No Threshold Dose for Estradiol-induced Sex Reversal of Turtle Embryos: How Little Is Too Much?

Daniel M. Sheehan,[1] Emily Willingham,[2] David Gaylor,[1] Judith M. Bergeron,[2] and David Crews[2]

[1]Division of Genetic and Developmental Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079 USA; [2]Institute of Reproductive Biology, Department of Zoology, University of Texas at Austin, Austin, TX 78712 USA

Risk assessments for nongenotoxic chemicals assume a threshold below which no adverse outcomes are seen. However, when an endogenous chemical, such as 17β-estradiol (E_2), occurs at a concentration sufficient to cause an effect, the threshold is already exceeded. Under these circumstances, exogenous estradiol is not expected to provide a threshold dose. This principle is demonstrated for E_2 in the red-eared slider, a turtle with temperature-dependent sex determination. In this species, gonadal sex is determined by egg incubation temperature; female development requires endogenous estrogen produced by elevated temperature. While normal production of females by endogenous estrogens is not an adverse effect, exogenous estrogens can sex reverse presumptive males, which can be an adverse effect. A large dose–response study was conducted using seven doses and a vehicle control (starting n = 300/group); a single E_2 dose was applied to the eggshell of recently laid eggs. Animals were sexed after hatching. The incubation temperature chosen, 28.6°C, generates a minority of females. Thus, the criteria for testing the threshold hypothesis were met, i.e., there is evidence that there is endogenous estrogen and that it generates an irreversible response. The lowest E_2 dose tested, 400 pg/egg (40 ng/kg), sex reversed 14.4% of the animals, demonstrating very low dose sensitivity. The data were fit with a modified Michaelis–Menten equation, which provided an estimate of 1.7 ng/egg for endogenous estradiol. The median effective dose (ED_{50}) was 5.0 ± 2.0 ng/egg (95% confidence limits), of which 1.7 ng/egg was endogenous estradiol and 3.3 ng/egg came from the applied estradiol. There was no apparent threshold dose for E_2. A smaller replication confirmed these results. These results provide a simple biologically based dose–response model and suggest that chemicals which act mechanistically like E_2 may also show no threshold dose. If so, even low environmental concentrations of such chemicals may carry risk for sex reversal. *Key words*: biologically based dose–response model, development, endocrine disruptors, estrogens, risk assessment, safety testing, sex determination, sex reversal, threshold, turtle. *Environ Health Perspect* 107:155–159 (1999). [Online 14 January 1999]
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Endogenous estrogens are required for proper growth and development of several mammalian tissues, including the male and female reproductive tracts. This organizational effect of estrogens programs not only certain features of normal adult structures but also responsiveness to estrogens in adulthood, the latter termed the activational effect. Additionally, the organizational effect of estrogens appears to act through the same mechanisms that activate genes in adults. Some estrogen responses are activated by endogenous estrogens during development (*1,2*). These features are important in understanding why exposure during critical periods of development to very low doses of a variety of chemicals with estrogenic activity can irreversibly alter reproductive tract development, leading to adverse effects.

Estrogens are involved in normal female development of many organisms with temperature-dependent sex determination (TSD), including turtles. In the red-eared slider turtle (*Trachemys scripta elegans*), sex is determined by the incubation temperature of the egg (rather than by sex chromosomes) during the middle third of incubation, a window of time called the temperature-sensitive period (TSP). An egg incubation temperature of 26.6°C produces all males, and a temperatures of 31°C produces all females (*3*). A male:female ratio of about 1:1 is found at 29.2°C (*4*). As temperature increases from 28.8 to 29.6°C, the sex ratio changes from 9:1 male:female to 9:1 female:male (*4*). Administration of exogenous estradiol (E_2) or hydroxylated polychlorinated biphenyls (PCBs) to eggs incubated at all-male or male-biased temperatures results in females, overriding the temperature effect (*5,6*). Additionally, when eggs are incubated at increasing temperatures that progressively produce a larger fraction of females, the dose of E_2 required to sex reverse 50% of the animals decreases significantly (*7*). Further studies of steroid production provide a case for the involvement of steroids in determining the sex of the red-eared slider turtle. Aromatase, the enzyme that metabolizes testosterone to estradiol, is inhibited by fadrozole and other inhibitors. When these inhibitors are applied to eggs during the TSP, incubation at a female-producing temperature results in males (*4*). These findings provide evidence that estrogens mediate the temperature sex determination of females in this species; inhibition of its synthesis results in males, and application of estrogens to presumptive males results in females.

