Effects of Polychlorinated Biphenyls in CD-1 Mice: Reproductive Toxicity and Intergenerational Transmission

Paola Pocar,[*,1] Nadia Fiandanese,[*] Camillo Secchi,[*] Anna Berrini,[*] Bernd Fischer,[†] Juliane-Susanne Schmidt,[†] Kristina Schaedlich,[†] Stewart M. Rhind,[‡] Zulin Zhang,[‡] and Vitaliano Borromeo[*]

Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Facoltà di Medicina Veterinaria, Università degli Studi di Milano, 20133 Milano, Italy; †Department of Anatomy and Cell Biology, Martin Luther University, Faculty of Medicine, 06108 Halle (Saale), Germany and ‡The James Hutton Institute, Aberdeen, AB15 8QH, U.K.

[1]To whom correspondence should be addressed at Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Facoltà di Medicina Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy. Fax: +39 0250318123. E-mail: paola.pocar@unimi.it.

Several studies indicate that *in utero* and perinatal exposure to polychlorinated biphenyls (PCBs) induces adverse reproductive effects, but it remains unclear whether such effects may be transmitted to subsequent generations. We therefore investigated the association between maternal exposure to PCBs and reproductive health in male and female offspring over three generations. Mouse dams were fed 0, 1, 10, and 100 μg/kg/day of a PCB mixture (101 + 118) during pregnancy and lactation. PCB levels were measured in the tissues of both dams and offspring. PCB concentrations at all doses investigated were greater in the offspring than in the dams ($p \leq 0.0001$) confirming that the progeny were exposed as a result of maternal exposure. In F1 offspring, exposure to PCBs resulted in reductions in (1) testis weight ($p \leq 0.05$) and seminiferous tubule diameter ($p \leq 0.05$), (2) sperm viability ($p \leq 0.0001$) and developmental capacity ($p \leq 0.05$), (3) ovary weight ($p \leq 0.05$), (4) oocyte developmental capacity ($p \leq 0.05$), and (5) increased follicular atresia ($p \leq 0.0001$). In females, adverse effects were observed only in the F1 animals. In contrast, male offspring exhibited reduced sperm viability and altered seminiferous tubule distribution up to the third generation, showing intergenerational transmission. In summary, our data indicate that exposure to PCBs at the time of gonadal sex determination perturbed, significantly, the reproductive physiology of male and female offspring in adulthood. Furthermore, male reproductive deficiencies may be observed in at least two further generations. These findings have significant implications for reproductive health and fertility of animals and humans.

Key Words: PCBs; maternal exposure; reproductive toxicity; mouse; intergenerational transmission.

Polychlorinated biphenyls (PCBs) are pollutants derived from multiple industrial processes and products; they have been produced for a long time and on a large scale. Even though the commercial production of PCBs was banned at the end of the 1970s, owing to their chemical stability and lipophilicity, they persist in the environment and bioaccumulate in food chains. They can be detected in plasma, in tissue samples, and in the breast milk of a wide variety of species, including humans (Agudo *et al.*, 2009; Arctic Monitoring and Assessment Programme [AMAP], 2009; Bachelet *et al.*, 2011; McFarland and Clarke, 1989; Thomas *et al.*, 2006). Their occurrence has been linked to adverse health effects on reproductive function in humans and wildlife (Colborn *et al.*, 1993; Lie *et al.*, 2004, 2005; Ropstad *et al.*, 2006; Safe, 2004).

Because PCBs accumulate in adipose tissue and breast milk (Safe, 1990) and can easily cross the placenta (Eyster *et al.*, 1983; Guvenius *et al.*, 2003; Jacobson *et al.*, 1984), mammalian offspring are likely to be exposed to high concentrations of these pollutants during prenatal development and breast feeding (Colciago *et al.*, 2006; Kaya *et al.*, 2002). Due to heightened sensitivity, the fetus is extremely vulnerable to environmental challenges, which can result in permanent structural and functional changes (Dostal *et al.*, 1987; Latini *et al.*, 2003). Worryingly, many of the reproductive abnormalities resulting from developmental exposure only become apparent after puberty (long-latency effect), and this is a significant obstacle to the identification of causal relationships.

Nevertheless, studies have correlated maternal PCB exposure with the onset of reproductive disorders in both male (Hauser *et al.*, 2003, 2005; Hsu *et al.*, 2007; Kuriyama and Chahoud, 2004; Richthoff *et al.*, 2003) and female (Sager and Girard, 1994) adult offspring.

Recent observations indicated that exposure to environmental chemicals at the time of gonadal development not only directly affects the reproductive health of the exposed individual but can also induce effects in subsequent generations that may differ from those associated with the primary exposure (Anway *et al.*, 2005; Fernie *et al.*, 2003; Shipp *et al.*, 1998). The mechanisms of transmission along multiple generations are still to be clarified. In this matter, a substantial distinction has to be made between
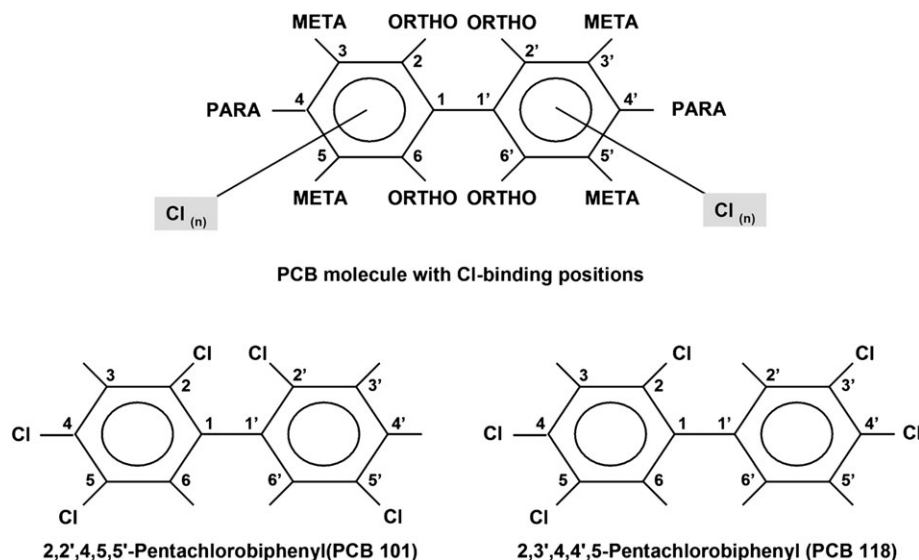
FIG. 1. (A) Structural formula of PCBs with Cl-binding positions. (B) Structural formula of PCB 101 (2,2′,4,5,5′-Pentachlorobiphenyl—CAS no. 37680-73-2) and PCB 118 (2,3′,4,4′,5-Pentachlorobiphenyl—CAS no. 31508-00-6).

intergenerational transmission involving direct exposure to the environmental factor and transgenerational effects involving germline transmission without direct exposure of the affected generation (Skinner, 2008).

