerful estrogen. For instance, 1 nm of BPA in vitro turns on proliferation in human prostatic adenocarcinoma cells that is androgen independent (Wetherill et al. 2002) and is equally as potent as 17-beta estradiol, activating the cAMP-regulatory element binding protein transcription factor in nuclear membrane estrogen receptors in pancreatic islet cells (Quesada et al. 2002). Besides turning on orphan receptors (McLachlan et al. 1992), dioxin turns on the aryl hydrocarbon (Ah[1]) receptor (Sanderson et al. 1997), which indirectly can lead to increases and/or decreases in a diverse selection of hormones such as LH (Bookstaff et al. 1990), testosterone (Gray et al. 1997), estrogens (Safe et al. 1998), and thyroid hormones (Schuur et al. 1997), to mention a few. Ah receptor activation also influences levels of enzymes and growth factors, sometimes directly and sometimes through interactions.

BPA is now identified as a selective estrogen receptor modulator (Gould et al. 1998; Kuiper et al. 1999; Long et al. 2000; Nagel et al. 2001) because its actions are tissue specific. The studies that uncovered the chameleon characteristic of BPA also revealed the sensitivity of perinatal tissue to BPA. When the CD-1 mouse was used, 100 mg/kg/day of BPA were required to obtain a uterotrophic response (Markey et al. 2001b), whereas after administering only 25 ng/kg/day from day 9 to the end of gestation, the ductal tissue in breast tissue responded (Markey 2001a). In this comparison, BPA was 4,000,000 times more potent in one tissue than the other (Markey et al. 2001a), In another study, to demonstrate further the unpredictability of a particular chemical in a system, a dose of 200 μg/kg/day of BPA stimulated uterine weight gain in Long Evans rats (Rubin et al. 2001), but only 25 μg/kg/day increased the size of fetal mouse mammary glands. Thus, effects at this low dose would not have been detected in a uterotrophic assay that measures only uterine weight gain to detect estrogenicity.

Several studies also revealed that prenatal exposure to low doses of BPA resulted in increased body weight in both sexes through puberty and into adulthood (Howdeshell et al. 1999; Rubin et al. 2001). Chemicals like BPA and dioxin that do not fit the traditional dogma concerning monotonic dose response curves and "no effect" levels are challenging the creativity and biological backgrounds of researchers attempting to design new screens and assays. Toxicologists, chemists, or biologists working alone cannot fill this need. Interdisciplinary collaborations that include developmental biologists and endocrinologists will be required to break through the roadblocks that have been holding up the validation and standardization of effective assays to detect endocrine disruptors.

Confounders

As new study designs have become more endocrinologically focused, and more and more adverse endocrine effects

are being linked with specific chemical products, a genuine need has arisen to replicate some of the low-dose studies, especially because some of these chemicals with low-dose effects are large-volume economically important chemicals for which there is widespread daily human exposure. In addition, until representatives of regulatory agencies have replicative evidence about a particular chemical, they will remain hesitant to take action. Now, after years of foiled attempts and the wasting of thousands of animals to replicate a number of low-dose BPA studies, attention is turning to what heretofore seemed to be innocuous contributors to the problem—the animal feed and the strain of animal used. The following discussion considers these factors because, undoubtedly, they have contributed in part to the difficulty of replication efforts.

A Look at Diet in Light of New Evidence

Diet has been the subject of toxicological debate for years. As early as 1987, it was recognized that diet can confound the results of research related to the role of estrogens in development, function, and the initiation of cancer (Thigpen et al. 1987b). At that time, the first evidence appeared in the literature to suggest that only standardized diets with a minimum of estrogens should be used for comparative studies (Thigpen et al. 1987c) when exploring the estrogenicity of substances. The same authors also stated that a valid bioassay must be able to demonstrate a significant increase in uterine weight using a diethylstilbestrol (DES[1])-spiked diet compared with a negative control diet (Thigpen et al. 1987b). In 1991, when the first list of endocrine disruptors was published (Colborn and Clement 1992), " . . . . soy products, and laboratory animal and pet food products" were included. However, for years this information was ignored during the design of large commercial laboratory investigations using animals.