Although endogenous estrogens are required for normal female turtle development (which is not an adverse effect) and thus normal sex ratios in turtle populations, exogenous estrogens can be regarded as developmental toxicants, irreversibly producing inappropriate development of females or, less frequently, intersexes at temperatures that normally produce males (*3,5*); both are adverse effects at the population level.

Estrogen mimics and E_2 have demonstrated sex-reversal effects similar to those shown by endogenous estrogens. When hydroxylated PCBs are applied to eggs incubated at all-male or male-biased temperatures, females result (*5*). Other species provide evidence of altered development as a result of exposure to estrogen mimics. Male fish swimming in effluent-polluted waters in Great Britain exhibited feminization (*8*), and a female-biased sex ratio is associated with exposure of gulls to environmental levels of DDT, a synthetic estrogenic chemical (*9*). This association has been confirmed in studies in which DDT applied to male gull eggs resulted in feminized male embryos exhibiting ovarian tissues and oviducts (*9*).

Risk assessments for virtually all chemicals, except genotoxic chemicals, assume a threshold dose below which no adverse effects occur (*10*). This assumption underlies the use of the no observed adverse effect level (NOAEL), which is the dose that provides no statistically significant increase in adverse effects. To account for imprecision and species extrapolation, this dose is divided by

Address correspondence to D.M. Sheehan, Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079 USA.

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an uncertainty factor (often 100) to provide the acceptable exposure values. The acceptable exposure, considered safe, is rarely tested; thus, safety depends on the validity of the threshold assumption. This assumption (threshold hypothesis) may not apply to chemicals that share a common mechanism with endogenous chemicals important to normal development if the threshold for the exogenous chemical already is exceeded by the endogenous chemical (*11–13*). Administration of the same chemical should lead to a curve showing no threshold dose, no matter how low the background incidence caused by the endogenous chemical (*11*). The criteria to refute the threshold hypothesis are 1) evidence of endogenous estrogen, 2) estrogen mediation of an irreversible effect (both satisfied by the data for the red-eared slider turtle), and 3) regression of the dose–response curve to the origin or to a positive value on the y- (response) axis (*11–13*). Using the red-eared slider turtle, we tested the threshold hypothesis for E_2 and developed a biologically based dose–response (BBDR) model.

Materials and Methods

Experimental. Freshly laid eggs obtained commercially (Robert Kliebert, Hammond, LA) were held at room temperature until we established viability by candling. Eggs were then placed in incubators in boxes (30 eggs each) containing vermiculite:water (1:1 by volume) and incubated at 28.6°C. This temperature was chosen to provide a large enough number of females to be detectable, but small enough to give a male-biased population. Temperature was monitored daily using a HOBO temperature logger (Onset Computer Corporation, Pocasset, MA) and frequent inspection of calibrated thermometers on different shelves (*14*). At Stage 17, the beginning of the temperature-sensitive window (*7*), each egg received one of seven doses (Table 1) of E_2 dissolved in 5 µl 95% ethanol. This was pipetted directly onto the eggshell. Each treatment group initially contained 300 eggs divided equally into 10 boxes, with a final sample size at hatch ranging from 253 to 267 eggs/group. A control group (final number = 240) was treated with 5 µl ethanol. After treatment, all eggs were returned to the incubator until they hatched. During the entire period of incubation, eggs were rotated daily from shelf to shelf to avoid any "shelf effect" of temperature. Within 2 weeks of hatching, turtles were anesthetized and sacrificed; gonadal sex and development of oviducts were examined, in a blinded manner, under a dissection microscope by two researchers and recorded. In all cases, macroscopic and histological assessments agreed and were consistent with the histological assessments previously described (*6*). Results from two boxes that were statistically significant outliers (out of a total of 80 boxes) were not used. In the next breeding season, a repeat study was conducted with the three lowest doses (and a control) as described above, with 55–62 animals (final number) in two boxes to determine replicability in an independent experiment.

Table 1. Comparison of estradiol dose-dependent sex reversal in 1996 and 1997

Dose (ng/egg)	Percent female ± SE 1996	1997
0	23.5 ± 2.0	7.0 ± 0.9
0.4	37.9 ± 3.5	25.5 ± 2.3
1.6	44.4 ± 4.0	53.2 ± 3.1
4.0	46.2 ± 3.1	76.8 ± 6.2
7.0	65.0 ± 2.1	ND
17	82.2 ± 2.5	ND
40	82.9 ± 2.3	ND
175	95.5 ± 1.6	ND

Abbreviations: SE, standard error; ND, not determined.