The aim of the present study was to determine whether or not exposure of mice to PCBs at the time of gonadal development can perturb reproductive function in the adult offspring of both sexes and to investigate the transmission of PCB effects through subsequent generations, along the female lineage. To address this objective, we exposed F0 dams to PCBs through pregnancy and lactation and examined the effects on reproductive performance in male and female offspring of F1, F2, and F3 generations. To our knowledge, only one study has reported intergenerational effects of maternal exposure to PCB in mammals. It involved rat offspring spanning two generations (Steinberg et al., 2008). However, unlike in the present study, exposure was limited to the time of sexual differentiation of the embryonic brain (day post coitum [dpc] 16–18) and therefore did not cover the entire period of development of the reproductive system in rodents.

In the present study, a mixture of two PCBs congeners, PCBs 101 and 118 was used. These two congeners were selected because (1) they represent a high proportion of the total PCB burden in biological samples. Both PCB 101 and 118 are part of the so-called ICES 7 and represent approximately 65% of the total PCBs in biological samples (Bernhoft et al., 1997; Cerna et al., 2008; Connor et al., 1997; International Council for the Exploration of the Seas [ICES], 1992; Shen et al., 2009). The proportions of individual congeners vary between matrices, but on average, PCB 101 and 118 contribute in a ratio of 1:1 to the sum of the seven indicators (Norwegian Scientific Committee for Food Safety [VKM], 2008). (2) Previous studies in sheep have shown that PCB 101 and 118 are

preferentially accumulated in fetal tissue, relative to that of their dams (Rhind et al., 2009, 2010), and therefore their effects may be particularly important during critical developmental stages.

Effects of PCB congeners are dependent on the chlorination of the ortho positions of the molecule, which influence the ability of the molecule to assume planar conformation. PCBs can be divided into non-ortho–substituted (coplanar) and ortho-substituted (noncoplanar) groups (Fig. 1); PCB 101 is a di-ortho–substituted PCB and is a typical noncoplanar PCB, whereas PCB 118, being a mono-ortho–substituted PCB, is able to assume a partial coplanar conformation and therefore may share some effects with non-ortho PCBs.

MATERIALS AND METHODS

Animals. The CD-1 mouse strain was selected due to its large litter size, high fecundity and robustness, all of which are useful traits in reproductive studies, and embryology. Virgin female, 5-week-old, CD-1 mice were purchased from Charles River (Calco, Italy), housed in the animal facilities of the Department of Animal Pathology and Health, Faculty of Veterinary Medicine, University of Milan, under controlled conditions (23 ± 1°C, 12-h light/dark cycle) and allowed to acclimatize for 2 weeks. Standard pellet food (4RF21, Charles River) and tap water were available *ad libitum*. Groups of two or three females were mated with one male overnight, and the day of the vaginal plug was considered to be day 0 of gestation (0 dpc). The pregnant mice were randomly assigned among the treatment groups and housed individually. Care and experimental procedures were in accordance with Italian national regulations and were approved by the University of Milan ethics committee.

Treatments. PCB congeners 101 and 118 were purchased from LGC Standards GmbH (Wesel, Germany) and were certified 99.8% pure. The two congeners, in a proportion of 1:1, were diluted in commercial sunflower oil and sent to a manufacturer of special and customized animal diets (Altromin, Lage, Germany), to prepare both PCB- and vehicle-treated chow. The amount of PCB

mixture added to the chow in order to obtain the desired doses (0, 1, 10, and 100 μg PCB/kg/day) was calculated on the basis of the mean daily food intake of mice. This was assessed in a preliminary study, under the same physiological conditions, and confirmed by literature (Johnson *et al.*, 2001). Each batch of diet was tested before use in an accredited laboratory (SGS laboratory GmBH, Hamburg, Germany). Pregnant mice were given diets formulated to contain known amounts of PCBs or a vehicle-containing diet from dpc 0 throughout lactation until weaning (postnatal day [PND] 21). The dose range was selected to overlap PCB concentrations reported in the breast milk of women in industrialized countries (19 ng/g/fat of PCB 118) (World Health Organization [WHO], 1996). The average daily PCB intake of the dams has been calculated based on the procedures proposed by U.S. Environmental Protection Agency (EPA) (2000). This approach assumes that the concentration in breast milk fat is the same as in maternal fat; the estimated intake of PCBs is calculated as follows: Daily maternal intake = (C_{milk} × maternal fat content). Considering that approximately 20% of the body weight (BW) of an adult female mouse is composed of adipose tissue (Kuriyama and Chahoud, 2004), such a concentration is achieved by a daily oral dose of 3.8 μg/kg BW. Therefore, the range of concentrations chosen for the present study (1–100 μg/kg/day) represented doses estimated to represent between 0.3 and 30× the mean concentration to which human infants are exposed.

F0 reproductive outcome. Dams and lactating offspring were examined daily for clinical signs of toxicity and during treatment dam BWs were recorded twice weekly. On PND 21, all dams were euthanized by CO_2 inhalation and BW was recorded. Liver, visceral fat, ovaries, and uterus were collected and organ weight recorded. The digestive tract, skin, and head were removed, and the remaining whole-animal body, including liver and fat, was wrapped in aluminum foil, freeze-dried, and stored at −20°C until it was analyzed to determine PCBs content. Variables including litter size, sex ratio, pup weight, and the number of viable pups were recorded.

F1 offspring data. On PND 21, all pups were sexed and BWs recorded. At least two offspring per litter were sacrificed. The digestive tract, skin, and head were removed, and the remaining whole-animal body was wrapped in aluminum foil, freeze-dried, and stored at −20°C until analysis for PCBs tissue content.

Remaining female and male pups from each litter were housed in groups for another 9 weeks (up to PND 84). Standard pellet food (Charles River 4RF21) and tap water were available *ad libitum*.

On PND 84, at least three animals of each sex per litter were randomly selected for measurement of BW and anogenital distance (AGD) and for autopsy. They were euthanized by CO_2 inhalation followed by cervical dislocation and AGD (defined as the distance between the center of the anus and the basis of the genital bud) was measured by a single investigator, using a manual caliper. The animals were handled carefully to avoid variation in the measurements due to stretching of the perineal region. AGD data were analyzed by the calculated AGD index, defined as AGD divided by the cube root of the BW (Gallavan *et al.*, 1999). Thereafter, reproductive organs were removed, weighed, and formalin-fixed for later analyses.

Sperm collection and dead:live ratio. Sperm was obtained from the cauda epididymis of adult offspring. Both cauda were dissected out from the body and transferred into 500 μl of previously equilibrated Whittingham medium (37°C at 5% CO2 in air). Sperm was passively released into the culture medium by puncturing the cauda 3–4 times with a 27G needle. Some of the samples were diluted (1:100) with water, and a sperm count was done in a Neubauer chamber. Other samples were diluted (1:20) with 0.9% NaCl and stained by a modified Kovács-Foote method (Kovács and Foote, 1992). Briefly, one drop of dilute sample was mixed on a microscope slide with one drop of iso-osmotic 0.2% Trypan blue (Sigma T-8154) and smeared with the edge of another slide. The slides were vertically air-dried, then fixed for 2 min with fixative solution (86 ml 1N HCL plus 14 ml 37% formaldehyde solution and 0.2 g neutral red [Fluka, 72210]), and rinsed with tap and distilled water. Finally, the slides were air dried and covered with Eukitt (Fluka, 03989) and a coverslip.

