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Effects of *In Utero* Exposure to 4-hydroxy-2,3,3′,4′, 5-pentachlorobiphenyl (4-OH-CB107) on Developmental Landmarks, Steroid Hormone Levels, and Female Estrous Cyclicity in Rats

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Previous studies have revealed that one of the major metabolites of PCBs detected in human blood, 4-OH-2,3,3′,4′,5-pentaCB (4-OH-CB107), accumulated in fetal liver, brain, and plasma and reduced maternal and fetal thyroid hormone levels after prenatal exposure to pregnant rats from gestational days (GD) 10–16. In the present study, the effects of 4-OH-CB-107 on developmental landmarks, steroid hormone levels, and estrous cyclicity of rat offspring after *in utero* exposure to 4-OH-CB107 was investigated. Pregnant rats were exposed to 0, 0.5, and 5.0 mg 4-OH-CB107 per kg bw from GD 10 to GD 16. Another group of rats was exposed to Aroclor 1254 (25 mg/kg bw) to study the differences between effects caused by parent PCB congeners and the 4-OH-CB107 alone. A significant, dose-dependent prolongation of the estrous cycle was observed in 75% and 82% of female offspring exposed to 0.5 and 5.0 mg 4-OH-PCB107, respectively, compared to 64% of Aroclor 1254 (25 mg/kg) exposed offspring. The diestrous stage of the estrous cycle was prolonged, resembling a state of pseudopregnancy, which might reflect early signs of reproductive senescence. Plasma estradiol concentrations in female rat offspring were significantly increased (50%) in the proestrous stage after exposure to 5 mg 4-OH-CB107 per kg bw. No effects on estradiol levels were observed in Aroclor 1254 treated animals. These results indicate that *in utero* exposure to 4-OH-CB107 leads to endocrine-disrupting effects, especially in female offspring. The possible impact on neurobehavior following exposure to 4-OH-CB107 will be reported elsewhere.

Key Words: **estrous cycle; anogenital distance; PCB; metabolite; reproductive senescence.**

INTRODUCTION

Polychlorinated biphenyls (PCBs) are environmental contaminants causing a broad range of toxic effects (reviewed in Brucker-Davis, 1998; Brouwer *et al*., 1998; Peterson *et al*., 1993; Safe, 1990, 1994; Seegal, 1996; Tilson and Kodavanti, 1997). Dependent on the number and position of the chlorine substituents, PCBs can be metabolized to hydroxylated PCBs (OH-PCBs) in animals via an arene oxide intermediate, catalyzed by cytochrome P450s 1A and 2B (Safe, 1994). Hydroxylated metabolites of PCBs (OH-PCBs) have been identified in the blood of marine mammals, polar bears, fish-eating birds, and humans (Bergman *et al*., 1994; Klasson-Wehler *et al*., 1998; Sandau *et al*., 2000; Sjödin *et al*., 2000), at concentrations of 10–30% of the PCB concentration in human blood (Sandau *et al*., 2000, Sjödin *et al*., 2000) but as high as 2–3 times the PCB level in polar bear blood (Sandau, 2000). Several potentially adverse effects of OH-PCBs on the endocrine system have been reported. OH-PCBs in human plasma were shown to competitively inhibit binding of the natural thyroid hormone thyroxine (T_4) to its transport protein transthyretin (TTR) (Lans *et al*., 1993). In addition, the activities of hepatic type I iodothyronine deiodinase (ID-1) and iodothyronine sulfotransferases (both enzymes involved in the intracellular metabolism of thyroid hormones) were inhibited by OH-PCBs (Adams *et al*., 1990; Lans *et al*., 1993; Rickenbacher *et al*., 1989 and Schuur *et al*., 1998).

Binding of OH-PCBs to TTR *in vivo* is thought to facilitate the transport of OH-PCBs across the placenta, thereby affecting maternal, but especially fetal, thyroid hormone levels (Brouwer *et al*., 1998). Earlier studies showed a selective accumulation of the PCB metabolite 4-hydroxy-2,3,3′,4′,5-pentachlorobiphenyl (4-OH-CB107) in fetal and neonatal rats after maternal exposure to Aroclor 1254 from gestation day (GD) 10 to GD 16 (Morse *et al*., 1996a). Thyroid hormone levels in the exposed fetuses and neonates were significantly decreased, and it was hypothesized that this decrease was caused by the competitive binding of 4-OH-CB-107 to TTR, which was observed for this compound *in vitro* (Meerts *et al*., 2002). We were able to support this hypothesis in an *in vivo* study, where exposure of pregnant rats to ^{14}C-4-OH-CB107 from GD 10 to GD 16 resulted in accumulation of 4-OH-CB107 in fetal livers, brain, and plasma

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measured at GD 17 and GD 20. Polyacrylamide gel electrophoresis of maternal and fetal plasma revealed the binding of 4-OH-CB107 to TTR. Consequently, maternal, but especially fetal, total thyroxine (TT_4) levels at GD 20 were significantly decreased by 38% and 89%, respectively (Meerts *et al*., 2002).

A prenatal or early postnatal hypothyroid state is known to severely affect the normal development of the brain and sexual organs. Effects on brain development include disorders of neuronal process growth (Stein *et al*., 1991), disruption of the expression pattern of neurotrophins, nerve growth factor, and brain-derived neurotrophic factor (Nevue and Arenas, 1996), as well as interference in neurotransmitter systems (Seegal, 1996). Exposure of rats to Aroclor 1254 resulted in alterations in regional brain serotonin metabolism and in glial and neuronal cell markers (Morse *et al*., 1996b). Exposure of rats to Aroclor 1016 from GD 8 through weaning caused elevations in regional dopamine concentrations in rat offspring. Studies with individual PCB congeners revealed that the structure of the congener and the age of the animal at the time of exposure were important variables for the observed effects on brain dopamine levels.

