Effects of Dibutyl Phthalate as an Environmental Endocrine Disruptor on Gonadal Sex Differentiation of Genetic Males of the Frog *Rana rugosa*

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To examine the effects of dibutyl phthalate (DBP) on gonadal sex differentiation, genetically male tadpoles of *Rana rugosa* were exposed to dilute solutions of DBP at concentrations of 0.1, 1, or 10 μM during days 19–23 after fertilization, which is the critical period of gonadal sex differentiation in *R. rugosa*. Tadpoles were necropsied on day 40. The genetically male tadpoles were produced from crossings between males (ZZ) of one local population, in which females are the heterogametic sex, and females (XX) of another local population, in which males are the heterogametic sex. As positive control groups, tadpoles were exposed to dilute solutions of 17β-estradiol (E_2) at concentrations of 0.01, 0.1, or 1 μM during the same period. The internal structure of the gonads was histologically examined in a total of 30 control tadpoles, 86 E_2-treated tadpoles, and 90 DBP-treated tadpoles. The gonads of the control tadpoles all showed the typical structure of testes. In contrast, 0.01, 0.1, and 1 μM E_2 treatments caused the undifferentiated gonads of 18, 63, and 100% of the tadpoles, respectively, to develop into gonads of complete or partial ovarian structure. After 0.1, 1, and 10 μM DBP treatment, 0, 7, and 17% of tadpoles, respectively, were similarly affected. These findings suggest that DBP was about 1,000-fold less potent than E_2. Nevertheless, DBP is an environmentally dangerous hormone that disrupts the pathways of testicular differentiation in genetically male animals. *Key words*: environmental endocrine disruptors, estrogens, frogs, gonadal sex differentiation, phthalate ester toxicity, plasticizers, sex chromosomes, sex-determining systems. *Environ Health Perspect* 108:1189–1193 (2000). [Online 15 November 2000]

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In recent years there has been growing concern that the estrogenicity or antiandrogenicity of certain chemical compounds released into the environment may have a harmful influence on the development and function of the male reproductive system in several animal species, including humans. The estrogenic or antiandrogenic effect of the chemical compounds bisphenol A (*1,2*), nonylphenol (*3,4*), DDT (*5*), polychlorinated biphenyls (PCBs) (*6*), various phthalate esters (*7,8*), parabens (*9*), and others, has been ascertained by *in vitro* and *in vivo* assays. Fact-finding inquiries have reported that serious effects on feral animals may have been caused by nonylphenol (*10*), DDT (*11*), and PCBs (*12*) in particular.

Dibutyl phthalate (DBP), one of the phthalate esters, was widely used as a plasticizer of polyvinyl chloride resins. The estrogenic potency of DBP is uncertain because different methods of analysis give different results. An *in vitro* yeast-based estrogen assay indicated that its estrogenic potency is 1,000,000-fold lower than that of 17β-estradiol (E_2), which is one of the most potent endogenous estrogens (*13*), whereas Milligan et al. (*14*) detected no estrogenic ability in DBP by an *in vivo* assay using ovariectomized mice. The risk of DBP to experimental animals may be different from one species to another because of their different levels of resistance to the chemical. Furthermore, the risk may be different

according to the method, duration, and magnitude of exposure in the screening trial. In any case, when the estrogenic or antiandrogenic impact of DBP is examined in vertebrates, the genetic sex of the experimental animals used should be standardized to male. From these points of view, the Japanese wrinkled frog, *Rana rugosa,* is a suitable experimental animal for the following reasons: *a*) in the gonadal sex differentiation of *R. rugosa,* exogenous estrogen induces ovarian formation in genetic males, and the estrogen-sensitive period is clearly defined during days 20–22 after fertilization; *b*) eggs from each spawn are numerous (about 700–2,000 eggs); *c*) the method of exposure is simple because tadpoles are aquatic; and *d*) genetically all-male tadpoles are easily produced. The sex of *R. rugosa* is under the control of sex chromosomes, and the sex-determining systems differ from one local population to another (*15*). Accordingly, crossings between males (ZZ) of the ZW/ZZ-type of sex-determining system and females (XX) of the XX/XY-type produce only genetically male (XZ) embryos.