Currently, animal dietary products are identified as open or closed, depending on whether the amounts of their ingredients are listed on the label or not, respectively. Feeds are also identified as to whether they contain large or small amounts of soy, which can contain variable amounts of phytoestrogens. Laboratory studies have shown that the amount of each phytoestrogen is generally consistent, but there can also be a wide range of variation between the amount of soy protein or alfalfa meal in the same product (Thigpen et al. 1999a,b, 2003, 2004). The quantity of ingredients in closed feed products are not labeled, and when several products were tested recently, it was revealed that there were significantly different amounts of the soy phytoestrogens daidzine and genistein between batches and even between bags from the same batch (Thigpen et al. 2003, 2004). The values were high enough in some of the tested closed diet samples to induce uterine weight gain in young adult females and accelerate vaginal opening (VO[1]) comparable to 4 ppb of DES added to a low phytoestrogen feed product. It is important to note that there were no uterotrophic and VO responses to DES in offspring of animals that had been exposed previously to the high phytoestrogen diet.

Corn-derived ingredients in animal feed can also confound study results. Test diets that included dextrose, corn starch, and corn oil were found to increase uterine weight in mice compared with mice on a negative control diet (Thigpen et al. 1987a). Results such as those described above clearly demonstrate that the odds for false-negatives would be high if closed feed diets such as these were used in assays testing for estrogenicity. Consequently, the diet of the maternal animals and their offspring, even if the offspring are purchased after they are born, should be as rigidly controlled as it would be during the testing period. This control could mean having to work closely with animal suppliers when purchasing animals for endocrine-related research.

Another factor, the amount of metabolizable energy (ME[1]) in the food, also complicates diet selection in studies looking at estrogenic effects. If one uses uterine weight gain as an endpoint, ME content contributes comparably to the actual weight gain as that of phytoestrogens. For the more sensitive estrogen endpoints such as VO, ME is less effective for advancing VO than it is for increasing uterine weight (Thigpen et al. 2002). Thus, diets should be standardized for both ME and phytoestrogen content to minimize potentially implicating effects and to maximize the possibility for replication.

A Look at Species and Strain in Light of New Evidence

Since the early 1990s, the literature on endocrine disruption has also revealed the importance of rigidly controlling the selection of animal species and strain, just as for diet. With respect to testing for estrogenicity, the evidence is especially convincing if the animals are to be used for comparative or regulatory purposes. For example, over the years the production of animals for laboratory use has resulted in marked differences among species and strains in sensitivity for estrogens (Long et al. 2000; Spearow et al. 1999). The CD-1 mouse has been bred for litter size, the Charles River CD Sprague-Dawley (CD-SD) rat for large litters, and the Wistar rat for both large litters and smaller animals. After 65 generations of inbreeding, the CD-SD rat now produces very large litters but is markedly less responsive to estrogenic compounds (e.g., uterotrophic response) than many other animal models. With respect to detecting male responses to estrogens, for example, the CD-SD rat required a dose of 200 μg/kg/day of ethinyl estradiol from 7 wk of age for 28 days to show a significant change in the male reproductive system, and 50 μg/kg/day for a uterine response (Yamasaki et al. 2002). In contrast, the CF-1 offspring mouse showed significant adult male responses to ethinyl estradiol at 0.002 μg/kg from day 1 of gestation through day 17 (Thayer et al. 2001.) The clinically effective dose of ethinyl estradiol in birth control pills is about 0.5 μg/kg/day,

confirming that the CD-SD rat is not an appropriate model for testing estrogenic chemicals and predicting response in humans.

