

Endocrine, metabolic and apical effects of *in utero* and lactational exposure to non-dioxin-like 2,2',3,4,4',5,5'-heptachlorobiphenyl (PCB 180): A postnatal follow-up study in rats

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ABSTRACT

PCB 180 is a persistent and abundant non-dioxin-like PCB (NDL-PCB). We determined the developmental toxicity profile of ultrapure PCB 180 in developing offspring following *in utero* and lactational exposure with the focus on endocrine, metabolic and retinoid system alterations. Pregnant rats were given total doses of 0, 10, 30, 100, 300 or 1000 mg PCB 180/kg bw on gestational days 7–10 by oral gavage, and the offspring were sampled on postnatal days (PND) 7, 35 and 84. Decreased serum testosterone and triiodothyronine concentrations on PND 84, altered liver retinoid levels, increased liver weights and induced 7-pentoxoresorufin O-dealkylase (PROD) activity were the sensitive effects used for margin of exposure (MoE) calculations. Liver weights were increased together with induction of the metabolizing enzymes cytochrome P450 (CYP) 2B1, CYP3A1, and CYP1A1. Less sensitive effects included decreased serum estradiol and increased luteinizing hormone levels in females, decreased prostate and seminal vesicle weight and increased pituitary weight in males, increased cortical bone area and thickness of tibial diaphysis in females and decreased cortical bone mineral density in males. Developmental toxicity profiles were partly different in male and female offspring, males being more sensitive to

Abbreviations: AHR, aryl hydrocarbon receptor; ANOVA, one-way analysis of variance; AR, androgen receptor; BMD, benchmark dose; BMDL, lower bound of the 90 % confidence interval of the BMD; BMR, benchmark dose response; bw, body weight; CAR, constitutive androstane receptor; CED, critical effect dose = benchmark dose; CES, critical effect size = benchmark dose response; CV, coefficients of variation; CYP, cytochrome P450; DL, dioxin-like; EFSA, European Food Safety Authority; E_{max}, maximum efficacy; ER, estrogen receptor; EROD, 7-ethoxyresorufin O-deethylase; FSH, follicle stimulating hormone; ft3, free triiodothyronine; ft4, free thyroxine; GD, gestational day; GLP, Good Laboratory Practice; IUL, *in utero* and lactational; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LH, luteinizing hormone; LOD, limit of detection; MoE, margin of exposure; NDL, non-dioxin-like; NOAEL, no-observable-adverse-effect level; PCB, polychlorinated biphenyl; PCDD/Fs, polychlorinated dibenzo-*p*-dioxins and furans; PND, postnatal day; PROD, 7-pentoxoresorufin O-dealkylase; PXR, pregnane-X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; TBG, thyroid-binding globulin; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TR, thyroid hormone receptor; TRIR, total RNA isolation reagent; TTR, transthyretin; UF, uncertainty factor; UGT, UDP glucuronosyltransferase.

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increased liver weight, PROD induction and decreased thyroxine concentrations. MoE assessment indicated that the 95th percentile of current maternal PCB 180 concentrations do not exceed the estimated tolerable human lipid-based PCB 180 concentration. Although PCB 180 is much less potent than dioxin-like compounds, it shares several toxicological targets suggesting a potential for interactions.

1. Introduction

Polychlorinated biphenyls (PCBs) are persistent and potent synthetic chemicals that were in commercial production since late 1920's until to the end of 1970's when they were banned [1]. It has been estimated that up to 1.5 million tons of PCBs were produced and used for a wide variety of closed and open commercial and military applications. They are still released to the environment from old depots, and as lipophilic compounds especially the higher chlorinated compounds accumulate in tissues and undergo biomagnification in the food chain. The main route of human exposure for the general population is through diet, but occupational exposure through the skin may also occur.

Based on their structure and toxicological properties, the 209 PCB congeners have been divided into 12 dioxin-like (DL-PCBs) and 197 non-dioxin-like (NDL-PCBs) congeners [2]. DL-PCBs have no or only one chlorine substitution in the *ortho* position, and consequently they can adopt a planar conformation, bind to the aryl hydrocarbon receptor (AHR) and exert DL toxic effects with high potency. NDL-PCBs have at least two *ortho* substitutions and do not bind to the AHR. They are much more abundant than DL-PCBs in the environment, and in mother's milk they account for about 90 % of total PCBs [3]. However, the toxic effects of NDL-PCBs are less characterized mainly because in most studies the presence of very potent and persistent DL impurities (DL-PCBs and polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs)) have not been controlled rigorously or quantified, although their contribution even at low concentrations may often be toxicologically significant overriding the effects of NDL-PCBs [2]. Therefore, until more recently, the specific toxic effects of NDL-PCBs have been far less characterized according to strict regulatory standards than those of DL congeners. The available data from *in vitro* and *in vivo* studies with high purity NDL-PCB congeners indicate that they have distinct, gender specific toxicological profiles [4–9], many of them share toxicological targets with DL-PCBs, such as decreased thyroid hormone levels, thyroid gland and liver pathology, effects on the hematopoietic system and altered behavior [4–9]. However, NDL-PCBs do not have several characteristic AHR dependent effects, including thymic atrophy, permanent body weight loss and typical CYP induction profile. In addition, in general they are far less potent than DL-PCBs.

PCB 180 is an abundant NDL-PCB both in the environment and in human tissues, and it belongs to the “six indicator PCBs” [2]. Due to long elimination half-life, it has a high potential for accumulation and biomagnification. The estimated elimination half-life is 90 days in rats [10] and 11.5 years in humans [11]. Based on recent studies with high purity PCB 180 in adult rats the biological effects include activation of the constitutive active (androstane) receptor (CAR) and the pregnane-X-receptor (PXR) resulting in the phenobarbital type induction of xenobiotic metabolism in liver (*i.e.* cytochrome P450 (CYP) 2B1/2, CYP3A1, UDP glucuronosyltransferase (UGT) 1A1, UGT1A6), behavioral changes, alterations in serum lipids and gonadotropins, hypothyroidism and associated changes in thyroid histopathology, alterations in tissue retinoid concentrations, and increased expression of biomarkers of DNA damage [6,9]. There were some differences in toxicological profile between males and females, *i.e.* males were more sensitive to most effects including most thyroid and liver related effects, such as induction of *Cyp2b1* mRNA and the associated 7-pentoxoresorufin O-dealkylase (PROD) activity.

Regarding retinoids, PCB 180 exposure for 28 days resulted in changes suggestive of increased mobilization of the storage forms of vitamin A, retinyl esters and retinol, into all-*trans*-retinoic acid [9]. The

retinoic acid signaling pathway regulates the homeostasis of the tissues through retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which form RAR-RXR heterodimers. In fact, the retinoid system is essential for a healthy organism, including its fertility, development and survival. Thus, hypervitaminosis, vitamin A deficiency as well as the disruption of the retinoid system due to exposure to chemical substances are potential causes of a variety of severe adverse effects including perturbations of bone development and maintenance [12,13]. Retinoids are also important regulators of thyroid and steroid hormone homeostasis, well-known targets of PCB 180. Retinoid signaling up-regulates steroidogenesis [14,15] and both RXR-thyroid hormone receptor (TR) and RAR-TR heterodimers mediate regulation of thyroid hormones [16].

Developmental exposure to NDL-PCB is a potential concern, not the least because of the demonstrated impact of PCB 180 on multiple endocrine pathways, which are well known to play fundamental roles in organ development and functional programming over the life-course [4, 5,9]. In experimental studies with the BeWo cell line and human placental perfusion *in vitro*, PCB 180 was able to rapidly pass the placental barrier [17]. PCB 180 has also been detected in human placenta [18] and cord blood [19,20].

The objective of the present study was, therefore, to determine the toxicological profile in developing offspring following *in utero* and lactational (IUL) exposure to PCB 180. The aim was also to carry out risk characterization and Margin of Exposure (MoE) calculations. The knowledge gaps have been identified for this compound by several scientific expert committees, such as the CONTAM panel of the European Food Safety Authority (EFSA [2]) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA [21]). To address these gaps, a regulatory relevant experimental protocol for rodents with extended observation time-points until early adulthood on postnatal day 84 was used as the core design [22–24]. We have already reported findings on neurodevelopmental and neurobehavioral alterations in the offspring of the present study, which included increased threshold of the brainstem auditory evoked potential in female offspring [7], increased preference of female offspring to drink solution sweetened with saccharin, which illustrates supernormal sexually dimorphic behavior [8], and pronounced and dose-dependent reduction in latencies to movement onset during haloperidol-induced catalepsy in male offspring, which suggests a dopaminergic effect of PCB 180 [25]. Skeletal and dental analysis indicated decreased facial length, shift in the palatal suture and altered molar size [26]. Furthermore, non-targeted metabolic profiling of serum samples from PND 84 offspring indicated gender and dose-related changes, males being more sensitive than females [27]. Altered endogenous metabolism included systematically decreased concentrations of glycerophospholipids (mainly lysophosphocholines), amino acids and their derivatives, and carnitines. These alterations were consistent with the observed neurodevelopmental, neurobehavioral and hepatic effects. The focus of the current study was therefore to identify and quantify additional PCB 180 sensitive and potentially apical effects and to find molecular links to endocrine and metabolic alterations including the retinoid system.

2. Materials and methods

2.1. Chemicals

PCB 180 (2,2',3',4,4',5,5'-heptachlorobiphenyl; CAS 35065-29-3; molecular weight 395.3 g/mol; batch No. 6115) was purchased from Chiron, Trondheim, Norway and analyzed. In brief, 20 mg PCB 180 was

dissolved in n-hexane and applied on an activated carbon column, flushed with n-hexane and then back-flushed with toluene to recover DL contaminants [28]. The toluene fraction was analyzed using a gas chromatograph interfaced with a high-resolution mass spectrometer tuned for identification of DL-PCBs and PCDD/Fs. The purity of PCB 180 as stated by the supplier was 98.9 % and the analyzed level of DL impurities as represented by sum of WHO-TEQ contamination was 2.7 ng/g PCB 180. The PCB was dissolved in purity-controlled corn oil (0.2 pg WHO-TEQ/g) applying the same protocol as described above for PCB 180 (Sigma Aldrich, Munich, Germany; batch No. 065K0077) which served also as control.

2.2. Animals

Outbred female and male Sprague Dawley rats were obtained from Harlan, Zeist, The Netherlands. During the study they were kept in a conventional laboratory animal unit subjected to regular health surveys consisting of serological and bacteriological screening as suggested by FELASA [29]. These surveys indicated that the animals were free of typical rodent pathogens. The rats were housed in stainless steel cages (floor area 1380 cm²) supplied with a bedding of aspen chips (Tapvei Co., Kaavi, Finland). Rooms had a 12 h light:12 h dark cycle, with the light phase starting at 7 am. The ambient temperature was 21 ± 2 °C, and relative humidity was maintained at 50 ± 20 %. Standard laboratory rodent diet (R36, Lactamin, Stockholm, Sweden; contains vitamin A 12000 IU/kg) and water were available ad libitum. For mating, females were observed for the stage of estrus cycle, one female was transferred to the cage of one male. The morning of the day on which females were sperm-positive was taken as gestational day 0 (GD 0). Thereafter, females were housed in single cages and acclimatized to the experimental conditions for one week before commencing with dosing.

2.3. Experimental design

All animal work was conducted in strict accordance with national, international and ARRIVE guidelines. The study protocol was approved by the National Animal Experiment Board of Finland (license No. ESLH-2006-07965/Ym23).

Pregnant females were randomized by body weight into 6 treatment groups, 7 dams per group (Table 1) using random numbers. The total number of animals in the study was 42 dams, 168 male offspring, 168 female offspring and 27 sires. Dams inseminated by the same male were allocated into different groups. Animals were individually marked with numbers on tail using a water-resistant pen, and the cages were labelled with color coded cage cards by treatment group. Total dose levels of PCB 180 were 0, 10, 30, 100, 300 or 1000 mg/kg body weight (bw). Total doses were divided into four equal daily doses and applied on GDs 7–10 by oral gavage. Dosing volume was 4 ml/kg bw. Controls received only corn oil (vehicle). Six dose levels were included to allow benchmark dose analysis of the results, which requires a modelling of dose-response relationship using the observed data. The dams were observed for

Table 1
Treatment groups, doses and number of animals.

Treatment group	Total dose (mg/kg bw)	Daily doses on GDs 7–10 (mg/kg bw)	Number of dams	Offspring / litter ^a	
				Males	Females
1. Control	0	4 × 0	7	4	4
2. PCB180	10	4 × 2.5	7	4	4
3. PCB180	30	4 × 7.5	7	4	4
4. PCB180	100	4 × 25	7	4	4
5. PCB180	300	4 × 75	7	4	4
6. PCB180	1000	4 × 250	7	4	4

^a One male and one female offspring were sampled on postnatal days 7, 35 and 84. In addition, one male and one female offspring were used for neurobehavioral testing [7,8,25]. GD, gestation day.

clinical signs twice daily except during weekends once daily, and they were weighed at the interval of 2–7 days as indicated in Fig. 1.