The aim of this study was to ascertain whether DBP can alter the intrinsic mode of testicular formation in genetically all-male *R. rugosa*. We also examined the rate of gonadal sex reversal caused by various concentrations of E_2 and compared the effect of DBP with that of E_2.

Materials and Methods

Production of genetically all-male tadpoles. To produce all-male tadpoles, three females and three males were used. The three females had the XX/XY-type of sex-determining system and belonged to the west Japan strain, named in our laboratory, of which the original frogs were collected from Hiroshima city. The three males had the ZW/ZZ-type of sex-determining system and belonged to the north Japan strain, of which the original frogs were collected from Kanazawa city. We induced ovulation of each female by injecting 0.5 mL Ringer's solution containing two pulverized pituitary bodies of *Rana catesbiana* into her abdominal cavity 10 hr before her eggs were used. The eggs were squeezed out from the anesthetized females onto glass slides and were inseminated by adequate sperm suspension prepared by crushing testes, removed from anesthetized male frogs, in a small amount of dechlorinated tap water with tweezers. Embryos produced from this insemination were subdivided into eight groups: three for dibutyl phthalate treatment, three for E_2 treatment, one for the vehicle control, and one for the control. Each group comprised 50 embryos.

Reagents for exposure. As stock solutions, 28 mg dibutyl phthalate (DBP; Sigma, St. Louis, MO) and 3.8 mg E_2 (Sigma) were dissolved separately in 10 mL absolute ethanol. Then, 1, 0.1, and 0.01 mL of these stock solutions were diluted with 1,000 mL of dechlorinated tap water to give final concentrations of 10, 1, and 0.1 μM DBP and 1, 0.1, and 0.01 μM E_2, respectively. We based the concentration of the DBP on the fact that 100 μM DBP-treated tadpoles always died within 5 min. For the vehicle control, 1,000 mL 0.1% ethanol solution was prepared.

Method of exposure. We exposed 50 male tadpoles in the 1,000 mL respective diluted solutions, which were poured into 2-L enameled containers, from the beginning of day 19 to the end of day 23 after fertilization. The tadpoles were reared at approximately 25°C temperature under white fluorescent light and fed on boiled spinach. Rearing water was changed every 2 or 3 days.

Preparation for microscopic examination. On day 40 after fertilization, the earliest time when ovarian structure was distinguished from testicular structure and the undifferentiated state of gonads, a total of 90 tadpoles treated with DBP, 86 treated with E_2, and 30 vehicle controls were fixed with Nawaschin fixing solution (solution A: 2 g chromic acid and 198 mL distilled water; solution B: 80 mL formalin and 20 mL acetic acid; equal parts of each solution were mixed just before use). The remaining tadpoles had been reared to examine the effects of DBP on the next generation of offspring. Then their gonads were removed with the mesonephros and embedded in paraffin after dehydration through an ethanol series. Samples sectioned successively to a thickness of 10 μm were stained with Mayer's Alum hematoxylin.

Results

Tadpoles produced from the crossings of females with two X chromosomes and males with two Z chromosomes were all genetically male. Of 30 tadpoles in the vehicle control, 28 showed the typical structure of testes in which germ cells intermingled with medullary somatic cells roughly uniformly (Table 1, Figure 1A). The remaining two tadpoles in this group had many meiotic germ cells in the peripheral area of the gonads, although the inside showed normal testicular structure (Figure 2A). In the offspring of *R. rugosa* collected from Kanazawa, the existence of meiotic germ cells in the gonads of tadpoles is usually a criterion for ovarian differentiation because meiotic germ cells in the testes only begin to appear in young frogs that have completed metamorphosis (*26*). However, in the offspring of *R. rugosa* collected from Hiroshima, meiotic germ cells are frequently found as testes-ova in the testes of tadpoles (*16*). Therefore, we do not regard the presence of meiotic germ cells in the testicular structure of Hiroshima frogs as a sign of feminization.