Several studies have discovered that the SD rat in particular is insensitive to BPA. While vaginal epithelium DNA synthesis responded to 37.5 mg/kg body weight of BPA in female F344 rats, there was no DNA response in SD rats at any dose tested up to 150 mg/kg of body weight, although BPA clearance and its affinity for the estrogen receptor was identical in both species (Long et al. 2000). Another study reported that BPA initiated posterior pituitary prolactin secretion in Fischer 344 females with similar efficacy in estrogen response as estradiol, whereas there was no response in SD rats (Steinmetz et al. 1997). F344 rats exposed to BPA at 0.3 mg/kg/day developed hypertrophy and hyperplasia in the uterus, and cornification of the vaginal epithelium. SD rats treated in the same manner did not respond (Steinmetz et al. 1998). Others discovered that the B6C3F1 hybrid mouse is more sensitive to uterotrophic stimulation than the CD-1 mouse, similar to the greater sensitivity of the F344 rat compared with the SD rat (Markey et al. 2001b; Papaconstantinou et al. 2001). In the meantime, others reached the broad conclusion that the F344 rat was more sensitive than the SD rat after examining the neonatal effects for age-related endpoints as the result of both low- and high-dose estrogen treatment (Putz et al. 2001). These studies reveal the importance of taking both sex and one or more specific endpoints into consideration when selecting animal species and strain.

Discussion

A great deal of interest focused on three publications in 1997 and 1998 in which the authors reported that the male pups of pregnant CF-1 mice exposed to low, environmentally relevant concentrations of BPA were born with permanently enlarged prostates (Nagel et al. 1997; vom Saal et al. 1997, 1998). Because billions of pounds of BPA are produced each year and used widely in everyday products, and it is estimated that one in seven men will develop prostate cancer, these papers initiated a series of "replication" studies that were unable to replicate the prostate effect (Ashby et al. 1999; Cagen et al. 1999a,b; Tyl et al. 2002; Yamasaki et al. 2002). Some of these were expensive, multigenerational studies that used large numbers of SD rats—more than 8000 in one study. Unfortunately, a great deal of energy and time has been wasted arguing over the results of these studies. Much has been learned over the past decade about the influence on study results of diet (Thigpen et al. 1975, 1987a,b,c, 1999a,b, 2001, 2002, 2003, 2004) and strain selection (Spearow et al. 1999). Different animal strains and species, feeds, and dosage administration were used; and the experience of the technicians with microscopic organ removal and tissue dissection was not identical. In addition, several of the replication studies did not use positive controls to determine whether the animals were already estrogenized and whether the species and strain of animal used was sensitive enough to respond to doses of estrogenic drugs that can initiate a response in women; and some studies used positive controls and obtained no response. In essence, the replications were not truly replications. A great deal has been learned from all of the studies involved, including the initial low-dose prostate study, about the design and precision required to detect hormonal effects. With this knowledge in mind, it is now time to move ahead.

Endocrine disturbances, although often not obvious on gross observation, are not rare events that require a large number of animals to detect. The use of vast numbers of animals to detect occasional overt damage such as malformations and/or change in organ weight can now be set aside. By incorporating endocrinological considerations into protocol design, far fewer animals will be needed for future screening as well as for validation and standardization without loss of statistical power. Granted, the endocrinological changes or endpoints that are now being measured may be expressed over a range of intensity; nonetheless, they will be expressed in a larger proportion of the exposed animals than in previous studies using cruder endpoints. In addition, as mentioned above, new designs have already demonstrated that there can be multiple options when selecting endpoints during the development of new protocols. It is now possible to increase the number of endpoints in a study or a screen, to save time and animals, and to avoid conducting single-endpoint assays one at a time to achieve the same knowledge. By utilizing all of the tissue from exposed animals (e.g., not simply the gonads and/or thyroid) and looking at changes in the pituitary, hypothalamus, hippocampus, adrenals, and pancreas, to mention a few, the assays will provide a more complete picture of the activity of a chemical. From an animal welfare perspective, the more endpoints in an assay, the better the assay.