The University of Texas at Austin has an approved Assurance from the Office of Protection from Research Risks of the National Institutes of Health (Assurance no. A-1496). All protocols used in these studies have been approved and reviewed by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Michaelis–Menten Model. A variant of the Michaelis–Menten equation was developed. Maximum response is fixed at 100% female, while the dose term consists of an endogenous dose (d_0) plus the administered dose (d); the dose providing a 50% response is the ED_{50} (or median effective dose). The Michaelis–Menten model is as follows: % female = $100(d + d_0)/[ED_{50} + (d+d_0)]$. This can be rewritten as a simple linear model in d where (% female/% male) = $(d_0/ED_{50}) + (d/ED_{50})$. The intercept is d_0/ED_{50} and the slope is $1/ED_{50}$. A noniterative weighted linear least-squares procedure was used to fit the model. The weights were the reciprocals of the standard deviations of % female/% male among boxes for each dose group. The sample size (n = 9 or 10 for the first experiment and n = 2 for the replicate) was the number of boxes used for a dose group. Because the standard deviation tended to increase with dose, the most weight was assigned to the control and low doses, the regions of most interest.

Results

Retrospective analysis of published data on sex reversal by varying doses of E_2 at three different incubation temperatures (*7,14*) revealed dose–response curves that fit a Michaelis–Menten equation (Fig. 1). While no threshold dose was observed, these results were obtained from a relatively small number of animals. Based on this outcome, we then designed a large (2,400-egg) prospective dose–response study to test further the threshold hypothesis and the fit of the Michaelis–Menten equation. The results are shown in Figure 2. The curve regressed to the y-axis at the control value (23.5%) for percent female and to the negative x-axis at the endogenous dose (d_0), which was 1.7 ± 0.9 ng/egg (approximate 95% confidence limits). The lower 95% confidence limit around d_0 was <0, indicating that regression to the origin or to the positive x-axis was unlikely (p<0.003). The value for d_0 is expressed in the same units as applied dose and may or may not reflect the actual concentration of E_2 in the egg. This is because in the egg, estradiol biosynthesis occurs over time, whereas we applied a single dose to the eggshell.

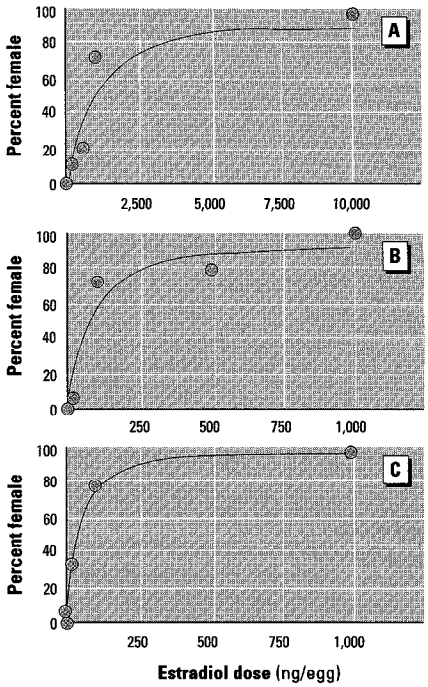

Figure 1. Retrospective analysis of the effect of exogenous estradiol applied to eggs of the red-eared slider turtle incubated at different temperatures (*7,12*). At 26.0°C (A), the r^2 and median effective dose values were 0.85 and 445 ng/egg, respectively; at 28.2°C (B), these were 0.995 and 38 ng/egg; and at 28.6°C (C), they were 0.998 and 31 ng/egg. Neither a line drawn by eye through the points nor a Michaelis–Menten fit showed a threshold dose.

Using the modified Michaelis–Menten equation, an ED_{50} of 5.0 ng/egg with 95% confidence limits of ± 2.0 ng/egg (endogenous dose = 1.7 ± 0.9 ng/egg; exogenous dose = 3.3 ± 1.8 ng/egg) and an r^2 of 0.90 were obtained for the fit of the equation to the data points. The lowest dose administered, 400 pg/egg (40 ng/kg egg weight), significantly increased the female fraction