Exposure to E_2

The gonads of 28 tadpoles treated with 1 μM E_2 all showed the typical structure of the ovary in which the majority of germ cells were in the prophase of meiotic division and an ovarian cavity was formed in the center (Table 1, Figure 1B). Of 30 tadpoles treated with 0.1 μM E_2, 5 had gonads showing ovarian

structure throughout. The gonads of 14 other tadpoles were composed of testicular and ovarian structure in their anterior and posterior parts, respectively (Figure 3), although the proportion of testicular and ovarian structure varied in each gonad. The gonads of 3 other tadpoles had far more meiotic germ cells in their peripheral parts than those of the vehicle control tadpoles, in addition to the testicular structure of the interior (Figure 2B). The remaining 8 tadpoles had typical testes with few if any meiotic germ cells. Of 28 tadpoles treated with 0.01 μM E_2, 1 had gonads showing ovarian structure throughout, 4 had gonads showing the coexistence of testicular and ovarian structure, 10 had gonads with many meiotic germ cells in the peripheral parts, and the gonads of the remaining 13 showed testicular structure throughout.

Exposure to DBP

Exposure of tadpoles to 10 μM DBP induced obvious ovarian formation in the gonads of genetic males. Of 30 tadpoles, 1 formed typical ovaries and 4 others had gonads consisting of testicular and ovarian structure (Table 1, Figure 4). In 30 tadpoles treated with 1 μM DBP, the gonads of 2 tadpoles also showed partial ovarian structure. However, no tadpoles treated with 0.1 μM DBP showed any signs of feminization in their gonads like those of the remaining 25 and 28 tadpoles treated with 10 and 1 μM DBP, respectively.

Discussion

Chemical compounds that mimic estrogenic activity in *in vitro* assays must be directly examined for their estrogenic or antiandrogenic action on living animal species. Genetic

Table 1. Number of genetically male *Rana rugosa* tadpoles with gonads showing various degrees of ovarian and testicular structure induced by DBP and E_2 treatment.

	Internal appearance of gonads				
Concentration	Ovarian throughout	Ovarian and testicular	Testicular (many meiotic germ cells)	Testicular (few if any meiotic germ cells)	Total
0.1 μM DBP*	0	0	1	29	30
1 μM DBP*	0	2	8	20	30
10 μM DBP*	1	4	14	11	30
0.01 μM E_2*	1	4	10	13	28
0.1 μM E_2	5	14	3	8	30
1 μM E_2	28	0	0	0	28
Vehicle control*	0	0	2	28	30

*No significant differences (χ^2-test, $p < 0.05$) between 0.1 μM DBP, 1 μM DBP, and vehicle control and between 10 μM DBP and 0.01 μM E_2.
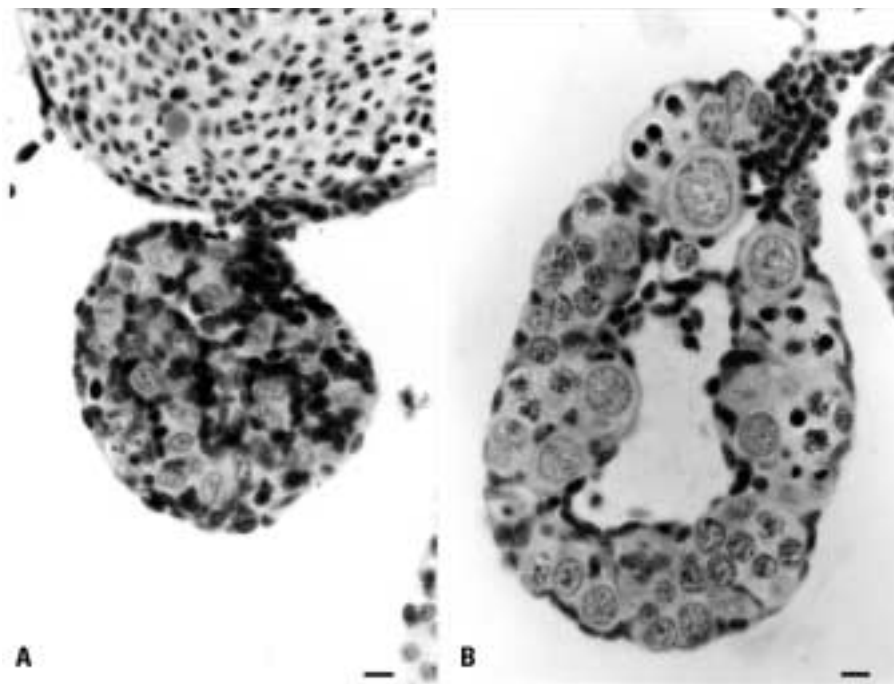