In addition to the above-mentioned effects of PCB-induced hypothyroidism on brain development, it is also possible that the relatively high concentrations of OH-PCB congeners in plasma or brain of fetal rats have a direct effect on brain development and/or reproduction. OH-PCBs are known to induce uncoupling of oxidative phosphorylation in mitochondria (Narasimhan *et al*., 1991), and inhibition of intercellular communication (de Haan *et al*., 1994). Some OH-PCBs also possess (anti-) estrogenic activities (Korach *et al*., 1988, Moore *et al*., 1997). The OH-PCBs identified in human serum were mostly anti-estrogenic (Moore *et al*., 1997). Kester *et al*. (2000) reported extremely potent inhibition of human estrogen sulfotransferase activity *in vitro* by environmentally relevant OH-PCBs, suggesting that OH-PCBs indirectly induce estrogenic activity by increasing estradiol bioavailability in target tissues.

The aim of the present study was to investigate the potential impact of *in utero* exposure to 4-OH-CB107 on the development of rat offspring and the possible long-term effects on sex steroid hormone levels and reproduction. Effects on thyroid hormone concentrations and brain development were also investigated, but are reported elsewhere (Meerts *et al*., 2004). Pregnant rats were exposed to 0.5 or 5 mg 4-OH-CB107 per kg bw from GD 10 to GD 16. Exposure of pregnant rats to a concentration of 5 mg 4-OH-CB-107 per kg bw per day from GD 10 to GD 16 resulted in a concentration of 4-OH-CB107 in the fetal compartment comparable to the concentration that was reached after *in utero* exposure to Aroclor 1254 (Meerts *et al*., 2002; Morse *et al*, 1996a). In fetal plasma at GD 20, concentrations of 37.2 ± 5.14 nmol/ml were measured after *in utero* exposure to 4-OH-CB107 (Meerts *et al*., 2002). A concentration of 0.5 mg/kg bw per day was additionally chosen to determine possible dose-dependent effects of 4-OH-CB-107. To determine if the effects observed in offspring exposed to Aroclor 1254 (as described by Morse *et al*., 1996a) can be explained mainly by the accumulation of the metabolite 4-OH-CB107 in fetuses and neonates, an additional group of rats was exposed to Aroclor 1254.

ANIMALS, MATERIALS, AND METHODS

Chemicals. 4-Hydroxy-2,3,3′,4′,5-pentachlorobiphenyl (4-OH-CB107) was synthesized as described by Bergman *et al*. (1995) and was at least 99.9% pure. The nomenclature used is adapted from Letcher *et al*. (2000). Aroclor 1254 was kindly donated by Prof. Dr. M. van den Berg (IRAS, University of Utrecht, the Netherlands). Dichloromethane and Tris were purchased from Merck Chemical Company (Darmstadt, Germany). ^{125}I-Estradiol, estradiol antiserum and goat anti-rabbit gamma globulin were obtained from Diagnostic Products Corporation (DPC, Breda, the Netherlands). Pregnen-(4)-dion-(3,20), 17β-estradiol and bovine serum albumin were obtained from Sigma Chemicals Co. (St. Louis, MO). [1,2,6,7–^{3}H]-progesterone was purchased from Amersham (Buckinghamshire, UK). Progesterone antiserum was produced as described in Van der Meulen *et al*. (1988) and Mattheij and Swarts (1995). Testosterone was obtained from DRG (Marburg, Germany). Ultima Gold liquid scintillation fluid was purchased from Canberra Packard (Packard, St. Louis, MO).

Animals and treatment. All experimental procedures involving animals were approved by the Animal Welfare Committee of Wageningen University. Wistar WU rats (60 females, 30 males; 14 weeks old) were purchased from Charles River (Sulzfeld, Germany) and allowed to acclimatize for 3 weeks. Throughout the experiment, animals were kept in an artificial 12 h:12 h light–dark cycle with lights on at 06:00 h. Room temperature was maintained at 21° ± 2°C and humidity at 50 ± 10%. Animals were provided rat chow (Hope Farms, Woerden, the Netherlands) and tap water *ad libitum*.

After the acclimatization period two females were placed in a cage with one male from 17:00 to 8:00 h the next day. Females were examined for copulation each morning by checking the presence of sperm in the vaginal smear. When spermatozoa were found, that day was designated as day 0 of gestation (GD 0), and females were housed individually. On GD 10 the pregnant rats were divided randomly into the different treatment groups and transferred to a Macrolon, stainless steel cage to facilitate the collection of PCB-contaminated feces. In total 52 pregnant rats (13 per exposure group) were dosed by oral intubation with 0 (corn oil), 0.5, or 5 mg 4-OH-CB107 per kg body weight per day dissolved in corn oil (2 ml/kg) from GD 10 to GD 16. For comparison of the effects of 4-OH-CB107 with effects caused by parent PCB congeners, a fourth group of rats was dosed with 25 mg Aroclor 1254 per kg body weight from GD 10 to GD 16. In an earlier study it was observed that this dose level of Aroclor 1254 resulted in an amount of 4-OH-CB107 in the fetal compartment in the same range as observed after *in utero* exposure to 5 mg/kg 4-OH-CB107 (Meerts *et al*., 2002).

Maternal body weights were monitored daily throughout gestation. On GD 20, pregnant females were transferred to bedding material. At birth, *i.e*., postnatal day (PND) 0, live offspring were counted and sexed. Individual pups and dams were weighed on PNDs 1, 4, 7, 14, and 21, and after weaning body weights of the offspring were monitored weekly until sacrifice. On PND 4, litters were adjusted to 4 males and 4 females. Generally, this required the termination of excess offspring. However, in a few cases where a litter contained fewer than eight pups or the sex distribution was not permissive, the standardized litter required pups from two dams. To maintain litter independence, no dam was allowed to contribute pups to more than one litter. In addition, pups transferred from one litter to another were not used for any analysis. The standardized litter became the experimental unit and all treatment mean values reported are litter based. Liver, kidneys, brain, and thymus of the excess offspring were weighed. The remaining offspring were numerically marked on their feet to identify individual animals within a litter.

