Effects of Chinese Domestic Polychlorinated Biphenyls (PCBs) on Gonadal Differentiation in *Xenopus laevis*

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To determine whether polychlorinated biphenyls (PCBs) influence gonadal differentiation in *Xenopus laevis,* tadpoles were exposed to two Chinese domestic PCBs (PCB_3 and PCB_5) from Nieuwkoop and Faber stage 46/47 to complete metamorphosis. Gonads were characterized using a dissecting microscope. The control *X. laevis* had normal ovaries or testes in gross morphology, whereas obviously abnormal testes including ovotestes were found in PCB_3- and PCB_5-exposed groups. Ovotestes were characterized by morphologic ovaries in the cranial and caudal parts and morphologic testes in the middle part. PCBs did not alter the percentage of females but reduced the percentage of males with morphologically normal testes. The histologic structure of gonads was examined by a series of sections. Morphologically normal and abnormal testes from a few frogs exposed to PCBs were interspersed with oocytes in histologic sections. These testes exhibited looser structure with fewer seminiferous tubes, spermatogonia, and spermatozoa than in controls. The findings suggest that PCB_3 and PCB_5 have significant feminization effects on gonadal differentiation in *X. laevis* and that this species is sensitive to endocrine disruption and may be used as a good model to study endocrine disruption. *Key words:* endocrine disruptor, gonadal differentiation, ovotestis, PCB_3, PCB_5, polychlorinated biphenyls, *Xenopus laevis. Environ Health Perspect* 111:553–556 (2003). doi:10.1289/ehp.5620 available via *http://dx.doi.org/* [Online 28 October 2002]

Considerable attention has been focused on some environmental chemicals that can disrupt the functions of endocrine systems of animals and humans in recent years, including industrial chemicals, pesticides, and natural substances such as phytoestrogens. These endocrine disruptors may cause a variety of problems with growth, development, reproduction, and behavior (Kavlock et al. 1996). Gonadal abnormality and reproductive impairment caused by endocrine disruptors have been documented in fish (Edmunds et al. 2000; Gimeno et al. 1997; Gray and Metcalfe 1997), turtles (Guillette et al. 1994, 1996; Willingham and Crews 1999), minks (Harding et al. 1999; Heaton et al. 1995), and birds (Fry et al. 1987; Halldin et al. 1999). However, information about gonadal differentiation in amphibians involving endocrine disruptors is relatively scarce.

Amphibians might represent potential sentinels for assessing adverse effects of environmental chemicals because of their permeable skins and biphasic life cycle (Van der Schalie et al. 1999). Ecotoxic effects of endocrine disruptors on amphibians as a taxonomic group in ecologic systems warrant attention. There is ample evidence that gonadal differentiation in amphibian species is highly sensitive to sexual hormones. Estrogens can induce a sex reversal in genetic males, where either an ovotestis is produced or complete and permanent feminization occurs (Lofts 1974). On the basis of the above information, in 1999 we proposed that gonadal differentiation in amphibians may be sensitive to endocrine disruptors of environmental chemicals, and we began this research in 2000. During our study, several authors reported effects of endocrine disruption on gonadal differentiation in amphibians. Kloas et al. (1999) reported that bisphenol A and 4-nonylphenol significantly altered the sex ratio in *Xenopus laevis*. Dibutyl phthalate and styrene induced gonadal feminization in *Rana rugosa* (Ohtani et al. 2000, 2001). Recently, hermaphroditic gonads induced by atrazine in *X. laevis* were reported by Hayes et al. (2002). In our present study, effects of Chinese domestic polychlorinated biphenyls (PCBs) on gonadal differentiation in *X. laevis* were studied.

