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ENDOCRINE DISRUPTORS: PRESENT ISSUES, FUTURE DIRECTIONS

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ABSTRACT

A variety of natural products and synthetic chemicals, known collectively as endocrine-disrupting compounds (EDCs), mimic or interfere with the mechanisms that govern vertebrate reproductive development and function. At present, research has focused on (i) the morphological and functional consequences of EDCs; (ii) identifying and determining the relative potencies of synthetic and steroidal compounds that have endocrine-disrupting effects; (iii) the mechanism of action of EDCs at the molecular level; and (iv) the recognition that in "real life," contamination usually reflects mixtures of EDCs. Future research must examine (i) the interactive nature of EDCs, particularly whether the threshold concept as developed in traditional toxicological research applies to these chemicals; (ii) when and how EDCs act at the physiological level, particularly how they may organize the neural substrates of reproductive physiology and behavior; (iii) the various effects these compounds have on different species, individuals, and even tissues; and (iv) how adaptations may evolve in natural populations with continued exposure to EDCs. Several predictions are offered that reflect these new perspectives. Specifically, (i) the threshold assumption will be found not to apply to EDCs because they mimic the actions of endogenous molecules (e.g., estrogen) critical to development; hence, the threshold is automatically exceeded with exposure. (ii) Behavior can compound and magnify the effects of EDCs over successive generations; that is, bioaccumulated EDCs inherited from the mother not only influence the morphological and physiological development of the offspring but also the offsprings' reproductive behavior as adults. This adult behavior, in turn, can have further consequences on the sexual development of their own young. (iii) The sensitivity of a species or an individual to a compound is related to species (individual)-typical concentrations of circulating gonadal steroid hormones. Related to this is the recent finding that alternate forms of the putative receptors are differentially distributed, thereby contributing to the different effects that have been observed. (iv) Except in extraordinary situations, populations often continue to exist in contaminated sites. One possible explanation for this observation that needs to be considered is that animals can rapidly adapt to the nature and level of

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contamination in their environment. It is unlikely that successive generations coincidentally become insensitive to gonadal steroid hormones fundamentally important as biological regulators of development and reproduction. Rather, adaptive alterations in the genes that encode steroid receptors may occur with chronic exposure to EDCs, allowing the sex hormone receptor to discriminate natural steroids from EDCs.

INTRODUCTION

IN THE PAST FEW YEARS, much has been learned about how certain natural products and synthetic chemicals, known collectively as endocrine-disrupting compounds (EDCs), can act early in development at low dosages to affect subsequent sexual differentiation of reproductive tissues and associated processes in a variety of animals. This newly emerging field is marked as much by heat as substance, which might be predicted from the nature of scientific revolutions *sensu* Thomas Kuhn. The traditional toxicological paradigm—with its emphasis on a carcinogenic/survival model, its single-compound testing based on the concept of additivity, and its presumption of a threshold dosage below which no adverse effect is evident—is gradually being supplanted by another paradigm developed largely by researchers from other disciplines. This endocrine disruptor paradigm recognizes four concepts that differ from the toxicological approach. First, exposure to EDCs in nature is chronic, not acute as occurs in laboratory dose-response paradigms. Second, the effects of exposure may be latent, sometimes not observable for years after exposure. Third, a "lowest" dose that causes no adverse effects may not actually exist because compounds that mimic biological molecules already present in the body will by definition exceed a threshold level. And fourth, EDCs in the environment rarely exist in isolation, usually occurring in conjunction with other compounds.

PRESENT ISSUES

Thus far, researchers that investigate endocrine disruption by chemicals in the environment have emphasized organismal studies that pinpoint the ability of these compounds to affect gonadal sex, normal sexual development, and fertility. Also, a great deal of progress has been made, establishing ways to identify EDCs and determine their mechanisms of action. But if the toxicological assessment paradigm is to change relative to EDCs, more studies must be done on environmentally and physiologically relevant mixtures of EDCs and their consequences.

TRADITIONAL TOXICOLOGICAL VS. ENDOCRINE DISRUPTOR PARADIGMS

The difficulties in demonstrating a clear cause-and-effect relationship between EDCs and reproductive anomalies arise from the fact that the wrong standards have been used (Table 1). Environmental toxicological paradigms typically focus on exposure of adult individuals to high pharmacological doses of EDCs that result in mutagenesis, cancer, and death as unequivocal indications of contaminant effects. Endocrine-disrupting chemicals, however, are characterized by a delayed response, often measured in years, after exposure to low, physiologically relevant dosages during sensitive periods of organ development in the embryo. Thus, while traditional toxicological paradigms require a clear causal relationship, traceable from exposure to the development of a cancer and death, those that study endocrine disruptors find that functional sterility results from the bioaccumulation of chemicals that persist in the environment at relatively low levels. In addition, few spills of EDCs occur as pure compounds; industrial contamination is often a potpourri of contaminants. Thus, understanding the effects of EDCs is predicated on knowledge about the interactive nature of these compounds at the biological level.

MIXTURES AND SYNERGY

Clearly, risk-assessment paradigms must more closely simulate what occurs in nature. Recent studies have shifted away from a single-exposure dose-response approach that uses individual compounds to assay the effects that combinations of these compounds can cause.

Lake Apopka in Florida has become a classic example of how environmental contamination can affect the reproduction of animals in nature. Gonadal and penile abnormalities of the American alligator (*Alligator mississippien-*

TABLE 1

Paradigm shift brought on by discovery of sex hormone mimics in the environment

Traditional Toxicological Approach	Endocrine Disruptor Approach
Carcinogenic model	Developmental model
Mortality/acute toxicity	Delayed dysfunction
Threshold	No threshold
Additive effects	Synergistic effects

Note differences in the indices that mark the traditional toxicological approach to risk assessment and the newly emerging approach for endocrine-disrupting chemicals.

sis) in this lake resembled those described in mice treated with the potent, synthetic estrogen DES (McLachlan 1977; McLachlan et al. 1982). This finding led to detailed studies that determined that chronic pollution by agricultural runoff—exacerbated by a chemical spill of dicofol—was the most likely cause for these observed reproductive abnormalities (Guillette et al. 1994, 1996; Matter et al. 1998). Dicofol and its components have been shown to bind the estrogen receptor (ER) (Vonier et al. 1996); therefore, these compounds may mimic estrogens in the alligator. In addition to o,p'-DDE/o,p'-DDT contamination, PCB mixtures resembling Aroclor 1242 and a variety of pesticides, including dieldrin, toxaphene, *cis/trans* nonachlor, chlordane, and p,p'-DDD, have been detected in alligator eggs (Heinz et al. 1991). Exposure of alligator (Guillette et al. 1999) and red-eared slider turtle (*Trachemys scripta elegans*) (Willingham and Crews 1999) embryos to combinations and concentrations of these compounds typically found in nature results in anomalous reproductive development.