Developmental landmarks. During the study, a number of developmental landmarks for all litters were recorded in a blind fashion. Treatment groups were

decoded only after termination of the animals when all analyses were completed. On PND 1 and PND 4, anogenital distance (AGD) and crown–rump length (CRL) were measured on each pup by means of a micrometer capable of resolution to 0.01 mm. The AGD was measured in both sexes as the distance from the anterior edge of the anus to the base of the genital tubercle. Measurements of AGD and CRL were done by one person, to avoid interindividual variations. Each pup was additionally examined for the following developmental landmarks: pinna detachment (starting on PND 1), age at the onset of hair growth, and age at bilateral eye opening (starting on PND 12).

Following weaning at PND 21, pups were housed with littermates in unisexual groups, two pups per cage. Dams were sacrificed at PND 21 and blood was collected via the *vena cava* in heparinized tubes for thyroid hormone measurements. Liver, kidneys, adrenals, thymus, brain, spleen, uterus, and ovary were collected, blotted dry, and weighed. All organs were frozen in liquid nitrogen and stored at −80°C.

Female pups were examined daily for vaginal opening, starting at PND 30. The age at preputial separation (Korenbrot *et al*., 1977) in male offspring was examined from PND 35 until a complete preputial separation in all males was achieved. After puberty, the offspring were split into two cohorts; one cohort ($n = 41$ litters with 2 males and 2 females per litter) was housed individually and used for behavioral studies (Meerts *et al*., 2004). From the other cohort ($n = 41$ litters, 2 males and 2 females per litter), females were also housed individually to study estrous cyclicity and reproduction as described below. Male offspring from this cohort were housed in unisexual groups with 2 animals per cage until dissections at about 11 months of age.

Reproductive capability of female offspring. Female vaginal estrous cyclicity was monitored for 21 days, starting at PND 210, by daily evaluation of the vaginal smears (between 8:00 and 10:00 h and at other times as needed). Differentiation of the cells during the 4 days of the estrous cycle was determined microscopically according to the method of Staples and Geils (1965). Because of the effects found on the length of the estrous cycle (cf. Results), a study was conducted to determine the reproductive capability of the female offspring. The females were split into two cohorts; one cohort stayed in unisexual groups with 2 females per group until necropsy at about 11 months of age to determine possible long-term adverse effects on sex steroid parameters. The other cohort ($n = 41$) was housed individually, and after 2 weeks mating with untreated males (16 weeks old, Charles River, Sulzfeld, Germany) was started (1:1). Females were examined for copulation each morning by checking the presence of sperm in the vaginal smear. When spermatozoa were found, that day was designated as GD 0. If no spermatozoa were found, the female was remated up to a maximum of 2 weeks with a stud male. The number of matings was recorded. Pregnant females were sacrificed at GD 20. Maternal blood was collected via the vena cava in heparinized tubes, and plasma was prepared and stored at −80°C for measuring thyroid hormones, estradiol, progesterone, and testosterone. Maternal body weight, ovarian weights, and the number of corpora lutea (examined with a microscope) implantation sites and embryos were recorded. Additionally, maternal liver, kidneys, spleen, brain, and thymus were weighed. From the fetuses, sex was determined and liver, brain, and thymus were removed and weighed.

Long-term effects on male and female hormone levels. Male offspring and the female cohort that was not used for the reproductive capability study were dissected at about 11 months of age (PND 310–320) to determine possible long-term adverse effects on sex steroid parameters. To avoid the effects of stress on serum steroid hormone levels, the animals were killed by decapitation within 15 s of removal from their cages. Dissections were conducted between 08:00 and 12:00 to minimize circadian influences on testosterone and estradiol levels. Trunk blood was collected in Eppendorf tubes (for serum preparation) and heparinized tubes (for plasma) for hormone analysis. Immediately after collection of the blood, the brains were dissected rapidly (within 5 min) and separated in different regions for neurotransmitter analyses (Meerts *et al*., 2004). Weights measured at dissection included body, liver, kidneys, adrenals, spleen, thymus, and pituitary gland. From the males, testes, prostate, seminal vesicle, and cauda epididymis were weighed additionally. The coagulating glands were detached from the seminal vesicles and care was taken to avoid expression of fluids from these organs. From the females, uteri and ovaries were weighed. Estrous cycle stage was estimated at dissection from the appearance of the uterus as either estrous (“ballooning”) or nonestrous.

Measurement of sex steroids. Before estradiol measurements, 100 µl plasma was extracted three times with 1.25 ml dichloromethane in glass tubes by vortexing for 30 s, centrifugation at 1,000 × g for 5 min, and collection of the dichloromethane phase. The dichloromethane phases were pooled, evaporated to dryness in a Savant Speed Vac vacuum concentrator, and 175 µl phosphate buffered saline (PBS) containing 0.1% (w/v) BSA was added to each tube. After thorough vortexing, estradiol concentrations were measured in triplicate as described by Palm *et al*. (1999). The extraction efficiency, determined by the addition of tracer amounts of ^{125}I-E_2 before extraction of the plasma, was 94.2 ± 2.84% for plasma from pregnant rats and 95.5 ± 2.32% for non-pregnant rats.

Progesterone concentrations were measured in triplicate in unextracted plasma, diluted 20 times in PBS containing 0.1% (w/v) BSA, as described by Van der Meulen *et al*. (1988) and Mattheij *et al*. (1995). Testosterone concentrations were measured in duplicate in extracted serum using a commercial ELISA kit (DRG, Marburg, Germany). Testosterone was extracted from 200 µl serum using the extraction method described above for estradiol analysis. The extraction efficiency was determined by comparing extracted standard samples with the non-extracted standards and was 95.3 ± 0.6%.

