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# Effects of PCB99 and PCB153 exposure on spermatogenesis in young adult C57BL6 mice

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**Abstract**

This study examined the effects of acute exposure to PCB99 (2,2′,4,4′,5-pentachlorobiphenyl), and PCB153 (2,2′,4,4′5,5′-hexachlorobiphenyl), on spermatogenesis in 8-week-old C57BL6 mice. The mice were randomly allocated to PCB99 and PCB153 and a single dose of respectively 10 and 100 mg/kg was given by oral gavage. During the 6-week experiment, six mice per treatment group were sacrificed weekly, body weights were recorded and samples with respect to the male reproductive system were collected until further analysis. None of the treatments, showed changes in body weight or reproductive endpoints. Flow cytometric analysis revealed spermatogenesis to be unaffected. However, PCB99 and PCB153 showed a significant increase in Leydig cell apoptosis. The results from the present study indicate that the male reproductive system is relatively refractory to PCB99 and PCB153 at levels exceeding those of wildlife and humans, when exposed during adult life. However, the finding of apoptotic Leydig cells merits further investigation.


*Keywords:* PCBs; Spermatogenesis; Flow cytometry; Leydig cells; Apoptosis

## 1. Introduction

Polychlorinated biphenyls (PCBs) used in commercial products are distributed worldwide as environmental pollutants. As a class of highly lipophilic chemicals, resistant to decomposition in the natural environment, PCBs are able to bioaccumulate and biomagnify at higher trophic levels of different food chains. Diet is the most important exposure source in both wild animals and humans [1,4–6]. Although a few temporal trend studies of PCBs for example in marine ecosystems, show declining concentrations that eventually level off, PCBs are still considered of great concern because of the wide spectrum of toxic effects on both animal and human health [6–9].

Both the potency and nature of toxicity of the PCBs are dependent on this molecular structure [8]. The group of non-*ortho* coplanar PCBs, including PCB126 (3,3′,4,4′,5-pentachlorobiphenyl), reveals similar toxic properties as 2,3,7,8-tetrachloridibenzo-*p*-dioxin (TCDD), and the mechanistic pathway for dioxins and dioxin-like PCBs is mediated through binding to the cytosolic aryl hydrocarbon (Ah) receptor. The mono-*ortho*-substituted PCB congeners, such as PCB99 (2,2′,4,4′,5-pentachlorobiphenyl), are less able to take a coplanar configuration yet can also bind to the cytosolic Ah-receptor but with lower affinity. PCBs with two or more *ortho*-substituted chlorines, including PCB153 (2,2′,4,4′5,5′-hexachlorobiphenyl), have low or no affinity for the Ah-receptor.

PCB99 and PCB153 are two of the major congeners detected in wildlife and humans, particularly in the Arctic environment [2,3,5,11–14] and each represent a subgroup of

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the, respectively mono-*ortho* and di-*ortho*-substituted group of congeners. As far as we know, only a limited number of human and wildlife studies indicate possible cause and effect relationships with PCB99 and 153. In this respect, a negative relationship was seen between the plasma concentrations of PCB153 as well as PCB99 and plasma testosterone levels in polar bears [15]. A similar negative relationship was seen for PCB153 and plasma testosterone levels in humans [16].

PCB126 is one of the most potent PCB congeners, and its toxic properties have been estimated to be about 1/10th as potent as TCDD. PCB126 has been intensively studied, and as a model congener for AhR-mediated responses, it has often been included in health and risk assessment studies. However, in comparison to PCB153 and PCB99, PCB126 is only present at fairly low concentrations in the environment. Most information concerning adverse effects of single congeners on the male reproductive system and perturbed reproductive function has been derived from experimental studies [17–19]. If only based on their toxic equivalency factor (TEF), which estimates their potency relative to TCCD toxicity, one may get the impression that compounds with no or low TEFs, such as PCB153 and PCB99 are associated with little if any adverse outcomes in wildlife and humans [20]. However, the high concentrations of prevalent congeners like PCB99 and PCB153 in mainly all biota, prompts further studies to determine possible toxic effects.

In humans and other long-lived mammals, environmental exposure may be described as a chronic exposure to mixtures of PCBs, both pre- and postnatally. On the other hand, accidental incidences [21] provide direct links between exposure and adverse health outcomes. Another aspect concerning both environmental exposure as well as the use of commercial PCB mixtures, or so-called Aroclors, in experimental studies is related to the fact that they consist of complex mixtures of PCB congeners, which show wide ranges of metabolic activities and toxic potencies [20].

Many studies on PCBs are based upon in utero and lactational exposure studies [22] and the majority of these studies have used commercial PCB mixtures [23]. Many of theses studies reported alterations in parameters such as plasma testosterone concentration, reduced testes weight, sperm count and in vitro fertilizing ability in the F1 generation. Thus, the in utero and lactational periods appear to be sensitive to treatment with PCBs [19,24,25].

The number of studies explicitly focusing on possible PCB-induced testicular toxicity in adult animals is rather small. In one study adult deer mice (*Peromyscus maniculatus)* were exposed to Aroclor 1254 supplemented in their diet (5 ppm) for 30 days. At the end of the treatment period the testes from the treated deer mice revealed significant degenerative alterations in the seminiferous epithelium [26]. In another investigation Hsu et al. [27] observed that postnatally exposed rats by a single i.p. injection of 9.6 or 96 mg/kg PCB132 or PCB149, the testes and epididymides weights, sperm counts, and testosterone concentration were unaffected, but there was a decrease in sperm motility. Furthermore, Faqi et al. [18] studied the effects in adult male rats after subcutaneous exposure to the single congener PCB77 (18 or 60 mg/kg). The relative testicular and prostate weights were slightly increased in the high dose group. Both treatment groups showed a permanently reduced daily sperm production during the whole experiment as well as a significant increase in the percentage of abnormal sperm.