The evidence that is now available concerning the significant impacts of strain and feed selection on study results should discourage the practice of using historical data for comparisons unless data are available about the diet of the animals in the earlier study. However, a great deal can be learned from prior studies to provide guidance for the creation of screens and assays that incorporated developmental and endocrinological expertise. The challenge now is how to implement this knowledge during the development of the new assays.

Looking back over the past decade, it appears that the research efforts to create screens and assays to detect endocrine disruptors have begun to take the path that leads directly to the crux of the need for the tests—to be able to detect chemicals that interfere with prenatal development. It is crucial to probe the earliest stages of development, when the greatest damage can occur, and avoid lifelong irreversible disorders. A major breakthrough took place December 11, 2003, when the EPA National Health and Environmental Effects Research Laboratory Office of Research and Development released preliminary results of a demonstration

study using pubertal animals exposed to a series of known endocrine disruptors (http://epa.gov/scipoly/oscpendo/edmvs.htm, docket control number OPPTS-2003-00176). Among the list of new, occult biochemical and physiological changes that were measured, many would have been missed using adult animals. Hopefully, this trend toward probing earlier in development will continue and will broaden the number of endpoints in a single assay. Future research with whole animals should complement efforts to develop rapid, inexpensive in vitro screens, some of which eventually will replace whole animal testing. Ultimately, this trend will significantly reduce the number of animals required for testing.

References

Ashby J, Tinwell H, Haseman J. 1999. Lack of effects for low dose levels of bisphenol A and diethylstilbestrol on the prostate gland of CF1 mice exposed in utero. Reg Toxicol Pharmacol 30:156-166.

Barthold JS, Kryger JV, Derusha AM, Duel BP, Jednak R, Skafar DF. 1999. Effects of an environmental endocrine disruptor on fetal development, estrogen receptor-α and epidermal growth factor receptor expression in the porcine male genital tract. J Urol 162:864-871.

Bookstaff RC, Moore RW, Peterson RE. 1990. 2,3,7,8-Tetrachlorodibenzo-p-dioxin increases the potency of androgens andestrogens as feedback inhibitors of luteinizing hormone secretion in male rats. Toxicol Appl Pharmacol 104:212-224.

Cagen SZ, Waechter JM Jr, Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, Harris LR. 1999a. Normal reproductive organ development in Wistar rats exposed to bisphenol A in the drinking water. Reg Toxicol Pharmacol 30:130-139.

Cagen SZ, Waechter JM Jr., Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, Harris LR. 1999b. Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. Toxicol Sci 50:36-44.

Colborn T. 2004. Neurodevelopment and endocrine disruption. Environ Health Persp 112:944-949.

Colborn T, Clement C, eds. 1992. Chemically induced alterations in sexual and functional development: The wildlife/human connection. In: Mehlman MA, ed. Advances in Modern Environmental Toxicology. Vol 21. Princeton: Princeton Scientific Publishing Co., Inc.

Duft M, Schulte-Oehlmann U, Weltje L, Tillmann M, Oehlmann J. 2003. Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgus antipodarum*. Aquat Toxicol 64:437-449.

Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T, Safe S, McDonnell DP, Gaido KW. 1998. Bisphenol A interacts with the estrogen receptor-α in a distinct manner from estradiol. Mol Cell Endocrinol 142:203-214.

Gray LE, Ostby JS, Kelce WR. 1997. A dose-response analysis of the reproductive effects of a single gestational dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin in male Long Evans Hooded rat offspring. Toxicol Appl Pharmacol 146:11-20.

Gupta C. 2000. Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. Proc Soc Exp Biol Med 224:61-68.

Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, vom Saal FS. 1999. Exposure to bisphenol A advances puberty. Nature 401:763-764.

Jobling S, Casey D, Rodgers-Gray T, Oehlmann J, Schulte-Oehlmann U, Pawlowski S, Baunbeck T, Turner AP, Tyler CR. 2003. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquat Toxicol 65:205-220.