Statistical analysis. Statistical analysis was performed using the SPSS statistical software package. Differences between the number of pups and organ weights were analyzed by means of analysis of variance (ANOVA). Levene’s test was used to evaluate homogeneity of variances, and the Bonferroni test was used to compare individual treatment means when ANOVA indicated that significant differences were present. For the evaluation of body weight development until sacrifice, ANOVA with repeated measures was used, with age as the factor. For hormone determinations, one pup per litter was used. Nonparametric analysis used the Kruskal-Wallis ANOVA by ranks. When this indicated significant differences, treatment ranks were compared to the control group by the Wilcoxon-Mann-Whitney test. Categorical data were analyzed by chi-squared analysis.

In all cases, the litter was the independent experimental unit, and data from individual male and female offspring were assumed to be representative for the litter. Where more than one male or female from a given litter was evaluated, the results were averaged to form a litter mean. In all cases significance was set at $p < 0.05$.

RESULTS

Effects of Maternal Exposure on Offspring

Of the 52 exposed females, 11 were false positive (*i.e*., spermatozoa were found on GD 0 but the female was not pregnant). Unfortunately, 5 false-positive females were randomly assigned to the lowest 4-OH-CB107 exposure group (0.5 mg/kg), resulting in only 8 litters in this group. The corn oil, 4-OH-CB107 (5 mg/kg) and Aroclor treated groups all contained 11 litters (Table 1). All pregnant dams delivered without complications, and no effects could be observed on length of gestational period, litter size, sex ratio (Table 1), number of live fetuses, late gestational death, number of resorptions, or postnatal death (data not shown). In addition, treatment of dams with 4-OH-CB107 or Aroclor 1254 caused no overt signs of toxicity in dams and offspring, as assessed by visual inspection (data not shown).

Growth and Development of Offspring

Male and female body weight gain of the offspring was slightly, but not significantly, reduced by maternal exposure

TABLE 1
Effects on Developmental Landmarks in Offspring Following *In Utero* Exposure to 4-OH-2,3,3′,4′,5-CB107 or Aroclor 1254 from Gestational Day 10 to Gestational Day 16

Parameters	Control (corn oil)	4-OH-CB107 (0.5 mg/kg)	4-OH-CB107 (5.0 mg/kg)	Aroclor 1254 (25 mg/kg)
No. of litters	11	8	11	11
Litter size	11.7 ± 0.3	10.9 ± 0.3	10.9 ± 0.4	10.5 ± 0.4
Gestational period	21.5 ± 0.1	21.8 ± 0.1	21.6 ± 0.2	21.7 ± 0.1
Male to female ratio (%)	1.26 ± 0.14	1.30 ± 0.53	1.27 ± 0.24	1.00 ± 0.17
Body weight PND 4 Male	8.60 ± 0.16	9.20 ± 0.26†	9.50 ± 0.46##	8.11 ± 0.17
Female	8.29 ± 0.18	8.87 ± 0.25	9.08 ± 0.41	8.20 ± 0.17
AGD PND 4 Male	3.69 ± 0.12	3.87 ± 0.17	4.04 ± 0.08#	3.63 ± 0.12
Female	1.52 ± 0.08	1.64 ± 0.06	1.64 ± 0.05	1.74 ± 0.07
CRL PND 4 Male	49.50 ± 0.42	50.52 ± 0.52#	50.52 ± 0.68	48.33 ± 0.28
Female	48.74 ± 0.41	49.51 ± 0.53#	49.44 ± 0.58	47.11 ± 0.34
AGD/CRL ratio Male	0.074 ± 0.002	0.074 ± 0.004	0.080 ± 0.001	0.077 ± 0.002
Female	0.031 ± 0.002	0.033 ± 0.001	0.033 ± 0.0001	0.036 ± 0.001*
Pinna detachment[a], Male	3.8 ± 0.2	4.1 ± 0.2	3.6 ± 0.2	4.0 ± 0.2
Female	3.9 ± 0.2	4.1 ± 0.2	3.7 ± 0.2	3.7 ± 0.1
Eye opening[b], Male	16.8 ± 0.1	16.6 ± 0.2	16.5 ± 0.2	16.1 ± 0.2*
Female	16.7 ± 0.1	16.6 ± 0.3	16.5 ± 0.2	15.8 ± 0.2**
Age at vaginal opening	36.4 ± 1.1	34.4 ± 0.6	33.9 ± 0.4	34.0 ± 0.6
Age at preputial separation	43.9 ± 0.5	44.0 ± 1.1	44.1 ± 0.5	43.4 ± 0.5

PND = postnatal day; AGD = anogenital distance (mm); CRL = crown–rump length (mm).
Data are given as mean ± S.E.M.
*Significant difference from control, $p < 0.05$; ** $p < 0.01$; #significant difference from Aroclor 1254, $p < 0.05$; ## $p < 0.01$.
[a]Age at pinna detachment (in days).
[b]Age at bilateral eye opening (in days).

to Aroclor 1254 (data not shown). At PND 4, male body weights from 4-OH-CB107 treated animals were significantly higher ($p < 0.01$) compared to Aroclor treated animals (Table 1). Crown–rump lengths (CRL) of male and female offspring exposed to 0.5 mg 4-OH-CB107 per kg bw were significantly higher compared to Aroclor treated offspring. When corrected for CRLs of the animals, female anogenital distances (AGD/CRL ratio) of the Aroclor treated animals were significantly increased by 16% compared to controls (Table 1).

The onset of bilateral eye opening was significantly earlier in male and female offspring exposed to Aroclor 1254 via the dams, as compared to the other exposure groups. The day of vaginal opening was not changed by either 4-OH-CB107 or Aroclor 1254 exposure (Table 1). In all groups, vaginal opening occurred at approximately PND 34–36 (8–11 litters). Male preputial separation was completed at PND 43–44 in all treatment groups.