The day of birth is the postnatal day (PND) 0. The number of pups (live births, stillbirths) was counted as soon as possible after delivery, and more detailed examination of sex and presence of gross defects were carried out on PND 1. To allow uniform postnatal exposure the litter size was randomly adjusted to 8 (4 males and 4 females), if possible, on PND 1. Offspring were observed for clinical signs twice daily except during weekend once daily. More detailed observations were carried out once weekly. Anogenital distance was measured on PNDs 1 and 14, eruption of the first upper incisor was monitored starting on PND 7 and opening of eyes starting from PND 13. In male offspring the presence of areolae/nipples was recorded once on PND 12, and balano-preputial separation was monitored starting from PND 42. In female offspring vaginal opening was monitored starting from PND 30. Body weights were recorded on PNDs 1, 4, 7 and once weekly thereafter.

Offspring were weaned on PND 28, marked individually by a tattoo on pinna and up to 5 littermates of the same sex were housed together in one cage. Dams were anesthetized with CO₂/O₂ (70/30 %), blood samples were drawn from the left ventricle using Venoject needles (Terumo) and Vacuette EDTA and serum blood collection tubes, and the rats were killed by exsanguination. Samples of uterus, brain, liver and perirenal adipose tissue were collected, and the number of uterine implantation sites counted. One offspring of each sex from each litter was killed on PND 7, 35 and 84, by decapitation on PND 7, and by CO₂ asphyxiation and exsanguination on later times. Blood samples were taken and a full necropsy was carried out. Tissues were sampled and the weights of prostate, seminal vesicles, epididymis, testis, ovaries, uterus, pituitary gland, adrenal gland, thyroid gland, liver, brain, heart, spleen, kidney, lung and thymus were measured. The organs were stored at -80 °C, for subsequent analyses. Half of the left testis of PND 84 males was fixed in Bouin's solution and stored in 70 % ethanol for histological evaluation. The fourth male and female offspring from each litter were used for neurobehavioral testing as reported separately [7,8,25].

2.4. Adipose tissue PCB 180 concentrations

Perirenal adipose tissue from dams and male and female offspring of each treatment group were analyzed for PCB 180 concentrations. Dams were sampled on postpartum day 28 and offspring on PNDs 35 and 84. Samples were pooled (5–7 individuals per pool), freeze-dried and dry weight was determined. The samples and blanks were extracted by accelerated solvent extraction using hexane:dichloromethane (1:1), followed by an acid silica column cleanup. PCBs 103 and 198 were used as internal standards. Two blanks and a reference material were measured in the series. The extracts were analyzed by gas chromatography with electron capture detection with a double column system (CP-SIL 8 CB and CP-SIL 19 CB). Concentrations were calculated using external calibration standards. The concentrations were corrected for the blank signal. Determination of total lipids was performed according to Bligh and Dyer [30]. Data are shown as lipid based adipose tissue PCB 180 concentrations and as estimated body burdens. PCB 180 body burdens were calculated by multiplying the lipid based adipose tissue PCB 180 concentrations with the amount of body lipid estimated according to the regression equation established by Schoeffner et al. [31] for 4–40 weeks old male Sprague-Dawley rats:

$$\text{Body lipid} = 0.0724 \times \text{body weight} - 2.85.$$

2.5. Circulating concentrations of thyroid hormones

Serum free triiodothyronine (fT3) and free thyroxine (fT4) were analyzed by enzyme linked immunosorbent assay using the fT3 and fT4 Enzyme Immunoassay Test Kit by Genway (San Diego, CA, USA).

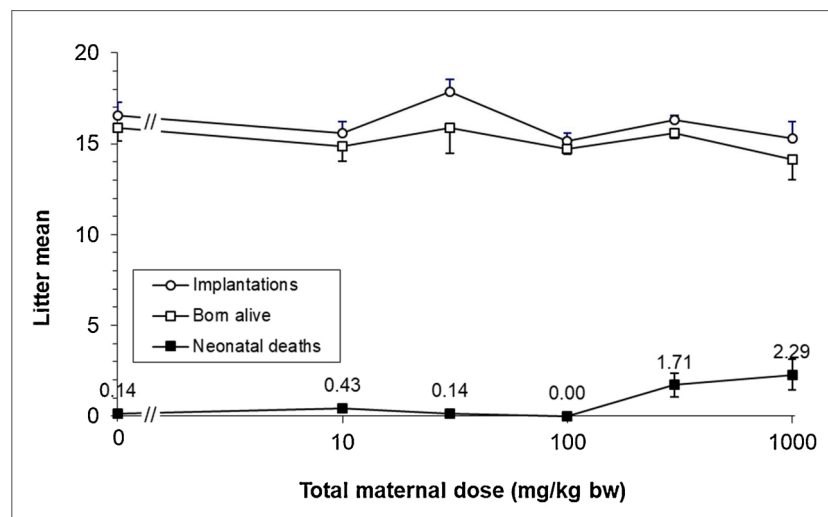


Fig. 1. Litter means of number of implantations, offspring born alive and neonatal deaths by postnatal day 1. Each datapoint represents mean \pm SE ($n = 7$), lin-log scale. The neonatal deaths were dose-dependently increased at 300 and 1000 mg/kg bw, benchmark dose (BMD_{10}) = 621 mg/kg bw, maximum efficacy (E_{max}) = 13 % and χ^2 Test's p-value < 0.001).

Samples were processed according to manufacturer's instructions and analyzed with a multilabel plate reader (Victor3, PerkinElmer, Finland) by comparing absorbances of the duplicate samples to the standard curve. The intra- and inter-assay coefficients of variation (CV) for T3 were 4.1 % and 5.2 %, respectively, and for T4 were 4.5 % and 3.7 %, respectively. Sensitivity was 0.05 pg/mL for both assays.

2.6. Circulating concentrations of steroid hormones and gonadotropins

Serum concentrations of steroid hormones, testosterone and estradiol, were measured by time-resolved immunofluorometric assay, DELFIA (PerkinElmer Life and Analytical Sciences, Turku, Finland) after diethyl ether (Merck KGaA, Darmstadt, Germany) extraction. Ether-extracted serum samples were reconstituted to DELFIA Diluent II buffer (PerkinElmer Life and Analytical Sciences, Turku, Finland) and used for analysis. The sensitivity of the assay was 100 pg/mL for testosterone and 13.6 pg/mL for estradiol. To enhance the sensitivity, commercial tracer and antiserum were additionally diluted 5:8 with assay buffer (PerkinElmer Life and Analytical Sciences, Turku, Finland) in testosterone assay. For estradiol, the dilution rate was 1:2.

Serum concentrations of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), were determined from unextracted samples by DELFIA as described previously [32,33]. The sensitivity of the rat FSH assay was 0.1 μ g/L, intra-assay CV 4.3 % and inter-assay CV 10.4 % at 4.8 μ g/L, and the sensitivity of the rat LH assay was 0.03 μ g/L, intra-assay CV 19 % at 0.04 μ g/L, 5 % at 1 μ g/L and inter assay CV 12.5 % at 0.24 μ g/L and 7.8 % at 0.78 μ g/L.

2.7. Testis histopathology and histomorphometry, cauda epididymal sperm density

Six-micrometer thick sections of paraffin embedded testes were mounted on microscopic slides (MENZEL-GLAZER, SuperFrost Plus, Germany) followed by clearing, rehydration, and staining with hematoxylin and eosin. Sections were examined under light microscope (DIAPLAN, Leitz, Wetzlar, Germany), and the diameters of the seminiferous tubules and the thickness of germinal epithelium at stage VII of seminiferous epithelial cycle were determined (10 measurements per testis) using a morphometric program (Leica IM500 Version: 4.0, Leica Microsystems Imaging Solutions Ltd., Cambridge, U.K.).

Frozen right cauda epididymis of control and the highest dose (1000 mg/kg bw) male offspring sampled on PND 84 were homogenized

for 2 min using Ultra Turrax homogenizer (model T25 basic, IKA-WERKE GmbH, Staufen, Germany) in 20 ml 0.9 % saline containing 0.05 % Triton X-100 and 0.01 % thiomersal. Homogenates were diluted to about 1×10^6 sperms/mL and counts from 4 hemocytometer chambers were counted and averaged.

2.8. Bone geometry, densitometry and biomechanics

Bone geometry, densitometry and biomechanics were assessed as described by Romero et al. [26]. Hind limbs were collected and frozen at -20°C . Dissected right tibias were cleaned from soft tissue and stored in Ringer solution at -20°C until analysis. The length of each bone was measured using an electronic sliding caliper to the nearest 0.01 mm (IP65, Sylvac SA, Crissier, Switzerland). The tibias were scanned using the peripheral quantitative computed tomography (pQCT) system (Stratec XCT Research SA+) with software version 5.50 (Norland Stratec Medizintechnik, GmbH, Birkenfeld, Germany). The growth plate was used as anatomical landmark to normalize analysis locations for metaphyseal trabecular bone and diaphyseal cortical bone. The cross-sectional images with voxel size of 0.07 mm were collected 10 and 45 % of femoral length distal from the growth plate for metaphysis and diaphysis, respectively. The thresholds for defining trabecular bone were 280 and 400 mg/cm^3 , while cortical bone was defined above a threshold of 710 mg/cm^3 .

For biomechanical testing hind limbs were defrosted and tibial shafts tested with a three-point bending test using the Instron universal testing machine (Instron 3366, Instron Corp., Norwood, MA). Each bone was placed on a support with a span length of 13 mm and bending load was applied with a constant speed of 0.155 mm/sec until failure and biomechanical properties were evaluated from load-displacement data. Bending stiffness was calculated as the slope of the linear part of load-displacement curve. Yield point was defined as a corresponding point where the recorded load-displacement data separated from the fitted slope for stiffness. Strength was evaluated as peak force, and to estimate toughness applied energies were analyzed at peak force and breaking point.

2.9. Hepatic and renal retinoid concentrations

Retinoids in liver and kidney were analyzed according to Schmidt et al. [34]. The polar retinoids 13-*cis*-4-oxo-retinoic acid, 9-*cis*-4-oxo-13, 14-dihydro-retinoic acid, all-*trans*-retinoic acid and the apolar retinoids

retinol and retinyl esters, predominantly retinyl palmitate, were extracted by a single liquid-extraction, separated from each other *via* solid-phase extraction using an aminopropyl phase, and then injected onto two different HPLC systems that were coupled with UV detection. The polar retinoids were separated on a Spherisorb ODS2 column (2.1×150 mm, $3 \mu\text{m}$ particle size, Waters, Eschborn, Germany) using a binary gradient and UV detection at 340 nm. The limits of detection (LOD) for all-*trans*-retinoic acid and 9-*cis*-4-oxo-13,14-dihydro-retinoic acid were 0.7 and 1.0 pmol/g tissue. retinol and retinyl palmitate were separated on a J'sphere ODS-H80 (4.6×150 mm, $4 \mu\text{m}$ particle size) obtained from YMC (Schermbach, Germany), and were detected at 325 nm. The LOD for retinol and retinyl palmitate were 5.6 and 5.5 pmol/g tissue.

2.10. Liver mRNA isolation and real-time PCR analysis of selected metabolic enzymes

Total mRNA was isolated from 0.5 cm^3 frozen liver samples according to the Total RNA Isolation Reagent (TRIR)-protocol from ABgene (Epsom, UK). The work was performed in an RNase free environment using RNase free agents either on ice or in a 4°C cooled centrifuge. Samples were homogenized with a homogenizer in the presence of 1.5 ml TRIR reagent. After separating the aqueous phase from the organic phase through adding chloroform to the homogenate and centrifugation at $12,000 \times g$ for 15 min, the mRNA was precipitated with isopropanol from the aqueous phase and centrifuged for 10 min at $12,000 \times g$. The resulting mRNA pellet was washed twice with 75 % ethanol, centrifuged each time for 5 min at $7500 \times g$ and briefly dried under vacuum. The pellet was resolved in 100 μl DEPC treated water and the RNA concentrations were quantified using a Nanodrop ND-1000 spectrometer (Thermo Scientific, Dreieich, Germany). The mRNA concentrations of samples were adjusted to a final value of 100 ng/ μl and reverse transcribed into cDNA using the iScript cDNA Synthesis kit from BIO-RAD (Munich, Germany). The focus was on genes representing enzymes related to CAR, PXR and AHR mediated metabolism of xenobiotics as well as retinoic acid. The primers for the Real-time PCR analysis, listed in Table S1, were designed using the Beacon Designer 3.0, a PREMIER Biosoft International software and blasted to avoid secondary products. Real-time PCR analysis was performed with the Absolute QPCR SYBR Green Fluorescein Mix from ABgene with a iCycler iQ (Bio-Rad, Munich, Germany). All primers were validated to assure an efficiency of nearly 100 %, determined by standard curve and PCR-product by melting curve analysis. The β -actin gene product used as a reference [35] was not affected by the treatment. The ratios were calculated using the $\Delta\Delta C_T$ method.