Stained smears were examined by light microscopy at ×400 magnification. The status of the head and tail of at least 100 spermatozoa was classified in each smear. Sperm with white or pale pink heads (intact plasma membrane) were classified as alive, and sperm with black to dark-purple heads (damaged membrane) were classified as dead.

In vitro *fertilization and embryo culture.* Females were superovulated by ip injection of 3.5 IU Folligon (PMSG, Intervet International, Netherlands), followed 48 h later by an ip injection of 5 IU Chorulon (hCG, Intervet).

Spermatozoa were collected as described above and capacitated for 60 min in Whittingham medium (37°C at 5% CO2 in air). Fourteen hours post-hCG cumulus-oocyte complexes were recovered from oviducts in M2 medium (Sigma-Aldrich). After rinsing in Whittingham medium, cumulus-oocyte complexes were inseminated with 2×10^6 capacitated spermatozoa. Putative fertilized eggs (6 h postinsemination) were then transferred to 250 μl drops of M16 medium (Sigma-Aldrich) covered with paraffin oil and incubated at 37°C at 5% CO2 in air for a further 96 h. Cleavage and blastocyst rate were assessed at 24 and 96 h postinsemination, respectively.

Intergenerational study. To study the transmission over multiple generations of PCB effects, at least seven F1 females from different litters were randomly selected and subsequently mated with CD-1 nonexposed males of proven fertility in order to obtain F2 offspring. F2 offspring were analyzed as described for F1 and at the same ages. Furthermore, at least seven F2 females from different litters were randomly chosen and mated as described for F1. The experiment ended when F3 offspring reached adult age (PND 84).

Determination of tissue PCBs concentrations. Tissue concentrations of the selected PCB congeners were determined using previously described methods (Rhind *et al.*, 2009). Tissue samples (1 g), together with internal standards, were extracted using dichloromethane, in a 40 ml, sealed glass tube heated to 55°C for 2 h. After filtering and evaporation under nitrogen, the sample was subjected to clean up by absorption chromatography (10 g acid modified silica column which was conditioned by 40 ml isohexane). The elute (100 ml isohexane) was collected and concentrated by rotary evaporation and gentle nitrogen flow and transferred to gas chromatograph (GC) vials, until further analysis. The analysis was conducted using an Agilent 5975C MSD (mass spectrometer detector) linked to 7890A GC with an autosampler (7683B). Separations were effected on a Zebron ZB5 fused silica capillary column (30 m × 0.25 mm [id]) coated with 95% dimethylpolylysiloxane/5% phenyl with a phase thickness of 0.25 mm (Phenomenex, Macclesfield, U.K.). The oven temperature program for PCB analysis started at 120°C for 1 min; the temperature was then ramped at 4°C/min to 280°C and held for 1 min and then ramped to 320°C at 30°C/min and held for 5 min. The carrier gas was helium, and samples were injected onto the GC column in splitless mode. The mass spectrometer was operated in the electron ionization (EI⁺) mode at 70 electron volts and a source temperature of 200°C. The limits of detection were calculated by dilution of a quality control sample until the signal to noise ratio was less than 3:1 (20 ng/kg for each congener). Linearity was demonstrated from 0.02 μg/kg to 50,000 ($R^2 > 0.99$).

Histological analysis. Ovaries collected from F1, F2, and F3 female offspring were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. Specimens were serially sectioned (8 μm), mounted on glass slides, and stained with hematoxylin and eosin (HE) according to standard procedures. Follicle counts were performed as described by Tomic *et al.* (2002). Briefly, a stratified sample consisting of 10 sections was used to estimate the number of different follicles class per ovary. The selected sections from each ovary were randomized, and follicles were counted on the entire section. Only follicles with a visible nucleolus in the oocyte were counted to avoid counting follicles twice. The number of follicles in the marked sections was then multiplied by 10 (because every 10th section was analyzed) and subsequently by 8 (accounting for section thickness) to obtain an estimate of the total number in each ovary. Follicles were categorized according to Flaws *et al.* (2001). They were classified as (1) primordial when they contained an intact oocyte surrounded by a one-layer ring of fusiform granulosa cells, (2) primary if they consisted of an oocyte and a single layer of cuboidal granulosa cells, (3) secondary if they contained an oocyte and more than

one layer of granulosa cells, (4) tertiary if they contained more than one layer of granulosa cells and an antral space, and (5) end-stage atretic follicles atretic if containing zona pellucida remnants. All sections were evaluated "blind," without knowledge of the treatment group of the animals.

Testes collected from F1, F2, and F3 animals were fixed in Bouin's fixative, dehydrated, and embedded in paraffin. Then, 5-μm serial microscopic sections were prepared and at least six slides from each testis were stained with HE for histological assessment. In cross sections of randomly selected tubular profiles that were round in shape, the percentages of each of three types of tubules were determined: (1) normal seminiferous tubule with sperm (type I), (2) normal seminiferous tubules without sperm (type II), and (3) depleted seminiferous tubule (type III); the diameters of the tubules and epithelium thickness were measured, also, using light microscopy, accordingly to the methods of Koruji et al. (2008). Each parameter measurement was based on examination of at least 45 fields in histological sections from at least six testes per group.

Statistical analysis. All data were analyzed using GraphPad Prism software (GraphPad Software 5.03, San Diego, CA). The mean numbers of follicles per ovary were calculated using ovaries from at least five different animals. Seminiferous tubule morphology was assessed using testes from at least six different animals. The mean number of pups per litter was calculated from at least six different mating pairs per treatment group, and mean body and organ weights were calculated for at least six different litters per treatment group. Because individual pups within a litter received the same treatments, for the purpose of statistical analysis, the experimental unit was the whole litter, and the unit of statistical analysis was the litter mean. Differences between the means were examined by one-way ANOVA, with statistical significance assigned at $p \leq 0.05$. When ANOVA gave a significant p value, the Newman-Keuls' test was used in the *post hoc* analysis.

Data for *in vitro* embryo culture were analyzed by binary logistic regression. Controls were taken as the reference group. Experiments were replicated at least three times, and each replicate was fitted as a factor. The log likelihood ratio statistic was used to detect between-treatment differences, and significance was set at $p \leq 0.05$.

RESULTS

PCB Congener Concentration in F0 and F1 Body Tissues

Total PCB concentrations in tissues of dams (F0) and of their offspring (F1) at weaning (PND 21) increased in a dose-dependent manner. Offspring accumulated about 1.4- to 1.9-fold more PCB per unit of weight than their mothers (Table 1).

The ratio of the PCB congeners 101 and 118 in the body of both dams and offspring in each of the 10 and 100 μg/kg/day groups was about 1:2–1:3, despite being present in the diet in a proportion of 1:1.

F0 Dam Reproductive Outcome

There were no signs of immediate toxicity in dams exposed to PCB. Necroscopy revealed no significant change in mean liver weight, visceral fat content, or reproductive organ weights in any of the treatment group, relative to the control. Furthermore, treatment with PCB did not affect the duration of gestation, litter size, sex ratio, or viability index (Table 2).

Morphological Indices in F1, F2, and F3 Male and Female Offspring

Tables 3–5 show the morphological indices of three generations of mice once they reached adulthood (PDN 84).