Figure 1. Cross-sections of the gonads of 40-day-old genetically male tadpoles. (*A*) Testis of a control tadpole; the germ cells (light and spherical) intermingle with the somatic cells (dark and slender) and are in the phase of proliferating gonia. (*B*) Ovary of a tadpole treated with 1 μM E_2; almost all the germ cells are in the phase of gametogenesis in which the chromosomes are meiotically dividing. There is an ovarian cavity lined with somatic cells in the center of the gonad. Bar = 10 μm.

males of oviparous animals should be used for such studies to avoid the effect of maternal estrogen. Willingham and Crews (*17*) performed an outstanding experiment using all-male embryos of the red-eared slider turtle, *Trachemys scripta.* In this turtle, gonadal sex is determined by incubation temperature during embryonic development. An incubation temperature of 26°C results in all male offspring, whereas an incubation temperature of 31°C results in all female offspring (*18*). Willingham and Crews (*17*) incubated the turtle eggs at a male-producing temperature and administered the PCB Aroclor 1242 and seven kinds of pesticide compounds at concentrations detected in alligator eggs from Lake Apopka, Florida, to examine their potential estrogenicity. They tested these compounds both singly and in combination. They found that significant sex reversal is induced by *trans*-nonachlor, *cis*-nonachlor, Aroclor 1242, *p,p'*-DDE, and chlordane when used singly.

In *R. rugosa,* all male offspring or all female offspring can be produced easily. Gonadal sex is determined by the combination of sex chromosomes, unlike sex determination in the red-eared slider turtle (*15*). The sex chromosomes consist of three variations of subtelocentric, telocentric, and metacentric types caused by two pericentric inversions and conduct the different sex determining systems (*19,20*). In western and eastern Japan, *R. rugosa* has homomorphic sex chromosomes of subtelocentric (X and Y) and telocentric (X and Y) types, respectively, in both sexes. *R. rugosa* in central Japan has heteromorphic sex chromosomes consisting of metacentric (X) and subtelocentric (Y) types in males, whereas in northern Japan it has heteromorphic sex chromosomes consisting of metacentric (W) and subtelocentric (Z) types in females. The complexity of X- and W-metacentric chromosomes is thought to result from the different potential of the female-determining factor, because the combination of Z- and metacentric X-chromosomes produces the same number of females and males, whereas that of Z- and subtelocentric X-chromosomes gives rise to all males (*21*). Naturally, the combination of Z- and metacentric W-chromosome produces all females. Such complicated sex-determining systems enable *R. rugosa* to produce all female and all male offspring; that is, female embryos with XX chromosomes and male embryos with ZZ chromosomes can change their gonadal sex permanently under the influence of exogenous testosterone and estradiol, respectively. The crossing of normal XX females with sex-reversed XX neomales produces all female offspring, and that of sex-reversed ZZ neofemales and normal ZZ males produces all male offspring. In addition, the offspring developed gynogenetically from XX females are all female, whereas the gynogenesis of ZW females produces only all male offspring because WW embryos die at an early stage of development. In this study, the sample of all male offspring was produced by crossing females with two subtelocentric X-chromosomes and males with two Z-chromosomes.