2.11. Hepatic PROD and EROD activities

The CAR-associated enzymatic activity of CYP2B1 was measured in liver microsomes as 7-pentoxoresorufin O-dealkylase (PROD) activity while the AHR-associated enzymatic activities CYP1A1, 1A2 and 1B1 were measured as 7-ethoxoresorufin O-deethylase (EROD) activity [36] using a modified method according to [37]. All steps were carried out at 4°C . Frozen liver samples, about 0.5 cm^3 , were homogenized in the presence of 4 ml of an isotonic extraction buffer (containing: 10 mM HEPES pH 7.8, 0.25 M saccharose, 1 mM EGTA, 25 mM KCl, in dd-H₂O pH 7.8, sterile filtered) containing a protease inhibitor cocktail (0.1 %). Samples were centrifuged at $1000 \times g$ for 10 min. The supernatants were centrifuged again at $12,000 \times g$ for 15 min. After this step the supernatants were centrifuged at $100,000 \times g$ for 60 min. The pellets were suspended in 100 μl Na/Pi-Buffer (containing: 47.4 mM Na₂HPO₄, 2.6 mM NaH₂PO₄, in dd-H₂O, pH 8.0), homogenized with an ultrasonic probe and stored at -80°C . CYP activity was determined in 96-wellplates with a Fluoroskan Ascent FL plate reader (Labsystems). For the assay, 3 μl of the isolated microsomes were added to each well and diluted with 122 μl of Na/Pi-Buffer. The substrates 7-ethoxoresorufin for EROD activity and

7-pentoxoresorufin for PROD activity were diluted in methanol to generate their respective 1.05 mM solutions. 25 μl of a 1:30 dilution in Na/Pi-Buffer of the 1.05 mM solution were added into the well for measuring the respective EROD and PROD activities. The plate was set into the plate reader and incubated for 10 min at 37°C . To start the reaction, 10 μl NADPH (Biomol, Hamburg, Germany) (13.4 mM in dd-H₂O) were added to the well. After 10 min, the reaction was stopped by adding 65 μl of a fluorescamine:acetonitrile solution (150 g/mL). The fluorescence of resorufin was measured at an excitation of 544 nm and an emission of 590 nm. For the fluorescence of the fluorescamine-protein complex, excitation at 390 nm and emission at 460 nm was used. The contents of resorufin and protein per well were extrapolated from calibrating curves of resorufin (from 0 to 135 pmol/well) and BSA (from 0 to 0.1 mg/well) standards, respectively.

2.12. Statistical analysis and dose-response modelling

Statistical calculations were performed with the Rcmdr package in the R software version 3.1.1 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). For comparisons between groups the equality of variances was first confirmed with Levene's test. If the variances were homogenous, one-way analysis of variance (ANOVA) was carried out. If the variances were heterogeneous even after appropriate transformations, the nonparametric Kruskal-Wallis test was carried out. The limit for statistical significance was $p < 0.05$ (two tailed). Statistical analyses were conducted separately for males and females.

Data were also analyzed using the benchmark dose (BMD) method, which is based on dose-response modelling of the full data set using a nested family of exponential models [38]. From this family, the software selects a model that differs significantly from the no-effect model ($E(y) = a$) and that optimally describes the dose-response. BMD calculations for continuous endpoints were carried out using the Proast 38.9 package (RIVM, Bilthoven, The Netherlands) in the R software version 3.1.1 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) [38,39]. The benchmark dose response (BMR) of 5 % was used as a default value to calculate BMD₀₅ for analysis of continuous variables since the lower bound of the 90 % confidence interval (BMDL) is generally close to the no-observable-adverse-effect level (NOAEL) [39]. However, this default BMR was modified based on statistical and toxicological considerations, *i.e.* parameters with a known high maximum response [38]. Thus, a BMR of 100 % was used to calculate BMD₁₀₀ for PCB-inducible EROD and PROD activities. A BMR of 200 % was used to calculate BMD₂₀₀ for expression of CYP genes [38].

BMD calculations for neonatal deaths, a quantal endpoint, were carried out using the Proast 70.0 package (RIVM, Bilthoven, The Netherlands) in the R software version 4.0.3 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). The recommended BMR default for quantal variables was used, *i.e.* an extra risk of 10 % [40]. Model averaging was applied by using two-stage, log-logist, Weibull, log-prob, gamma and latent variable models of exponential and Hill families [41]. The BMD confidence intervals were obtained from the selected models. For comparisons, the BMD₁₀ of the model with the highest weight was reported.

2.13. Margin of Exposure (MoE) calculations and risk characterization

For MoE calculations the dose based BMDL values of sensitive endpoints in the offspring (Table 2) were first transformed to lipid based BMDL values using the following regression equation on total doses (Table 1) vs maternal adipose tissue PCB 180 concentrations determined on postpartum day 28:

$$\text{Adipose tissue concentration} = 0.179 \times e^{(3.74 \times [\text{oral dose}]^{0.148})}$$

Endpoints with the lowest BMDL values representing adversity and/

Table 2

Significant dose-response relationships and the outcome of benchmark dose-response modeling.

Parameter	Sex	PND	Model	BMR	BMD (mg/kg bw)	BMDL (mg/kg bw)	Ratio BMD/BMDL	E _{max}
Neonatal deaths			Average ^a	10 %	621	400	1.55	13 % ^b
Organ weights								
Liver absolute weight	M	7	E4	5 %	11.9	5.05	2.36	35 %
Liver relative weight	M	7	E3	5 %	9.96	1.41	7.09	46 %
Liver absolute weight	F	7	E4	5 %	3.54	0.54	6.52	42 %
Liver relative weight	F	7	E4	5 %	35.0	7.99	4.38	37 %
Liver absolute weight	M	35	E4	5 %	30.5	4.68	6.52	29 %
Liver relative weight	M	35	E4	5 %	47.1	25.8	1.83	31 %
Liver absolute weight	F	35	E4	5 %	42.8	16.4	2.60	30 %
Liver relative weight	F	35	E5	5 %	47.1	7.23	6.52	33 %
Liver absolute weight	M	84	E4	5 %	29.0	8.42	3.44	21 %
Liver relative weight	M	84	E2	5 %	280	228	1.23	19 %
Heart absolute weight	M	7	E3	5 %	841	51.8	16.2	–25 %
Lungs relative weight	F	35	E2	5 %	396	225	1.76	13 %
Lungs absolute weight	M	84	E2	5 %	276	177	1.56	–17 %
Lungs relative weight	M	84	E2	5 %	222	144	1.55	–21 %
Thymus absolute weight	M	7	E2	5 %	133	81.8	1.63	–32 %
Thymus relative weight	M	7	E2	5 %	227	148	1.53	–20 %
Thymus absolute weight	F	7	E2	5 %	158	97.9	1.61	–28 %
Thymus relative weight	F	7	E2	5 %	241	167	1.44	–19 %
Pituitary relative weight	M	84	E2	5 %	391	256	1.53	13 %
Adrenal absolute weight	M	84	E2	5 %	458	267	1.71	–11 %
Adrenal relative weight	M	84	E4	5 %	89.1	35.0	2.55	–11 %
Prostate absolute weight	M	35	E2	5 %	115	76.8	1.50	–36 %
Prostate relative weight	M	35	E2	5 %	122	82.0	1.49	–34 %
Prostate absolute weight	M	84	E2	5 %	222	148	1.50	–21 %
Prostate relative weight	M	84	E2	5 %	242	154	1.58	–19 %
Seminal vesicles absolute weight	M	84	E2	5 %	345	212	1.63	–14 %
Seminal vesicles relative weight	M	84	E2	5 %	398	228	1.74	–12 %
Bone parameters of tibial diaphysis								
Cortical density	M	84	E3	–	–	–	–	–2.3 %
Cortical area	F	84	E2	5 %	839	519	1.62	6.0 %
Cortical thickness	F	84	E2	5 %	953	598	1.59	5.3 %
Serum hormone levels								
Testosterone	M	35	E2	5 %	66.0	46.9	1.41	–54 %
Testosterone	M	84	E4	5 %	8.42	3.23	2.61	–50 %
Follicle stimulating hormone	M	35	E2	5 %	204	119	1.71	–22 %
Estradiol	F	35	E2	5 %	111	65.8	1.69	–37 %
Luteinizing hormo	F	35	E2	5 %	93.2	55.6	1.68	69 %
Free triiodothyronine	F	Dams	E4	5 %	40.7	27.3	1.49	–33 %
Free triiodothyronine	F	35	E2	5 %	131	80.2	1.63	–32 %
Free triiodothyronine	M	84	E4	5 %	36.8	22.7	1.62	–36 %
Free triiodothyronine	F	84	E4	5 %	16.2	7.84	2.07	–40 %
Free thyroxine	F	Dams	E2	5 %	434	279	1.56	–11 %
Free thyroxine	M	35	E3	5 %	781	522	1.50	–18 %
Free thyroxine	M	84	E2	5 %	434	264	1.64	–11 %
Hepatic cytochrome P450 activities								
EROD	M	7	E2	100 %	173	147	1.18	5442 %
EROD	F	7	E2	100 %	176	157	1.12	5016 %
EROD	M	35	E2	100 %	308	275	1.12	847 %
EROD	F	35	E2	100 %	264	232	1.14	1287 %
EROD	M	84	E2	100 %	840	685	1.23	128 %
EROD	F	84	E4	–	–	–	–	50 %
PROD	M	7	E5	100 %	10.8	7.57	1.43	673 %
PROD	F	7	E5	100 %	9.10	6.18	1.47	852 %
PROD	M	35	E4	100 %	12.1	7.97	1.52	1592 %
PROD	F	35	E4	100 %	22.3	14.5	1.54	1903 %
PROD	M	84	E2	100 %	347	293	1.19	637 %
PROD	F	84	E2	100 %	989	658	1.50	102 %
Retinoid concentrations								
Liver retinyl palmitate	M	84	E2	5 %	119	91.0	1.31	–35 %
Liver retinyl palmitate	F	84	E2	5 %	142	103	1.38	–30 %
Liver retinol	M	35	E4	5 %	24.8	14.9	1.66	–58 %
Liver retinol	F	35	E4	5 %	7.00	2.45	2.86	–45 %
Liver all- <i>trans</i> -retinoic acid	M	35	E2	5 %	112	71.9	1.56	55 %
Liver all- <i>trans</i> -retinoic acid	F	35	E4	5 %	9.76	2.71	3.60	83 %
Liver 9- <i>cis</i> -4-oxo-13,14-dihydro-retinoic acid	M	35	E2	5 %	78.5	53.2	1.48	–48 %
Liver 9- <i>cis</i> -4-oxo-13,14-dihydro-retinoic acid	F	35	E2	5 %	61.7	40.7	1.52	–56 %
Liver 13- <i>cis</i> -4-oxo-retinoic acid	M	35	E4	5 %	6.22	2.09	2.98	112 %
Kidney retinyl esters	M	35	E2	5 %	63.1	41.6	1.52	117 %
Kidney retinyl esters	F	35	E2	5 %	57.6	39.5	1.46	133 %
Kidney retinol	M	35	E2	5 %	430	244	1.77	12 %
Hepatic gene expression								
<i>Cyp1a1</i>	F	7	E3	200 %	61.4	22.5	2.73	13128 %

(continued on next page)

Table 2 (continued)

Parameter	Sex	PND	Model	BMR	BMD (mg/kg bw)	BMDL (mg/kg bw)	Ratio BMD/BMDL	E _{max}
<i>Cyp1a1</i>	M	35	E2	200 %	950	710	1.34	218 %
<i>Cyp1a1</i>	M	84	E2	–	–	–	–	79 %
<i>Cyp2b1</i>	F	7	E5	200 %	8.44	5	1.58	3320 %
<i>Cyp2b1</i>	M	35	E5	200 %	8.18	4	1.85	29996 %
<i>Cyp2b1</i>	F	35	E5	200 %	12.8	7	1.83	13900 %
<i>Cyp2b1</i>	M	84	E5	200 %	27.8	17	1.60	26536 %
<i>Cyp2b1</i>	F	84	E5	200 %	41.2	25	1.64	38298 %
<i>Cyp3a1</i>	F	7	E5	200 %	44.1	18	2.51	2200 %
<i>Cyp3a1</i>	M	35	E4	200 %	123	53	2.31	1465 %
<i>Cyp3a1</i>	F	35	E2	200 %	534	440	1.21	681 %
<i>Cyp3a1</i>	M	84	E2	–	–	–	–	125 %
<i>Cyp3a1</i>	F	84	E2	200 %	921	670	1.37	230 %

PND, postnatal day; BMR, benchmark dose response; BMD, benchmark dose; BMDL, lower bound of the 90 % confidence interval of the BMD; E_{max}, maximum efficacy; PROD, 7-pentoxeresorufin O-dealkylase; CYP, cytochrome P450.

^a Model averaging of two-stage, log-logist, Weibull, log-prob, gamma and latent variable models of exponential and Hill families. BMD of log-probit (model 8): 621 mg/kg bw, which was the model with the highest weight. BMD confidence interval: 400–1110 mg/kg bw.

^b Maximum response calculated as extra risk for quantal variables.

or different endocrine or metabolic effects were selected for MoE calculations. Maternal dosimetry was considered as an appropriate basis of MoE calculations because maternal dose is critical for later life responses, tissue levels in the offspring are less constant and tissue level data on newborn or children are difficult to obtain. For MoE calculations, lipid-based BMDL values were divided by human lipid-based PCB 180 concentrations of different representative cohorts as shown in Table 3. Uncertainty factors (UF) established by WHO [42] for estimating tolerable intake levels of chemicals were used. As lipid-based adipose tissue concentrations of PCB 180 were available for both rat dams and humans, no interspecies toxicokinetic UF was needed, and an UF of 2.5 for toxicodynamics was applied to extrapolate the observed endpoints from rats to humans. An additional UF of 10 was used for inter-individual variability in human general populations. Hence, the UF of 25 was used for comparisons against calculated MoEs.