TABLE 1
PCB 101 and 118 Tissue Concentration in F0 Dams and F1 Offspring Exposed From DPC 0.5 to PND 21

	PCBs 101 + 118			
	0 μg/kg/day	1 μg/kg/day	10 μg/kg/day	100 μg/kg/day
Total PCBs (μg/kg)				
Dams	0.9 ± 0.2^a	4.6 ± 0.4^b	34.42 ± 3.6^c	412.1 ± 24.6^d
Offspring	1.7 ± 0.2^a	$7.7 \pm 0.3^{b*}$	$66.3 \pm 2.0^{c*}$	$561.2 \pm 21.4^{d*}$
PCB 101:118 (%)				
Dams	60:40	33:67	27:63	28:72
Offspring	47:53	44:56	31:69	27:73

Note. Different superscript letters within a row indicate statistical differences for $p \leq 0.05$.
*Indicates statistical difference between columns for $p \leq 0.05$.

In F1 offspring of the three PCB treatment groups, testes and ovaries weights (absolute and relative) were significantly smaller than controls ($p \leq 0.001$). Furthermore, at a dose of 100 μg/kg/day PCB, a reduction in AGD was observed in male offspring ($p \leq 0.0001$).

F2 male offspring of all three PCB treatment groups, as for F1 animals, had significantly lighter testes compared with controls ($p \leq 0.0001$). Conversely, in F2 female offspring, the mean ovary weight was unaffected, although the 100 μg/kg/day dose was associated with a nonsignificant reduction in mean ovarian weight.

In F3 animals, there were no significant differences in morphological indices between treated and control groups.

Histological Analysis of Male and Female Gonads

In utero and lactational exposure to PCBs at 10 and 100 μg/kg/day increased significantly the proportion of type II tubules at the expense of type I tubules in all generations (Fig. 2). The

TABLE 2
Reproductive Outcome of F0 Dams Treated With PCB Throughout Pregnancy and Lactation

	PCBs 101 + 118			
Parameter	0 μg/kg/day	1 μg/kg/day	10 μg/kg/day	100 μg/kg/day
Number of dams	10	9	10	10
Pregnancy at term (%)	100	100	100	100
Litter size	13.5 ± 0.4	13.0 ± 0.4	14.4 ± 0.5	13.3 ± 0.4
Sex ratio (female:male, %)	48:52	49:51	52:48	54:46
Viability index	97.1 ± 2.0	98.7 ± 1.3	97.3 ± 1.6	98.1 ± 1.9

TABLE 3
Morphological Indices in Male and Female F1 Offspring (PND 84)

	PCB			
	0 µg/kg/day	1 µg/kg/day	10 µg/kg/day	100 µg/kg/day
Males				
Number of litters (individuals)	10 (52)	9 (50)	10 (41)	10 (54)
BW (g)	32.71 ± 0.87	33.05 ± 0.40	30.97 ± 0.52	32.82 ± 0.75
AGD index	0.517 ± 0.009[a]	0.509 ± 0.009[a]	0.514 ± 0.005[a]	0.478 ± 0.004[b]
Testis (g) (% of BW)	0.107 ± 0.002[a]	0.093 ± 0.003[b]	0.087 ± 0.003[b]	0.091 ± 0.001[b]
	(0.301 ± 0.005[a])	(0.281 ± 0.005[b])	(0.274 ± 0.009[b])	(0.279 ± 0.004[b])
Seminal vesicle (g)	0.182 ± 0.006	0.169 ± 0.009	0.193 ± 0.003	0.192 ± 0.005
Females				
Number of litters (individuals)	10 (50)	9 (42)	10 (34)	10 (51)
BW (g)	30.31 ± 0.60	29. 34 ± 0.62	28.33 ± 0.44	28.16 ± 1.65
AGD index	0.249 ± 0.004	0.246 ± 0.004	0.254 ± 0.005	0.247 ± 0.009
Ovary (g) (% of BW)	0.0086 ± 0.0008[a]	0.0070 ± 0.0005[b]	0.0067 ± 0.0004[b]	0.0065 ± 0.0004[b]
	(0.033 ± 0.006 [a])	(0.025 ± 0.003[b])	(0.023 ± 0.002[b])	(0.023 ± 0.002[b])
Uterus (g)	0.134 ± 0.014	0.106 ± 0.006	0.119 ± 0.008	0.128 ± 0.003

Note. Different superscript letters indicate statistical differences within rows for $p \leq 0.05$.

incidence of depleted (type III) tubules was unaffected by treatment at any dose or generation investigated.

In F1 animals, tubule diameter was significantly reduced with all PCB doses, compared with controls, without changes in epithelium height (Table 6). Similar changes in tubule morphometric indices were observed in F2 testes, but no treatment differences were observed in either variable in the F3 generation (Table 6).

In F1 adult females, exposure of F0 dams to PCB treatment increased the incidence of follicular atresia without affecting the proportions of preantral or antral follicles (Fig. 3).

No differences in follicular distribution nor in the incidence of atretic follicles were observed in F2 and F3 generations, irrespective of treatment.

Semen Characteristics in F1, F2, and F3 Generations

PCB exposure of F0 dams significantly depressed the sperm viability of offspring of all generations, generally by about 20–30%, relative to controls of the same generation ($p \leq 0.0001$). There was no effect of PCB dose (Fig. 4).

Sperm concentration was unaffected by PCB exposure in any generation.

TABLE 4
Morphological Indices in Male and Female F2 Offspring (PND 84)

	PCB			
	0 µg/kg/day	1 µg/kg/day	10 µg/kg/day	100 µg/kg/day
Males				
Number of litters (individuals)	8 (36)	7 (25)	7 (26)	7 (19)
BW (g)	34.52 ± 0.86	34.17 ± 0.54	34.11 ± 0.51	36.30 ± 0.53
AGD index	0.537 ± 0.007	0.528 ± 0.006	0.533 ± 0.008	0.523 ± 0.004
Testis (g) (% of BW)	0.113 ± 0.001[a]	0.100 ± 0.003[b]	0.100 ± 0.001[b]	0.101 ± 0.004[b]
	(0.315 ± 0.011[a])	(0.276 ± 0.001[b])	(0.288 ± 0.003[b])	(0.271 ± 0.002[b])
Seminal vesicle (g)	0.179 ± 0.005	0.183 ± 0.007	0.153 ± 0.001	0.162 ± 0.009
Females				
Number of litters (individuals)	8 (38)	7 (27)	7 (31)	7 (18)
BW (g)	27.39 ± 0.30	27.99 ± 0.11	27.85 ± 0.69	29.41 ± 0.63
AGD index	0.268 ± 0.005	0.277 ± 0.006	0.266 ± 0.003	0.261 ± 0.006
Ovary (g) (% of BW)	0.0082 ± 0.0002	0.0081 ± 0.0004	0.0080 ± 0.0004	0.0071 ± 0.0002
	(0.032 ± 0.002)	(0.031 ± 0.006)	(0.032 ± 0.003)	(0.028 ± 0.009)
Uterus (g)	0.143 ± 0.007	0.135 ± 0.019	0.124 ± 0.005	0.118 ± 0.008

Note. Different superscript letters indicate statistical differences within rows for $p \leq 0.05$.