DBP, widely used as the plasticizer of polyvinyl chloride resins, was screened for its estrogenic properties for the first time by Jobling et al. (*7*) using mammalian estrogen
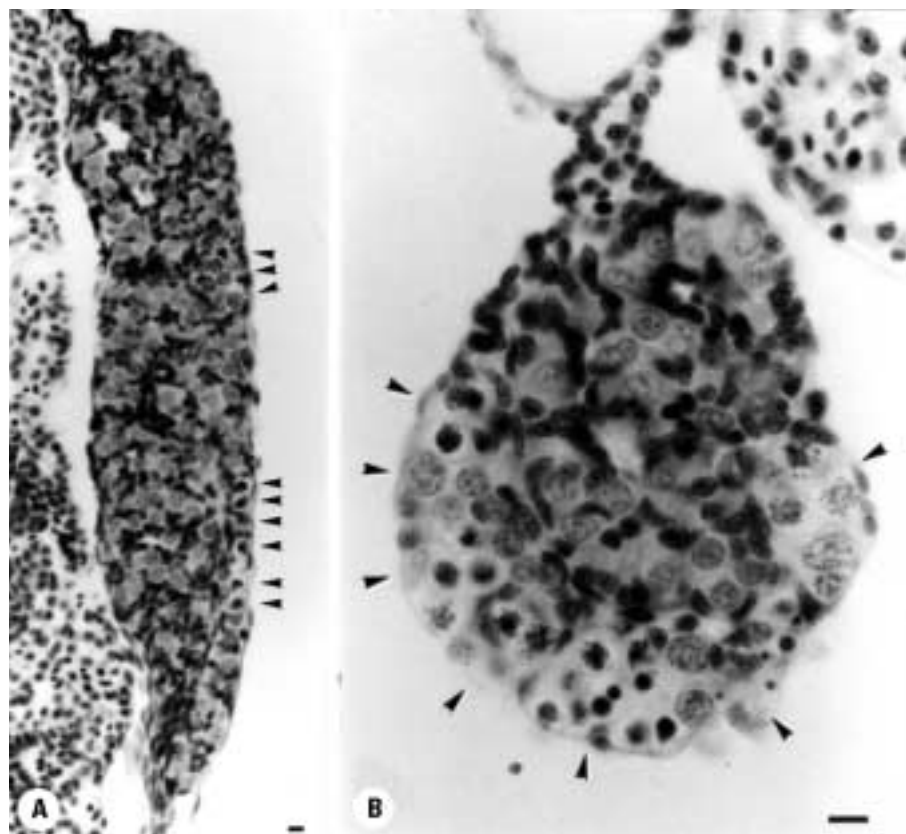

Figure 2. Sections of the gonads from 40-day-old genetically male tadpoles. (*A*) Vertical section of the testis from a control tadpole; there are some germ cells (arrowheads) entering the prophase of meiotic division in the ventral side of the gonad. (*B*) Cross-section of the testis of a tadpole treated with 0.1 μM E$_2$; the germ cells (arrowheads) in the peripheral area of the gonad are in the prophase of meiotic division. Bar = 10 μm.
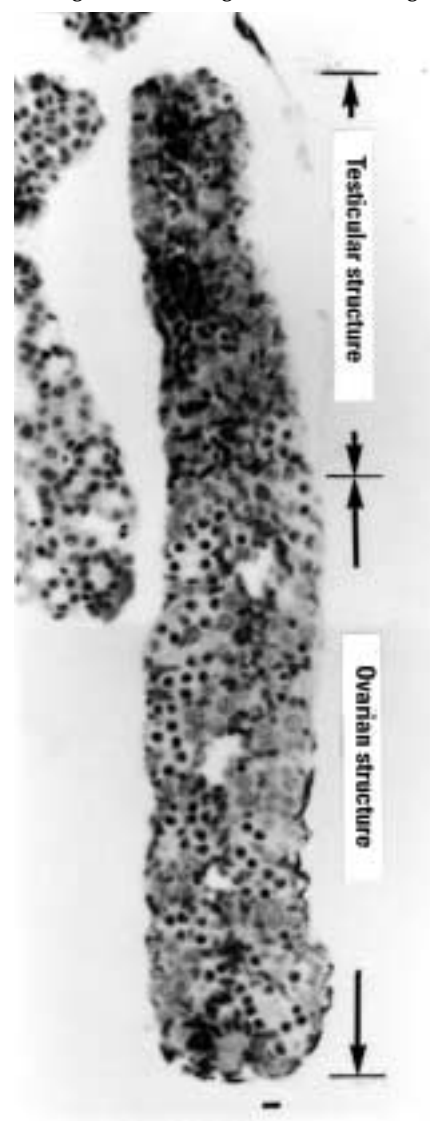