Kuiper GGJM, van den Bemd G-JCM, van Leeuwen JPTM. 1999. Estrogen receptor and the SERM concept. J Endocrinol Invest 22:594-603.

Levy G, Lutz I, Krüger A, Kloas W. 2004. Bisphenol A induces feminization in *Xenopus laevis* tadpoles. Environ Res 94:102-111.

Long X, Steinmetz R, Ben-Jonathan N, Caperell-Grant A, Young PCM, Nephew KP, Bigsby RM. 2000. Strain differences in vaginal responses to the xenoestrogen bisphenol A. Environ Health Persp 108:243-247.

Mably TA, Moore RW, Bjerke DL, Peterson RE. 1991. The male reproductive system is highly sensitive to in utero and lactational TCDD exposure. In: Gallo MA, Scheuplein RJ, van der Herijden CA, eds. Biological Basis for Risk Assessment of Dioxins and Related Compounds. Cold Springs Harbor: Cold Springs Harbor Laboratory Press. Banbury Report 35. p 69-78.

MacLusky NJ, Brown TJ, Schantz S, Seo BW, Peterson RE. 1998. Hormonal interactions in the effects of halogenated aromatic hydrocarbons on the developing brain. Toxicol Ind Health 14:185-208.

Markey CM, Luque EH, Munoz de Toro M, Sonnenschein C, Soto AM. 2001a. In utero exposure to bisphenol A alters the development and tissue organization of the mouse mammary gland. Biol Reprod 65: 1215-1223.

Markey CM, Michaelson CL, Veson EC, Sonnenschein C, Soto AM. 2001b. The mouse uterotrophic assay: A reevaluation of its validity in assessing the estrogenicity of bisphenol A. Environ Health Persp 109: 55-60.

McLachlan JA, Newbold RR, Teng CT, Korach KS. 1992. Environmental estrogens: Orphan receptors and genetic imprinting. In: Colborn T, Clement C, eds. Chemically Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection. Princeton: Princeton Scientific Publishing Co., Inc. p 17-84.

Nagel SC, Hagelbarger JL, McDonnell DP. 2001. Development of an ER action indicator mouse for the study of estrogens, selective ER modulators (SERMs), and xenobiotics. Endocrinology 142:4721-4728.

Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. 1997. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. Environ Health Persp 105:70-76.

Olsen H, Enan E, Matsumura F. 1994. Regulation of glucose transport in the NIH 3T3 L1 preadipocyte cell line by TCDD. Environ Health Persp 102:454-458.

Papaconstantinou AD, Fisher BR, Umbreit TH, Goering PL, Lappas NT, Brown KM. 2001. Effects of beta-estradiol and bisphenol A on heat shock protein levels and localization in the mouse uterus are antagonized by the antiestrogen ICI 182,780. Toxicol Sci 63:173-180.

Peterson RE, Moore RW, Mably TA, Bjerke DL, Goy RW. 1992. Male reproductive system ontogeny: Effects of perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. In: Colborn T, Clement C, eds. Chemically Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection. Princeton: Princeton Scientific Publishing Co., Inc. p 175-193.

Putz O, Schwartz CB, LeBlanc GA, Cooper RL, Prins GS. 2001. Neonatal low- and high-dose exposure to estradiol benzoate in the male rat: II. Effects on male puberty and the reproductive tract. Biol Reprod 65: 1506-1517.

Quesada I, Fuentes E, Viso-León MC, Soria B, Ripoll C, Nadal A. 2002. Low doses of the endocrine disruptor bisphenol-A and the native hormone 17-β-estradiol rapidly activate transcription factor CREB. FASEB J 16:1671-1673.

Romkes M, Piskorska-Pliszczynska J, Safe S. 1987. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on hepatic and uterine estrogen receptor levels in rats. Toxicol Appl Pharmacol 87:306-314.

Rubin BS, Murray MK, Damassa DA, King JC, Soto AM. 2001. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. Environ Health Persp 109: 675-680.