TABLE 5
Morphological Indices in Male and Female F3 Offspring (PND 84)

	PCB			
	0 µg/kg/day	1 µg/kg/day	10 µg/kg/day	100 µg/kg/day
Males				
Number of litters (individuals)	7 (25)	7 (30)	6 (20)	7 (28)
BW (g)	31.74 ± 0.89	32.98 ± 0.91	30.05 ± 0.58	32.50 ± 1.27
AGD index	0.548 ± 0.005	0.537 ± 0.006	0.558 ± 0.008	0.536 ± 0.007
Testis (g) (% of BW)	0.102 ± 0.002	0.101 ± 0.002	0.101 ± 0.001	0.097 ± 0.001
	(0.316 ± 0.008)	(0.302 ± 0.005)	(0.322 ± 0.005)	(0.309 ± 0.007)
Seminal vesicle (g)	0.147 ± 0.011	0.168 ± 0.016	0.164 ± 0.001	0.146 ± 0.009
Females				
Number of litters (individuals)	7 (32)	7 (32)	6 (21)	7 (33)
BW (g)	28.32 ± 1.07	28.87 ± 1.08	28.63 ± 0.56	30. 28 ± 0.61
AGD index	0.263 ± 0.007	0.267 ± 0.005	0.251 ± 0.005	0.266 ± 0.005
Ovary (g) (% of BW)	0.0085 ± 0.0006	0.0082 ± 0.0010	0.0078 ± 0.0004	0.0079 ± 0.0006
	(0.032 ± 0.002)	(0.030 ± 0.002)	(0.030 ± 0.001)	(0.029 ± 0.001)
Uterus (g)	0.124 ± 0.005	0.125 ± 0.014	0.123 ± 0.011	0.141 ± 0.010

Gamete Quality in F1 Generation

PCB exposure compromised sperm developmental capacity but not its fertilization capacity. In tests using oocytes from untreated mice and *in vitro* fertilization protocols, the sperm from the 10 and 100 µg/kg/day groups resulted in zygotes with the same ability to complete first mitotic division, but with a significantly reduced capacity to reach the blastocyst stage, compared with controls ($p \leq 0.05$; Fig. 5A).

The oocytes of the PCB groups of the F1 generation, like the sperm of this generation, when fertilized with gamete from untreated animals, produced embryos with a significantly reduced capacity to reach the blastocyst stage but with the same ability as controls to complete the first mitotic division. This effect was significant in the 100 µg/kg/day group compared with control ($p \leq 0.05$; Fig. 5B); values in the 10 µg/kg/day group were intermediate.

PCB did not affect the number of ovulated mature oocytes per animal.

Reproductive Outcome of Female Mice of the F1 and F2 Generations

All females of both generations became pregnant and exhibited normal lengths of gestation. Nevertheless, F1 dams of all three PCB treatment groups gave birth to significantly smaller litters (about two pups fewer than controls) (Table 7).

DISCUSSION

The present study shows that prenatal and perinatal exposure of mice to a mix of PCBs 101 and 118, at doses designed to simulate human exposure, induced permanent morphological and functional reproductive alterations in both male and female adult offspring. Furthermore, the results indicate that reproductive deficiencies in male may be transmitted intergenerationally.

PCBs tissue concentration increased with the dose administered in a broadly linear manner, suggesting that the desired differences in treatment were actually achieved. Lower burdens of PCB 101 compared with PCB 118 could be explained by differences in clearance and redistribution rates; these processes are more rapid for PCB 101 than for PCB 118 (24 h vs. 1 week) (Oberg *et al.*, 2002).

PCBs concentration measured in dams were in the range of those found in adipose tissue in the human population (up to 134 µg/kg) (Kiviranta *et al.*, 2005). In fact, multiplication by a factor of 5 of the whole-body burden measured in the present study (mouse body fat content being approximately 20% of the total body mass) indicates that the concentrations in dams treated with the low and medium doses overlap PCB human levels.

The finding of preferential accumulation of PCBs in the offspring, compared with their dams, confirms previous studies in sheep exposed to contaminated pastures (Rhind *et al.*, 2009, 2010) and indicates that the most vulnerable developmental stages are subject to the greatest PCB burden following maternal exposure, an observation of particular concern.

In the present study, a significant reduction in ovarian weight, together with increased follicular atresia, was observed in PCB-exposed F1 female offspring. These data are in agreement with studies indicating that reduction in mammalian ovary weight may be related to morphological changes, such as a reduction in the number of antral follicle and/or increase in atretic follicle number (Rao and Kaliwal, 2002; Shirota *et al.*, 2006). The observation of increased atresia without effects on total follicle count or on the proportion of follicles of each class is interesting. In fact, it has been suggested that maternal
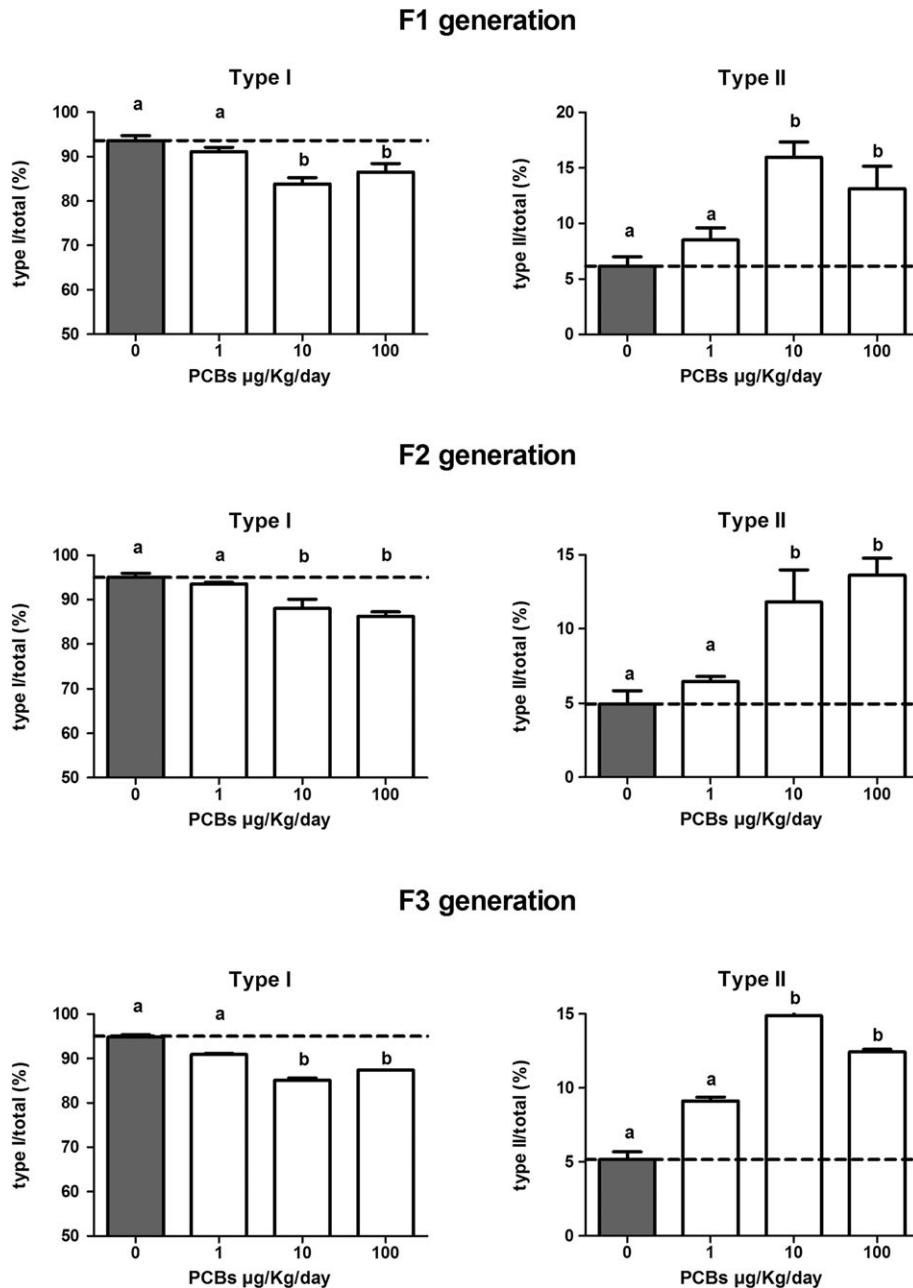
FIG. 2. Seminiferous tubules distribution in testes of the F1, F2, and F3 offspring. Type I: normal seminiferous tubule with sperm and type II: normal seminiferous tubules without sperm. Each column represents the mean ± SE of at least three separate experiments. Horizontal dashed lines represent mean control levels. Different superscripts denote significant differences between columns ($p < 0.05$).