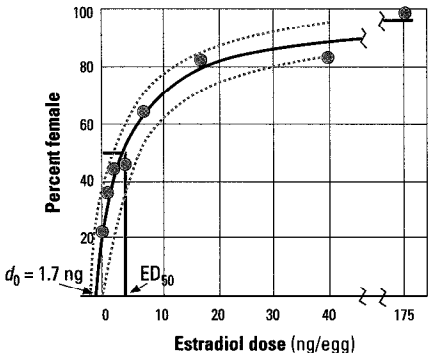

Figure 2. A large prospective test of the no threshold model using 2,400 red-eared slider turtle eggs incubated at 28.6°C and treated with 17β-estradiol (0, 0.4, 1.6, 4.0, 7.0, 17, 40, and 175 ng/egg). The Michaelis–Menten fit was as follows: % female = $100(d+1.7)/5 + (d+1.7)$. The solid line, calculated from the equation, fits with an $r^2 = 0.90$. Dashed lines indicate 95% confidence intervals. The line strikes the dose axis at -1.7 ng/egg, providing the predicted endogenous dose (d_0) in units of applied dose (ng/egg). The median effective dose (ED_{50}) of 5.0 ng is the sum of the endogenous and applied doses ($ED_{50} = 3.3 + 1.7 = 5.0$ ng).

by 14.4%. A curve drawn by eye is virtually identical to that found with the Michaelis–Menten equation.

The replication of the three lowest doses and the control is shown in Table 1. In 1997 the control showed a lower fraction of female turtles and a steeper dose–response slope as compared to 1996, with an ED_{50} of 1.3 ± 0.6 ng/egg and an endogenous dose of 0.08 ± 0.28 ng/egg ($p<0.15$). Thus, there is a 15% chance that there is not an endogenous dose in the repeat experiment. The smaller number of eggs and boxes per group and the smaller number of doses may account for the inability to conclude at $p<0.05$ that an endogenous dose exists. Another potential explanation for these differences is that the eggs were collected in the late summer in 1996 and in the late spring in 1997. Combining the p-values from the two experiments, the endogenous dose of estradiol was significantly different from zero ($p< 0.01$).

Discussion

In this unusually large study, we tested the hypothesis that, even if a threshold exists, no threshold dose is expected because endogenous estrogens are at a sufficiently high concentration to exceed the threshold for sex reversal of red-eared slider turtles. This is a variant of the hypothesis that if an adverse effect occurs due to an endogenous chemical, no threshold dose is expected (*11*). In turtles, endogenous estrogens program normal sex reversal to provide an evolutionary-derived appropriate sex ratio. While the no-threshold hypothesis has been in the toxicology literature for decades (*11–13*), this is the first robust prospective study in an experimental system that meets the criteria for evaluation of a threshold. Over 70,000 man-made chemicals that have an aggregate value of billions of dollars are found in food, water, air, or soil (*15*). Given the central role of the threshold assumption in evaluation of health safety, the exposure of all organisms to synthetic chemicals, the importance of these chemicals in modern society, and their huge production volume and economic value, it is surprising that the threshold assumption has been so widely accepted and so rarely tested. It is important that the assumption of a threshold is not a theory or fact, but simply a pragmatic step in risk assessments. The threshold assumption is made in the absence of data contradicting it, but has not been subjected to robust direct experimental testing for any developmental toxicant.

The threshold assumption is based on an infinite regress. If a dose–response curve appears to show no threshold, it can be argued that the threshold could be defined if a sufficient, perhaps up to an infinite, number of animals were put on study. Contrariwise, if the curve appears to show a threshold, it can again be argued that there were insufficient numbers of animals on the study to demonstrate that a threshold exists. The solution to this dilemma is to test the hypothesis as described above and determine which model (threshold or no threshold) is supported by the simplest explanation (Occam's Razor) (*16*). This hypothesis is not an infinite regress, as we are interpolating between an endogenous dose and the lowest applied dose using a model of biological relevance that utilizes all the data. The threshold/NOAEL approach only uses one dose, the NOAEL. Furthermore, simply connecting the data points by eye with a smooth curve generates an almost perfect replica of the Michaelis–Menten curve. For the first experiment, the interpolation traverses a very small dose difference; the control endogenous E_2 dose is 1.7 ng/egg, whereas the lowest dose of 0.4 ng/egg provides a total dose of 2.1 ng/egg (the sum of the endogenous and exogenous dose). The increase in E_2 above the control value is 23%. This should be contrasted with the situation in cancer and radiation risk analysis, where a no-threshold model is frequently used. The difference between the lowest dose tested and the background exposure is about 100,000-fold (the lowest dose will generally give a response rate of about 10%, and the curve is extrapolated to a risk of one in a million), and different statistical models lead to differences of 100-fold or higher for an estimated acceptable exposure (*17*). These differences arise because the models assume different curve shapes. For the turtle data, it would not be cogent to draw a significantly different curve than that presented, given the data points and the knowledge that endogenous estrogens are responsible for producing the female phenotype. Because the extent of interpolation in our model is so small (23%) as compared to that in cancer and radiation risk analyses and because the curve shape is model independent, the objections to the large uncertainties found in the former extrapolations do not apply to our model.