2.14. Quality assurance

The in-life phase of the study was carried out according to the principles of Good Laboratory Practice (GLP) at the National Institute for Health and Welfare. The Institute does not have an official GLP status, but the study was audited and site-visited by the internal Quality Assurance Unit.

3. Results

Group mean values (\pm SD) and statistically significant differences from controls for most of the analyzed parameters are presented in Supplementary Tables S2–S9. Significant dose-response relationships and BMD modeling parameters for all effects where the characteristics of the dose-response data made it technically possible to establish a BMD are listed in Table 2. Therefore, Table 2 includes adverse, potentially adverse, reversible as well as mechanistic endpoints as analyzed in the present study.

3.1. General observations and developmental milestones

There were no remarkable clinical signs and no mortality in dams or in offspring. No dose-related or consistent changes were observed in fetal mortality and male/female ratio of the newborn. Number of offspring born alive was not affected by the treatment, but neonatal deaths observed by PND 1 were dose-dependently increased at 300 and 1000 mg/kg bw (BMD₁₀ 621 mg/kg bw, maximum efficacy (E_{max}) 13 % and χ^2 Test's p-value < 0.001) (Fig. 1, Table 2).

No statistically significant differences between controls and treated groups were observed in body weights of dams and offspring at any time point. However, body weight of dams at the highest dose of 1000 mg/kg

bw was slightly and transiently decreased at the end of the dosing period, but it was caught up by study day 18 (Fig. 2). In offspring, body weight was very similar in all treatment groups on PND 1, but thereafter the body weight at 1000 mg/kg bw started to decrease slightly and transiently in both genders (Fig. 3). Mean body weight values were close to control animals towards the end of the observation period. On PND 84, mean body weight of control males was lower than in the other groups, and the highest body weight was at 300 mg/kg bw, 13 % above controls (ANOVA, $p = 0.021$) followed by 100 mg/kg bw. A similar pattern was observed in females.

Analysis of developmental milestones revealed slight delays in the frequency of balano-preputial separation at 1000 mg/kg bw between PNDs 44–49, after which the frequency reached the control level (Fig. S1A). At lower dose levels the frequency of balano-preputial separation was slightly faster than in controls. Vaginal opening was quite similar in all treatment groups until PND 38 after which the frequency of opening was delayed at 1000 mg/kg bw (Fig. S1B). Vaginal opening was complete in all other groups on PNDs 39–40 except at 1000 mg/kg bw/day on PND 47. Tooth eruption, eye opening, presence of areolae/nipples or ano-genital distance and ano-genital index (ratio of ano-genital distance to cube root of body weight) were not affected by the treatment (data not shown).

3.2. Adipose PCB 180 tissue concentrations

Analysis of adipose tissue PCB 180 concentration of control dams on postpartum day 28 as well as control male and female offspring on PNDs 35 and 84 revealed low concentrations within the range of human background exposure levels indicating lack of contamination of the animals (Fig. 4A, Table S2). In PCB 180 treated groups, concentrations reflected the administered doses showing an increase with increasing dose. Overall, female offspring showed higher concentrations than male offspring, and the difference was larger on PND 84 than on PND 35. Mean concentrations in offspring on PND 35 were about 50 % and 25 % lower than in dams on postpartum day 28 at maternal total doses of 100 and 1000 mg/kg bw, respectively. The corresponding figures on PND 84 were about 14 % and 8 %, respectively. In the offspring the adipose tissue concentrations on PND 84 were about 27 % and 47 % of the values on PND 35 at 100 and 1000 mg/kg bw, respectively.

Although female offspring had clearly higher adipose tissue PCB 180 concentration than male offspring, this difference disappears when comparing PCB 180 body burdens (concentration adjusted with estimated lipid mass) (Fig. 4B). This suggests that the gender difference in adipose tissue PCB 180 concentration is due to faster growth of males (dilution by growth) rather than difference in rate of elimination. Elimination of PCB 180 seems to be very slow and quite similar in male and female offspring.

Table 3

Margins of exposure (MoE¹) for some human cohorts on the basis of selected sensitive endpoints determined in rat offspring after *in utero* and lactational exposure to PCB 180.

Endpoint	Sex	PND	BMDL ₀₅	Human cohort, MoE								
				Mother's milk (Europe) ²		Women (Spain) ³			Pregnant women (Italy) ⁴		Baltic Sea Fishermen ⁵	
			(µg/g lipid)	Median	Max	50 th	75 th	95 th	Median	Max ⁶	Median	Max ⁶
<i>Liver weights</i>												
Liver absolute weight	F	7	6	131	18	36	30	16	77	14	13	5
Liver absolute weight	M	7	21	459	62	125	105	54	266	47	46	18
Liver absolute weight	F	35	51	1114	151	309	260	134	659	117	111	43
Liver absolute weight	M	35	20	437	59	119	100	51	253	45	43	17
Liver absolute weight	M	84	30	655	89	182	153	79	387	69	65	25
<i>Serum hormone concentrations</i>												
Testosterone	M	84	15	328	45	92	78	40	197	35	33	13
Free triiodothyronine	F	84	68	1485	202	408	344	177	870	154	148	57
Free triiodothyronine	M	84	29	633	86	172	145	75	367	65	63	24
<i>Hepatic retinoid concentrations</i>												
Retinol	F	35	13	280	38	77	65	33	164	29	28	11
Retinol	M	35	47	1035	141	286	241	124	609	108	103	40
Retinol	F	84	15	338	46	93	79	40	199	35	34	13
All-trans-retinoic acid	F	35	14	298	41	82	69	36	175	31	30	11
13-cis-4-oxo-retinoic acid	M	35	12	253	34	70	59	30	149	26	25	10
<i>Hepatic enzyme activity</i>												
PROD	F	7	24	524	71	144	122	63	308	55	52	20
PROD	M	7	28	611	83	168	141	73	357	63	61	23
PROD	F	35	46	1004	136	277	234	120	591	105	100	38
PROD	M	35	29	633	86	175	147	76	372	66	63	24

F, females; M, males; BMDL₀₅, lower confidence limit of the benchmark dose; PROD, pentoxoresorufin-O-dealylase. MoE values were calculated with 50th (median), 75th (percentile 75th), 95th (percentile 95th), and Max (maximum) of PCB 180 adipose tissue concentration in the human cohort. MoE values ≤25 are bolded.

PND, postnatal day; BMDL, lower bound of the 90 % confidence interval of the BMD; PROD, 7-pentoxoresorufin O-dealkylase.

¹ MoE = BMDL₀₅ (µg/g lipid) in dams / human lipid-based PCB 180 concentration. Oral dose based BMDL₀₅ values (Table 2) were transformed to lipid-based BMDL₀₅ values using the following regression model on total dose vs maternal adipose tissue PCB 180 concentrations: $Adipose\ tissue\ concentration = 0.179 \times e^{(3.74 \times [oral\ dose]^{0.148})}$.

² Mother's milk, 58 milk pools from 18 European countries, 2001–2002, median 45.8 ng/g lipid, range 6.1–337 ng/g lipid. EFSA (2005).

³ Women, n = 20. Median = 166 ng/g lipid; percentile 75th = 197 ng/g lipid; percentile 95th = 383 ng/g lipid. Fernández et al., (2008).

⁴ Pregnant women, n = 70. Median = 77.9 ng/g lipid; Max = 439.3 ng/g lipid. Bergonzi et al., (2009).

⁵ Baltic Sea fishermen, 1997–1999, median 460 ng/g lipid, range 190–1200 ng/g lipid. Kiviranta et al., (2002).

⁶ Maximum concentrations are from single individuals and therefore not used for risk characterization.

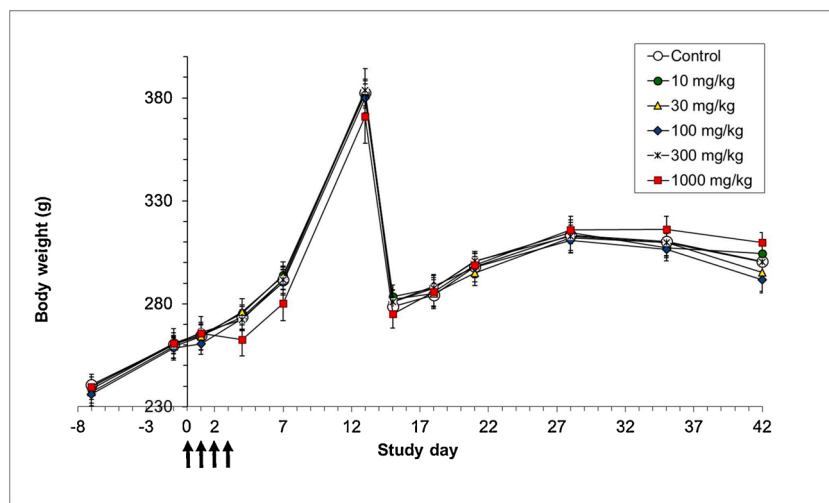


Fig. 2. Body weight of dams throughout the study. The arrows indicate dosing. Each datapoint represents mean ± SE (n = 7). After the dosing period the body weight was transiently and non-significantly decreased at 1000 mg/kg bw/day. Study day 0 = gestational day 7 and study day 15/16 = postnatal day 0.

3.3. Organ weights

Absolute and relative organ weights of dams sampled on postpartum day 28 and those of offspring sampled on PNDs 7, 35 and 84 are shown in Table S3. No significant changes of organ weights were observed in dams.

In the offspring, absolute and relative liver weights were increased at

all time points and in both sexes, except in female offspring on PND 84 (Tables 2, S3). The increase attenuated over time as adipose tissue PCB 180 concentration decreased. On PND 84 the liver weight remained elevated in males, but not in females despite of the 2.5-fold higher adipose tissue PCB 180 concentration in females (Tables S2, S3). On PND 7, BMD₀₅ of increased absolute liver weight was 11.9 and 3.54 mg/kg bw in males and females, respectively, and BMD₀₅ of increased relative liver

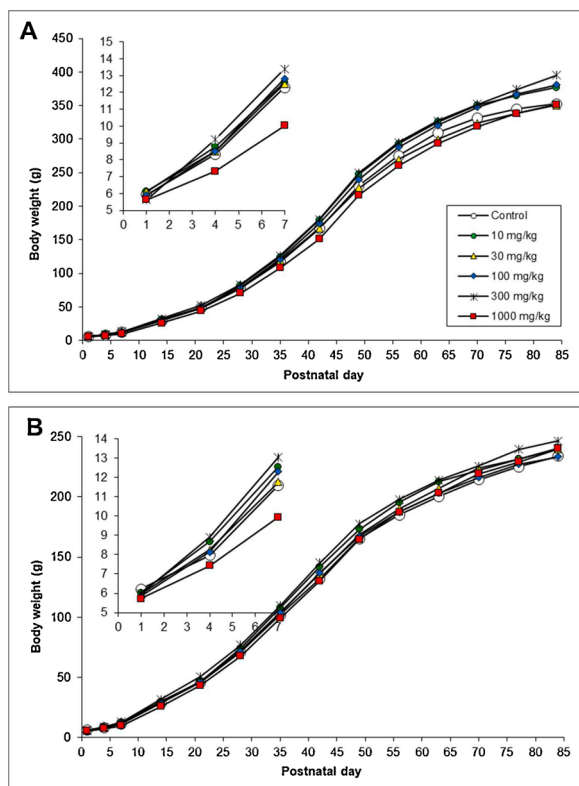


Fig. 3. Body weight of male (A) and female (B) offspring on postnatal days 1–84. Each datapoint represents mean \pm SE ($n = 7$ –28). Body weight was transiently and non-significantly decreased at 1000 mg/kg bw during the few first weeks after birth.

weight 9.96 and 35 mg/kg bw (Tables 2, S3). The E_{\max} for absolute liver weight was 35 and 42 % and for relative liver weight 46 and 37 %, respectively. On PND 35, BMD₀₅ of increased absolute and relative liver weight was higher, i.e., 30.5 and 47.1 mg/kg bw in males and 42.8 and 47.1 mg/kg bw in females, respectively. E_{\max} for absolute liver weight was 29 and 31 % and for relative liver weight 30 and 33 %, respectively. On PND 84, BMD₀₅ of increased absolute and relative liver weight in males was 29 and 280 mg/kg bw, and the E_{\max} 21 and 19 %, respectively.

On PND 7, absolute heart weight was decreased in male offspring at 1000 mg/kg bw (BMD 841 mg/kg bw, E_{\max} –25 %) (Tables 2, S3). Likewise, on PND 7 absolute and relative thymus weights were decreased at 1000 mg/kg in male (BMD₀₅ 133 and 227 mg/kg bw, E_{\max} –32 and –20 %, respectively) and female offspring (BMD₀₅ 158 and 241 mg/kg bw, E_{\max} –28 and –19 %, respectively) (Tables 2, S3). On PNDs 35 and 84, heart and thymus weights of PCB 180-treated groups did not differ from controls.