Mixtures of EDCs may behave differently, compared to their individual behavior, and it becomes necessary to determine whether their actions are additive or synergistic. There is much controversy as to whether EDCs exhibit synergistic activity. The warmth of this debate stems from the fact that a number of EDCs have a lower potency than natural steroidal estrogens (Korach et al. 1988; McLachlan 1993; Soto et al. 1994) and, when considered individually, these chemicals may exist in the environment in concentrations believed to be too low to be of concern. If two compounds alone have no detectable effect, but the two together have an effect, it is difficult to determine whether this is a result of additivity or synergism. One approach is to consider the cumulative effect of the two compounds compared to the minimum detectable level of the assay system. For example, let us assume that the response is being measured in a system that has a minimum level of detection of 1.0 ng. In some instances additive effects are obvious; if one-half of the animals exposed to compounds *A* or *B* alone exhibit a positive effect, yet when *A* and *B* are combined all of the individuals exposed display the effect, this is evidence of additivity. The difficulty arises when compounds alone have but a small detectable effect, but together have a robust effect. Continuing with our example, if only a few of the animals respond when exposed to compounds *C* or *D*, yet when these compounds are combined all of the individuals exhibit the effect, then the two must be acting synergistically. However, if none of the animals exposed to compounds *E* or *F* alone exhibit an effect, but when animals are exposed to these compounds together they show an effect, then the result could be either additive or synergistic (since it is possible that each compound alone causes an effect at 0.75 ng, suggesting additivity ($0.75 + 0.75 = 1.5$)). On the other hand, it is also possible that each compound alone could cause an effect at 0.1 ng, which together suggests synergism if they caused a cumulative effect of 1.5 ng. In the latter instance, the limits of detectability of the assay will not allow one to distinguish between the possibilities.

Is there evidence in nature that dosages of certain compounds in combination act synergistically to produce a strong estrogenic response? Low-dose synergy was first shown with polychlorinated biphenyls (PCBs), using an in vivo sex determination assay in the red-eared slider. As metabolites of other PCBs, hydroxylated PCBs may exist in steady-state concentra-

tions in aquatic environments, potentially exposing wildlife to their effects via direct contact or through the food chain (McKinney et al. 1990). Some PCBs can act as estrogens in the red-eared slider, overriding a male-producing temperature and causing female development, presumably through their action on gonad-determining genes. Using the all-or-nothing nature of the response of red-eared slider embryos to exogenous estrogen, 11 common PCBs were assayed by Bergeron and coworkers (Bergeron et al. 1994). Only two of the compounds tested—2′,4′,6′-trichloro-4-biphenylol (3-PCBOH) and 2′,3′,4′,5′-tetrachloro-4-biphenylol (4-PCBOH), both hydroxylated PCBs—were found to have estrogenic activity, indicated by the production of female or intersex hatchlings from eggs incubated at a male-producing temperature. In tests of mouse tissue, these same two compounds show an appreciable affinity for ER, due in part to their conformational properties as hydroxybiphenyls (Korach et al. 1988; McKinney et al. 1990). When eggs were treated with various mixtures of PCBs except 3- and 4-PCBOH, there was no evidence of an effect on sex ratio. When 3- and 4-PCBOH were combined, however, the compounds acted synergistically, resulting in a significant increase in female and intersex hatchlings at a dose of 10 μg or less than 1 ppm; when administered alone, 3-PCBOH and 4-PCBOH required a dose at least a tenfold higher to show sex reversal.

Natural estrogens also exhibit synergy in the red-eared slider. Bergeron et al. (1999) found that estradiol-17β synergizes with estriol, resulting in a rate of sex reversal that is twice that shown by estradiol-17β alone (36% reversal for estradiol-17β alone vs. 69% reversal for 0.2 μg estradiol-17β + 0.01 μg estriol). The discovery that sex steroid hormone receptors have various forms, each encoded by different genes, may provide a mechanism by which this synergism could occur. Thus, separate studies with both synthetic and steroidal EDCs performed with the red-eared slider have revealed synergy between compounds, demonstrating the need to examine *combinations* of compounds rather than assaying each compound singly.

MECHANISM OF ACTION

Several different mechanisms of action have been proposed for EDCs. EDCs may alter circulating sex steroid levels by affecting steroidogenic enzymes and second-messenger systems in the gonads and brain. For example, PCBs have been shown to interfere with pituitary gonadotropin secretion by altering hypothalamic serotonergic activity (Kahn and Thomas 1997). Other proposed mechanisms center on steroid receptor binding, where the EDC acts as an agonist or an antagonist. Other studies point to activation of the steroid receptor in the absence of ligand binding. Thus far, steroid receptor activation remains the focus of study for elucidating the mechanism of action for these compounds.

Several in vitro assays have established the ability of a number of the compounds to compete with estradiol-17β for the estrogen receptor (ER) and with DHT for the androgen receptor (AR) (Kelce et al. 1995; Vonier et al. 1996; Danzo 1997). For example, nonylphenol and DDE are two estradiol-17β competitors (Danzo 1997). However, some compounds seem to exhibit estrogenic or antiandrogenic effects, yet have a low affinity of binding to ER and AR.