In order to provide insight into possible reproductive toxic effects, the present study was conducted using PCB99 and PCB153 to investigate possible effects on spermatogenesis and sperm chromatin in young adult mice. Single doses of each congener were selected on literature survey and the basis of the results of a two-week preliminary study using gavage as the route of exposure. Additionally, PCB126 was included in a preliminary study to serve as a possible, positive control group since it was observed to cause reproductive toxic effects following gestational and lactational treatments [17]. To investigate possible direct effects on spermatogenesis or indirect effects on sperm chromatin structure, conventional reproductive endpoints as well as flow cytometric (FCM) assessments were performed.

## 2. Materials and methods

### 2.1. Experimental design

Three groups of 8-week-old mice were randomly allocated to two different PCB congeners: PCB99 and PCB153, and one vehicle control. During the 6-week experiment, six mice per treatment group were sacrificed weekly on the same day of the week. In addition three mice per group were sacrificed 24 h after exposure. The rational of choosing this schedule was to cover the complete process of spermatogenesis in order to investigate if any of the PCB congeners was able to exert adverse effects.

### 2.2. Animals and housing

C57BL6 inbred SPF mice were purchased from Harlan, UK. The animals were acclimated to the animal facility 2 and 4 weeks, respectively prior to the preliminary and final study. The mice were housed individually in Macolon type III cages on quality-controlled aspen bedding at approximately 21 °C, on a 12:12 h light–dark cycle. Food (Rat & Mouse Standard Diet No. 1; Scanbur BK) and tap water were available ad libitum. The experiment was carried out in compliance with the provisions enforced by the National Animal Research Authority (NARA).

### 2.3. Preliminary study

PCB99 and PCB153 in powder form, purity respectively 98.6 and 99.7%, were purchased from Promochem Standard Supplies AB (Kungsbacka, Sweden). To prepare doses, the PCB congeners were dissolved in cyclohexane (Sigma Chem-

ical Co., St. Louis, MO, USA) and most of the solvent was evaporated under a stream of nitrogen. Corn oil was added and the suspension was subjected to ultrasonic waves (sonicated) for 5 min. The resulting PCB concentrations were confirmed by high-resolution gas chromatography. The corn oil vehicle was also analyzed for contamination with trace amounts of PCBs.

A preliminary study was conducted, including PCB99, PCB153 and PCB126, and the doses used were chosen according to available literature, including documented PCB levels measured in polar bears from the Norwegian Arctic [15,28–30], to avoid overt toxicity. The selected doses dissolved in corn oil (per kg body weight) were as follows: PCB99: 0.1, 0.3, 3.0 and 10 mg/kg; PCB153: 10, 30 and 100 mg/kg; PCB126: 0.1, 0.3 and 1.0 mg/kg. For each concentration three mice received a single PCB dose by oral gavage. The control group was treated with corn oil only. The preliminary study lasted for 2 weeks and was concluded by necropsy of all the animals. Body and liver weights were recorded, a gross pathological examination and FCM analysis of spermatogenesis was performed. Based on the preliminary study the following doses were selected for the final study: 10 mg/kg for PCB99 and 100 mg/kg for PCB153.

### 2.4. Final study and sample collection

The dose of the test chemical was adjusted to the mouse body weight on the day of treatment. During the experiment, body weights were recorded once weekly. Three mice per group were sacrificed 24 h after exposure to look specifically for apoptotic events in the testes. For the remaining 6 weeks, six randomly selected mice per treatment and the control group were sacrificed and body weights were determined just prior to sacrifice. The mice were anesthetized with isoflurane and blood samples were collected by cardiac puncture with a heparin-rinsed 1 ml syringe. Blood samples were transferred into Lithium Heparin sample tubes and directly stored on ice. After centrifugation (1000 × *g*, 15 min, 4 °C) the supernatant was transferred to an Eppendorf tube and frozen at −20 °C for subsequent plasma serum testosterone analysis.

The animals were killed by cervical dislocation under isoflurane anesthesia. The abdominal cavity was then opened and examined for gross pathology. The liver was removed and weighed. A small sample of peri-testicular fat was removed and placed in a cryovial, quickly frozen on dry ice and stored at −70 °C until further analysis of PCB concentrations. The seminal vesicles were dissected, placed into plastic zip-lock bags on dry ice for storage at –70 °C. Later the seminal vesicles were weighed after thawing at room temperature. Testes, including epididymis, were removed separately and cleaned of excess tissue. The left and right epididymis together with the vas deferens were carefully dissected from the testes and directly frozen into plastic zip-lock bags on dry ice to be stored at −70 °C. Epididymides were weighed and at week 3 and 6 after exposure, the vas deferens were transferred in a TNE buffer (0.15 M NaCl, 0.01 M Tris–HCl, 1 mM EDTA, pH 7.4) as described by Evenson et al. [31] for subsequent Sperm chromatin structure assay (SCSA) analyses. One testis was transferred into Bouin's fixative for 24 h, dehydrated in ethanol 70% and embedded in paraffin for future histological evaluation as well as and in situ DNA 3′ end labeling. Immediately after dissection the other testis was placed into a vial with TNE buffer and placed on crushed ice for subsequent flow cytometric analyses of testis cell populations.

### 2.5. PCB analysis

The organochlorine analyses were performed at the Environmental Toxicology Laboratory at the Norwegian School of Veterinary Science, Oslo. The laboratory is accredited by the Norwegian accreditation as a testing laboratory for analyses of PCBs and other organochlorines according to the requirements of NS-EN 45001 (1989), ISO/EC Guide 25 (1990) and NS-EN ISO/IEC 17025 (2002), TEST 051.

Fat samples (∼0.1 g) were weighed and internal standards (PCB29 and 112) were added. The samples were homogenized with an IKA Ultra Turrax (Janke & Kunkel, GmbH & Co., KG, Germany) and extracted twice with cyclohexane (HPLC grade, Rathburn Chemicals Limited, Scotland) and acetone (Glass distilled grade, Rathburn Chemicals Limited, Scotland). The percent extractable fat was determined gravimetrically. The cyclohexane fraction was washed with ultra pure sulphuric acid 96% (Scanpure, Chemscan AS, Elverum), according to Brevik [32].