Absolute and relative prostate weight was decreased both on PND 35 (BMD₀₅ 115 and 122 mg/kg bw, E_{\max} –36 and –34 %) and PND 84 (BMD₀₅ 222 and 242 mg/kg bw, E_{\max} –21 and –19 %). In addition, absolute and relative seminal vesicle weight was decreased on PND 84 (BMD₀₅ 345 and 398 mg/kg bw, E_{\max} –14 and –12 %). Likewise, absolute and relative adrenal weights were decreased in male offspring on PND 84 (BMD₀₅ 458 and 89.1 mg/kg bw, respectively, E_{\max} –11 % for both weights). On PND 84 relative pituitary weight was slightly increased in male offspring (BMD₀₅ 391 mg/kg bw, E_{\max} 13 %).

On PND 35, relative lung weight was slightly increased in female offspring (BMD₀₅ 396 mg/kg bw, E_{\max} 13 %), but on PND 84 absolute and relative lung weight was decreased in male offspring (BMD₀₅ 276 and 222 mg/kg bw, E_{\max} –17 and –21 %). These changes showed significant dose-response relationships according to BMD modelling, but none of the groups differed significantly according to ANOVA.

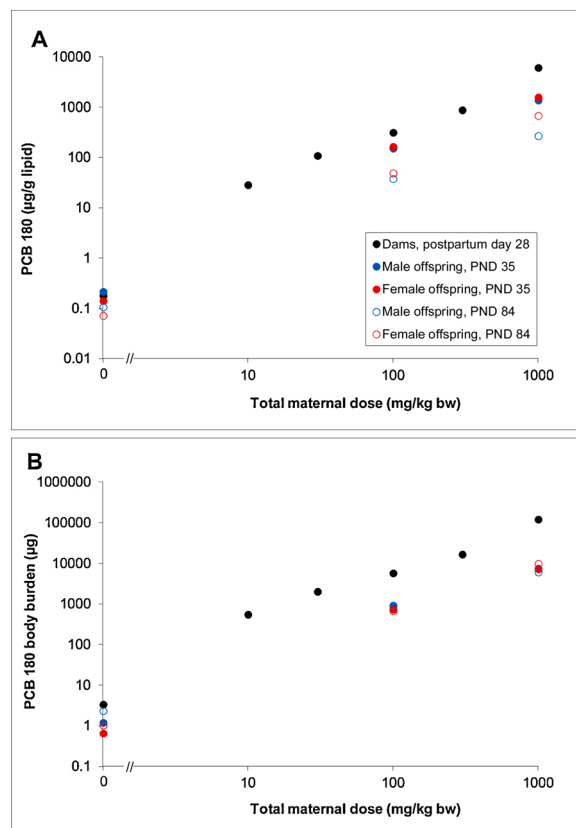


Fig. 4. Lipid based adipose tissue PCB 180 concentrations (A) and estimated body burdens (B) of dams and male and female offspring at sampling time points. Pooled samples of treatment groups, 5–7 individuals per pool, log-log scale. PCB 180 tissue concentrations reflected accurately the administered doses and indicated slow elimination. PND, postnatal day.

No significant changes were observed in weights of brain, spleen, kidney, thyroid gland, testis, epididymis or ovaries at any sampling time points (Table S3).

3.4. Circulating hormone concentrations

Serum concentrations of the thyroid hormones fT3 and fT4, the steroid hormones testosterone in males and estradiol in females as well as gonadotropins LH and FSH are shown in Table S4. Thyroid hormones were analyzed in dams and in the offspring on PND 35 and 84, and steroid hormones and gonadotropins only in the offspring (estradiol and gonadotropins of females were not analyzed on PND 84).

In dams, serum fT3 and fT4 concentrations were significantly decreased; fT3 decrease occurred at much lower dose (BMD₀₅ 40.7 mg/kg bw, E_{\max} –33 %) than that of fT4 (BMD₀₅ 434 mg/kg bw, E_{\max} –11 %) (Fig. 5, Tables 2, S4). In PND 35 offspring, serum fT3 concentrations were significantly decreased only in females (BMD₀₅ 131 mg/kg bw, E_{\max} –32 %) and serum fT4 concentrations only in males (BMD₀₅ 781 mg/kg bw, E_{\max} –18 %). On PND 84, serum fT3 concentrations were decreased in both male and female offspring (BMD₀₅ 36.8 and 16.2 mg/kg bw, E_{\max} –36 and –40 %, respectively), and fT4 concentrations only in male offspring (BMD₀₅ 434 mg/kg bw, E_{\max} –11 %). Overall, fT3 decrease was more sensitive and pronounced than fT4 decrease, PND 84 offspring were more affected than PND 35 offspring and fT4 decrease was limited to male offspring.

In male offspring, serum testosterone levels were decreased on PND 35 (BMD₀₅ 66 mg/kg bw, E_{\max} –54 %) and PND 84 (BMD₀₅ 8.42 mg/kg bw, E_{\max} –50 %) (Fig. 6, Tables 2, S4). In addition, serum FSH was decreased on PND 35 (BMD₀₅ 204 mg/kg bw, E_{\max} –22 %), but on PND

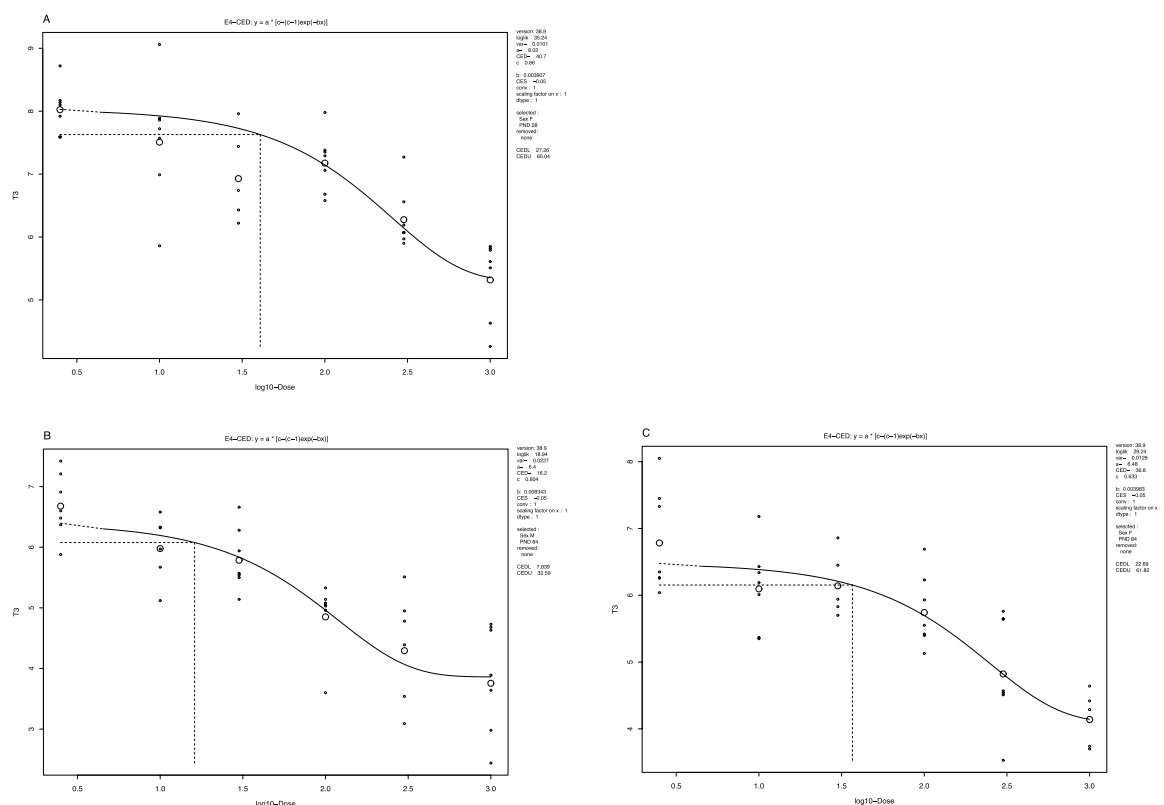


Fig. 5. BMD analysis of serum free triiodothyronine (T3) concentration (pmol/L) in dams on postpartum day 28 (A), male (B) and female offspring (C) on postnatal day (PND) 84. Small symbols indicate individual samples, large circles the group mean; the vertical dotted line indicates the critical effect dose (CED = BMD₀₅) calculated at a critical effect size (CES = BMR) of 5 % decrease (CES = −0.05) compared to background (a parameter). PND, postnatal day; BMD, benchmark dose; CEDL = BMDL, lower bound of the 90 % confidence interval of the BMD; BMR, benchmark dose response.

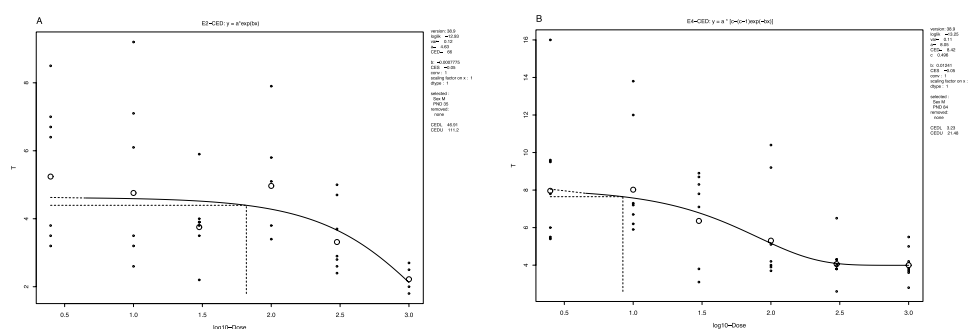


Fig. 6. BMD analysis of serum testosterone (T) concentration (ng/mL) in male offspring on PND 35 (A) and PND 84 (B). Small symbols indicate individual samples, large circles the group mean; the vertical dotted line indicates the critical effect dose (CED = BMD₀₅) calculated at a critical effect size (CES = BMR) of 5 % decrease (CES = −0.05) compared to background (a parameter). PND, postnatal day; BMD, benchmark dose; CEDL = BMDL, lower bound of the 90 % confidence interval of the BMD; BMR, benchmark dose response.

84 FSH concentrations of 3 high dose males were exceptionally high (>10 ng/mL) while the rest of the values were normal. This difference did not reach statistical significance. The male with highest FSH value (27.32 ng/mL) also had very high LH value (4.35 ng/mL). In female offspring, serum estradiol concentrations were decreased (BMD₀₅ 111 mg/kg bw, E_{\max} −37 %) and serum LH levels increased (BMD₀₅ 93.2 mg/kg bw, E_{\max} 69 %) on PND 35 (no data on PND 84) (Tables 2, S4). These changes were limited to the highest dose level of 1000 mg/kg bw.

3.5. Testis histopathology and cauda epididymal sperm density

Histopathological evaluation of testis sampled on PND 84 revealed adverse effects limited to 2 out of 7 male offspring at 1000 mg/kg bw. They included local damage and vacuolization of germinal epithelium and decreased number of germ cells of seminiferous tubules and one case of Sertoli cell-only syndrome showing complete absence of germ

cells (Fig. 7). These individuals had the lowest body weights of the dose group.

Cauda epididymal sperm counts indicated a remarkable decrease in sperm density in 3 individuals, 2 of which had histopathologically identified damage of seminiferous tubules (Fig. 8, Table S5). The rat with Sertoli cell-only syndrome had not sperm cells, and this individual had also exceptionally high circulating FSH (27.3 ng/mL, control mean \pm SD 8.34 \pm 1.41 ng/mL) and LH (4.35 ng/mL, control mean \pm SD 1.14 \pm 0.30 ng/mL) concentrations, while circulating testosterone (4.2 g/mL) did not differ from the group mean (group mean \pm SD 4.08 \pm 0.93 ng/mL) but was lower than the control range (control mean \pm SD 8.53 \pm 3.75 ng/mL).

3.6. Bone geometry, densitometry and biomechanics

Bone geometry, densitometry and biomechanics data are mainly from Romero et al. [26], and are shown in Table S6. Some of these data

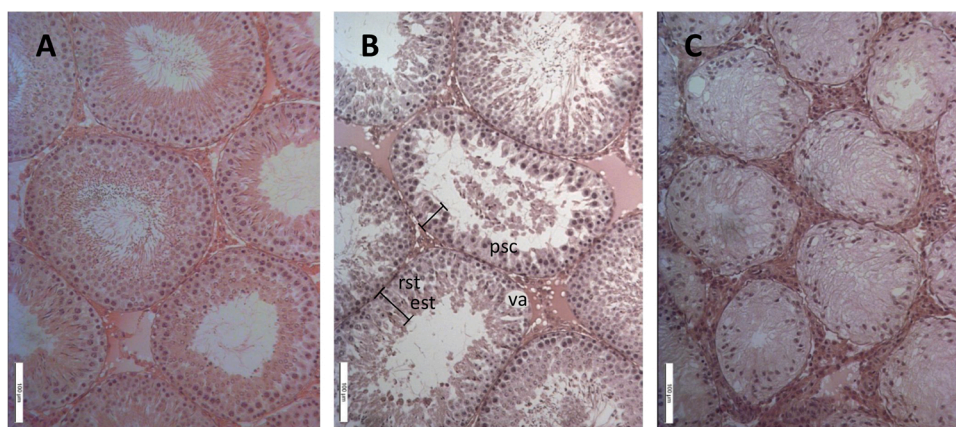


Fig. 7. Microphotographs of testis on postnatal day 84 stained with hematoxylin and eosin. A, control rat testis (004-03), B a testis with local damage of germinal epithelium (—) of seminiferous tubules (va, vacuolization) and decreased number of germ cells: est, elongated spermatids; rst, round spermatids; psc, primary spermatocytes (PCB 180 1000 mg/kg bw, 058-02) and C, a testis with Sertoli cell-only syndrome characterized by absence of germ cells (PCB 180 1000 mg/kg bw, 055-03). Bar: 100 µm.