Organ Weights of Dams (P_0) and Neonates (F_1)

At PND 21, maternal body weights and absolute or relative weights of the collected organs (liver, kidneys, adrenals, thymus, brain, spleen, uterus, ovary) of treated animals showed no differences compared to the control group. Absolute and relative liver weights from male and female offspring at PND 4 were significantly increased after exposure to Aroclor 1254 (44% and 38%, respectively, for relative liver weights; Table 2). Relative thymus weight at PND 4 was significantly reduced in both male (23%) and female (27%) offspring exposed to Aroclor 1254 *in utero*.

Estrous Cyclicity in F_1

The average estrous cycle length of female offspring (F_1) monitored at PND 210–231 was significantly prolonged in animals exposed *in utero* to 5 mg 4-OH-CB107 per kg body weight (Fig. 1A). The prolongation of the estrous cycle was caused by an increased length of the diestrous stage (normal length 2 days), which lasted more than 4 days in 50% and 64% of the females in the 0.5 and 5 mg/kg 4-OH-CB107 treatment groups, respectively (Fig. 1B). The total estrous cycle was prolonged in 75% (0.5 mg/kg 4-OH-CB107) and 82% (5.0 mg/kg 4-OH-CB107) of the female offspring, compared to 64% of Aroclor 1254 exposed offspring (Fig. 1C).

F_1 Fertility

The percentage of mated female F_1 offspring (age ± 260 days) with litters was 64% (corn oil), 88% (0.5 mg/kg 4-OH-CB107), 73% (5.0 mg/kg 4-OH-CB107), and 91% (Aroclor 1254). No effects were observed on the number of matings attempted, the number of resorptions or implantation sites, the number of dead or live fetuses, total litter weight, mean fetal body weight, or sex

TABLE 2
Organ Weights of Male and Female Offspring at Postnatal Day 4 (PND4)

Organ	Control	4-OH-CB107 (0.5 mg/kg)	4-OH-CB107 (5.0 mg/kg)	Aroclor 1254 (25 mg/kg)
PND 4, male	$n = 8$	$n = 0$[a]	$n = 6$	$n = 3$
Liver (g)	0.28 ± 0.01		0.30 ± 0.02	0.36 ± 0.02*
Rel. liver weight (%)[b]	3.40 ± 0.06		3.48 ± 0.08##	4.91 ± 0.10†
Kidney (g)	0.11 ± 0.002		0.12 ± 0.006‡	0.10 ± 0.006
Rel. kidney weight (%)	1.26 ± 0.03		1.25 ± 0.02	1.23 ± 0.04
Thymus (mg)	22 ± 0.8		24 ± 1.5##	13 ± 0.3†
Rel. thymus weight (%)	0.26 ± 0.01		0.26 ± 0.01#	0.17 ± 0.01†
PND 4, female	$n = 8$	$n = 8$	$n = 7$	$n = 5$
Liver (g)	0.29 ± 0.02	0.32 ± 0.01‡	0.30 ± 0.02‡	0.40 ± 0.03***
Rel. liver weight (%)	3.56 ± 0.23	3.60 ± 0.12#	3.38 ± 0.12##	4.93 ± 0.16†
Kidney (g)	0.11 ± 0.004	0.12 ± 0.004	0.12 ± 0.006	0.11 ± 0.009
Rel. kidney weight (%)	1.28 ± 0.02	1.34 ± 0.03	1.32 ± 0.02	1.35 ± 0.07
Thymus (mg)	21 ± 1.2	24 ± 0.1§	22 ± 1.8‡	15 ± 0.1**
Rel. thymus weight (%)	0.25 ± 0.01	0.27 ± 0.01§	0.25 ± 0.02§	0.19 ± 0.01†

Data are given as mean ± S.E.M.
*Significant difference from control ($p < 0.05$; ** $p < 0.01$; ***$p < 0.005$; †$p < 0.0001$). ‡Significant difference from Aroclor 1254 ($p < 0.05$; §$p < 0.01$; # $p < 0.001$; ## $p < 0.0001$)
[a]At postnatal day 4 (PND 4), no males were left for autopsy after standardization of the litters.
[b]Percentage of total body weight.

ratio (data not shown). No significant differences were observed in the number of corpora lutea in the left and right ovaries of pregnant female offspring exposed *in utero* to 0.5 mg/kg 4-OH-CB107 (Fig. 2). Maternal absolute and relative organ weights (liver, spleen, kidneys, adrenals, thymus) of female offspring at PND 260 exposed *in utero* to 4-OH-CB107 or Aroclor 1254 were not different from corn oil treated females (data not shown). In addition, no effects could be observed on F_2 fetal absolute and relative organ weights (liver, kidney, brain, thymus) at GD 20.

Organ Weights of F_1 Males and Females at 11 Months

At 11 months of age, no significant differences were observed in body weights or absolute and relative weights of adrenals, kidneys, liver, spleen, and thymus in both male and female offspring exposed *in utero* (data not shown). In addition, male accessory sex organ weights (prostate, testis, seminal vesicle, and cauda epididymis) were not affected (Table 3). In F_1 females exposed to 5 mg 4-OH-CB107 per kg, the weights of the left ovary were significantly increased by 21% compared to females from the low 4-OH-CB-107 dose group (Table 3).

Sex Steroid Hormone Levels

Plasma estradiol concentrations of pregnant F_1 offspring (PND 260) showed no significant differences, although levels in animals treated *in utero* with 0.5 mg/kg 4-OH-CB107 showed an increase in estradiol concentrations of approximately 56% (data not shown). As a result of high standard deviations between animals from the same group, this increase was not significant.

At 11 months of age, plasma estradiol concentrations in female F_1 offspring in the proestrous stage (determined by the appearance of a swollen uterus at necropsy) were significantly increased in the 5 mg/kg 4-OH-CB107 treatment group by approximately 230% compared to control animals (Fig. 3). Estradiol concentrations of female F_1 offspring in which the uterus was not swollen showed no significant differences.