exposure to coplanar and noncoplanar PCBs may exert opposite effects on follicular dynamics. Baldridge *et al.* (2003) reported that maternal exposure to a mixture of noncoplanar PCBs reduced preantral and antral follicle numbers in rat ovaries while concurrently causing an increase in atresia. In contrast, *in utero* exposure to coplanar PCBs has been demonstrated to increase follicle number in different species (Kraugerud *et al.*, forthcoming; Ronnback and de Rooij, 1994). Considering that the mono-ortho PCB 118 may

share some effects with coplanar PCBs whereas the di-ortho PCB 101 is a typical noncoplanar congener, it is postulated that the interaction of the two congeners could have a stimulatory effect on follicle count that would be masked by increased rate of follicular loss through atresia. This hypothesis is further supported by the observation that, despite increased atresia, no differences in mean numbers of ovulated oocytes were observed in any of the treatment groups. Analyses of the underlying molecular mechanisms would be required to

TABLE 6
Tubule Diameters and Epithelium Height in F1, F2, and F3
Generations (PND 84)

	PCBs 101 + 118			
	0 μg/kg/day	1 μg/kg/day	10 μg/kg/day	100 μg/kg/day
Generation F1				
Number of fields	79	58	79	66
Tubule diameter (μm)	256.7 ± 2.2^a	234.9 ± 3.1^b	242.8 ± 1.9^b	244.8 ± 2.9^b
Epithelium height (μm)	67.20 ± 0.79	63.85 ± 1.18	67.42 ± 0.96	66.17 ± 1.37
Generation F2				
Number of fields	45	48	45	46
Tubule diameter (μm)	264.2 ± 5.8^a	231.7 ± 3.1^b	230.4 ± 4.3^b	236.0 ± 4.0^b
Epithelium height (μm)	72.40 ± 1.27	66.44 ± 1.31	67.89 ± 2.00	66.73 ± 1.95
Generation F3				
Number of fields	47	51	46	48
Tubule diameter (μm)	251.3 ± 3.9	244.7 ± 2.7	245.1 ± 3.1	238.0 ± 2.6
Epithelium height (μm)	65.46 ± 1.81	64.57 ± 1.37	66.35 ± 1.30	64.58 ± 1.65

Note. Different superscript letters indicate statistical differences within rows for $p \leq 0.05$.

determine the relative contributions of PCB 101 and 118 to the modification of F1 follicular dynamics.

In view of the observed patterns of follicle populations in treated animals, no alteration in fertility rate might be expected, but in fact, a significant reduction in mean litter size was observed in PCB-treated F1 parental animals. Animal and human studies have shown that PCBs cause implantation failure, resorption, and reduced litter size (Jonsson *et al*., 1975; Seiler *et al*., 1994). Reduced litter size is also consistent with impaired developmental competence of oocytes derived from F1-treated females observed in the present study.

Several studies have shown that exposure to PCBs reduces oocyte maturation, fertilization, and blastocyst development (Campagna *et al*., 2002; Kholkute and Dukelow, 1997; Kholkute *et al*., 1994a,b; Krogenaes *et al*., 1998; Kuchenhoff *et al*., 1999; Lindenau and Fischer, 1996; Pocar *et al*., 2001). Studies in women showed preferential accumulation of PCB 118 in the follicular fluid (Pauwels *et al*., 1999), which may explain the adverse effects on oocyte developmental competence observed in the current study. Further studies are needed to clarify the mechanisms underlying adverse effects on oocyte developmental competence and the reduction in litter size observed.

Effects of PCB were not confined to the female offspring. In adult F1 male offspring, reduced mean testis weight and reduced sperm quality and developmental capacity were observed, suggesting an antiandrogenic effect attributable to maternal exposure to PCBs. This hypothesis is consistent with the reduced AGD observed in the highest dose group. In agreement with present data, rats and guinea pigs prenatally exposed to PCBs showed reduced testis weight and limited reproductive capability (Brouwer *et al*., 1995; Kuriyama and Chahoud, 2004; Lundkvist, 1990).

Histological analysis of PCB-treated animals has also been shown to be associated with a significant increase in sperm-depleted seminiferous tubules and reduced diameter of seminiferous tubules, findings that may indicate spermatogenic cell loss and tubules disorganization (Creemers *et al*., 2002). Thus, while no significant alteration in epididymal sperm count was observed, the possibility of a subclinical reduction in sperm production cannot be ruled out.

Our data are consistent with cohort studies indicating that men exposed to PCBs (directly or *in utero*) had a higher percentage of oligospermia, abnormal sperm morphology, and reduced sperm penetration capacity (Guo *et al*., 2000; Hsu *et al*., 2003). In rodents, sperm production can be reduced by up to 90% without compromising fertility (Aafjes *et al*., 1980; Faqi *et al*., 1997), whereas in men, relatively small changes in sperm concentration and quality may have severe consequences because the sperm count is near the critical lower threshold for fertility (Zenick and Clegg, 1989).

Of particular concern is the observation that PCB effects on seminiferous tubules distribution and of sperm viability were detected in later generations, up to the F3, an observations consistent with other physiological systems and environmental toxicants (Anway *et al*., 2005; Wolf *et al*., 1999). To our knowledge, this is the first study reporting long-lasting reproductive effects of PCBs spanning three generations in mammals. Nevertheless, other environmental chemicals have been shown to induce reproductive abnormalities for multiple generations (Anway *et al*., 2006b). Different mechanisms have been postulated to explain multigenerational reproductive adverse phenotype, including epigenetic changes in the germline (Anway *et al*., 2006a). Specifically, imprinting defects of paternally or maternally methylated genes have been associated with altered spermatogenesis (Marques *et al*., 2010; Roeleveld and Bretveld, 2008) and have been observed in oligospermic or azoospermic patients.