Safe S, Wang F, Porter W, Duan R, McDougal A. 1998. Ah receptor agonists as endocrine disruptors: Antiestrogenic activity and mechanisms. Toxicol Lett 103:343-347.

Sanderson JT, Janz DM, Bellward GD, Giesy JP. 1997. Effects of em-

bryonic and adult exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin on hepatic microsomal testosterone hydroxylase activities in great blue herons (*Ardea herodias*). Environ Toxicol Chem 16:1304-1310.

Schulte-Oehlmann U, Tillmann M, Casey D, Duft M, Mrkert B, Oehlmann J. 2001. Östrogenartige Wirkungen von Bisphenol A auf Vorderkiemenschnecken (*Mollusca: Gastropoda: Prosobranchia*). [Estrogenic effects of bisphenol A in prosobranchs (*Mollusca: Gastropoda: Prosobranchia*)]. Z Umweltchem Ökotox 13:319-333.

Schuur AG, Boekhorst FM, Brouwer A, Visser TJ. 1997. Extrathyroidal effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on thyroid hormone turnover in male Sprague-Dawley rats. Endocrinology 138: 3727-3734.

Sheehan DM. 2000. Activity of environmentally relevant low doses of endocrine disruptors and the bisphenol A controversy: Initial results confirmed. Proc Soc Exp Biol Med 224:57-60.

Skakkebaek NE, Rajpert-De Meyts E, Main KM. 2001. Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. Hum Reprod 16:972-978.

Spearow JL, Doemeny P, Sera R, Leffler R, Barkley M. 1999. Genetic variation in susceptibility to endocrine disruption by estrogen in mice. Science 285:1259-1261.

Steinmetz R, Brown NG, Allen DL, Bigsby RM, Ben-Jonathan N. 1997. The environmental estrogen bisphenol A stimulates prolactin release in vitro and in vivo. Endocrinology 138:1780-1786.

Steinmetz R, Mitchner NA, Grant A, Allen DL, Bigsby RM, Ben-Jonathan N. 1998. The xenoestrogen bisphenol A induces growth, differentiation, and c-fos gene expression in the female reproductive tract. Endocrinology 139:2741-2747.

Takai Y, Tsutsumi O, Ikezuki Y, Hiroi H, Osuga Y, Momoeda M, Yano T, Taketani Y. 2000. Estrogen receptor-mediated effects of a xenoestrogen, bisphenol A, on preimplantation mouse embryos. Biochem Biophys Res Comm 270:918-921.

Takai Y, Tsutsumi O, Ikezuki Y, Kamei Y, Osuga Y, Yano T, Taketan Y. 2001. Preimplantation exposure to bisphenol A advances postnatal development. Reprod Toxicol 15:71-74.

Talsness C, Fialkowski O, Gericke C, Merker H-J, Chahoud I. 2000. The effects of low and high doses of bisphenol A on the reproductive system of female and male rat offspring. Congen Anom 40:S94-S107.

Thayer KA, Ruhlen RL, Howdeshell KL, Buchanan DL, Cooke PS, Preziosi D, Welshons WV, Haseman J, vom Saal FS. 2001. Altered prostate growth and daily sperm production in male mice exposed prenatally to subclinical doses of 17 alpha-ethinyl oestradiol. Hum Reprod 16:988-996.

Thigpen JE, Faith RE, McConnell EE, Moore JA . 1975. Increased susceptibility to bacterial infection as a sequela of exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Infect Immunol 12:1319-1324.

Thigpen JE, Haseman JK, Saunders H, Locklear J, Caviness G, Grant M, Forsythe D. 2002. Dietary factors affecting uterine weights of immature CD-1 mice used in uterotrophic bioassays. Cancer Detect Prev 26:381-393.

Thigpen JE, Haseman JK, Saunders H, Setchell DR, Grant M, Forsythe D. 2003. Dietary phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. Comp Med 53:477-485.