Plasma progesterone levels were unaltered in pregnant F_1 animals (data not shown). In addition, progesterone levels at 11 months of age showed no differences between the exposure groups (Table 4). However, estradiol/progesterone ratios were increased (not significantly) in the 5 mg/kg 4-OH-CB107 exposed female offspring in the proestrous stage (Table 4).

Serum testosterone levels were decreased by 26% (though not significantly) in male offspring at 11 months of age of the 5 mg/kg 4-OH-CB107 dose group (data not shown). Testosterone levels measured in pregnant female F_1 offspring showed no differences between the treatment groups (data not shown).

DISCUSSION

In the present study we investigated if the effects caused by *in utero* exposure to the PCB-metabolite 4-OH-CB107 could mainly explain the effects observed in rats following *in utero* exposure to a commercial PCB-mixture (Aroclor 1254). The PCB-metabolite used, 4-hydroxy-2,3,3′,4′,5-pentaCB

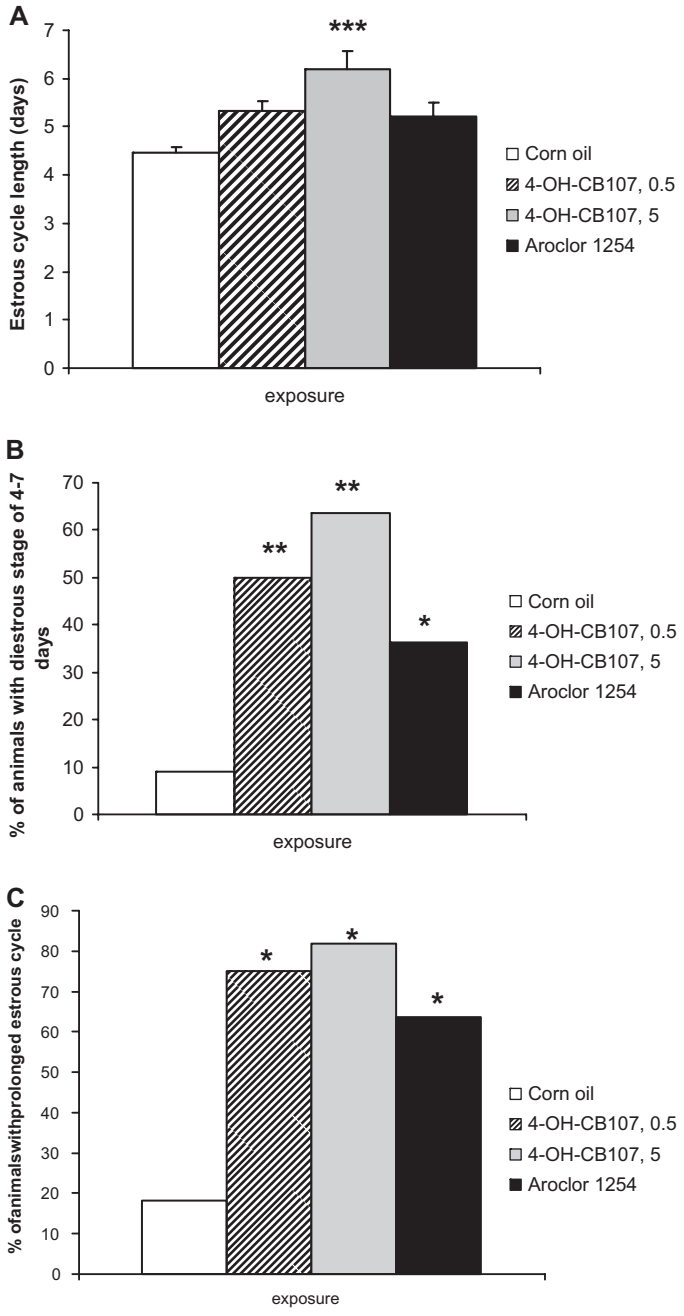

FIG. 1. Estrous cycle length (A), percentages of females with a diestrous stage of 4–7 days (B), and percentages of females with a prolonged estrous cycle (C) in offspring, monitored from 210 to 231 days postpartum, following *in utero* exposure to corn oil (CO), 4-OH-CB107 (PCB-OH, 0.5 and 5 mg/kg per day) or Aroclor 1254 (AC 1254, 25 mg/kg per day) from GD 10 to GD 16. Statistically significant differences from control are given by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

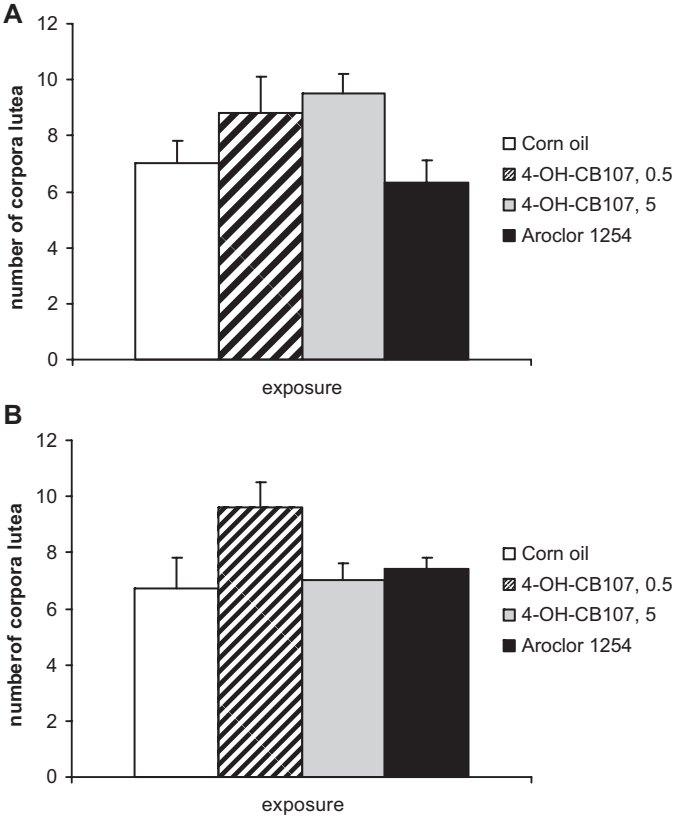

FIG. 2. Number of corpora lutea in the left (A) and right (B) ovarium of pregnant female offspring at the age of ± 260 days, following *in utero* exposure to corn oil (CO), 4-OH-CB107 (PCB-OH, 0.5 and 5 mg/kg per day) or Aroclor 1254 (AC 1254, 25 mg/kg per day) from GD 10 to GD 16.