PCBs are a class of persistent organic pollutants that exist ubiquitously in the environment. They are resistant to degradation and tend to accumulate in adipose and other high-lipid tissues of animals. Although worldwide commercial production has declined since the 1970s and the level of PCBs residues has decreased slowly in various environmental and biologic samples since the 1980s, animals and humans still continue to be exposed to PCBs. Extensive studies *in vivo* and *in vitro* have shown that PCBs have endocrine-disrupting actions such as binding estrogen receptors (ERs), promoting ER-positive cells, MCF-7 proliferation, and affecting enzymes involving in thyroid hormone (Brouwer et al. 1999; Hansen 1998; Jansen et al. 1993). There is increasing evidence that PCBs can influence gonadal differentiation and development in several species. It was reported that PCBs could alter sexual differentiation and produce gonadal abnormalities in females in fish (Karmaus et al. 2002; Matta et al. 1998). Gonadal sex reversal caused by PCBs has been demonstrated in turtles (Bergeron et al. 1994; Willingham et al. 2000). Male rats treated with PCBs were markedly impaired in histologic structure of testes (Hany et al. 1999; Kaya et al. 2002; Kim et al. 2001). However, no laboratory study involving PCBs in gonadal differentiation in amphibians has yet been reported, despite the fact that a field survey from PCBs and polychlorinated dibenzofuran (PCDF)-polluted sites suggested that intersexuality in cricket frogs might be related to PCB and PCDF contamination (Reeder et al. 1998).

In China, about 10,000 tons of PCBs were produced from 1965 to 1974, some of which were released into the environment and resulted in local pollution (Li and Jiang 1995). PCB_3 and PCB_5 are two main industrial mixtures produced in China. The former contains relatively abundant low-chlorinated congeners, and the latter contains relatively abundant high-chlorinated congeners. Using a gas chromatography–electron capture detector (GC-ECD), a GC–mass spectrometer (GC-MS), GC–microconductivity, thin-layer chromatography, ultraviolet-spot *in situ* scanning spectrum, and element analysis, we identified PCB_3 and PCB_5 as close to Aroclor 1242 and Aroclor 1254, respectively (Wang et al. 1981). Our previous study showed that PCB_3 and PCB_5 had estrogenic activity in the MCF-7 proliferation assay (Du et al. 2000). The aim of the present study is to determine whether PCB_3 and PCB_5 influence gonadal differentiation in *X. laevis.*

Materials and Methods

Breeding and housing. *X. laevis* were obtained from the Institute of Developmental Biology of the Chinese Academy Sciences. Mature female and male frogs were maintained separately in glass tanks containing dechlorinated water at 22 ± 2°C with a 12-hr light/12-hr dark cycle and fed once a week on chopped

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pork liver. Breeding was induced by subcutaneous injection of human chorionic gonadotrophin. Mating occurred at night and in the morning. After eggs were laid, the females and the males were taken out of the breeding tank. Fertilized eggs hatched at 22 ± 2°C with a 12-hr light/12-hr dark cycle. On day 5 after fertilization, tadpoles were fed on *Daphnia* daily.

Exposure to chemicals. On day 6 after fertilization (stage 46/47 of Nieuwkoop and Faber 1956), healthy tadpoles among the offspring of a pair of parental frogs were randomly selected for the exposure experiment. PCB_3 and PCB_5 were obtained from the Institute for Environmental Chemistry of the Chinese Academy of Sciences. PCBs and estradiol (E_2; positive control) were dissolved in ethanol to produce stock solutions. The experiment water was prepared by adding the stock solution to dechlorinated water. In the preexperiment, tadpoles exposed to a series of PCB concentrations (5, 10, 20, 40, 80 μg/L) did not exhibit acutely toxic response within 10 days. Therefore, we chose 10 μg/L, a higher concentration than that in the environment, as the final exposure concentration of PCBs in order to detect possible effects of PCBs. The final concentration of E_2 in water was 100 μg/L. The control group received the same amount of ethanol used as solvent for PCBs. Seventy tadpoles in every experimental group were raised in a glass tank containing 18 L water. All tanks were the same size (30 cm × 20 cm × 25 cm). The experiment water was changed twice weekly.