Thigpen JE, Lebetkin EH, Dawes ML, Richter CB, Crawford D. 1987a. The mouse bioassay for the detection of estrogenic activity in rodent diets: III. Stimulation of uterine weight by dextrose, sucrose and corn starch. Lab Anim Sci 37:606-609.

Thigpen JE, Li LA, Richter CB, Lebetkin EH, Jameson CW. 1987b. The mouse bioassay for the detection of estrogenic activity in rodent diets: I. A standardized method for conducting the mouse bioassay. Lab Anim Sci 37:596-601.

Thigpen JE, Li LA, Richter CB, Lebetkin EH, Jameson CW. 1987c. The mouse bioassay for the detection of estrogenic activity in rodent diets: II. Comparative estrogenic activity of purified, certified and standard open and closed formula rodent diets. Lab Anim Sci 37:602-605.

Thigpen JE, Locklear J, Haseman JK, Saunders H, Grant MF, Forsythe DB. 2001. Effects of the dietary phytoestrogens daidzein and genistein on the incidence of vulvar carcinomas in 129/J mice. Cancer Detect Prev 25:527-532.

Thigpen JE, Setchell KDR, Ahlmark KB, Locklear J, Spahr T, Caviness GF, Goelz MF, Haseman JK, Newbold RR, Forsythe DB. 1999a. Phytoestrogen content of purified, open- and closed-formula laboratory animal diets. Lab Anim Sci 49:530-536.

Thigpen JE, Setchell KDR, Goelz MF, Forsythe DB. 1999b. The phytoestrogen content of rodent diets. Environ Health Persp 107:A182-A183.

Thigpen JE, Setchell KDR, Saunders HE, Haseman J, Grant MG, Forsythe DB. 2004. Selecting the appropriate rodent diet for endocrine disruptor research and testing studies. ILAR J 45:401-416.

Tyl RW, Myers CB, Marr MC, Thomas BF, Keimowitz AR, Brine DR, Veselica MM, Fail PA, Chang TY, Seely JC, Joiner RL, Butala JH, Dimond SS, Cagen SZ, Shiotsuka RN, Stropp GD, Waechter JM. 2002. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicol Sci 68:121-146.

USC [US Congress]. 1996a. 1996 Food Quality Protection Act, Implementation Plan. Washington DC: GPO.

USC [US Congress]. 1996b. 1996 Safe Drinking Water Act, Amendments. Washington DC: GPO.

Vandenbergh JG. 2004. Animal models and studies of in utero endocrine-disruptor effects. ILAR J 45:438-442.

vom Saal FS. 1989. Sexual differentiation in litter-bearing mammals: Influence of sex of adjacent fetuses in utero. J Anim Sci 67:1824-1840.

vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, Welshons WV. 1998. A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol Ind Health 14:239-260.

vom Saal FS, Montano MM, Wang MH. 1992. Sexual differentiation in mammals. In: Colborn T, Clement C, eds. Chemically Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection. Vol XXI. Princeton: Princeton Scientific Publishing Co., Inc. p 17-83.

vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, Dhar MD, Ganjam VK, Parmigiani S, Welshons WV. 1997. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc Natl Acad Sci U S A 94:2056-2061.

Watts MM, Pascoe D, Carroll K. 2003. Exposure to 17 alpha-ethinylestradiol and bisphenol A—Effects on larval moulting and mouthpart structure of *Chironomus riparius*. Ecotoxicol Environ Saf 54:207-215.

Wetherill YB, Petre CE, Monk KR, Puga A, Knudsen KE. 2002. The xenoestrogen bisphenol A induces inappropriate androgen receptor activiation and mitogenesis in prostatic adenocarcinoma cells. Mol Cancer Ther 1:515-524.

Yamasaki K, Sawaki M, Noda S, Imatanaka N, Takatsuki M. 2002. Subacute oral toxicity study of ethynylestradiol and bisphenol A, based on the draft protocol for the "Enhanced OECD Test Guideline no. 407." Arch Toxicol 76:65-74.