With the discovery that ER can be activated in the absence of ligand binding (Bunone et al. 1996; Curtis et al. 1996), another possible mechanism has been revealed. Some apparent estrogen mimics may operate independent of ER binding and work via a signaling pathway that involves cell membrane receptors. Research has demonstrated that ER activation can take place via the binding of epidermal growth factor (EGF) to its cell membrane receptor (Bunone et al. 1996; Curtis et al. 1996). This binding triggers a signaling cascade that involves the MAP kinase pathway and phosphorylation of ER. It is possible that some EDCs may interact with components of such signaling pathways, thereby affecting steroid receptors via an indirect route rather than directly binding to the steroid receptor.

FUTURE DIRECTIONS

DO EDCS HAVE A THRESHOLD?

Traditional toxicological protocols use single doses of a single chemical at different concentrations, seeking the lowest dose at which

no adverse effects are observed in the animal subject (the no-observed-adverse-effect level or NOAEL). Thus, risk assessments are founded on the presumption that for every chemical there exists a threshold dose below which no adverse effects occur. In practice, the NOAEL is derived from a two-year chronic study in rodents: establishing the highest dose that does not elicit an adverse response and then applying an uncertainty factor (i.e., dividing the dose by 100). This figure is then used as a surrogate for the threshold dose. The concept of a threshold dosage—just like the past model that compares EDC action to a single compound with immediate, observable threshold effects—may be changing in response to recent studies.

PREDICTION: ***The threshold assumption cannot be applied to EDCs since they mimic or antagonize the actions of an endogenous molecule important to development, and in such cases the threshold is automatically exceeded with exposure.***

Gaylor et al. (1988) suggested that the concept of a threshold dose might not apply to EDCs. This hypothesis was recently tested in red-eared sliders. First, a retrospective analysis was conducted on published data that describe the effects of varying doses of estradiol-17β at different incubation temperatures in relation to female sex determination (Sheehan et al. 1999; Figure 1). The results revealed that the Michaelis-Menten equation fit all dose-response data, suggesting that both estrogen and temperature act on a single protein-molecule driving a reversible process. A power-analysis approach was then used to conduct a large (2400 eggs) dose-response study designed to test whether the threshold hypothesis applies to estradiol-17β in this animal model system, and to determine whether the Michaelis-Menten equation can predict organismal response. This equation provided an ED_{50} of 5.0 ng with a 95% confidence limit of ± 2.0 ng (endogenous dose = 1.7 ± 1.3 ng; exogenous dose = 3.3 ± 1.7 ng), and an $r^2 = 0.90$ for fit of the modified Michaelis-Menten equation. The lowest dose, 0.4 ng/10 g egg, significantly increased the female fraction by 11.4%. An important feature is that the curve becomes increasingly more linear as dose decreases relative to ED_{50}, reinforcing the failure to find a

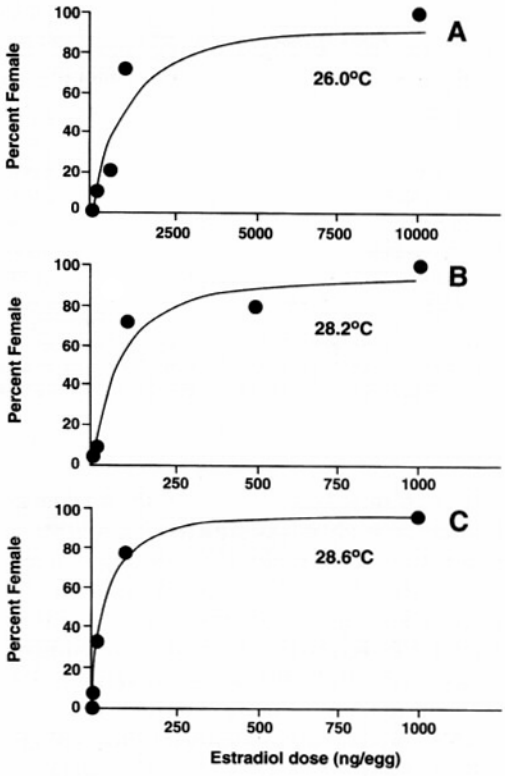

FIGURE 1. RETROSPECTIVE ANALYSIS OF THE EFFECT OF EXOGENOUS ESTRADIOL-17β APPLIED TO EGGS OF THE RED-EARED SLIDER TURTLE (*TRACHEMYS SCRIPTA*).

Data points (from Crews et al. 1996) were fitted to a Michaelis-Menten equation with the line calculated from the equation. Here, V_{max} is fixed at 100% female; v = % female; substrate concentration = dose; and $K_m = ED_{50}$ or the estimated dose to sex-reverse 50% of the eggs. Dose consists of an effective endogenous dose (d_o) plus the administered dose (d). The Michaelis-Menten model is $v = 100\ (d + d_o)$ divided by $K_m + (d + d_o)$. This can be rewritten in the form of a simple linear model in d where the intercept is d_o/K_m and the slope is $1/K_m$. **A**) At 26.0°C, the r^2 and ED_{50} value were 0.850 and 445 ng/egg, respectively; **B**) at 28.2°C these were 0.995 and 38 ng/egg; **C**) at 28.6°C they were 0.998 and 31 ng/egg. Neither an empirical nor Michaelis-Menten fit showed a threshold dose (modified/adapted from Sheehan et al. 1999).

TABLE 2

Effect of endocrine-disrupting chemicals on sex determination in the red-eared slider turtle

Compounds	Amount/egg in ng	Concentration (μM)
Dieldrin	1.197	0.63
Toxaphene	0.415	0.22
p,p'-DDD	4.200	2.60
cis-Nonachlor	0.356	0.16*
Aroclor 1242	0.848	0.53*
trans-Nonachlor	0.556	0.25*
chlordane	0.451	0.22*
p,p'-DDE	28.200	18.00*

Amount (ng) administered to each egg is based on Heinz et al. (1991) report of concentrations in egg yolk of alligators at Lake Apopka. Conversion of amount to actual concentration (μM) indicates actual dosage applied to egg. * Indicates significant sex reversal for compounds applied singly. Adapted from Willingham and Crews 1999.

threshold dose by an empirical fit. A replication of the study was conducted the following year with similar results. Considering that approximately 2% of the estradiol-17β administered to an egg gets into the yolk, and of that only 0.1% is accumulated in the embryo (Crews et al. 1991), the dosages that influence sex determination can be extremely low.