Aliquots (2 μl) of the lipid free organochlorine extracts were analyzed as described by Bernhoft et al. [2]. Standard procedures were used to ensure adequate quality assurance and control. Percent recoveries and coefficient of variance (CV) of OCs in spiked matrix varied from 104 to 116% and 3.3 to 15.5% for PCB153 and PCB99, respectively. Detection limits for individual PCB congeners were determined as three times the noise level and were between 633 and 725 ng/g wet weights. All calculations were performed within the linear range of the detector's 5-level calibration curve. The reproducibility was tested continuously by analyzing the PCB levels in the laboratory's own reference sample (seal blubber), which was within the mean coefficient of variance (8.7%) for the year 2000.

The repeatability of the GC-performance was tested by repeated injection of standard PCBs at regular intervals and blank samples were included to test for interference. Concentrations of PCB99, PCB126 and PCB153 were quantified in all samples of the different treatment groups.

### 2.6. Testosterone analysis

Plasma levels of total testosterone were determined by a solid-phase radioimmunoassay kit (Coat-A-Count, total testosterone, Diagnostic Products Corporation, LA, USA). The assay was validated for use with mouse plasma by demonstrating parallelism between dilutions of mouse plasma samples and the standard curve, and no modifications

of the standard procedures were needed. The detection limit of the assay was 0.04 ng/ml. Intra- and interassay variation coefficients were less than 10%.

### 2.7. Histological evaluation of testes

Hematoxylin and eosin (H–E) staining was performed on two cross sections (6 μm thickness). For each testis section seminiferous tubules (ST) were evaluated by light microscopy (200× magnification) by three morphological criteria, namely the percentages of seminiferous tubules revealing normal spermatogenesis, loose epithelium, and apoptotic cells. Intratubular apoptotic cells and apoptotic Leydig cells within the interstitium were identified by the following criteria: intense uniform basophilic nucleus with either chromatin condensation or nuclear shrinkage or with the fragmentation of the nucleus into several uniformly basophilic masses. For each mouse an entire cross section of the testis stained with H–E was examined for the presence and number of apoptotic Leydig cells.

### 2.8. In situ detection of apoptosis

In order to confirm the presence of apoptotic Leydig cells observed in the H–E stained sections, the TUNEL labeling using the Apop Tag Plus In Situ Apoptosis Detection Kit (peroxidase) (Oncor, Gaithersburg, MD, USA) was used. This method uses terminal deoxynucleotidyl transferase (TdT) to end label 3′-OH ends of DNA with digoxigenin-labeled nucleotides. The sections were then treated with anti-digoxigenin antibody peroxidase conjugate, stained with diaminylbenzidine (DAB) and counterstained with hematoxylin, mounted and examined microscopically. For each batch a positive control section from the mammary gland of weaning mice was included. Apoptotic Leydig cells in the interstitium revealed intensely stained nuclei and were easy to detect. Since apoptosis is a common mechanism in the regulation of cell numbers, testis sections with >1 apoptotic Leydig cell were defined as positive, thus representing an increase in the normal apoptotic rate. The prevalence (percent positive mice per time point) and number (mean and S.E.) of apoptotic cells per section during the study period are presented.

### 2.9. DNA FCM analysis of testis cells

One testis was transferred to a 60 mm petridish containing 1–2 ml TNE buffer, the capsule was removed and the testis was minced with curved surgical scissors to liberate cells. Cell suspensions were pipetted into test tubes and the tissue fragments were allowed to settle for 1 min. The supernatant was gravity filtered through a 55 μm nylon filter and testicular cell samples were analyzed immediately [33].

For Hoechst 33258 staining, we incubated 1–2 × $10^6$ cells in TNE medium containing 0.1% Triton X-100 and 1.0 μg Hoechst 33258 $mL^{-1}$ for 15 min. Blue fluorescence was measured using an Argus 100 flow cytometer (Skatron, Lier, Norway). The percentages of cells in the 1C, 2C, S-phase and 4C populations were estimated from DNA cytograms using the Multicycle Program (Phoenix Flow System, San Diego, CA, USA).

Chromatographically-purified acridine orange (AO) (Polysciences Inc., Warrington, Pennsylvania) was used for differential staining of DNA and RNA [33]. The DNA and RNA content of the testis cells was measured using a Coulter EPICS XL flow cytometer (Beckman Coulter Ltd., Luton, England) equipped with a 15 mW argon laser with excitation at 488 nm. Both light-scatter and fluorescence data were collected in linear mode. Green fluorescence was detected using a 505–545 nm BP filter (FL1) while red fluorescence was detected using a 660–900 nm BP filter (FL4). Measurements were recorded 3 min after staining; fluorescence data were acquired on 5000 cells/sample at a flow rate of 100–200 cells/s. During the FCM analysis, testicular cell suspensions from a single control mouse were used as internal control in order to ensure reproducibility of sample manipulation and instrument performance. The percentage of different testicular cell types was analyzed using EXPO32$^{TM}$ ADC (Beckman Coulter version 1.1c) and allows the different cell subpopulations to be determined as a function of exposure and time.

### 2.10. SCSA of vas deferens sperm cells

Frozen epididymides and vas deferens were quickly thawed in a 37° C water bath and transferred into 2 ml of ice-cold TNE buffer. Sperm were squeezed out of the vas deferens and expelled several times through a Pasteur pipette, filtered through 153 μm nylon mesh into test tubes, and kept on crushed ice until analyzed by FCM, strictly following the procedure described by Evenson et al. [31]. Abnormal chromatin structure, here defined as showing an increased susceptibility to acid denaturation, was measured in individual cells. When excited with blue laser light (488 nm), AO intercalated into double-stranded DNA and fluorescence green representing sperm with low levels of fragmented DNA. When associated with single-stranded nucleic acids the red fluorescence represents sperm that have moderate to high levels of fragmented DNA yielding a higher percentage of the DNA fragmentation index (DFI). The samples were analyzed on a Coulter EPICS XL flow cytometer (see above). Recorded measurements were begun 3 min after staining with a measurement rate about 200 cells/s and a total of 1 × $10^4$ cells were processed for each measurement.