The mechanisms underlying transmission of PCB effects observed in the present study could involve intergenerational and/or transgenerational transmission. In fact, considering the congeners half-lives (between 54 and 124 days [Oberg *et al*., 2002]), PCB burden in F1 females at time of mating could still be significant. Therefore, remaining chemicals could represent a source of direct exposure for both F2 and F3 generations; that is, intergenerational transmission may be the result of transfer of maternal PCB burdens to the fetuses, in conjunction with preferential accumulation by them. However, since PCB-induced effects were present up to the F3, which is the first one that can exhibit true transgenerational effects (Skinner, 2008), transmission to subsequent generations of permanent molecular
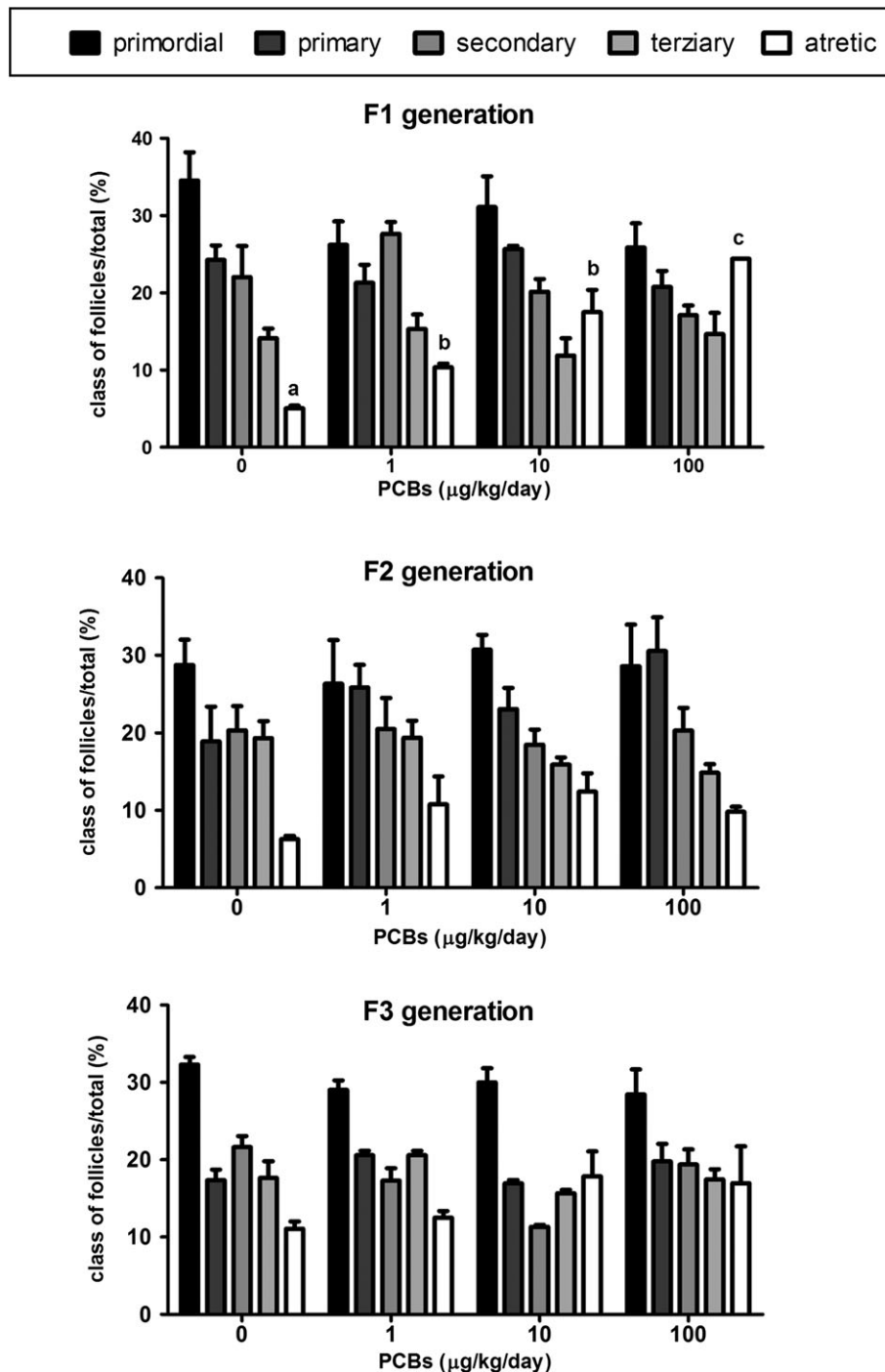
FIG. 3. Percentages of follicles in each follicular stage in ovaries of the F1, F2, and F3 offspring. Each column represents the mean ± SE of at least three separate experiments. Different superscripts denote significant differences between columns ($p \leq 0.0001$).

changes induced in the F1 cannot be ruled out. Because abnormal reproductive phenotype was highly consistent between individuals and among litters, genetic DNA sequence mutations are not likely the cause for intergenerational transmission of adverse effects. (Barber *et al.*, 2002; Dong *et al.*, 2004). In contrast, epigenetic variations involving the germline could

result in the high frequency observed (Anway *et al.*, 2005). Analyses are required to identify epigenetic patterns, which might explain intergenerational transmission of PCB effects.