This work has been extended using ecologically relevant dosages of EDCs. The alligators of Lake Apopka are a now famous case study in endocrine disruption. Analysis of yolk from alligator eggs identified a variety of compounds in different amounts (Heinz et al. 1991) (Table 2). Applying similar concentrations of these compounds to red-eared slider turtle eggs caused a significant number to sex reverse, producing female development at an otherwise male-biased incubation temperature (Willingham and Crews 1999). If an EDC in question acts like estradiol-17β and only 0.1% of the exposure is accumulated in the embryo, dosages of endocrine disruptors in the environment that have sex-reversing effects can be extremely low (Table 2). These same dosages influence steroid hormone levels after hatching (Willingham et al. 2000), and are low when compared to the amount of natural estrogen required to achieve the same result (Crews et al. 1991, 1996).

When taken together, such data suggest that even very low concentrations of endocrine-disrupting, receptor-dependent chemicals presently found in the environment carry risk, and there may be no threshold for such compounds.

BRAIN, BEHAVIOR, AND EDCS

The brain controls sexual behavior and acts as a secondary sex structure, much as the reproductive ducts do. The neuroendocrine mechanisms that regulate reproduction in all groups of vertebrates share a basic similarity. The core of this system consists of the hypothalamus-pituitary-gonadal (HPG) axis. Steroid hormones secreted by the gonads not only affect the differentiation of accessory and secondary sex structures but also act directly on the brain. Specific nuclei in the brain concentrate these steroid hormones, and these neuroendocrine areas serve as the final common pathway for the integration of internal and external cues important for reproduction.

The fact that individuals of each gonadal sex have the ability to develop and behave as the opposite sex implies that morphological and neural substrates of the opposite sex exist in each individual. Two distinct neural circuits appear to mediate sexual behaviors in vertebrates. Studies on representative species of *all* vertebrate classes show that portions of the anterior hypothalamus and preoptic area (AH-POA) are involved in the control of mounting and intromission behavior, whereas portions of the ventromedial hypothalamus (VMH) are involved in sexual receptivity. Neurons residing in these areas concentrate sex hormones and, further, implantation of minute amounts of the appropriate sex hormones directly into these areas of gonadectomized individuals restores mounting and receptive behaviors (Crews and Silver 1985; Meisel et al. 1987). Studies of

genetic anomalies and manipulation experiments with exogenous hormone treatments have shown that the relative development of these circuits in each individual is what distinguishes males from females in their sexual behavior. There is now clear evidence that shows numerous structural differences in the male and female brain, and further suggests that sex steroid hormones early in life play a critical role in both the development of these structural sex differences and the sexual differentiation of behavior.

The absence of evidence that EDCs affect behavior reflects a lack of research, not the lack of a behavioral effect. The last 50 years of research on the organizational actions of sex steroid hormones and sexual differentiation would suggest that EDCs have a profound influence on behavior. For example, yolk is a significant repository of circulating hormones, and mirrors the hormonal profile of the mother at the time of deposition. Maternal hormones in the yolk have a major impact on the development of the young (Bern 1990; Schreck et al. 1991). In the zebra finch and the Japanese quail, circulating steroid levels in the female are correlated with steroid levels in the yolk of eggs (Adkins-Regan et al. 1995; Schwabl 1996b). In the zebra finch and the canary, the testosterone (T) content in the yolk varies predictably across eggs within a clutch, and these differences correlate with subsequent behavioral differences in the adults (Schwabl 1993; see also Schwabl 1997). Eggs laid on later days have higher T levels than eggs laid earlier which, in turn, correlates positively with the subsequent growth and social rank of the individual; that is, males hatched from eggs laid later tend to grow faster and achieve higher social status (Schwabl 1993, 1996a).

It should be kept in mind that many pesticides were designed to interrupt the endocrine processes of insects. Although the idea that insects have sex hormones has been disputed, recent evidence indicates that insects do share some steroidal background with vertebrates (de Loof and Huybrechts 1998). The steroidogenic factor-1 gene (SF-1), which plays a role in sex differentiation (Parker and Schimmer 1997), and is regulated by estrogen in the red-eared slider (Fleming et al. 1999), shares a high degree of similarity with FTZ-F1 of *Drosophila* (Ueda et al. 1990). Ecdysone, long known as a molting hormone in insects, may actually behave as an androgen in high concentrations, causing male development in female insects (de Loof and Huybrechts 1998). Additionally, gonadotropins secreted by female locusts elicit and maintain vitellogenin production in two species of locust examined (Girardie et al. 1996, 1998). Mosquitoes secrete compounds that can inhibit vitellogenic ovarian development in several mosquito species (Brown et al. 1998), and the male gypsy moth (Loeb et al. 1997; Wagner et al. 1997; Loeb et al. 1998) secretes a brain hormone that stimulates ecdysone biosynthesis in the moth testis. Clearly, the mechanism by which some EDCs operate in insects may translate to other, noninsect species, which may cause them to act in many different ways, affecting everything from sex determination to behavior.

There is now considerable evidence that environmental contaminants that mimic steroid hormones can interfere with the functioning of the vertebrate HPG axis. For example, DES and methoxychlor delay ovulation in adult female rats by interfering with the cyclic release of luteinizing hormone (LH) by the pituitary (Cooper et al. 1986; Faber et al. 1991). Dicofol, a pesticide containing o,p'-DDT, retards reproductive development in alligators and alters circulating concentrations of sex steroid hormones (Guillette et al. 1999). PCB exposure can modify normal sex determination processes in some turtles (Bergeron et al. 1994). Perinatal administration of the antiandrogen vinclozolin (Gray et al. 1994) can increase both LH and testosterone levels (Kelce et al. 1994; Murakami et al. 1995), while p,p'-DDT and o,p'-DDE causes the development of nipples and a vaginal pouch (Kelce et al. 1995), in male rats. The pesticides vinclozolin and methoxychlor delay puberty in male rats (Gray et al. 1989, 1994), and can cause early reproductive senescence in female rats (Bal 1984).