Figure 3. Vertical section of the gonad of a genetically male tadpole treated with 0.1 μM E$_2$. The anterior half of the gonad shows testicular structure and the posterior half ovarian structure. Bar = 10 μm.
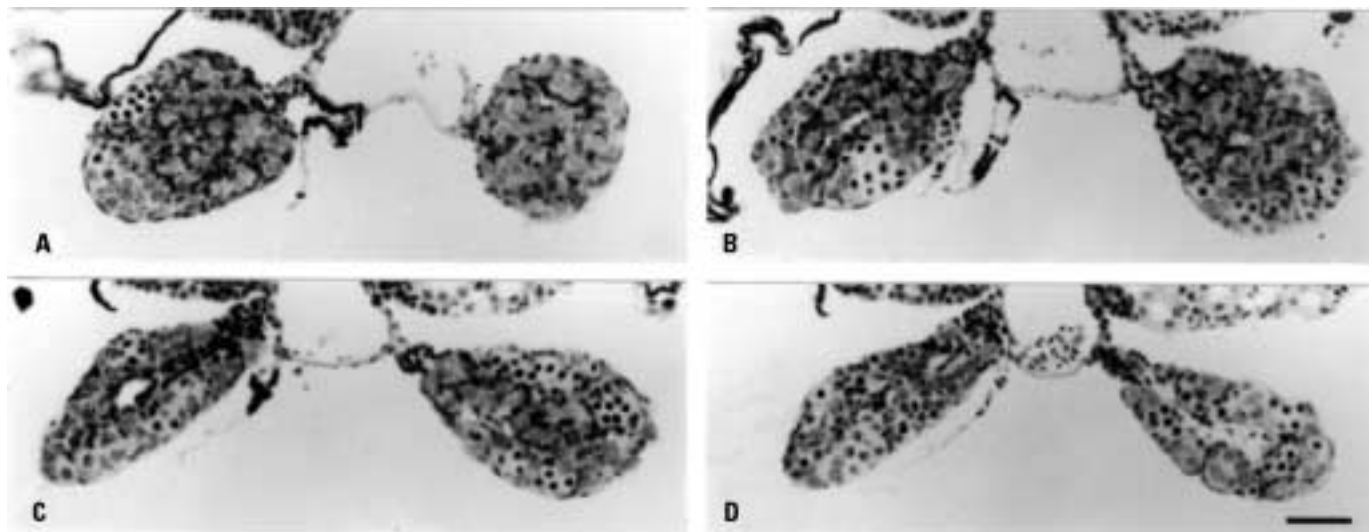
Figure 4. Cross-sections of the gonads of a tadpole treated with 10 µM DBP. Photographs are arranged from anterior (A) to posterior (D) parts of the gonads. (A) The left gonad shows typical testicular structure, and the right one shows testicular structure with meiotic germ cells in the peripheral area. (B) Both gonads show testicular structure, with meiotic germ cells in the peripheral area. (C) The left gonad is similar to that in (B), but the right gonad is transformed into ovarian structure. (D) Both gonads show typical ovarian structure. Bar = 50 µm.