The single-dose lowest observed adverse effect level (LOAEL) for estradiol in the turtle (40 ng/kg) is slightly lower than the total dose LOAEL in the developing fetal mouse prostate in which diethylstilbestrol (DES) doses were fed over 7 days (20 ng/kg × 7 = 140 ng/kg) (*2*). Additionally, the relationship between the increase in dose and response is similar to that found in the turtle. A 50% increase in dose above endogenous levels leads to a 30% increase in mouse prostate weight, whereas in the turtle, a 23% increase provides a 14.4% increase in response. These are very low doses as compared to most of those used in toxicology experiments and are a consequence of the exogenous dose supplementing endogenous doses that are already active in inducing responses. This emphasizes the need for low environmental concentrations of endocrine disruptors to be studied with the most dose-sensitive end points.

We have used a biologically based equation to generate a BBDR model that can be used for dose–response and subsequent risk assessments. This should be distinguished from equations that carry no underlying biological significance, but only fit the line based on a mathematical/statistical approach, such as a polynomial equation with the associated statistical parameters. The Michaelis–Menten equation as normally used has no threshold term (*18*), nor are thresholds observed in analysis of receptor binding. While low concentration curvature can be found in Michaelis–Menten equations with Hill coefficients >1, such curvature does not represent a threshold, but a lower than expected response compared to the unmodified equation. More complex BBDR models are under development (*10,19,20*). These use Michaelis–Menten kinetics to model the pharmacokinetic and biological response data. A theoretical receptor-mediated model (*19*) and modeling of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*20*) data sets strongly suggest that thresholds are not generated by hormone–receptor interaction and are unlikely when an endogenous chemical is active. Our model predicts the endogenous

dose when it exists. The close fit of organismal data to the Michaelis–Menten equation suggests that sex reversal is driven by a single rate-limiting, reversible binding event analogous to enzyme kinetic studies showing that substrate binding is the rate-limiting step, not the conversion of substrate to product. For receptor-mediated responses, the rate-limiting step is assumed to be liganded estrogen receptor binding to DNA response elements (*19,20*). While numerous events such as absorption, distribution, metabolism, and elimination occur between the application of the dose to the eggshell and the arrival of hormone to the receptor target, they will not influence the shape of the dose–response curve if they are not rate-limiting, but they can have an impact on potency.

The BBDR model demonstrates that no exogenous E_2 is without risk (as opposed to the threshold assumption) and requires that an acceptable risk level be defined and the corresponding exposure calculated directly, in contrast with the traditionally derived exposure levels. Just as a threshold is not predicted to lie between two exogenous doses on the ascending portion of the dose–response curve, it is illogical to assume that a threshold exists between the control endogenous dose and the lowest exogenous dose in our model. The fit of the Michaelis–Menten model was slightly better with a Hill coefficient of 1 compared to 2, but because most of the curvature in the latter case is in the low-dose region of the dose–response curve (i.e., in the endogenous dose region), good resolution was not feasible. However, in the replication, the control value was lower and the curve showed no low-dose curvature. These considerations, taken with the fit of the large data set to the Michaelis–Menten equation, the three analyses in Figure 1, and the replication study, argue for our conclusions that no threshold dose exists. In the absence of a threshold dose, there will be risk at any dose, no matter how low (*11*).

Risk assessment assumptions are often made in the absence of data; this is the first report of a robust prospective experimental test of the threshold hypothesis for a developmental toxicant. These results are consistent with the finding that no clear threshold exists either for exogenous E_2-induced enlargement of the fetal mouse prostate, the size of which is controlled, in part, by endogenous estrogens (*2*) or for adverse effects of estrogens on thyroid hormone in the developing rat (*21*). Likewise, a large conventional teratology experiment with 2,4,5-trichlorophenoxyacetic acid was conducted using five mouse strains with 4–10 replicates each and 548–1,154 pregnant dams per strain experiment; no threshold was discernable (*22*). The criteria we have established were used to examine published data from endocrine-active chemicals; responses ranging from the expression of single genes through physiological responses to adverse effects were found to fit a Michaelis–Menten equation with high correlation coefficients for 15 chemicals (*23*). These criteria should be used to prospectively examine other endocrine disruptors and outcomes across diverse species to determine the extent to which no-threshold models are applicable. There are clear circumstances in which this would be inappropriate: if the endogenous hormone is absent or induces no response, a threshold would be expected. However, if any level of response occurs, even if experimentally undetected due to insufficient resolving power, no threshold is expected (*11*). The procedure described here allows risk assessments to be conducted with knowledge of actual or estimated risk in the observed or interpolated dose range, respectively. Our approach allows a more informed decision by risk assessors, in contrast with the NOAEL/uncertainty procedure in which no risk is estimated.