### 2.11. Statistical analysis

The control group was defined as the reference category. The differences between the PCB groups and the control group were estimated with multiple linear regressions, using Proc Reg in SAS [34], including the biological and reproductive parameters as the dependent variables and the PCB

groups as independent dummy variables. The variables were tested for normality and in case the normality assumption was rejected the log transformed of the variable was used in the analysis. *P*-values less than 0.05 were considered as statistically significant.

## 3. Results

### 3.1. Preliminary study

A range-finding study was conducted in order to select the PCB doses for the final study. During this period it was found that the general well being of all groups was normal, and necropsy did not reveal any signs of overt toxicity. In the preliminary study, necropsy findings of the PCB126-treated mice showed macroscopically visible fatty degeneration in the liver for all doses, indicating overt toxicity. However, even at the toxic doses no perturbation of any of the reproductive endpoints was observed (unpublished data), and therefore, it was decided not to include PCB126 in the final study.

### 3.2. Chemical analysis of PCBs

For both exposure groups, the PCB concentrations were quantified in peri-testicular fat tissue (Fig. 1). The corn oil did not contain any detectable concentrations of PCBs. The mean and standard error (S.E.) of the concentrations of PCB99 was 81.3 (11.6), 73.5 (4.6), 217.8 (150.6) and 24.8 (1.7) μg/g wet weight on respectively day 1, week 1, 2 and 6 after exposure. This indicates that after 6 weeks, the concentrations were reduced to approximately 25% of that on day 1. For the PCB153 treatment, the concentration (mean and S.E.) was 312.7 (93.8) μg/g wet weight one day after exposure. There was an obvious increase from day 0 to week 2, after which the concentrations declined (Fig. 1).

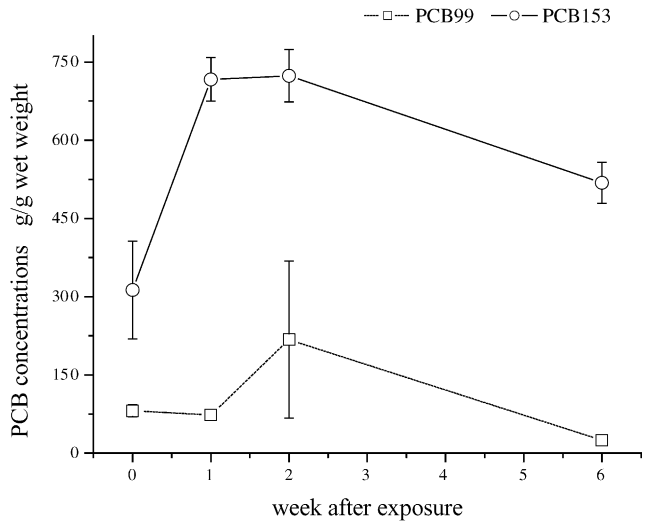


Fig. 1. Concentrations (μg/g wet weight) of PCB99 (□) and PCB153 (○) measured in peri-testicular fat. Sampling at day 1 (week 0) and during week 1, 2 and 6 after exposure of male C57BL6 mice. Data are expressed as the mean and standard error of the mean (S.E.). S.E. for PCB99 is very small on week 0, 1 and 6, thus not presented.

Table 1
Body and organ weights in young adult male C57BL6 mice after oral gavage with vehicle (corn oil), PCB99 (10 mg/kg) and PCB153 (100 mg/kg)

| | Difference | S.E. | *P*-value[a] |
|---|---|---|---|
| Body weight gain[b] | | | |
| Control group mean; 2.3% | | | |
| PCB99 | −0.54 | 0.34 | 0.12 |
| PCB153 | −0.03 | 0.34 | 0.92 |
| Relative epididymis weight[c] | | | |
| Control group mean: 0.23 | | | |
| PCB99 | −0.005 | 0.009 | 0.57 |
| PCB153 | −0.0007 | 0.008 | 0.28 |
| Relative liver weight | | | |
| Control group mean: 5.8 | | | |
| PCB99 | −0.05 | 0.10 | 0.63 |
| PCB153 | 0.22 | 0.10 | <0.05* |
| Relative seminal vesicle weight | | | |
| Control group mean: 0.71 | | | |
| PCB99 | 0.04 | 0.04 | 0.38 |
| PCB153 | 0.02 | 0.04 | 0.61 |
| Logarithm testosterone | | | |
| Control group mean: 0.28 ng/ml | | | |
| PCB99 | 0.12 | 0.16 | 0.45 |
| PCB153 | 0.06 | 0.16 | 0.71 |

Data are expressed as the difference and S.E. with the control group, for PCB99 and PCB153 during 6 sampling weeks.
[a] *P*-value for the comparison between treatment and control group.
[b] Difference between initial body weight and body weight on day of sacrifice.
[c] All relative organ weights calculated as: organ wt/body wt × 100%.
* Significantly different compared to the control group.

### 3.3. General health and reproductive parameters

During the experiment the control animals as well as PCB99 and PCB153 showed similar body weight gains (Table 1). The PCB153 group showed a slightly but significantly higher relative liver weight (Table 1) whereas no significant changes in relative weights of epididymides or seminal vesicles, nor in the plasma testosterone concentrations were observed for the PCB99 or PCB153 group in comparison to the control group (Table 1).

### 3.4. Histological evaluation and TUNEL analysis of the testes

Evaluation of the testes for the presence of alterations in spermatogenesis in the treated groups compared to the control group showed no significant differences. In all groups more than 95% of the ST revealed normal spermatogenesis. The presence of loose epithelium in the seminiferous tubules was rare and mostly associated with the method of histological preparation. All testes revealed small numbers of apoptotic cells within the seminiferous tubules.