screens *in vitro,* and they proved that DBP is estrogenic. Harris et al. (*13*) found, using an *in vitro* yeast-based estrogen assay, that the estrogenic ability of DBP is 1,000,000-fold less potent than E_2. However, no estrogenic action by DBP was detected in an *in vivo* assay using ovariectomized mice (*14*). In contrast, the developmental toxicity of DBP was evaluated using pregnant rats given DBP at 250–750 mg/kg/day by Ema et al. (*22*), Mylchreest et al. (*23,24*), and Gray et al. (*25*). These researchers found that the male offspring display an unusually high incidence of reproductive tract malformations—decrease of anogenital distance, testicular and epididymal atrophy, widespread germ cell loss, absence of prostate gland and seminal vesicles, etc. Because these lesions are similar to those elicited by the antiandrogen flutamide, which disrupts androgen signaling necessary for male sexual differentiation, Mylchreest et al. (*23,24*) concluded that the effect of DBP is not estrogenic but antiandrogenic.

In this study we investigated whether the toxicity of DBP alters the process of gonadal sex differentiation in genetically male *R. rugosa* tadpoles. The critical period of gonadal differentiation in this frog is days 20–22 after fertilization because exogenous E_2 was effective only during this period in altering the testicular formation of male tadpoles with two Z-chromosomes (*26*). The results of 1 µM E_2 and 0.5 µM aromatase inhibitor treatment were different between the western and the northern Japan frog populations in the gonadal differentiation of *R. rugosa* (*26*). The genetically male tadpoles with subtelocentric X- and Y-chromosomes formed impermanent ovaries when treated

with E_2 alone and together with aromatase inhibitor, whereas those with two Z-chromosomes formed permanent ovaries after E_2 treatment alone, but E_2 in combination with aromatase inhibitor did not alter the testicular formation of male tadpoles (*26*). These findings suggest that the gonadal sex reversal of ZZ male tadpoles induced by E_2 is not necessarily due to the activation of estrogen receptors. Consequently, we cannot identify whether the effect of DBP is estrogenic or antiandrogenic until the target receptors for DBP have been examined. When genetically male tadpoles were exposed to DBP during days 19–23 after fertilization, 10 and 1 µM DBP induced complete or partially developed ovaries in the gonads of 17% and 7% of tadpoles, respectively. The level of gonadal alteration induced by 10 µM DBP is similar to that brought about by 0.01 µM E_2. This result indicates that DBP is about 1,000-fold less potent than E_2. This result is similar to the results obtained in mammalian-based gene expression assays performed by Zacharewski et al. (*27*). According to Zacharewski et al., 10 µM DBP exhibited 36% activity, compared with the 100% response using 0.01 µM E_2. The current findings highlight the danger of DBP as an environmental endocrine disruptor.

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27. Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB. Examination of the *in vitro* and *in vivo* estrogenic activities of eight comm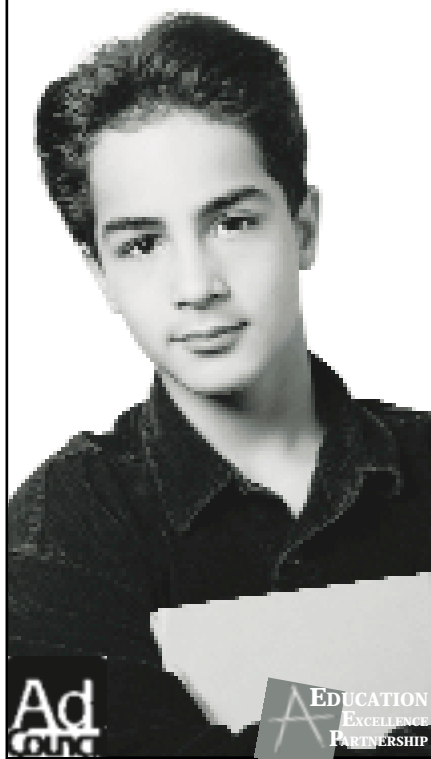ercial phthalate esters. Toxicol Sci 46:282–293 (1998).