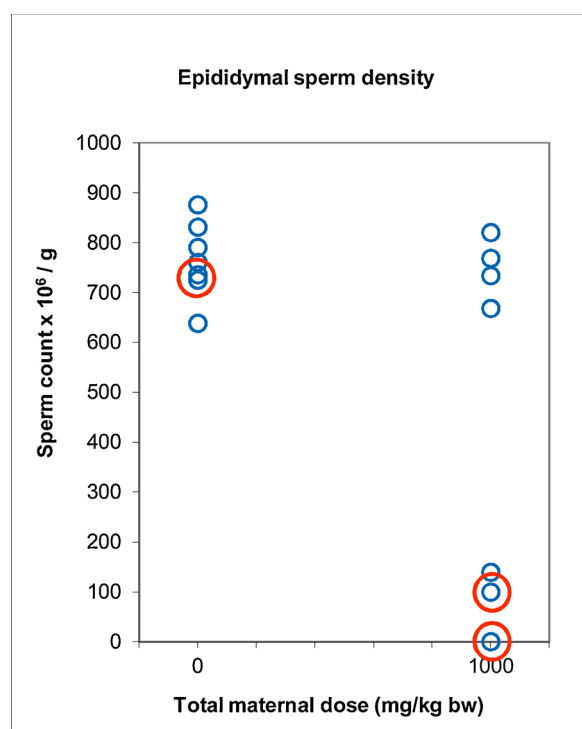


Fig. 8. Individual cauda epididymal sperm density of control and high dose male offspring on postnatal day 84. Red circles indicate the individuals shown in Fig. 7; the animal with Sertoli cell-only syndrome (Fig. 7C) had no sperm cells (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

have been published [26]. Cortical area and thickness of tibial diaphysis were increased in female offspring on PND 84 (BMD₀₅ 839 and 953 mg/kg bw, E_{max} 6.0 and 5.3 %, respectively), while in male offspring there was a tendency to decreased cortical bone mineral density (significant ANOVA and dose-response relationship with a E_{max} −2.3 %) (Table 2). A similar, but non-significant tendency of decreased cortical bone mineral density was observed in female offspring on PND 35, while in male offspring there were no bone effects on PND 35. Biomechanical properties of tibial diaphysis (stiffness, maximum force,

toughness and energy absorbed at maximum force) were not affected by the treatment.

3.7. Hepatic and renal retinoid concentrations

Hepatic and renal retinoid concentrations in male and female offspring on PND 35 are shown in Table S7. Hepatic retinol concentrations were decreased in male and female offspring on PND 35 (BMD₀₅ 24.8 and 7 mg/kg bw, E_{max} −58 and −45 %, respectively (Table 2). Hepatic all-*trans*-retinoic acid concentrations were increased in male and female offspring on PND 35 (BMD₀₅ 112 and 9.76 mg/kg bw, E_{max} 55 and 83 %, respectively) (Table 2). Hepatic 9-*cis*-4-oxo-13,14-dihydroretinoic acid concentrations were decreased in male and female offspring on PND 35 (BMD₀₅ 78.5 and 61.7 mg/kg bw, E_{max} −48 and −56 %, respectively) (Table 2). Hepatic 13-*cis*-4-oxo-retinoic acid concentrations were increased in male offspring on PND 35 (BMD₀₅ 6.2 mg/kg bw, E_{max} 112 %). Renal retinyl ester concentrations were increased in male and female offspring on PND 35 (BMD₀₅ 63.1 and 57.6 mg/kg bw, E_{max} 117 and 133 %, respectively) and renal retinol concentrations were increased in male offspring (BMD₀₅ 430, E_{max} 12 %) (Table 2). Although hepatic retinyl ester concentration was not affected by PCB 180 exposure on PND 35 (Table S7) there were retinyl palmitate decreases in the liver of male and female offspring on PND 84 (BMD₀₅ 119 and 142 mg/kg bw, E_{max} −35 and −30 %, respectively) (Fig. S2).

3.8. Hepatic PROD and EROD activities

Hepatic PROD and EROD activities of offspring on PND 7, PND 35 and PND 84 are shown in Table S8. In terms of BMD₁₀₀ values PROD induction was more sensitive than EROD induction. Male and female offspring were similarly sensitive to induction of both enzymes, and the BMD₁₀₀ for induction of both enzyme activities increased over time along with decreasing adipose tissue PCB 180 concentrations (Tables 2, S2, S8). Yet, on PND 84 both PROD and EROD activities remained more elevated in male offspring (Table S8), despite of the 2.5-fold higher adipose tissue PCB 180 concentration in females (Table S2). In male offspring, BMD₁₀₀ of hepatic PROD activity was 10.8, 12.1 and 347 mg/kg bw, and fold-induction 9, 15 and 8 on PND 7, 35 and 84, respectively, and in female offspring 9.10, 22.3 and 989 mg/kg bw, and 8, 16 and 2-fold, respectively (Table 2). EROD activity was induced quite similarly both in male and female offspring at all time points, and the induction decreased over time. In male offspring, BMD₁₀₀ was 173, 308 and

840 mg/kg bw and fold-induction 67, 12 and 2 on PND 7, 35 and 84, respectively (Table 2). The same figures of female offspring were 176 and 264 mg/kg bw on PND 7 and 35, respectively (on PND 84 the maximum efficacy was only 50 %), and 43, 17 and 1.4-fold, respectively (Table 2).

3.9. Hepatic gene expression of metabolic enzymes

Expressions of hepatic genes are shown in Table S9⁴. *Cyp2b1* and *Cyp3a1* were induced in males and females at all time points (Table 2). In male offspring, BMD₂₀₀ for *Cyp2b1* expression was 8.18 and 27.8 mg/kg bw on PND 35 and 84, respectively. BMD₂₀₀ for *Cyp3a1* expression was 123 mg/kg bw on PND 35 whereas E_{max} was 125 % on PND 84. In female offspring, BMD₂₀₀ for *Cyp2b1* expression was 8.44, 12.8 and 41.2 mg/kg bw on PND 7, 35 and 84, respectively. BMD₂₀₀ for *Cyp3a1* expression was 44.1, 534 and 921 mg/kg bw, respectively. Expression of *Cyp1a1* was induced in females on PND 7 with a BMD₂₀₀ of 61.4 mg/kg bw. In males, BMD₂₀₀ for *Cyp1a1* expression was 950 mg/kg bw on PND 35 whereas E_{max} was lower than 200 % on PND 84.

4. Discussion

The present study is the first comprehensive report on toxic effects of the NDL-PCB 180 on the offspring after IUL exposure. Due to long elimination half-life ($t_{1/2}$ 90 days [10]) maternal dosing on GD 7–10 results in continuous exposure of the offspring [43]. It starts at the late gastrula stage, completion of implantation (GD 7–8), covering the primitive streak (GD 8.5) and neurula stage (GD 9–11) and most of the organogenesis [44], lactation and slowly decreasing beyond weaning. The selected maternal doses resulted in a non-significant and temporary decrease of body weight at the highest dose level in the dams and dose-dependently decreased serum FT3 concentrations, while a large spectrum of effects in the offspring was observed, some of which persisted into adulthood. Only at the highest dose level there were transient, non-significant decreases in body weight during the first postnatal weeks, a slight delay in some developmental milestones and a slight increase in neonatal mortality at the two highest dose-levels. Significant effects at lower dose levels included increased liver weight, decreased concentrations of circulating testosterone and thyroid hormones, alterations in hepatic and renal retinoid concentrations as well as induction of hepatic PROD and EROD activities. The results are further discussed with the aim to provide a toxicological characterization of IUL exposure to PCB 180, which include comparisons between the identified sensitive, adverse or potentially adverse, as well as mechanistic endpoints. These considerations are further brought into the study-based assessment of potential health risks of maternal PCB 180 exposure to offspring using the MoE approach (Section 5).

4.1. Adipose tissue PCB 180 concentrations

Lipid based adipose tissue PCB 180 concentrations of the control dams and offspring were 0.07–0.22 µg/g lipid, which is within the range of human background exposure level. In the WHO mother's milk survey PCB 180 concentrations were 0.006–0.337 µg/g lipid (median 0.046 µg/g lipid) [2,3]. In the PCB 180 exposed groups the adipose tissue concentrations ranged from 28.8 µg/g lipid at 10 mg/kg bw to 6160 µg/g lipid at 1000 mg/kg bw. For comparison, in PCB exposed Baltic Sea fishermen the range of PCB 180 concentrations were 0.19–1.2 µg/g lipid, and the range sum of PCBs were 0.95–8.70 µg/g lipid [45]. This indicates that at the lowest dose level the PCB 180 concentration was 24-fold higher than the highest measured concentration of the Baltic fishermen.

⁴ No data are available on hepatic gene expression of PND 7 males, because the samples were sufficient only for analysis of PROD and EROD activities.

Adipose tissue PCB 180 concentrations in dams on postpartum day 28 of the present study were 65–73 % of the concentrations analyzed at the end of the 28-day toxicity study in nulliparous female rats that received the same total dose [9]. This difference is explained mainly by increased elimination due to parturition and lactation. A similar decrease in concentrations of DL compounds has been reported in women after childbirth and breastfeeding [46].

4.2. General observations and developmental milestones

At the highest dose level body weight was non-significantly and temporarily decreased in dams after the second dose of PCB 180; a complete recovery took place soon after parturition. A similar temporary decrease has been reported in pregnant rats after exposure to the highest total dose of 448 mg/kg bw of NDL-PCB 153 on GDs 10–16 [47]. In the offspring of both genders a similar temporary and non-significant decrease was observed soon after birth. This is consistent with the increased exposure after the commence of lactation as reported for DL compounds both in rats [48–50] and humans [46]. On PND 84 body weights of males at 300 mg/kg bw were significantly above controls suggesting a possibility for an obesogenic effect that has been shown in NDL-PCB 153 -treated mice [51,52]. The sporadic and slight delays in balano-preputial separation and vaginal opening were also limited to the highest dose of 1000 mg/kg bw and observed on PNDs 43–49 and 39–46, respectively. These delays were associated with decreased body weight.

4.3. Thyroid hormones

Thyroid hormones showed decreases both in dams and in offspring. BMD₀₅ values indicate that the decrease in FT3 was evidently more sensitive than that of FT4. In the 28-day study with PCB 180, decreased serum FT4 and to a lesser extent serum FT3 together with altered thyroid gland weight, depletion of thyroid follicles and hypertrophy of follicular epithelial cells were observed, and males were more sensitive than females [9]. Typical features after IUL exposure were progressive T3 hypothyroidism in the offspring and rather similar sensitivity of both genders as well as dams.

Decreased levels of thyroid hormones are characteristic effects of DL-PCBs, NDL-PCBs and PCDD/Fs, mainly due to increased elimination of thyroid hormones secondary to the induction of hepatic UGTs. UGTs responsible for glucuronidation of thyroid hormones are activated via AHR by DL compounds and via CAR and PXR by NDL-PCBs [6,53–55]. In addition to enhanced metabolism of thyroid hormones there are other mechanisms by which PCBs may disturb thyroid homeostasis. Because PCBs and especially their hydroxylated metabolites are structurally close to thyroid hormones, they compete with thyroid hormones for binding to thyroid hormone transport proteins transthyretin (TTR) and thyroid-binding globulin (TBG) as well as to thyroid hormone receptors (TR). A binding assay indicated that 4 monohydroxyl metabolites of PCB 180 (3'-OH-PCB 180, 4'-OH-PCB-172, 3'-OH-PCB 182 and 5-OH-PCB 183) efficiently displaced T4 from TTR at relative potencies 3.1–4.6 times higher than T4 [9]. As TTR is responsible for transport of thyroid hormones through placental and blood-brain barriers [56,57] the high affinity of hydroxylated PCB 180 metabolites to TTR potentially results in accumulation of these metabolites into the fetus and brain at the cost of thyroid hormones [58]. There is also evidence that PCBs suppress transcription of TR dependent genes by inhibiting binding of T3 to TR [59] or by dissociating the TR/RXR heterodimer complex from the thyroid response element [60].

Experimental and human studies have indicated that thyroid hormone insufficiency may result in adverse neurodevelopmental and cardiovascular effects. The developing nervous system is particularly sensitive and even a transient decrease of thyroid hormone levels may result in adverse outcome [53,54]. This mode-of-action is relevant in humans, and recent studies suggest that NDL-PCBs play key roles in

developmental neurotoxicity associated with PCBs [61]. Neurodevelopmental and neurobehavioral effects were also observed in siblings of the offspring of the present study. These include increased threshold of the brainstem auditory evoked potential in female offspring [7], increased preference of female offspring to drink solution sweetened with saccharin, which indicates supernormal sexually dimorphic behavior [8] as well as dose-related and pronounced reduction in latencies to movement onset during haloperidol-induced catalepsy [25]. Impaired learning ability in the Y maze task was also reported in male and female offspring exposed *in utero* and lactationally to PCB 180 at the total dose of 36 mg/kg bw [62]. As a sensitive and toxicologically relevant endpoint decreased serum FT3 (PND 84) was selected for risk characterization and MoE estimation (Section 5, Table 3).