How EDCs affect behavior has become an increasingly important question, since sociosexual behaviors associated with mate selection, courtship and mating, aggression, and parental care, as well as presexual behaviors (e.g., homing/migration) and homeostatic behaviors (e.g., eating and drinking), are modulated by steroid hormones. For example, in

rats DES both masculinizes and defeminizes the female brain and behavior; the sexually dimorphic nuclei of the preoptic area are larger, and cyclic secretion of pituitary LH is abolished, in female rats exposed to perinatal treatments of DES (Crisp et al. 1998). Long-term DES exposure during development results in an increase in masculinized juvenile behaviors in rhesus monkeys (Goy and Deputte 1996). In male Japanese quail, mounting behavior is decreased by DES or o,p'-DDT (Adkins-Regan and Garcia 1986; Bryan et al. 1989). The finding that certain pesticides and fungicides that act as antiandrogens and cause the development of nipples in male rats (Gray et al. 1994) raises the question of whether such animals exhibit related nursing and receptive behavior. Maternal treatment with low doses of DES, o,p'-DDT, and methoxychlor result in male offspring that exhibit an increased rate of urine-mark deposition when exposed to a novel environment (vom Saal 1995). In another experiment, male mice exposed prenatally to doses of o,p'-DDT and DES were found to display increased levels of aggressive behavior in resident-intruder tests (vom Saal et al. 1998).

PREDICTION: ***Bioaccumulated EDCs inherited from the mother influence not only the reproductive development and physiology of the offspring, but also the offsprings' reproductive behavior as adults, thereby magnifying the effects of EDCs through subsequent generations.***

Questions that need investigation are whether EDCs interfere with or modify the attractiveness of an individual, whether they affect an individual's motivation to mate (e.g., female proceptivity), its ability to execute appropriate mating behaviors (e.g., mounting and receptivity), and its choice of potential mates (sexual preference). While it is well established that neuroendocrine systems that subserve sexual behavior are organized early in life by sex-typical hormone patterns, relatively little is known about how these systems influence subsequent preferences for sexual partners. Bakker et al. (1993) found that male rats that had been treated embryonically with ATD, an aromatase inhibitor, prefer to associate with males instead of females when castrated as adults and treated with exogenous estrogen. Also worthy of consideration is the idea that the social environment early in life can combine with perinatal exposure to steroid hormones to shape an individual's preference for sexual partners in adulthood. If zebra finch females are treated with estrogen shortly after birth and then reared with other females from adolescence to adulthood, they will prefer females as sex partners when given androgen in adulthood; this preference does not occur if females are raised in mixed sex groups (Mansukhani et al. 1996). Other studies with zebra finches indicate that young fed seeds laced with ecologically relevant concentrations of EDCs have altered singing behavior and differences in brain nuclei that are part of the song control system as adults (Fry 1999).

Such results are particularly significant when considering EDCs because of their potential effects on sexual partner preference in animals that inhabit contaminated areas. For example, in the late 1970s the population of the California gulls (*Larus californicus*) on Santa Barbara Island was found to be profoundly affected by o,p'-DDT contamination; hatchlings had altered reproductive tract development and most adult males failed to exhibit courtship and copulatory behavior (Fry and Toone 1981). Similar responses to o,p'-DDT have been observed in herring gulls (the Great Lakes) for female-female pair bonds (Fry et al. 1987). Several hypotheses have been proposed for the female-female pairing, and most of them can be traced back to the purported effects of pollutants. The gulls may simply have undergone a shift in primary sex ratio because of a differential production of gametes, or a secondary sex ratio shift caused by sex reversal or creation of "fragile" males. Hormone levels in the parent also could have affected sex ratio—especially the operational sex ratio—by causing the production of compromised males or differential mortality weighted towards males. o,p'-DDT may have affected mate preference on the part of the male or female gulls, forcing females to resort to female-female pairing because of a lack of available mates.

It is now evident that the behavior of the adult to the young can have organizational effects on the behavior and physiology of the offspring and, further, that such effects are transgenerational in nature. Moore (1995;

Moore et al. 1997) discovered that mother rats behave differently toward male and female pups, and these differences reinforce and accentuate subsequent sex differences in behavior when the pup reaches adulthood. Individual differences (variation) in maternal care can also influence the development and endocrine physiology of the offspring (Levine 1957; Liu et al. 1997) and are the mechanism for a nongenomic or behavioral transmission of individual differences, including underlying differences in gene expression in the brain, across generations (Francis et al. 1999). Thus, the perturbations that derive from altered parental behavior could combine with the contamination itself to magnify the effect of EDCs through successive generations.

SPECIES AND INDIVIDUAL DIFFERENCES

It is not unusual to find that concentrations of sex steroid hormones vary by an order of magnitude in different species. Is this related to species differences that have been observed in sensitivity to different EDCs? For example, it is known that species with low estradiol-17β levels are more sensitive to estrogen than are species with relatively high estrogen titers (reviewed in Young and Crews 1995). The "sensitivity compensation" model was developed to explain such differences (Young and Crews 1995). Earlier research suggested that hormone level variation in different species might reflect the sensitivity of the species to the hormone, and that this, in turn, relates to the abundance of hormone receptor mRNA in neuroendocrine-controlling areas of the brain. For example, it is known that rats, guinea pigs, and hamsters have different levels of estradiol-17β, with the rat having the lowest and the hamster the highest. Behavioral sensitivity and estrogen binding capacity to exogenous estrogen in the brain is also proportional to these levels in the same order (Feder et al. 1974; Feder 1985; Lipsett et al. 1985; Young et al. 1995). This difference appears to be due to ER abundance in the brain, which is inversely proportional to estrogen levels.