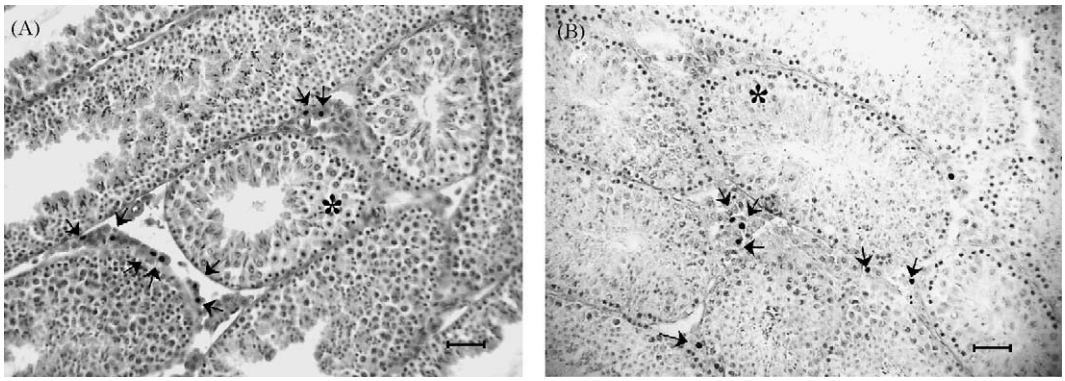


Fig. 2. Histological section from an adult C57BL6 mouse-treated with PCB153 (100 mg/mg). (A) H–E stained sections revealing apoptotic Leydig cells in the interstitium indicated by arrows. (B) Histological section of the same mouse after TUNEL staining demonstrating apoptotic Leydig cells. (*) indicates seminiferous tubules. Bars represent 50 μM.

Evaluation of the H–E stained testis sections revealed that some individuals had several apoptotic Leydig cells within the interstitium. The identification of apoptotic cells in the interstitium and within the seminiferous tubules was confirmed using a TUNEL technique (Fig. 2). Leydig cell apoptosis was observed in PCB99 and PCB153-treated mice as well as in the control group; however, this was never observed in the mice sacrificed at 24 h. The control as well as the treated animals revealed a significant increase in both the prevalence and number of apoptotic Leydig cells per section from week 3 to 6 (Fig. 3). The PCB99 group demonstrated significantly more apoptosis with a number of apoptotic Leydig cells (mean and S.E.) of 7.3 (3.7), 20.2 (8.6), 8.0 (5.6) and 16.1 (11.3) from week 3 to 6, than the controls with a mean (S.E.) of 1.3 (0.9), 5.5 (2.6), 0.0, and 3.0 (1.5), respectively. The PCB153 group, on the other hand, showed significantly more Leydig apoptosis than the controls during week 2, 5 and 6 with a mean (S.E.) number of 8.8 (3.6), 3.5 (1.6) and 21.3 (9.2), respectively (see Fig. 3).

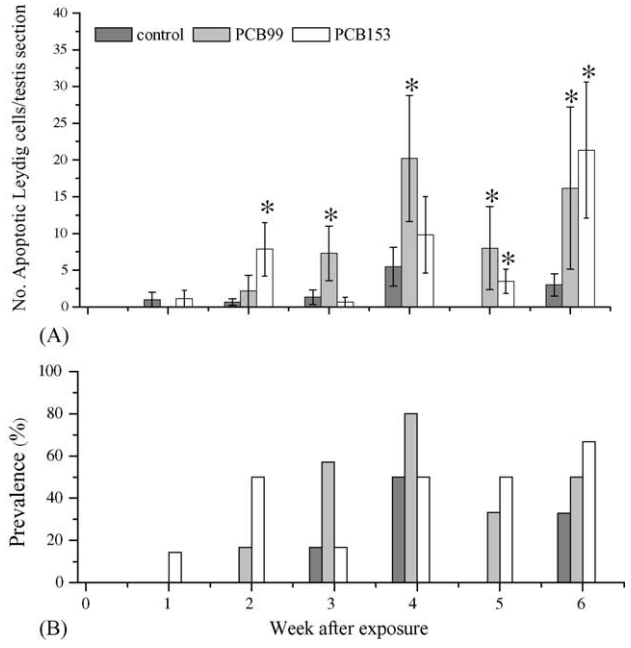


Fig. 3. Leydig cell apoptosis in histological sections of mouse testis stained by H–E. PCB99 (10 mg/kg) and PCB153 (100 mg/kg) with corn oil as vehicle control were given to young adult mice by a single gavage dose. H–E staining was quantified by measuring the number of apoptic Leydig cells per cross section. Testis sections with >1 apoptotic Leydig cell were defined as positive. Data are expressed as the number (mean and standard error of the mean) of apoptotic cells per cross section (A) and the prevalence of positive (%) mice per time point (B) are presented. (*) Significantly different from the control group: $P < 0.05$.

### 3.5. FCM analysis

In this study, Hoechst 33258 was used to measure possible spermatogenic alterations in testicular cell populations during weeks 1–6 after exposure. Fig. 4 shows the relative proportions of haploid, diploid and tetraploid cells for the three groups during the 6-week period. The haploid cell population consisting of two sub populations with round and elongating/elongated spermatids is presented in panel A, and the diploid and tetraploid cell populations are presented in panel B. Neither PCB99 nor PCB153 caused significant changes in the testicular cell populations during the 6-week period covering the process of spermatogenesis.

Acridine orange analysis of the testicular cell suspensions allows the characterization of seven cell populations, in comparison to Hoechst 33258 where basically five cell populations are detected (Fig. 5). The results from the AO anal-

Table 2
Sperm chromatin structure assay (SCSA) of vas deferens spermatozoa in adult male C57BL6 mice 3 and 6 weeks after oral gavage of vehicle (corn oil), PCB99 (10 mg/kg) and PCB153 (100 mg/kg)

| %DFI | Difference | S.E. | *P*-value[a] |
|---|---|---|---|
| Control group mean: 1.55% | | | |
| PCB99 | −0.22 | 0.15 | 0.45 |
| PCB153 | 0.52 | 0.14 | 0.08 |

Mean DNA fragmentation index (%DFI) expresses the percent of sperm with fragmented DNA week 3 and 6 combined. Data are expressed as the difference and S.E. in each treatment group with the control.

[a] *P*-value for the comparison between treatment and control group.