4.4. Steroid hormones, gonadotropins and reproductive effects

Dose-dependently and progressively decreased serum testosterone levels by more than 50 % and with rather low BMD₀₅ values in male offspring both on PND 35 and PND 84 was a remarkable finding. LH values were not significantly affected, but FSH levels were decreased at the higher dose levels on PND 35, and on PND 84 markedly increased without statistical significance only in a few animals. As expected, decreased serum testosterone was reflected in decreased weights of the androgen dependent organs prostate and seminal vesicle (Subsection 4.7). Distinctly lower BMD₀₅ values for decreased serum testosterone than for decreased prostate and seminal vesicle weights as well as FSH levels indicate that the former is more sensitive and therefore potentially the primary effect. Decreased serum testosterone became more sensitive over time while there was some recovery in weights of androgen dependent organs. Pituitary LH secretion was not increased despite low testosterone values, suggesting impaired capacity to compensate failing Leydig cell function. High FSH level in the Sertoli cell-only animal was an expected finding, since there is minimal negative feedback to the pituitary in the adult animals lacking germ cells.

The effects of PCB 180 on testosterone, LH and FSH of young adult male rats in the 28-day study shared the features observed in the present study but were generally milder [9]. The decrease in serum testosterone was non-significant and limited to the highest total dose of 1700 mg/kg bw. The decrease in FSH was of similar magnitude but had a higher BMD₀₅ than in the present study, and also LH levels were dose-dependently decreased. In accordance with our findings, long-lasting (up to PND 310!) and dose-dependent decrease in serum testosterone levels were observed in rat offspring after IUL exposure to a reconstituted PCB mixture with a congener pattern of human breast milk (14 congeners, including PCB 180) [63]. Similarly, IUL exposure of goats to PCB 153 resulted in decreased serum testosterone concentrations in male offspring around and after puberty, but conventional sperm parameters and testis histology were not affected [64].

Several epidemiological studies have reported the inverse relationship between exposure to PCBs (including NL-PCBs) and serum testosterone level [65–71]. Importantly, in the Duisburg cohort on healthy mother-infant pairs (2000–2002), lipid based maternal serum and milk concentration of PCBs (including 6 indicator PCBs) was inversely associated with cord blood testosterone and estradiol concentrations of infant boys and girls [66]. The blood concentration (pg/g lipid) of the 6 indicator PCBs were as follows: 5th percentile 49, 95th percentile 327, geometric mean 140 and median 149 (n = 104). Recently, Leong et al. [71] showed an association between a decrease in serum testosterone with increasing serum concentration of NDL-PCB 153 using the National Health and Nutrition Examination Survey (NHANES) database collected from 1999 to 2000 and 2001 to 2002. As a sensitive and toxicologically relevant endpoint decreased serum testosterone was selected for risk characterization and MoE estimation (Section 5, Table 3).

PCBs may potentially have both direct interactions with androgen receptor activation and indirect effects on circulating levels of testosterone due to decreased steroidogenesis and/or increased metabolism. It

has been shown that high purity PCB 180 antagonizes androgen receptor activation with a moderate potency in the AR-CALUX *in vitro* assay [4]. Other *in vitro* and *in vivo* studies have reported antiandrogenic effects of PCBs and their mixtures. Aroclor 1248 was shown to inhibit testicular androgenesis due to inhibition of 3 β -hydroxysteroid dehydrogenase, 17 α -hydroxylase/lyase and 17 β -hydroxysteroid dehydrogenase activities *in vitro* [72]. Furthermore, neonatal exposure of rats to Aroclor 1254 altered the activity of testosterone hydroxylases and decreased the circulating testosterone levels [73]. Testosterone is a well-known substrate of CYP 3A enzymes [74]. Thus, induced CYP 3A1 could have contributed to decreased testosterone in the present study, but because the induction has higher BMD₂₀₀ and diminished while testosterone levels decreased further towards the end of the study, it is not the primary reason.

Despite decreased testosterone levels, ano-genital distance, a sensitive indicator of prenatal androgen action [75], was not affected on PNDs 1 or 14. This is in accordance with findings in male or female rat offspring after IUL exposure to PCB 153 up to total maternal dose of 448 mg/kg bw [47]. The few cases of histopathologically observed disrupted germinal epithelium, decreased number of germ cells of seminiferous tubules and decreased spermatogenesis were only observed on PND 84 and limited to the highest maternal dose level of PCB 180 (1000 mg/kg bw). However, these cases were not associated with exceptionally low testosterone values. In general, male reproductive effects of PCB 180 warrant further studies.

The male offspring with the Sertoli cell-only syndrome and complete lack of epididymal sperm had a serum testosterone level close to the group mean value of rats with normal testicular histology and sperm count. FSH and LH values, however, were exceptionally high, testicular weight only about 20 % of the normal weight and the weights of epididymis, prostate and seminal vesicles only about 40–60 % of normal. Sertoli cell-only syndrome is characterized by a complete lack of germ cells but presence of Sertoli cells in seminiferous tubules, and elevated concentrations of FSH in serum [76]. Testosterone and LH levels are usually normal, but testosterone/LH ratio may be decreased. Etiology of the Sertoli cell-only syndrome is not fully understood, but both genetic and environmental factors are involved. Gestational exposure to antiandrogenic dibutyl phthalate has been shown to induce Sertoli cell-only tubules in rat testis [77]. The possible underlying causes of the present case of Sertoli cell-only syndrome may include disturbed development of spermatogonial stem cells, abnormal maturation of Sertoli cells or direct damage of spermatogenic epithelium.

In female offspring, serum estradiol levels were decreased, and LH levels increased (data only on PND 35) only at the highest dose level, but these changes were not reflected in weights of ovaries or uterus. Potentially induced activity of CYP 3A could result in decreased estradiol levels [78–80], although in young adult females of the 28-day study the decrease in estradiol was less clear and LH was not affected despite higher PCB 180 exposure than in the present study [9]. Also, inhibition of aromatase activity by hydroxyl metabolites of PCB 180 [81] could contribute to decreased estradiol levels. It is also of interest that in the ER-CALUX assay PCB 180 was a weak antagonist of estrogen receptors (ERs) [4], and several other *in vitro* studies also reported about weak antiestrogenic activity [82–84]. In an epidemiological study, high placental concentrations of PCDDs/PCDFs and PCBs were linked to lowered serum estradiol levels and to shorter fundus uteri and uterus lengths in a cohort of 33 8-year-old girls [85].

4.5. Hepatic and renal retinoids

The response to PCB 180 exposure comprised increased biodegradation and biosynthesis of all-*trans*-retinoic acid along with an increased mobilization of storage retinoid species. The results are compatible with a PCB 180 mediated CAR activation, as PCB 153, a structurally related NDL-PCB congener, disrupted retinoid homeostasis in a CAR-dependent manner [52]. Such CAR activation is corroborated by the induced PROD

activity and *Cyp2b1* gene expression, as discussed below (Subsection 4.8). In addition, xenobiotic metabolizing enzymes CYP 2B and 3A are involved in the oxidation of all-*trans*-retinoic acid [86,87] and further into retinoyl- β -glucuronide metabolites by UGTs [88].

Increased hepatic levels of all-*trans*-retinoic acid on PND 35 indicate its higher rate of biosynthesis than degradation, and females were more sensitive than males in terms of potency and efficacy. Interestingly, other studies also reported increased all-*trans*-retinoic acid levels following IUL exposure to Aroclor 1254 (both sexes on PND 35) [89], to a mixture of 27 contaminants including PCBs, organochlorine pesticides, and methylmercury (males on PND 35) [90], as well as to bisphenol A (10 μ g/kg bw/day on GDs 9–16; male mice on PND 30) [91]. Additionally, in the 28-day study with PCB 180, increased all-*trans*-retinoic acid levels were found in livers of males [9]. All-*trans*-retinoic acid concentrations are strictly regulated as this endogenous ligand of RAR is involved in the homeostasis of the tissues, including the liver. Disruption of the retinoic acid signaling pathways is known to be involved in a number of conditions causing liver disease [92].

The sustained biosynthesis of all-*trans*-retinoic acid as a result of the PCB 180 induced hepatic enzymes requires a significant mobilization of storage retinoid species in order to produce all-*trans*-retinoic acid precursors. Accordingly, increased mobilization of retinoid forms was indicated by reduced levels of retinyl palmitate (both sexes on PND 84) and 9-*cis*-4-oxo-13,14-dihydro-retinoic acid (both sexes on PND 35). Similarly, decreased retinyl ester levels in livers of both sexes and 9-*cis*-4-oxo-13,14-dihydro-retinoic acid levels in females were observed in the 28-day study with PCB 180 [9]. Retinyl palmitate is the predominant storage retinyl ester in the liver that is accumulated in lipid droplets in stellate cells [93]. In turn, 9-*cis*-4-oxo-13,14-dihydro-retinoic acid is a retinoic acid metabolite with the ability to activate RAR [94]. Noteworthy, both retinyl palmitate and 9-*cis*-4-oxo-13,14-dihydro-retinoic acid showed increasing trends following vitamin A supplementation [95], whereas decreasing trends were observed in the previously mentioned IUL studies following exposure to Aroclor 1254 [89] or the mixture of 27 contaminants [90]. Thus, decreased 9-*cis*-4-oxo-13,14-dihydro-retinoic acid levels were more sensitive to PCB 180 than retinyl ester levels, and they are indicative of mobilization of retinoid stores. In line with that, adipose tissue concentrations of PCBs 138, 153 and 180 in humans of the GraMo cohort were associated with reduced levels of retinol in adipose tissue [96]. This agrees with our result, because retinoid levels in adipose tissue correlate with those of hepatic storage retinoids [101]; an established hepatic biomarker for vitamin A status assessment [97]. Interestingly, PCB 180 was the congener, which contributed most (52 %) to the overall mixture effect [96]. On PND 35, in spite of the mentioned mobilization, retinol levels were reduced, as found in the offspring of the above-mentioned gestational studies [89, 90] and in the 28-day study with PCB 180 [9] which is compatible with its increased conversion into the functional all-*trans*-retinoic acid [98].

An additional mechanism that could result in decreased retinol levels in the liver was the discussed (Subsection 4.3) interaction between PCB metabolites and TTR in serum [9,56,57]. Briefly, retinol is distributed in the circulation by the retinol binding protein (RBP), which forms a protein complex with TTR. Thus, the reduced retinol levels might also be due to an increased delivery of retinol from the liver to extrahepatic tissues as a response to the disruption of the TTR-RBP complex by PCBs, which has been shown to be associated with the loss of RBP-retinol by glomerular clearance [99,100]. As sensitive and mechanistically relevant endpoints altered hepatic levels of retinol, all-*trans*-retinoic acid and 13-*cis*-4-oxo-retinoic acid were selected for MoE estimation (Section 5, Table 3).

In the kidney, the increased levels of retinol (males on PND 35) and retinyl esters (males and females on PND 35) might reflect an increased RBP reabsorption in the renal proximal tubular epithelium [101] followed by esterification of retinol into retinyl esters by lecithin: retinol acyltransferase [102], as an adaptive response against the retinoid losses. Increased retinoid levels in the kidney have also been observed in

studies reporting hepatic retinoid mobilization [9,34,89,90,102,103].

4.6. Bone geometry, densitometry and biomechanics

An increase in cortical bone area and cortical thickness was seen in female offspring, which contrasts with decreased cortical area following exposure of adult male rats to PCB 180 [9]. This difference indicates different responses depending on whether the bone was affected already during growth (as in the case of IUL exposure) or if only remodeling of mineralized mature bone was affected, as previously shown after IUL [104] and adult exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [105]. In line with the present findings, *in utero* exposure to NDLC-PCB 153 throughout pregnancy increased cortical thickness in sheep fetuses [106], and tissue concentration of sumPCBs in environmentally exposed otters was linked to increased cortical bone area and cortical thickness [107]. Decreased cortical bone mineral density as observed in male offspring on PND 84 has also been reported in rat offspring after IUL exposure to TCDD [50,104]. The fact that more bone effects are observed at later time points suggests that IUL exposure to both NDLC (this study) and DL [104] compounds interfere with normal bone maturation processes that manifest later in life.

4.7. Organ weights

Both absolute and relative liver weights of male and female offspring showed a sensitive, dose-dependent and significant increase at all time points that attenuated over time reflecting the decreasing adipose tissue PCB 180 concentration. Increased liver weight was quite similar in both genders except that on PND 84 the weight remained significantly elevated only in the male offspring. Increased liver weight was related to the induction of xenobiotic metabolism in the liver both in terms of sensitivity and time course (see Subsection 4.8). A comparable increase was observed in the 28-day toxicity study in rats with PCB 180, and again males were clearly more sensitive than females [6]. Males were also more sensitive to centrilobular hypertrophy in the 28-day study, but clinical chemistry revealed no indication of major disturbances in liver function. The same type of gender-related response pattern was also observed for the induction of hepatic PROD and EROD activities.