PREDICTION: ***The sensitivity of a species (or an individual) to a compound is related to the typical circulating concentrations of steroid hormone and the physiological effects these hormones may exert.***

Can the sensitivity compensation model explain the variations in sensitivity to EDCs that have been observed in different species? Figure 2 depicts two possible scenarios. Site 1 is an uncontaminated situation where animals have normal endogenous sex hormone/sex hormone receptor interactions and feedback mechanisms that maintain typical circulating concentrations of sex hormones. Site 2 depicts a situation where EDCs in the environment lead to lower concentrations of circulating sex hormones through several possible mechanisms. In the first, the mimic and endogenous hormone form heterodimers, which inhibit the release of gonadotropin releasing hormone (GnRH) and ultimately lower the level of sex hormones in the blood. The second mechanism involves the destruction of the receptor—caused by the low-affinity binding of the EDC—coupled with the lack of activation by the hormone receptor element on the GnRH gene; this combination of events leads to lower concentrations of circulating sex hormones.

Can the sensitivity compensation model be used to make predictions as to which EDCs an animal may be particularly sensitive? Table 3 suggests that species with relatively low levels of estradiol-17β normally in their circulation may be more sensitive to estrogen mimics, and may demonstrate equivalent physiological responses when exposed to them. Sex hormone levels in the alligator (Guillette et al. 1996) and red-eared slider (Willingham et al. 2000) exposed to EDCs are abnormally low. Determination of sex hormone levels in related species and their response to EDCs would test this prediction. If supported, circulating concentrations of hormones in animals from uncontaminated areas could serve as predictors for potential susceptibility to EDCs.

Since circulating concentrations of steroid hormones are correlated with the amount of sex hormone receptors in the target organs, receptor abundance may also be useful as an index for measuring the effects of EDCs (Lauber et al. 1991; Salbert et al. 1993). That is, species that have low concentrations of circulating estradiol-17β and hence a greater abundance of ER in the brain are likely to be more sensitive to exogenous estrogen mimics than species with relatively high estrogen titers

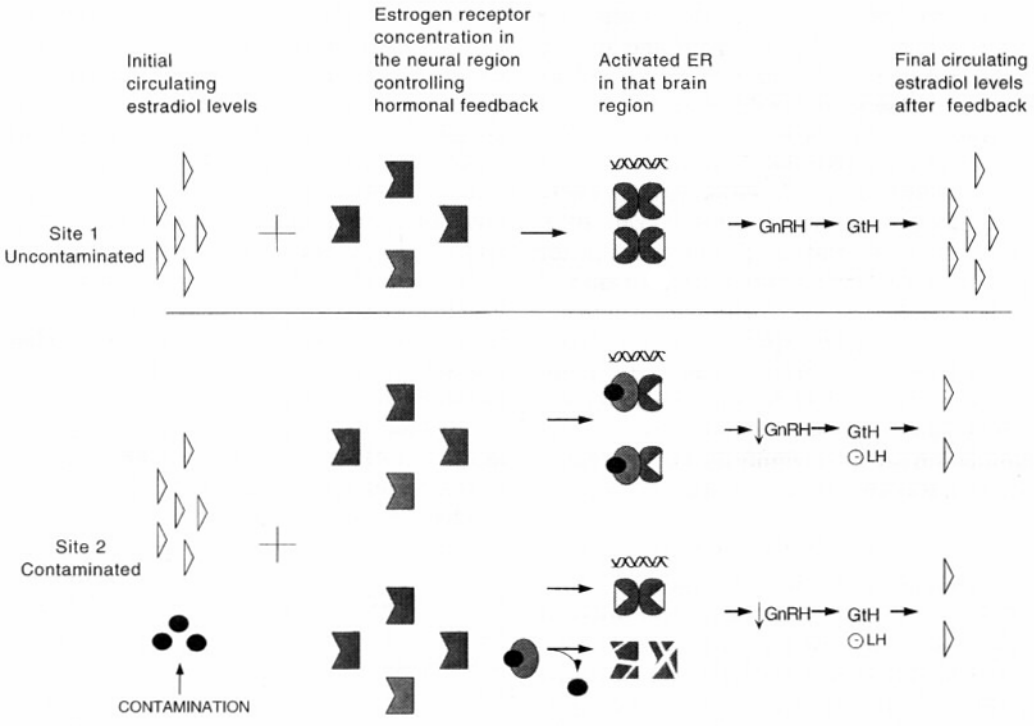

FIGURE 2. "SENSITIVITY COMPENSATION" MODEL FOR SPECIES AND INDIVIDUAL DIFFERENCES IN THE CONCENTRATIONS OF CIRCULATING SEX STEROID HORMONES APPLIED TO ENDOCRINE-DISRUPTING CHEMICALS.

Two different situations (Site 1 vs. Site 2) are presented. Site 1 depicts an uncontaminated situation where endogenous sex hormone/sex hormone receptor interactions and feedback mechanisms in the brain maintain species-typical concentrations of sex hormones. Site 2 depicts a situation where endocrine-disrupting chemicals (EDCs) in the environment lead to lower concentrations of sex hormones by several possible mechanisms. In the first, the mimic and endogenous hormone form heterodimers, which inhibit the release of gonadotropin releasing hormone (GnRH) and ultimately lower the circulating concentration of pituitary gonadotropins (GtH). In the second mechanism, low-affinity binding of the EDC to the hormone receptor destroys the receptor, and the lack of activation by the hormone receptor element leads to lower levels of GnRH and GtH production.

(Table 3). We would therefore predict that EDCs may also affect hormone receptor levels in brain areas that are important for the regulation of reproduction.

Physiological effects of EDCs may involve neuroendocrine feedback mechanisms, so that EDCs affect the concentration of circulating reproductive hormones, which in turn influences receptor abundance. For example, LH levels in female rats are lowered or delayed after exposure to chlordecone (Uphouse et al. 1984); similar results were found with 1,2-dibromo-3-chloropropane (DBCP) in male rats (Warren et al. 1988) and chlordimeform in female hamsters (Goldman et al. 1993). Other EDCs—including procymidone and vinclozolin (Kelce et al. 1994; Murakami et al. 1995)—cause an increase in LH levels in rats. Such changes in LH affect the concentration of other endogenous hormones and thus affect the abundance of hormone receptor. Thus, three avenues that measure the endocrine effects of EDCs—hormone levels, receptor abundance, and LH levels—can all serve as markers for sensitivity.