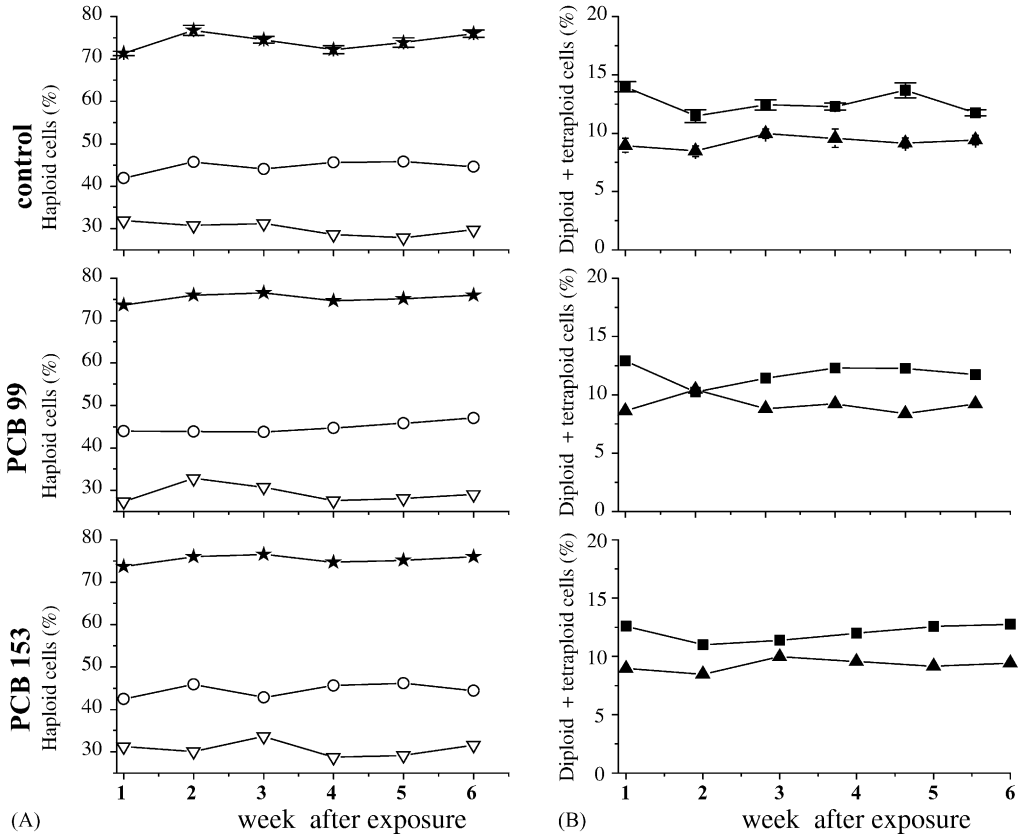


Fig. 4. Changes in percentage of (A) haploid cells (⋆), elongated spermatids (▽), round spermatids (◯). (B) Diploid (■) and tetraploid cells (▲) in testicular single cell populations stained with Hoechst 33258, from young adult C57BL6 mice. Control: corn oil; PCB99: 10 mg/kg; PCB153: 100 mg/kg. The mean and standard error of the mean are presented for the control group, whereas only the mean for the treatment groups.

yses during weeks 3–6 were very similar to the Hoechst 33258 results, and no significant differences were found between the control and two treatment groups (data not shown).

Sperm samples were extracted from the vas deferens from all three groups during week 3 and 6 after treatment, and were analyzed by the SCSA (Table 2). The percentages of vas deferens sperm with fragmented DNA are presented as

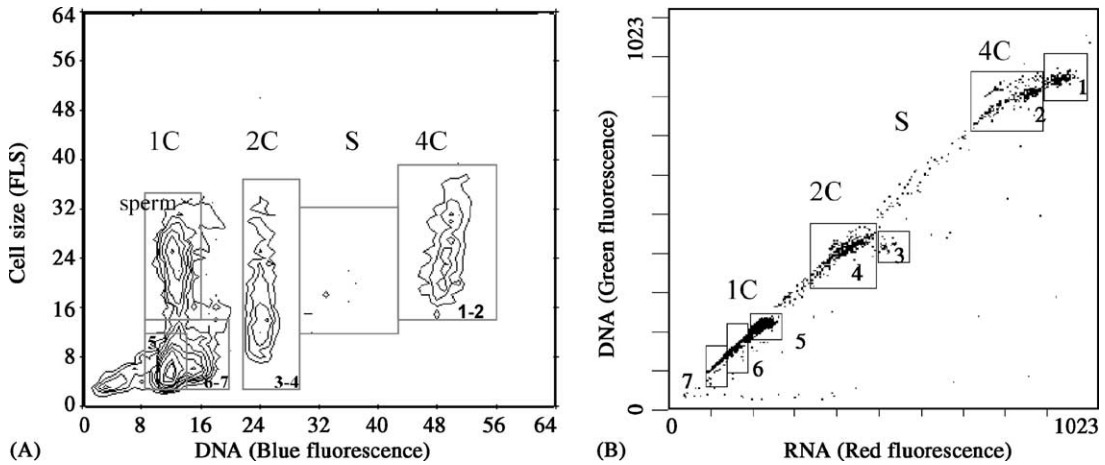


Fig. 5. Representative cytograms of the two FCM techniques used to study changes in testicular cell populations. (A) Hoechst 33258-staining allows the discrimination of five populations plus sperm and (B) acridine orange-staining shows seven populations in addition to S-phase cells (sperm is gated out). The boxes present the following testicular cell populations: (1C) haploid cells: round, elongating and elongated spermatids (boxes 5–7) and sperm (not included in the analyses). (2C) Diploid cells: spermatogonial $G_1$ cells, secondary spermatocytes, binucleated spermatids and somatic cells (boxes 3, 4). (S) S-phase: multinucleated spermatids. (4C) Tetraploid cells: primary spermatocytes and spermatogonial $G_2$ cells (boxes 1, 2).

percentages of sperm with single-stranded DNA. The analyses of all of the treatment groups showed low values for both treatment groups, and these were not significantly different in comparison to the control group (Table 2).