Gonadal identification and histologic examination. All animals were observed daily, and growth, development, and survival were recorded. Frogs at stage 61/62 and 1 month postmetamorphosis were killed by immersion in MS 222 at 3 g/L. Frogs were sexed by gonadal examination using a dissecting microscope. Gonads (from frogs at 1 month postmetamorphosis) attached to kidneys were fixed with Bouin's fixative. For examining histologic structure, fixed gonads were then embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Sections were examined with a light microscope. All procedures were conducted in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (National Research Council 1996).

Statistical methods. The *t*-test was used to evaluate statistical significance. A value of $\alpha = 0.05$ was chosen to detect significant difference.

Results

There was no significant difference in survival between the PCB-exposed and the control frogs. Through gonadal examination using a dissecting microscope, we identified all frogs exposed to E_2 as females. Abnormal males including intersexes were found in the PCB-exposed groups, whereas the control *X. laevis* had ovaries or testes with normally gross morphology. The percentage of males was 59% in the control group, and it was higher than the percentages of morphologically normal males in the PCB-exposed groups despite the lack of statistically significance ($p > 0.05$) (Figure 1). Of 25 frogs in the PCB_3-exposed group, three individuals (12%) had abnormal testes in the gross morphology. Of the three frogs with abnormal testes, one individual had a small left testis and a large right one, and the other two were intersexes with ovotestes, which had morphologic ovaries in cranial and caudal parts and morphologic testes in middle parts (Figure 2). Of 23 frogs in the PCB_5-exposed group, there were four individuals (17%) with abnormal testes in gross morphology. Of the four frogs with abnormal testes, two individuals were intersexes similar to the intersexes in the PCB_3-exposed group, and the testes of the other two were composed of several parts that were linked by thin tubes (Figure 3). It was obvious that PCB_3 and PCB_5 induced gonadal feminization in *X. laevis*.

Feminization induced by PCB_3 and PCB_5 was evident based on histologic sections (Figure 4). Oocytes occurred in sections of morphologically abnormal testes induced by PCB_3. In sections of three testes with normally gross morphology, oocytes were also found to intersperse in testis tissue. In these testes, there were large interstices in some parts. These testes exhibited looser structure with

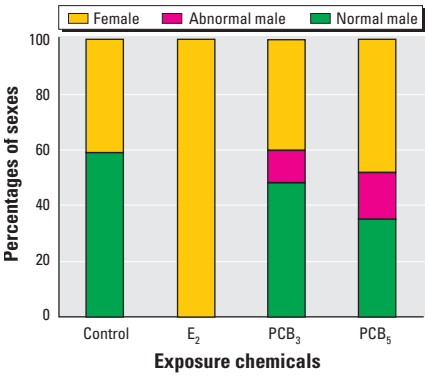

Figure 1. Sexual percentages based on gonadal examination using a dissecting microscope in the control group and groups exposed to E_2, PCB_3, and PCB_5.