(4-OH-CB107), is one of the major OH-PCBs detected in human blood (Bergman *et al*., 1994), and it has been shown to accumulate in the blood and brain of fetuses and neonates exposed *in utero* to Aroclor 1254 (Morse *et al*., 1996a). To our knowledge, this is the first study investigating the possible long-term effects after *in utero* exposure to a PCB metabolite on development, sex steroid hormone levels, and female reproduction.

The most pronounced developmental effect observed following exposure to 4-OH-CB107 was a striking and dose related prolongation of the estrous cycle in female offspring, measured between PND 210 and PND 231. The estrous cycle was monitored at this age in order to select the appropriate animals for the behavioral studies (described in Meerts *et al*., 2004). Because differences in the estrous cycle were noticed between the different treatment groups, a thorough investigation of the estrous cycle was performed. Although no data are available about the estrous cyclicity during the life cycle of the female animals, the differences observed at the age of 210–231 days between the different 4-OH-CB107 treatment groups are very pronounced. The prolongation in the estrous cycle was also observed in the Aroclor 1254 treated animals, but it was less pronounced. The total length of the estrous cycle was significantly increased in female offspring exposed to 5 mg 4-OH-CB107. It is known that a hypothyroid state is associated

TABLE 3
Sex Organ Weights of Male and Female Offspring (F_1) at 11 Months of Age

Organ	Control (corn oil)	4-OH-CB107 (0.5 mg/kg)	4-OH-CB107 (5.0 mg/kg)	Aroclor 1254 (25 mg/kg)
Males	$n = 11$	$n = 8$	$n = 11$	$n = 11$
Prostate	0.42 ± 0.02	0.47 ± 0.02	0.48 ± 0.03	0.39 ± 0.02
Seminal vesicle	1.58 ± 0.06	1.69 ± 0.10	1.61 ± 0.07	1.51 ± 0.07
Testis left	1.76 ± 0.09	1.57 ± 0.18	1.85 ± 0.03	1.81 ± 0.03
Testis right	1.83 ± 0.03	1.59 ± 0.18	1.85 ± 0.04	1.80 ± 0.03
Females	$n = 11$	$n = 8$	$n = 11$	$n = 11$
Ovarium left (mg)	46 ± 1.1	43 ± 1.2*	52 ± 1.9	45 ± 2.6
Ovarium right (mg)	46 ± 2.4	48 ± 2.0	48 ± 1.7	43 ± 1.6
Paired ovarian weight (mg)	92 ± 2.1	91 ± 3.5	99 ± 3.3	88 ± 3.7
Uterus (swollen)	1.43 ± 0.14 (7)	1.10 ± 0.09 (7)	1.36 ± 0.15 (6)	1.06 ± 0.11 (6)
Uterus (not swollen)	0.71 ± 0.05 (7)	0.68 ± 0.06 (4)	0.75 ± 0.05 (6)	0.68 ± 0.04 (10)

Data are given as mean ± S.E.M. In cases where the number of animals was different, the number is presented in parentheses.
*Significantly different from 4-OH-CB107 (5 mg/kg), $p < 0.05$.

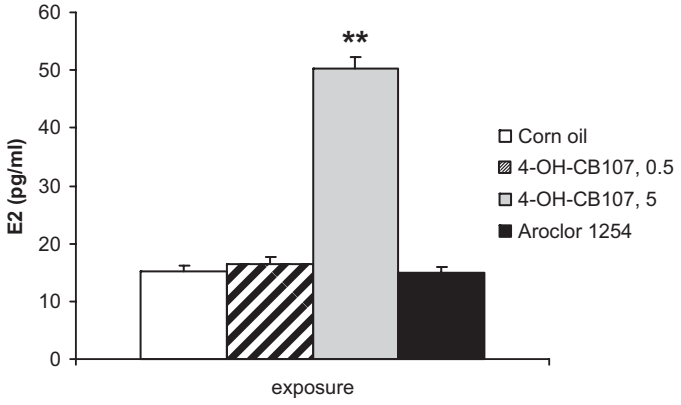

FIG. 3. Plasma estradiol concentrations in 11-month-old female offspring in proestrous stage, exposed *in utero* to corn oil (CO), 4-OH-CB107 (PCB-OH, 0.5 and 5 mg/kg per day) or Aroclor 1254 (AC 1254, 25 mg/kg per day) from GD 10 to GD 16. Statistically significant differences from control are given by **($p < 0.01$).