## 4. Discussion

Effects of PCBs on the male reproductive system have been extensively studied in laboratory animals [17,18,35–38]. Most of these investigations have in common that they are based on maternal exposure during the vulnerable pre and/or early postnatal periods. However, data revealing adverse reproductive effects after PCB exposure during adult life are very limited. The most common congeners detected in wildlife and humans are unfortunately among the least studied. These include some of the non-coplanar PCBs amongst which PCB99 and PCB153 reach the highest concentrations in wildlife and humans [2,3,10,13,39,40]. A recent epidemiological study pointed out that men with the highest serum concentrations of PCB153 tended to have lower testosterone levels [16]. Similar relationships were observed in polar bears from the Svalbard region, for both PCB99 and PCB153 [15]. This prompted us to study these congeners for possible reproductive toxic effects in adult mice. As a mono-*ortho*-substituted PCB congener, it was possible that PCB99 might be able to exert Ah receptor-related toxicity, and therefore, the C57BL6 mouse strain was used since it is an Ah positive strain [41,42]. The single exposure by oral intubation with relatively high doses represents acute exposure in order to study possible adverse effects whereas additionally, the oral route mimics the most relevant route of exposure in wildlife and humans [8].

Since very few studies have investigated possible reproductive effects caused by exposure in adult life to PCB congeners in general, we decided to include PCB126 as a possible, positive control because a number of in utero and early post natal studies showed that this congener could cause a variety of reproductive toxic effects. In the preliminary study, even at doses that caused overt toxicity, we did not observe any changes in relative epididymis or seminal vesicle weights, or in the plasma testosterone concentrations 14 days after treatment. Furthermore, FCM assessment showed no indications of alterations in spermatogenesis. This lack of effects on spermatogenesis is similar to the results from an in vitro study by Fukuzawa et al. [44], who reported that PCB126 did not alter the proliferative activity of spermatogenetic cells and Sertoli cells in the neonatal testis of mice. However, they did observe that PCB126 affected gene expression involved in steroidogenic enzymes. On the basis of the lack of effects on spermatogenesis and other reproductive toxic endpoints in mice at doses causing general toxicity, we decided not to include PCB126 in the final study.

The levels for PCB99 and PCB153 measured in peritesticular adipose tissue (Fig. 1) were as expected considerably higher than concentrations measured in blubber from seals and polar bears [2,5,6,45] or concentrations measured in adipose tissue in humans [4]. Thus, all PCB-treated mice were exposed to high concentrations throughout the study, but the fat concentrations for these congeners revealed two different trends. During the 6-week study period the PCB99 concentrations in fat were reduced by more than 75% of the starting concentration, whereas the concentrations of PCB153 showed an early large increase followed by a slow decline. A similar pattern for fat accumulation of PCB153 was observed in non-pregnant mice [46]. Due to their lipophilic character, the elimination of PCBs is primarily dependent based on metabolism [8,47]. Based on its molecular structure, PCB153 is known to be metabolized very slowly, and this is also reflected in the present study. After 6 weeks the peri-testicular fat concentrations of PCB153 were approximately twice as high as the concentrations on day 1. The residual levels from PCB99 in this study suggest that this congener was metabolized more readily; however, further studies are needed. It must also be taken into consideration that the high concentrations in adipose tissue do not mirror the actual PCB concentrations in the serum and body fluids to which the reproductive organs are exposed.

Body weight gain from both treatment groups did not significantly differ from the control group. This was also observed in other studies where adult rats were treated with PCB153 [28,49]. On the other hand, body weight seems to be a more sensitive indicator of toxicity when PCB treatment occurred during prenatal and early postnatal stages [37,49].

The non-*ortho* substituted PCBs are known to induce hepatic toxicity, which in fact undoubtedly was the case for the PCB126-treated mice in the preliminary study. Although not as potent, the mono-*ortho*-substituted congener PCB99 has also been reported to be hepatotoxic however, necropsy findings and unchanged liver weights did not indicate any sign of toxicity. On the other hand, the relative liver weights were slightly increased in the PCB153-treated mice (Table 1). Moore et al. [50] observed increased liver weights in male rat pups after orally dosed female rats during gestation. PCB153 induces mainly the 2B type of cytochrome P450 enzymes. Holene et al. [28] and Bouwman et al. [51] reported the dose-related induction of cytochrome P450 D (CYP2B1) in orally exposed rats, after in utero and lactational exposure. After a single i.p. injection with PCB153, Fadhel et al. [52] reported cytochrome P450 induction as well as the induction of oxidative damage in the rat liver by increased hepatic lipid peroxidation. Therefore, it is possible that the induction of cytochrome P450 enzymes by PCB153 can cause a decrease in testosterone as a result of enhanced degradation.

A number of organochlorines have been shown to affect sex hormones, which in turn have led to alterations in androgen-dependent organs [53–55]. In fetal life, epididymal growth is dependent on, among other things, androgen production. Alterations in epididymal weight after PCB exposure during pregnancy may be due to androgen deficiency and/or a decrease in androgen responsiveness of the epididymis in male offspring [48,49,54,56]. In mature males, the measure-

ment of epididymis weight covering the total process of spermatogenesis has shown to be a gross, but useful parameter for the evaluation of sperm production and possible changes in spermatogenesis. In the present study, neither of the congeners was found to alter the relative epididymis weight in comparison to the control animals.

Another endpoint in males that has been evaluated in a number of reproductive toxicity studies is the seminal vesicle weight. During fetal development, as well as in adult life, the accessory sex organs are androgen-dependent and may serve as indicators of Leydig cell function and/or androgen production. Weight of the accessory sex glands, e.g. relative seminal vesicle weight can be used as a gravimetric indicator for plasma testosterone levels [54,57]. For both PCB groups no changes were observed in seminal vesicle weight in comparison with the control, which was in agreement with the unchanged plasma testosterone levels also found in this study.

Plasma testosterone concentrations reflect the Leydig cell function, but they only mirror possible changes since intratesticular testosterone levels may be 10–100 times higher, depending upon species [58]. In the present study, no differences in plasma testosterone levels were observed among the groups. This may indicate that no changes occurred, but it may also be due to the large variations in plasma testicular concentration, which result from the episodic testosterone secretion pattern in [59]. These inherent variations make it difficult to find possible relationships with the results from the other endpoints investigated in the present study. It has been reported that intratesticular testosterone concentrations must be reduced by at least 80% in rodents before spermatogenesis becomes affected [60]. However, the fact that no alterations were observed in spermatogenesis in any of the groups provides an indication that there were no treatment related changes in the intratesticular testosterone concentrations.