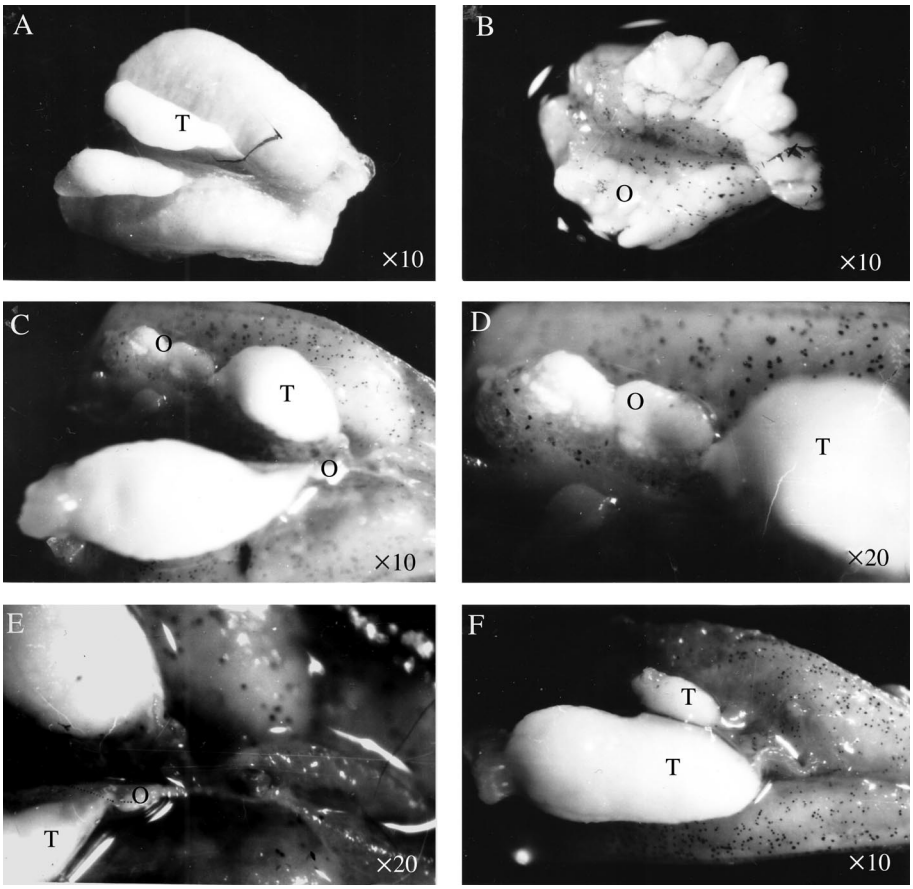

Figure 2. Abnormal testes induced by PCB_3 compared with control gonads. (*A*) Control testes (T). (*B*) Control ovaries (O). (*C*–*E*) Ovotestes induced by PCB_3. Ovotestes have morphologic ovaries in cranial and caudal parts and morphologic testes in middle parts. (*F*) A pair of malformed testes with a small left testis and a large right one. Magnification ×10 or ×20.

fewer seminiferous tubes, spermatogonia, and spermatozoa (Figure 4D,E). Testes of frogs with exposure to PCB_5 had the same abnormalities as did frogs with exposure to PCB_3. Among morphologically normal testes from frogs treated with PCB_5, testes with oocytes were also found. Figure 4F presents a typical ovotestis caused by PCB_5. Ovaries of frogs with exposure to PCBs or E_2 were not different in appearance from the control ovaries.

Discussion

In general, gonadal differentiation in amphibians corresponds to their genetic sex constitution (Hayes 1998). Natural intersexuality exists among some juvenile amphibians. Intersexuality shown by the temporary appearance of oocytes in testes sometimes occurs in some juvenile male *X. laevis,* but grossly morphologic ovotestes are not considered a normal variant (Gallien 1974). However, estrogen treatment during embryonic development can produce gonadal sex reversal in genetic males (Gallien 1953). In the process of gonadal differentiation, primary germ cells in presumptive males migrate from cortical region into medullary region, where they will form spermatogonia. In presumptive females, the contrary process occurs: Primary germ cells translocate from medullary region into cortical region, where they will form oocytes. If gonads are disrupted by estrogen in the process, intersexuality will occur (Villalpando and Merchant-Larios 1990).

In this study, we investigated the effects of PCB_3 and PCB_5 on gonadal differentiation in *X. laevis*. In a previous study, 100% *X. laevis* with estrogen treatment from stage 50 became females (Villalpando and Merchant-Larios 1990). Therefore, in our study, chemicals exposure began from stage 46/47, at which it was early enough to detect possible effects on gonadal differentiation because gonads had not yet begun to differentiate. Exposure to PCB_3 or PCB_5 did not significantly alter the percentage of females and the histologic structure of ovaries, but they induced feminized testes in gross morphology and in histologic structure. In addition to decreasing the percentage of males with morphologically normal testes, these results suggest that gonadal differentiation was disrupted and feminized by PCBs. Some testes were induced to ovotestes in gross morphology; some were altered in the histologic structure and occurred oocytes in local tissue, and some were affected in the histologic structure but without oocytes. This was the first report that PCBs affected gonadal differentiation in amphibians. It highlights the danger of PCBs as endocrine disruptors. Moreover, the results also support the proposal that intersexuality in cricket frogs from Illinois (USA) is caused by PCB and PCDF contamination (Reeder et al. 1998).