Absolute and relative thymus weights were quite similarly decreased in male and female offspring of the present study only at the highest dose level on PND 7. This decrease differs from the severe and sensitive thymus weight decrease characteristic PCDD/Fs and DL-PCBs [108]. However, the thymus weight decrease was associated with transient increases in hepatic EROD activity and *Cyp1a1* expression on PND 7 and PND 35 suggesting that the observation could reflect a weak early postnatal AHR activation. Furthermore, thymus weight was not affected in the 28-day study with PCB 180 [9].

The weights of androgen dependent tissues prostate and seminal vesicles were decreased most likely as a consequence of decreased serum testosterone concentrations (Subsection 4.4) on PND 35 (prostate only) and PND 84, although only at high dose-levels. Decreased prostate and seminal vesicle weights as such may be potentially adverse, although in lack of histopathological observations their toxicological significance is difficult to assess [109]. Decreased weights of heart, lungs, pituitary, adrenals, prostate and seminal vesicles of the present study were not observed in the 28-day study. On the other hand, thyroid gland weight was not affected in the present study, but significantly increased in males and decreased in females of the 28-day study. Despite the presence of abnormal seminiferous tubules and decreased sperm counts in some individuals the testis mean weight was not decreased in the present study nor in the 28-day study. For comparison, however, a relatively high maternal dose of TCDD (1 μ g/kg bw) decreases slightly testis weight of the offspring [110].

Overall, PCB 180-induced changes in organ weights were more sensitive after IUL exposure than after adult exposure [9]. Also, more organ weights were affected after IUL than adult exposure. As sensitive

or potentially adverse endpoints increased absolute liver weights and decreased absolute prostate weights were selected for risk characterization and MoE estimation (Section 5, Table 3).

4.8. Hepatic metabolism

Liver PROD and EROD activities were increased in PCB 180 exposed male and female offspring at all the time-points. Similar findings were reported after repeated dose exposure of adult rats to PCB 180 [6,9] and PCB 153 [111]. CYP2Bs are mainly responsible for the PROD activity, and their induction is mediated by CAR, which is known to be induced by PCB 180 and other NDL-PCBs [6,55]. These findings are consistent with the highly induced *Cyp2b1* mRNA. AHR activates CYP1A1 and CYP1B1, which results in induction of EROD activity. Although dioxins and DL-compounds are characteristic AHR ligands resulting in strong and sustained activation, some CAR activators have also been shown to activate AHR [112], which may explain the EROD induction by PCB 180 also without DL impurities. In addition, both *Ahr* and *Cyp1a1* are highly expressed in livers of young rats, whereas *Car* and *Cyp2b1* are not [113, 114], which could explain the stronger EROD induction (E_{max}) and lower PROD induction in PND 7 offspring compared to the later time points. Also, the PROD activity in control animals increased over time. As a sensitive and mechanistically relevant endpoint increased PROD activity was selected for risk characterization and MoE estimation (Section 5, Table 3).

5. Risk characterization

The broad range of adverse, potentially adverse, reversible, as well as mechanistic endpoints, which were analyzed in the present study showed widely different dose-response, time course, and gender characteristics. BMD and BMDL-values as listed in Table 2 allow for sensitivity comparisons between different endpoints and their change over time. Among dose-related adverse effects in Table 2, which were not taken forward to MoE calculations, are neonatal deaths, which were dose-dependently increased at the two highest dose levels (BMD₁₀ 621 mg/kg bw). Decreased body weight and delays in developmental milestones, which were limited to the highest dose level of 1000 mg/kg bw, were transient and observed only during the neonatal period. Delays in developmental milestones are likely non-specific consequences of high dose exposure. Decreased serum estradiol and increased LH in females were less sensitive reproductive endpoints than the decreased testosterone, however, these results suggest that also female reproduction is affected by PCB 180 IUL exposure.

The sensitive endpoints, which were taken forward for MoE calculations and risk characterization, are listed in Table 3. They cover a range of endocrine, metabolic, male reproductive, and hepatic effects that manifest postnatally and can be viewed as adverse, potentially adverse, reversible, as well as mechanistic endpoints. Sensitive endpoints for MoE calculations were identified based on BMDL values (Table 2) for male reproductive endpoints including decreased serum testosterone, hepatic endpoints including increased liver weight, hepatic PROD activity, and altered hepatic concentrations of retinol, all-*trans*-retinoic acid and 13-*cis*-4-oxo-retinoic acid, and decreased ft3 as a functional thyroid marker.

Of these endpoints, decreased serum testosterone and ft3 levels are toxicologically significant critical effects with specific later life health effects. Consequences of early life hypogonadism characterized by low testosterone include altered differentiation and development of the male phenotype, delayed puberty, growth and maturation, impaired spermatogenesis and poor muscle development [115]. Low testosterone due to congenital hypogonadotropic hypogonadism is also associated with decreased bone mineral density [116]. In this study the decreased serum testosterone concentration by up to more than 50 % was highly sensitive, progressive over time, and therefore considered adverse. The most sensitive time point was PND 84 suggesting a potential for later life

health consequences and adverse effects on male reproduction. Decreased prostate weight was more sensitive and more pronounced on PND 35 than on PND 84 and the effect was considered potentially adverse.

Thyroid hormones and the retinoid system are essential for normal development and maintenance of most physiological functions of the body as well as for the normal development and growth, including development of the central nervous system and maturation of the skeleton [117,118]. Hypothyroidism has well characterized neurological consequences that are dependent on timing of thyroid hormone insufficiency during development. In general, they include impaired brain development associated with different neuropsychological manifestations. Decreases in serum ft3, which is among the potentially adverse and/or mechanistic sensitive endpoints in this study, were more sensitive in male than in female offspring. ft3 decreases were observed only on PND 84. Decreases in serum ft4 were less sensitive than those in ft3. They were only observed in male offspring and were more sensitive on PND 84 than on PND 35. Both ft3 and ft4 were decreased also in dams, and the sensitivity was close to that of the offspring.

Similar to thyroid hormone defects, also alterations in the retinoid system have well characterized and serious developmental consequences, and recent regulatory attention has been directed towards the need to capture the retinoid system as a general target of relevance for most organs in toxicological guideline studies [119,120] [This issue]. Observations in the current study shows that decreased liver retinol and increased all-*trans*-retinoic acid concentrations are among the sensitive endpoints on PND 35, especially in females. Increase in liver 13-*cis*-4-oxo-retinoic acid was also very sensitive, but only in males on PND 35. Decreased hepatic retinyl palmitate on PND 84 and increases in kidney retinyl esters and retinol (only in males) concentrations observed on PND 35 only were less sensitive than the other changes in retinoid concentrations. Taken together with the induced hepatic *cyp 2b1*, *3a1*, and *1a1* expressions these retinoid data suggest that PCB 180 IUL exposure impacts on hepatic and renal retinoid homeostasis in early postnatal life as assessed on PND 35. In addition, later in adult life on PND 84, there is an impact also on the predominant retinoid storage form, retinyl palmitate, both in males and females.

Induction of xenobiotic metabolizing enzymes together with increased liver weight are generally considered as adaptive and temporary responses to increased xenobiotic load with less clear toxicological significance in adults. However, in the IUL exposure during critical windows of sensitivity continuous induction of several enzymes could potentially have developmental or endocrine effects, which would not exist or manifest following postnatal and/or adult exposure alone. Our observations show that the most sensitive endpoints were induction of hepatic PROD and EROD activity, the associated induction of *Cyp1a1*, *2b1*, and *3a1* expression and increased liver weight, and the most sensitive time point was PND 7 after which the responses attenuated reflecting PCB 180 adipose tissue concentration.

BMDL₀₅ of increased liver weight by 35 %, the most sensitive endpoint of Table 3, is 6 µg PCB 180/g lipid which is equivalent with the total dose of 0.54 mg/kg bw. Using UF 25 the corresponding tolerable human PCB 180 concentration is 200 ng/g lipid. For comparison, BMDL₀₅ values of decreased serum testosterone as well as altered hepatic retinol, all-*trans*-retinoic acid and 13-*cis*-4-oxo-retinoic acid, which all are very similar, are 12–15 µg PCB 180/g lipid (Table 3), which is equivalent with the total doses of 2.09–3.23 mg/kg bw. Of these endpoints, decreased serum testosterone (BMDL₀₅ = 15 µg/g lipid) is considered as the well-established endocrine endpoint with an associated comprehensive human knowledge base. Therefore, based on the outcome of this study decreased serum testosterone by 50 % on PND 84 following PCB 180 IUL exposure was selected as the critical effect of this study.

Risk characterization was carried out by using MoE values of several sensitive endpoints as measured in the offspring and human lipid-based PCB 180 concentrations from different representative cohorts (Table 3).

The selected human cohorts were relevant for risk characterization of developmental endpoints, and they included the European mother's milk cohort [2], a cohort of Spanish women [121], a cohort of Italian pregnant women [122] and, for comparison, a high-exposure Baltic Sea fishermen cohort [45]. For the Italian and the Baltic Sea fishermen cohorts the 95th percentile PCB 180 concentrations were not available. Therefore, the maximum concentrations are shown in Table 3, but because they are from single individuals, they were used for descriptive purposes only and hence they were not intended to be a comprehensive exposure assessment for risk characterization.

MoE values indicate that when using the WHO default UF of 25 [42] and the toxicologically sensitive endpoint (decreased serum testosterone concentration) are not likely to occur in any of the human cohorts (Table 3). For comparison, when increased liver weight is used, MoEs were below the WHO default UF of 25 [42] for the 95th percentile PCB 180 concentration of the Spanish cohort and the median of the Baltic fisherman cohort.

According to JECFA [21] a group MoE evaluation for the most abundant NDL-PCBs in human blood is currently not possible because of lacking *in vivo* toxicology data for two of these abundant NDL-PCB congeners, *i.e.* PCB 101 and PCB 138. JECFA also paid attention to the lack of reproductive and IUL toxicology data and recommended to develop a more complete toxicological database for such individual NDL-PCB congeners that contribute substantially to human background exposures. Additionally, JECFA highlighted concerns related to the simultaneous human exposure to a mixture of several DL-PCBs and PCDD/Fs in addition to the NDL-PCBs. JECFA noted that these compound groups, despite of partly different mechanisms (*e.g.* in terms of the initial chemical interaction with cellular receptor molecules and the associated molecular initiating event), share several toxicological endpoints, including steroid and thyroid hormones, retinoids, neurodevelopmental effects, and metabolic effects in the liver. These are all toxicological endpoints, which have been addressed in the present study and in previous publications [6–9,17,25–27] from the same research effort to address regulatory needs of new knowledge on NDL-PCBs in general and PCB 180 in particular.

6. Conclusions

Developmental toxicity profile of PCB 180 is characterized by endocrine, metabolic, male reproductive, and hepatic effects that manifest postnatally. The male reproductive and endocrine effects include decreased serum testosterone concentrations as well as decreased prostate and seminal vesicle weights, and decreased serum fT3 concentrations. Changes in hepatic concentrations of retinol, all-*trans*-retinoic acid, and 13-*cis*-4-oxo-retinoic acid in association with hepatic induction of *Cyp1b1*, *3a1*, and *1a1*, are suggestive of metabolic as well as transcriptional impact on the liver, such as mobilization of storage forms of retinoids from the stellate cell compartment and increased biosynthesis and degradation of the biologically active form all-*trans*-retinoic acid. The hepatic enzyme induction profile suggests activation of CAR and PXR that may interact with other receptors, including TRs, RXRs, RARs, androgen receptors (ARs) and ERs, potentially leading to altered function. Based on *Cyp1a1* induction involvement of AHR cannot be excluded, although there is no indication of sustained high-affinity AHR activation characteristic for DL compounds. Previous studies have shown that PCB 180 is also able to antagonize AR and ER, and its hydroxyl metabolites can efficiently displace thyroid hormones from TTR. These phenomena are likely to contribute to the observed endocrine and skeletal effects of PCB 180.

Developmental toxicity profiles were partly different in male and female offspring. Males were more sensitive to increased liver weight, induction of hepatic PROD activity and decreased serum fT4 concentrations on PND 84. These observations are in line with findings from male and female rats of the 28-day study with PCB 180 [6,9]. In addition, females alone had alterations in bone geometry parameters on PND

84 only, and PND 35 data suggest that females were more sensitive to increased liver all-*trans*-retinoic acid, decreased liver retinol and increased kidney retinyl ester concentrations. An additional gender-related observation is the 2.5 times higher adipose tissue PCB 180 concentration in females at the highest dose-level on PND 84 as compared to males despite of a comparable total PCB 180 body burden between males and females.

MoE assessment for developmental endpoints showed that the current human PCB 180 tissue concentrations (up to 95th percentile) do not exceed the estimated tolerable human lipid-based PCB 180 concentration. Although PCB 180 is classified as a NDL-PCB it shares several toxicological targets relevant for developmental effect, such as testosterone, thyroid hormones, retinoid system, bone and liver, suggesting potential for interactions.

Future studies should continue clarifying the toxicological significance and later life health consequences of the observed male reproductive and liver weight changes with associated endocrine and metabolic alterations, including CYP and other enzymes, steroid and thyroid hormones, and the retinoid system. Additional and more detailed mechanistic data on molecular and biochemical effects can play key roles in building adverse outcome pathways for CAR and PXR mediated hepatic effects of developmental origin and support regulatory toxicology efforts to assess chemicals with mixed type enzyme induction mode of action.

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Conflict of Interest

The authors declare no conflict of interest

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.reprotox.2021.04.004>.

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