The issue of whether sex reversal is an adverse effect is important. The definition of a response as adverse is frequently controversial in risk assessments. We need to distinguish traditional adverse effects, which may be based on clear histopathology, from nonpathological events that are adverse. In the case of turtle sex reversal by xenoestrogens, the females should be fertile, assuming no other adverse effects of the xenoestrogen on reproduction. However, sex reversal leading to reproductively capable females can have adverse demographic consequences. Clearly, at high rates of sex reversal, the reproduction of populations will be compromised. This is a clear adverse effect. But what about lower rates of sex reversal? These, too, could be adverse effects, depending on whether other events such as habitat loss, predation, global warming (which is particularly applicable in TSD animals), or other stressors occur at the same time. A principle of ecology is that four characteristics determine population density: age structure, survival rate, fecundity, and sex ratio (*24,25*). Sex reversal by xenoestrogens clearly alters the sex ratio and can thus impact population density and survival. Importantly, exposure to a mixture of estrogenic endocrine disruptors could lead to a high rate of sex reversal, although each chemical by itself may induce a low sex reversal rate. For xenoestrogens, the large majority of chemicals examined act as full or partial agonists, with the exception of antiestrogenic drugs (*26*). Additionally, chemicals can act as agonists in one tissue but antagonists in another (*27*). Given this, it is unlikely that antagonists in a mixture would contribute significantly to decreased adverse effects, although this will depend entirely on the components of the mixture and their concentration. The EPA has developed a toxic equivalency (TEQ) factor approach for mixtures such as dioxins (*28*). The TEQ is the sum of the concentrations of each chemical times their potency, where TCDD has a potency of 1. Risk assessments are then based on the mixture's TEQ. This avoids the possibility of setting an acceptable exposure level for each chemical individually, with the potential consequence that exposure to a mixture would result in adverse effects. The same approach would be appropriate for risk assessments for xenoestrogen-induced sex reversal.

It can be argued that the increase in the female fraction at low doses is within the normal fluctuation in sex ratio in this species and is therefore not adverse. However, no matter the extent of fluctuation, there will always be an added effect of estrogen exposure such that the female fraction is increased. Consequently, the population distribution is shifted to a higher proportion of females, which can interact with other population stressors to contribute to population declines. Thus, the real issues are the increased risk due to exposure to xenoestrogens, either from an individual chemical or a mixture, and how little exposure is too much for population stability under a variety of environmental conditions.

All crocodilians, many turtles, and some lizards have TSD (*4*). Thus, the potential for sex reversal by environmental estrogens has implications for many wildlife species. Additionally, the threshold hypothesis should be tested in other non-TSD animal models for chemicals that mimic the actions of estrogens and other endogenous signaling chemicals. Naturally occurring and synthetic chemicals found in the environment, such as coumestrol, DDT, and PCBs, produce classical estrogenlike effects in animals (*5,9,29–31*). Many estrogenic endocrine disruptors operate through the same mechanism as E_2 (*24*); importantly, at low doses, such chemicals can therefore increase rates of normal, active endocrine processes. The red-eared slider model, which provides a sensitive bioassay for these chemicals, should be used to explore the threshold issue for a variety of estrogenic endocrine disruptors.

Based on current risk assessment procedures, global environmental chemical concentrations are generally thought to be too low to exert adverse affects when compared to their traditionally calculated acceptable

exposure levels. We demonstrate dose-dependent risk at any dose of exogenous E_2, no matter how low, thereby providing a biologically justified explanation for low-dose effects of endocrine disruptors. Numerous endogenous chemicals serve critical determinative roles at specific times in organismal growth and differentiation; these are the frequently encountered circumstances in which no threshold dose is expected. Therefore, we expect our conclusions are not idiosyncratic, but rather are widely applicable. Our findings and their implications reinforce our concern for the health of humans and wildlife exposed to these low doses.

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