The morphologic and structural abnormalities in gonads would likely lead to reproductive dysfunction in *X. laevis*; thereby, ecotoxic effects would occur in species. Therefore, a multigeneration experiment should be conducted. Information from studies in the wild has shown that amphibians have markedly declined because of several potential factors in the past decades, including habitat loss, parasites, climate change, ultraviolet B radiation, and chemical contamination (Baustein and Wake 1990, 1995; Wake 1991). A survey showed that the levels of gonadtropin-releasing hormone and androgen in malformed frogs were significantly lower than those in normal frogs (Sower et al. 2000). Our present data on

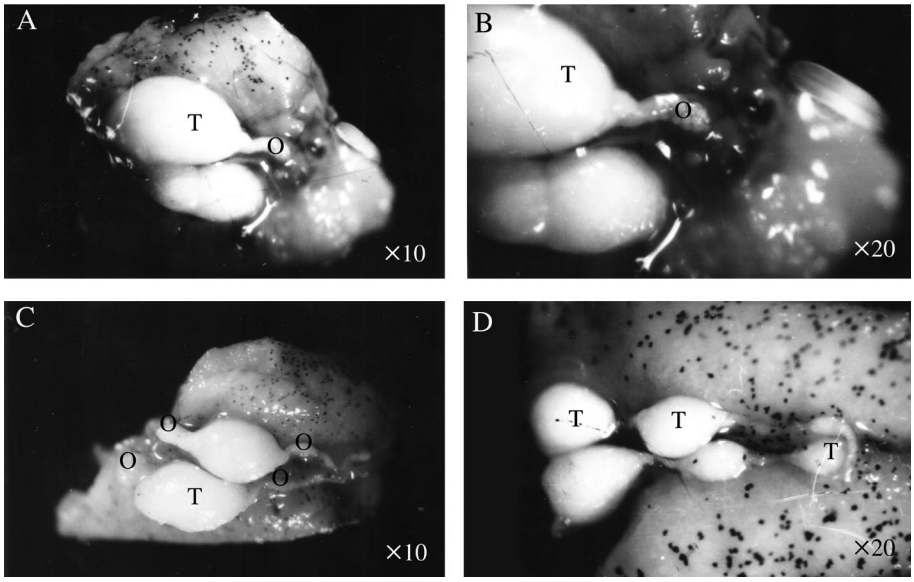

Figure 3. Abnormal testes induced by PCB_5. (*A–C*) Ovotestes with ovaries (O) and testes (T). (*D*) A pair of malformed testes. Each testis is composed of three parts, which are linked by thin tubes. Magnification ×10 or ×20.