A related issue is the relative potencies of different EDCs, including natural steroids. It is well known that species differ in the nature

TABLE 3

Species comparisons may predict the sensitivity of different species to estrogenic chemicals

Estrogen hormone level	Estrogen receptor abundance	ER binding (affinity/ specificity)	Behavioral sensitivity (receptivity)	Predicted response to estrogenic EDCs
High	Low	Low	Low	Insensitive
Low	High	High	High	Sensitive

Note: The comparisons reveal consistent relationships between hormone levels, estrogen receptor (ER) abundance, affinity and specificity of hormone binding, and behavioral sensitivity.

and pattern of sex steroid hormones they secrete. In some species testosterone may be the predominant steroid in males, but in other species it may be dihydrotestosterone or 11-ketotestosterone. In females, estrone, estriol, and estradiol-17β are commonly found, and research in mammals has led to the supposition that estradiol-17β is the most potent of these steroidal estrogens. While this may be true for human beings, it does not follow for other vertebrates, or even for all mammals. For example, in the red-eared slider sex determination assay, estriol is far more potent than estradiol-17β in overcoming the effects of a male-producing temperature (Bergeron et al. 1999). Similarly, o,p'-DDE and its metabolites have been implicated in a variety of reproductive anomalies, but not in all species. In the red-eared slider, o,p'-DDE will cause female development when applied in concentrations found in contaminated environments (Willingham and Crews 1999), but it has no detectable effect in the green sea turtle, *Chelonia mydas* (Podreka et al. 1998), and is antiestrogenic in the tiger salamander, *Ambystoma tigrinum* (Clark et al. 1998). In the rat, methoxychlor causes delayed anovulatory syndrome (Gray et al. 1989), but in hamsters it is without apparent effect on estrous cycling (Gray et al. 1985). On the other hand, the pesticide Kepone masculinizes the behavior of female hamsters (Gray 1982), and, in rats, seems to inhibit female sexual behavior when administered at proestrus (Brown et al. 1991), fails to induce lordosis in ovariectomized females (Uphouse et al. 1986), and accelerates puberty (Gellert 1978). Thus, rather than concentrating on a few species to serve as standards for defining a chemical as an EDC, a more instructive approach might be to document the effects and then understand why different species vary in their sensitivity to specific compounds.

Finally, the individual and its response to EDCs must be considered. Recent work by Howdeshell et al. (1999) has shown that an individual's response to EDCs as an adult can depend upon its particular embryonic environment. Previous work indicates that the female offspring of rats fed bisphenol-A during their pregnancy exhibit a subtle but significant increase in postnatal growth and an advancement of the onset of puberty. However, when the intrauterine position of the fetus is considered, the effect is restricted to those individuals that have the highest background levels of endogenous estradiol-17β during fetal life; that is, females that develop between female neighbors. Because the hormone milieu differs during development in relation to intrauterine position, such results indicate that the background hormonal profile influences an individual's subsequent sensitivity to bisphenol-A.

EVOLUTION OF CHEMICAL RESISTANCE

In the world today, generations of animals are constantly exposed to unique compositions and concentrations of chemical mixtures. With the exception of extraordinary spills, the fact is that populations of resident animals continue to reproduce in these environments. In some instances this persistence is due to immigration of unaffected individuals, adaptive enzymatic responses that affect catabolism of the EDCs, or proteins in the blood that sequester EDCs. Another possibility that needs to be considered is that these individuals are more resistant genetically to the chemical environment. Since sex steroid hormones are biological regulators fundamental to develop-

ment and reproduction, it is unlikely that such resistant individuals in successive generations in contaminated sites coincidentally become insensitive to their own endogenous ligands.

The evolution of resistance to pesticides in insects is well known, but it has not been studied adequately in vertebrates. Is it possible that with time vertebrates also become buffered in some way to the chemical environment? This buffering almost certainly occurs through genetic changes that render the animals less sensitive to the chemical. For example, sheep grazing on clover, which contains phytoestrogen, lose their sensitivity in four generations (Kaldas and Hughes 1989). And the development of o,p'-DDT tolerance has been documented in natural populations of cricket frogs, grackles, red-winged blackbirds, starlings, and ring-necked pheasants.

Early attempts to establish genetic resistance yielded promising results. Ozburn and Morrison (1962) produced o,p'-DDT tolerant mice after eight generations of selection, and Poonacha et al. (1973) selected for resistance to o,p'-DDT in the Japanese quail (*Coturnix coturnix japonica*). In the latter instance two selected lines of quail were fed a diet containing 200 ppm of o,p'-DDT during the quails' first 30 days of life. It was evident that the development of resistance started after the third generation of selection, as indicated by lower mortality among the selected lines when compared to a control line fed o,p'-DDT. Interestingly, there was a sex difference in resistance, with females being less resistant than males.

Resistance can be defined as the development of the ability in a lineage to tolerate doses of a toxicant which would prove lethal to the majority of individuals in a normal (i.e., susceptible) population (cf., Scott 1995). This definition raises the question of whether genetic change in the form of novel mutations underlies the development of resistance to EDCs. Resistance genes are more likely to result from selection pressures on existing genotypes that regulate or enhance particular defense mechanisms rather than from novel mutations that create new genes. One of the major mechanisms of pesticide resistance in insects is target-site insensitivity, which refers to an alteration of the molecule(s) that directly interacts with the pesticide to reduce toxicity (Scott 1995). For example, Mutero et al. (1994) has suggested that decreased sensitivity for acetylcholinesterase in *Drosophila melanogaster* results from point mutations that structurally change the enzyme.

There can be no doubt that sex steroid hormones and their nuclear receptors coevolved. The evolution of the steroid hormone molecule is mirrored in its biosynthetic pathway, with cholesterol giving rise to pregnenolone, which in turn is metabolized to progestagens; progestagens metabolize to androgens, which finally metabolize to estrogens. Similarly, the structural organization of the steroid hormone receptors suggests an evolutionary process, although the sequence is not as obvious. Phylogenetic analysis of the nuclear receptors indicates that the sex steroid hormone receptors evolved from an SF-1-like progenitor with estrogen and estrogen-related receptors diverging first, followed by androgen and progesterone receptors (Thornton and Kelley 1998; de Mendonca et al. 1999; Thornton and deSalle 2000).