A histological evaluation of the testis from the control group as well as two treatment groups showed that all had normal spermatogenesis, and that the seminiferous epithelium was normal in more that 95% of the seminiferous tubules in all individuals. As expected, this confirmed the results of the flow cytometric analyses. Testes from all three groups revealed small numbers of apoptotic cells within the seminiferous tubules. No single individual revealed significant increases in numbers of apoptotic germ cells. This is in accordance with the results from other studies indicating that a moderate rate of germ cell apoptosis in adult animals is an important mechanism for successful spermatogenesis [61]. It is therefore, concluded that treating young, adult mice with high concentrations of PCB99 or PCB153 did not cause toxic effects in the seminiferous epithelium.

During the histopathological examinations within the treatment groups as well as the control group there were some individuals observed with apoptotic Leydig cells. Since this is a rare event in mice [62–64], it was decided to re-examine each H–E-stained histological section for the presence of apoptotic Leydig cells and to confirm this phenomenon using the TUNEL-technique. No apoptotic Leydig cells were observed in any of the animals sacrificed 24 h post treatment; however, during the third week and onwards there was a general tendency in both the control and PCB groups towards increases in both the prevalence of animals with apoptotic Leydig cells, and the number of apoptotic cells per histological section. (Fig. 3). Since Leydig cell apoptosis is unusual in mice, we did not expect to observe an increase in the number of apoptotic cells in the corn oil-treated mice, even though apoptosis occurred to a lesser extent than the PCB-treated mice. Chemical analysis of the corn oil demonstrated that there were no detectable amounts of PCBs, but it may be possible that there were some other unknown contaminants that were able to cause the observed effects. Another possibility is that some of the saturated fatty acids present in the corn oil may have caused Leydig cell apoptosis since one in vitro study has indicated such effects [65]. In this study either palmitic or stearic acid was added to Leydig cell cultures, causing apoptosis by ceramide production in these cells. Palmitic and stearic acids account for around 13–14% in corn oil. However, it is not possible to make direct comparisons between in vitro and in vivo studies.

From week 3 and onwards the PCB99-treated mice had significantly greater numbers of apoptotic cells per section than the control animals. Similarly, the PCB153 testes had significantly more apoptotic cells than the controls during week 2, 5 and 6 (Fig. 3). Thus, both congeners were able to promote apoptosis in Leydig cells. A few compounds have been shown to cause Leydig cell apoptosis in rats, including ethane dimethanesulfonate (EDS), and corticosterone, the major glucocorticoid in rodents [62,66]. EDS is a unique testicular toxicant, and following a single dose the Leydig cell population is destroyed and subsequently regenerates, apparently from mesenchymal fibroblast-like precursors [67]. Glucocorticoid treatments have been used to simulate stress, and the resulting Leydig cell apoptosis occurred through a direct mechanism at the level of the cell, rather than through the suppression of LH [63]. This same group also reported that immobilization stress, 3 h/day for 7 day caused both decreases in the circulating levels of testosterone as well as significant increases in Leydig cell apoptosis [63]. In a similar fashion, PCB99 and PCB153 may be able to reduce testosterone production via Leydig cell apoptosis; however, this relationship was not observed in the present study where adult animals were exposed. Earlier studies on PCB153 dosed during the gestational and lactational periods, have led to decreases in plasma testosterone concentrations. A recent study from Sweden has reported a weak negative association between plasma PCB153 and testosterone levels in men [16]. A similar relationship has been suggested for male polar bears from the Svalbard region [15]. The pathways by which PCB congeners elicit Leydig cell apoptosis in mice is not known, but merits further study.

The reproductive endpoints used in this study as described above, provide a crude assessment of the animals' ability to

produce spermatozoa and undergo the process of maturation in the epididymis. When studying testicular toxicity, a number of studies have corroborated the conventional evaluation of spermatogenesis by FCM measurements [68,71]. In the FCM DNA content analysis, changes in the relative percentage of testicular cell populations are used to assess cytotoxic effects of a variety of toxicants, including organochlorines [69–70]. However, as far as we know, no data are available on the characterization of PCB99 and PCB153 cytotoxicity on mouse germ cells. As can be concluded from the results of the present study, treatment with high concentrations of PCB99 or PCB153 did not cause any alterations in spermatogenesis when exposure occurred in adult mice.

The sperm chromatin structure assay (SCSA) monitors the susceptibility of sperm chromatin DNA to acid-induced denaturation, as reflected in the %DFI, and has proven to be a sensitive tool when studying fertility and reproductive toxicity [31]. However, treating adult mice with high doses of PCB99 or PCB153 was not detrimental to the sperm chromatin structure of animals exposed for 3 or 6 weeks, as indicated by the low percentage of DFI in the SCSA (Table 4). However, we cannot rule out that other parameters of sperm biology, such as forward motility, might be affected by these congeners since other studies have shown that motility was altered by other PCB congeners [27].

To summarize, no adverse effects have been observed when a single high dose of either PCB99 or PCB153, at levels exceeding the exposure levels of wildlife and humans, was administered to young, adult mice. During the 6-week study period the body growth and relative sizes of a number of reproductive organs remained unchanged. Histological examination and flow cytometric analyses of the testis cell population revealed that spermatogenesis was not affected. Furthermore, the SCSA that measures effects on sperm chromatin showed that sperm from the treated and control mice were unaltered. However, although it was not reflected in the plasma testosterone levels, both congeners were associated with a significant 2–3-fold increase in the number of apoptotic Leydig cells.

The results from the present study indicate that the male reproductive system in mice is relatively refractory to PCB99 and PCB153 even at relatively high doses, during adult life. Whether or not the findings of apoptotic Leydig cells should be interpreted as an adverse reproductive effect warrants further investigation.

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