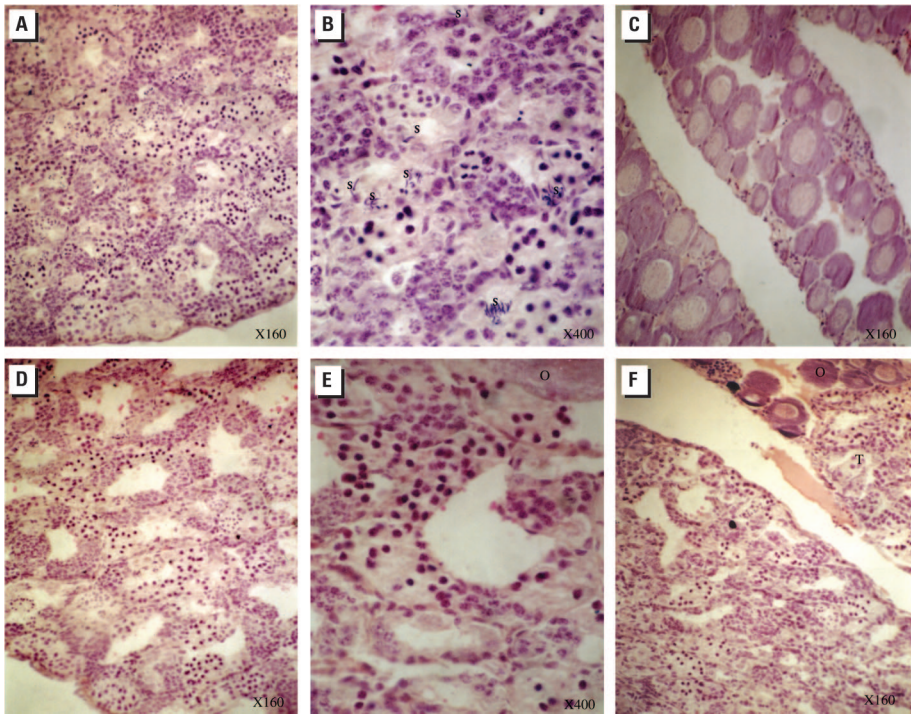

Figure 4. Photomicrographs of gonadal sections. (*A,B*) Control testis tissue, which exhibits compact structure with many spermatozoa (S). (*C*) Control ovary tissue. (*D,E*) Testis tissue of a frog exposed to PCB_3. Testis tissue exhibits looser structure with fewer seminiferous tubes, spermatogonia, and spermatozoa. (*F*) Typical ovotestis tissue of a frog exposed to PCB_5. Ovotestis tissue consists of ovary tissue (O) and testis tissue (T). Magnification ×160 (*A,C,D,F*) or ×400 (*B,E*).

intersexuality induced by PCB_3 and PCB_5 in *X. laevis* as well as previous studies suggest that endocrine disruptors can affect gonadal differentiation in amphibians and that this kind of chemical may be a factor leading to population declines (Hayes et al. 2002; Kloas et al. 1999; Ohtani et al. 2000, 2001). Therefore, it is necessary to develop a model using a representative species to study the effects of endocrine disruptors on amphibians.

X. laevis is an amphibian species used widely in laboratory study. It has being used as a model for developmental biology in many laboratories. A rich literature on growth, development, and reproduction exists, and it is available to establish a set of end points for assaying endocrine disruption. Exogenous estrogens can induce feminization in *X. laevis*, and the estrogen-sensitive period is clearly defined. Moreover, offspring of *X. laevis* are numerous and are raised easily. The method of exposure to chemicals is simple in water. Therefore, it is reasonable to propose that *X. laevis* would be a good model to study endocrine disruption in amphibians. In addition to the report by Hayes et al. (2002), our study suggests that this species is sensitive to endocrine disruptors that affect gonadal differentiation and development. This study also responds to the proposal of the Endocrine Disruptor Screening and Testing Advisory Committee that amphibian development and reproduction are promising end points for evaluating endocrine disruption (U.S. EPA 1998). Certainly, other amphibian species might have also their own advantages. For example, genetic males in *Rana rugosa* can be induced (Ohtani et al. 2000), and feminization in *Hyperolius argus* can be identified directly by sex coloration (Noriega and Hayes 2000). However, the most serious disadvantage of these species is that they have not been used broadly as typical experimental animals.

In conclusion, this study discovered and determined feminization effects of PCB_3 and PCB_5 on gonadal differentiation in *X. laevis*. The results highlight the danger of PCBs as endocrine disruptors and demonstrate the sensitivity of amphibians to endocrine disruptors. It is suggested that *X. laevis* can be a good model to study effects of endocrine disruptors on gonadal differentiation and development in amphibians and even in other vertebrate species.

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