While it is now rare for a new sex steroid hormone to be discovered in vertebrates, this is not the case for hormone receptors. There is a host of transcriptional activators with no known ligand called "orphan" receptors. If we assume that steroid molecules did not become endocrine signals until there was a means of detecting them, then we can view orphan receptors as the background against which the steroid molecule evolved to assume its function. That is, the now well-established steroidogenic pathway, representing repeated alterations of the basic steroid molecule, provided a ready source of endogenous signals because they were associated with important physiological functions such as gametogenesis, sex determination, and sexual differentiation. In this scenario, primitive orphan receptors co-opted circulating estrogen and androgen as activators, hence becoming the estrogen and androgen receptors that are known today (de Mendonca et al. 1999; Thornton and deSalle 2000). Thereafter, genetic mutations in one of the functional domains of nuclear transcription factors created new receptor subtypes (e.g., estrogen receptor α and β). In this way the function of sex steroid hormones and the receptor molecules that bind them represent a coevolutionary

process—which might be termed steroid exploitation—operating in a similar manner as the sensory exploitation hypothesis of Ryan (1998), in which sensory systems are predisposed to specific stimuli, thereby leading to present-day sender-receiver acoustic signals.

PREDICTION: ***Chronic exposure to EDCs selects for genetic changes that buffer species from further deleterious effects.***

Molecular studies will be needed to determine how genomic changes might protect receptors from the transactivational effects of EDCs. Some of the scenarios focus on alterations in the steroid receptor itself. Changes in amino acid arrangements or composition may alter affinity of binding, making the affected steroid receptor protein more specific to its natural ligand and reducing its affinity for other ligands. Alternatively, the receptor's genetic code may be altered so that the receptor discriminates between EDCs and natural ligands. Other explanations might include mutations that preclude dimerization unless a specific (i.e., natural) ligand is present.

The estrogen receptor is unique among steroid receptors in its capacity to bind different estrogen analogues as well as structurally diverse nonsteroidal compounds (Anstead et al. 1997). Studies on the structure of the receptor indicate that this promiscuity can be explained in part by the large size of the steroid binding cavity and its plasticity (Brzozowski et al. 1997). The ligand-binding cavity is about twice the volume of estradiol-17β, and there are especially large cavities opposite the B and C rings of the steroid. Alterations in protein structure that fill these cavities and/or result in a less plastic ligand binding site would be expected to lead to an ER with a greater binding specificity.

Because of the separated cassette structure of the DNA binding domain and the ligand binding domain (LBD), it is unlikely that mutations in the LBD would influence the ability of the ER to bind to the estrogen response elements. However, the unoccupied receptor is held in an inactive state by molecular chaperonins, such as heat shock protein 90, until a ligand capable of inducing a conformational change sufficient to dissociate the Hsp90 binds to the receptor. Some alterations in structure may make this dissociation step more dependent on natural ligands. In effect, the first step in receptor activation would not occur unless natural ligands were bound.

Another consequence of estrogen binding is that helix 12 at the C-terminus swings across the ligand binding cavity to form a lid (Brzozowski et al. 1997). This precise positioning of helix 12 is a ligand-induced conformational change that is involved in forming the active transactivation function 2 (TF2). A hydrophobic cavity is created that allows the receptor to be bound to coactivator proteins, which are required for the receptor to interact with the core transcriptional machinery (Feng et al. 1998). Mutations in ER structure that fail to activate TF2 when EDCs are bound, but activate TF2 in the presence of natural ligands, would also buffer the effect of EDCs on reproductive physiology.

CONCLUDING REMARKS

Compounds capable of interfering with the endocrine system have become ubiquitous in natural environments. Long-lived organisms feeding at the top of the food chain are particularly vulnerable to these contaminants. Numerous studies indicate that while these compounds may not adversely affect the adult organism, they can be passed to the fetus where—even at extremely low doses—they can disrupt normal sexual development. Such sterile individuals occupy space and use resources but cannot contribute to the growth of the population, as their genes will not transmit to subsequent generations, hence leading to evolutionary death.

It has become evident that assumptions based on traditional toxicological research may not apply to chemicals that mimic the action of ligands important to sexual development. Certain combinations of steroidal and nonsteroidal compounds may act synergistically rather than additively. Current risk assessments for virtually all chemicals except genotoxic carcinogens assume that there is a dose below which no adverse effects occur (the threshold dose). Assessing risk becomes more complicated, however, when a chemical mimics or antagonizes the action of an endogenous substance like a hormone important in development. The threshold-dose hypothesis, the

basis of current safety limits, may not apply to EDCs since, upon exposure, the threshold for the endogenous ligand is already exceeded; hence no threshold dose may exist for EDCs. How these compounds affect the brain and, in turn, the reproductive behavior and physiology of animals, is largely unexplored. Finally, it should be possible to study the evolution of resistance to chemicals that mimic the effects, or interfere with the actions, of steroid hormones by selecting for genotypes that are resistant at low frequencies of the EDC while maintaining sensitivity to the natural steroid. Molecular analysis may reveal whether or not EDC resistance derives from structural changes in the hormone receptor gene that decrease the ability of the chemical to physically bind to its site of action and thereby prevent activation of the putative receptor(s).

The benefits of many EDCs in industry and agriculture ensure that they will continue to be used. Thus they will continue to pose a threat, forcing individuals and populations to adapt in order to survive. It would be a step forward to develop and implement alternative manufacturing processes for certain human-use products. As our understanding of the biology of EDCs improves, it may also be possible to develop biochemical interventions which protect the embryo from hormone disruptive effects. For example, it should theoretically be possible to treat pregnant females with compounds that bind and sequester hormone disruptors and protect the developing embryo. Finally, better understanding of genetic changes that occur as a consequence of chronic exposure to EDCs may reveal mechanisms by which the evolution of the genome may protect transactivation of the sex hormone receptors.

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