In the present study, no obvious dose-response relationships were found for any of the endpoints analyzed. This may reflect the multiple mechanisms of action of the PCB mixture and the
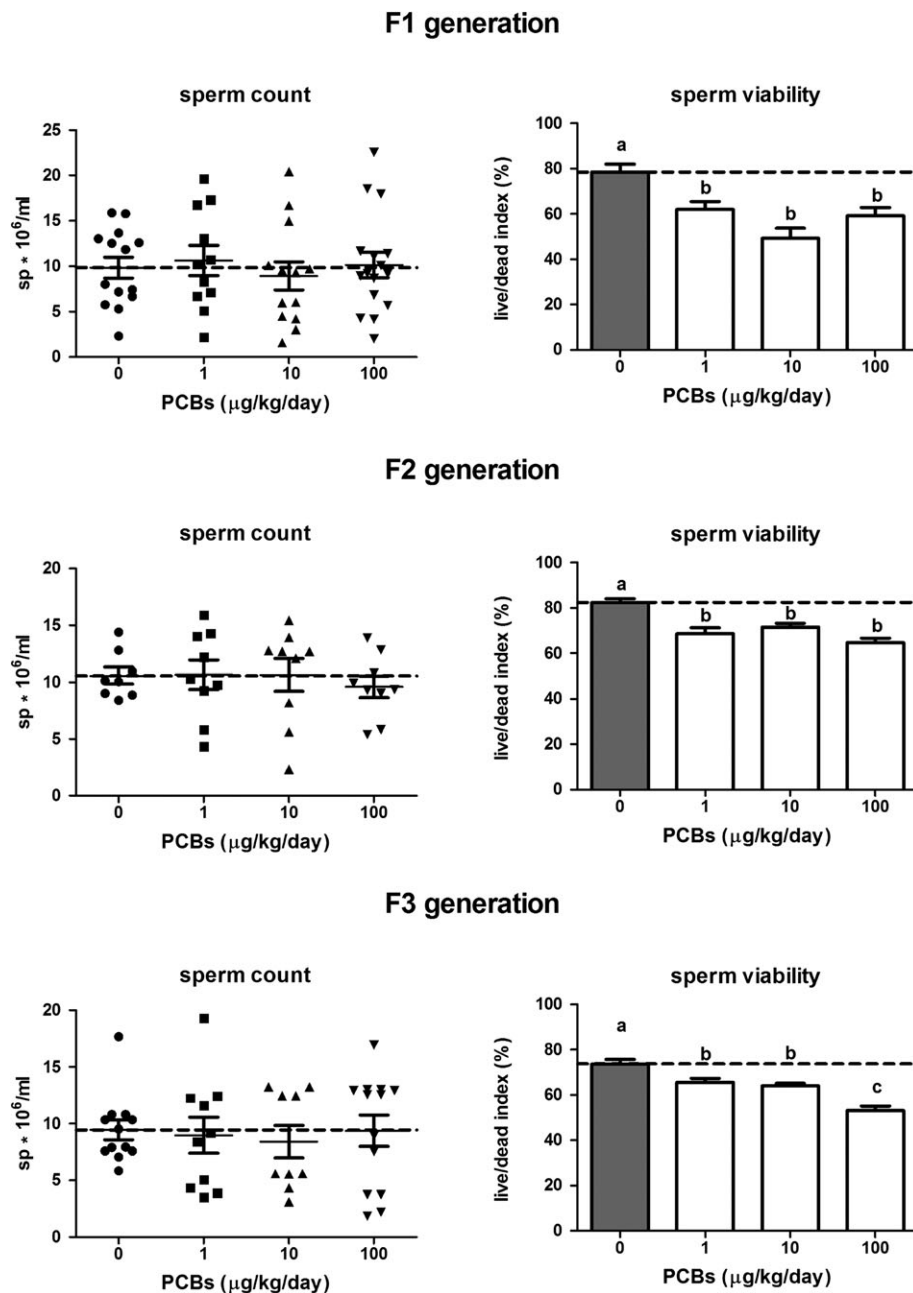
FIG. 4. Counts and viability of epididymal caudal sperm from offspring of the F1, F2, and F3 generations. Horizontal dashed lines represent mean control levels. Different superscripts denote significant differences between columns ($p \leq 0.0001$).

different, sometimes opposite, actions of the congeners. Furthermore, the induction of enzyme activity at high doses may alter toxicokinetics and this, in turn, may change the effective dose at the site of action resulting in nonlinear dose-response (Kupfer, 1987; Li and Hansen, 1996).

In conclusion, our study demonstrates that *in utero* and lactational exposure to a congener mixture of PCBs 101 and 118 has multiple effects on the reproductive system in adult offspring

of both sexes. In F1, several effects, including reductions in gonadal weight, impaired gamete quality, and reduced developmental competence were similar in both male and female offspring, which may suggest a common mechanism of action in both sexes. The observation that reproductive abnormalities in males occurred in subsequent generations, up to F3, suggests vertical transmission of PCB-mediated adverse effects. Finally, it needs to be emphasized that the adverse effects have been
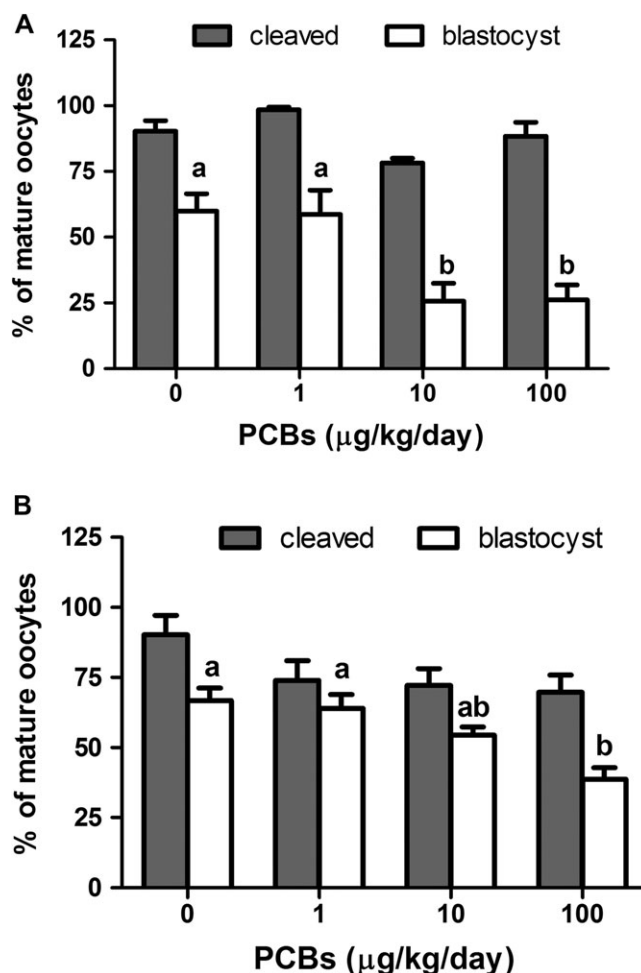
FIG. 5. (A) Fertility and developmental ability of epididymal caudal sperm from mice treated *in utero* and during lactation with PCBs 101 and 118. Embryo cleavage (gray bars) and blastocyst development (white bars) following *in vitro* fertilization of untreated oocytes. Each column represents the mean ± SE of at least three separate experiments. Different superscripts denote significant differences between columns ($p < 0.05$). (B) Fertility and developmental ability of oocytes from mice treated *in utero* and during lactation with PCBs 101 and 118. Embryo cleavage (gray bars) and blastocyst development (white bars) following *in vitro* fertilization with untreated sperm. Each column represents the mean ± SE of at least three separate experiments. Different superscripts denote significant differences between columns ($p < 0.05$).

TABLE 7

Reproductive Outcome of Female Offspring of the F1 and F2 Generation

	PCBs 101 + 118			
	0 μg/kg/day	1 μg/kg/day	10 μg/kg/day	100 μg/kg/day
Generation F1				
Number of dams	8	7	7	8
Pregnancy at term (%)	100	100	100	100
Litter size	14.71 ± 0.64^a	12.60 ± 0.24^b	12.20 ± 0.49^b	11.67 ± 0.67^b
Sex ratio (female:male, %)	49:51	50:50	53:47	57:43
Viability index	92.06 ± 4.88	96.08 ± 3.92	91.96 ± 5.45	91.38 ± 5.27
Generation F2				
Number of dams	7	7	7	8
Pregnancy at term (%)	100	100	100	100
Litter size	13.57 ± 0.87	13.43 ± 0.69	14.83 ± 0.70	13.17 ± 0.31
Sex ratio (female:male, %)	45:55	49:51	49:51	50:50
Viability index	95.99 ± 1.64	98.75 ± 1.25	97.92 ± 2.08	98.90 ± 1.10

Note. Different superscript letters indicate statistical differences within rows for $p \leq 0.05$.

observed in a dose range relevant to human exposure and that preferential accumulation of PCBs was observed in the offspring, pointing to the progeny as a target of maternal exposure to PCB.

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