TABLE 4
Plasma Progesterone Levels (ng/ml) and Estradiol/Progesterone Ratios in Female Offspring at 11 Months Following *In Utero* Exposure to 4-OH-CB107 or Aroclor 1254

Exposure	Corn oil (2 ml/kg)	4-OH-CB107 (0.5 mg/kg)	4-OH-CB107 (5 mg/kg)	Aroclor 1254 (25 mg/kg)
Proestrous stage (ballooning uterus)				
Progesterone	30.1 ± 4.3	36.6 ± 8.8	36.2 ± 8.9	23.6 ± 2.1
E/P ratio[a]	0.65 ± 0.21	0.50 ± 0.13	1.55 ± 0.5	0.44 ± 0.12
Diestrous stage (not swollen uterus)				
Progesterone	59.5 ± 9.3	47.6 ± 8.2	75.6 ± 8.5	68.5 ± 9.2
E/P ratio	0.25 ± 0.09	0.29 ± 0.08	0.13 ± 0.03	0.12 ± 0.04

The data are presented as means ± standard error (SEM).
[a]E/P-ratio = estradiol/progesterone ratio (10^{-3}).

with a higher incidence of pseudopregnancy (Tohei *et al.*, 2000). However, it is unlikely that the increase in the estrous cycle length in the current study is caused by hypothyroidism, since the prolongation of the estrous cycle was observed in offspring at the age of 11 months, and these animals did not show differences in thyroid hormone or TSH levels. Decreases in thyroid hormone levels (TT_4 levels) following *in utero* exposure to 4-OH-CB107 were only visible in fetuses (Meerts *et al.*, 2002) and neonates at PND 4 (Meerts *et al.*, 2004).

Aroclor 1254 and aryl hardrocarbon (Ah) receptor binding PCB congeners have been reported to induce several adverse effects on mammalian endocrine function. For example, prolongation of the estrous cycle (by an increasing length of the diestrous stage) and a delay in the first estrous was observed in female rats after transplacental and translactational exposure to 30 mg/kg Aroclor 1254 for one month (Brezner *et al.*, 1984). The increased length of the estrous cycle in female offspring in our study was also a result of a prolonged diestrous stage, determined by the appearance of large amounts of leucocytes in the vaginal smears. A stage representing 11–20 days of diestrous is known as *pseudopregnancy* (De Feo, 1967). In the present study, the total length of the diestrous stage did not exceed 7 days. In addition, the prolongation of the diestrous stage had no effects on the fertility of the females in this study. However, the disturbances in estrous cycle length may indicate that females exposed *in utero* to 4-OH-CB107 may show signs of reproductive senescence at an earlier stage in life compared to corn oil treated females. The first stage of reproductive senescence in rodents is an increase in mean cycle length. Most aging rats then enter a stage of persistent vaginal cornification (PVC), which is often followed by a repetitive pseudopregnancy and finally persistent anestrous (Finch *et al.*, 1984).

Strikingly, plasma estradiol (E_2) levels in 11-month old female offspring were significantly increased by 230% in the

5 mg/kg 4-OH-CB107 exposed group (in the proestrous stage). High plasma E_2 values are often observed in aging rats with a prolonged estrous cycle (Lu *et al*., 1994). It is demonstrated that higher amounts of plasma E_2 in middle-aged rats during successive estrous cycles gradually diminishes the neuroendocrine responsiveness to the positive feedback effect of E_2 on luteinizing hormone (LH) secretion (LaPolt *et al*., 1988). Another possible explanation may be an effect of OH-PCB-107 on estrogen sulfotransferase activity. Kester *et al*. (2000) showed that OH-PCBs, including 4-OH-CB107, are extremely potent inhibitors of the human estrogen sulfotransferase (hEST) *in vitro*. In fact, 4-OH-CB107 was one of the strongest of the 32 tested compounds with an IC_{50} of 0.15–0.25 nM. Estrogen sulfation is a normal route of reversible inactivation of estradiol. As a result of the inhibition of estrogen sulfation, OH-PCBs may increase the bioavailability of E_2 in target tissues, thereby exerting an indirect estrogenic effect or mimicking the increase in plasma E_2 levels observed in aging female rats.

4-OH-CB107, used as a model PCB metabolite in this study, is also known to possess anti-estrogenic activity *in vitro* in HeLa cells, or in human T47D breast tumor cells transfected with an estrogen-responsive luciferase gene construct (Moore *et al*., 1997; Meerts, unpublished results). In addition, Moore *et al*. (1997) showed that 4-OH-CB107 significantly displaced [^{3}H]E_2 from the rat uterine cytosolic estrogen receptor, though $<50\%$ displacement was observed at the highest concentration used (10^{-3} M). It is thus very unlikely that the observed increases in E_2 in our study are caused by binding of the metabolite to the estrogen receptor.

Next to the above-mentioned effects of 4-OH-CB107 on the estrous cycle length and estradiol concentrations, all other developmental effects observed were caused by the parent compound (Aroclor 1254) only. These effects include a significant increase in the female anogenital distance/crown-rump length ratio (AGD/CRL-ratio), an indicator of circulating androgen concentrations over time, or of decreased androgen responsiveness. This may indicate a possible partial ''masculinization'' of female offspring by Aroclor 1254 treatment. In addition, exposure to 25 mg/kg Aroclor 1254 significantly accelerated eye opening in the offspring by one day. Similar effects have been observed using either TCDD (Gray *et al*., 1997; Theobald and Peterson, 1997) or Aroclor 1254 (Goldey *et al*., 1995a). The effect on eye opening is most likely caused by a direct effect of the compound used (*i.e*., PCBs or TCDD) and not by the accompanying hypothyroidism observed in treated offspring, because hypothyroidism is typically associated with a delay in this developmental landmark (Adams *et al*., 1989; Goldey *et al*., 1995b). From the present study, it can be concluded that accelerated eye opening is most likely an effect of parent PCB congeners, and not their metabolites.

In conclusion, maternal exposure to the PCB metabolite 4-OH-CB107 results in a significant increase of the estrous cycle length and increased estradiol/progesterone ratios. The effects of the PCB metabolite are sex-related, because no effects could be detected on male accessory sex organ weights or testosterone levels at postnatal days 310 to 325. The well-known developmental effects of Aroclor 1254 (accelerated eye opening in treated offspring, increased AGD/CRL-ratio in female offspring), also shown in this study, could not be observed in offspring exposed to 4-OH-CB107 only. The adverse effects of 4-OH-CB107 on neurotransmitter levels and brain development in rat offspring exposed *in utero* are published elsewhere (Meerts *et al*., 2004).

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