CHAPTER 2: EXPERIMENTAL

In this chapter the experimental design and methods are described. Two independent experiments were designed to investigate the effects of pre- and postnatal (via milk) PBDE 99 exposure on offspring development. In the first experiment, tissue distribution of PBDE 99, effects on thyroid hormonal status and hepatic enzyme activities were evaluated in dams and offspring during lactation. The second experiment was designed to investigate the possible long term effects of PBDE 99 that can become apparent during puberty and

adulthood. These include the investigation of neurobehavioral changes (spontaneous postnatal reflexes, sexual dimorphic behavior and locomotor activity) and male reproductive health (reproductive performance, sexual behavior, sperm and spermatid counts, testicular morphometric analysis and Sertoli cell count, flow cytometry of germ cells and sexual hormone levels).

2.1 EXPERIMENT I

2.1.1 ANIMALS AND TREATMENT

Acclimatization of Wistar rats (HsdCpb:WU; Fa. Harlan-Winkelmann, Borchen, FRG), weighing 200 ± 15g, was performed for two weeks. The rats were exposed to constant light/dark periods of 12 hours each, a temperature of 21 ± 1°C and 50 ± 5% relative humidity. Rodent chow (Altromin 1324) obtained from Altromin GmbH (Lage, FRG) and tap water were available *ad libitum*. The mating procedure was performed as proposed by Chahoud and Kwasigroch (1977). Two nongravid females were placed with one male for three hours per day and the day of sperm detection in the vaginal smear was considered as day 0 of gestation. The gravid females were housed in Type III macrolon cages with stainless steel covers and wood shavings obtained from Altromin GmbH. 2,2´,4,4´,5-pentabromodiphenyl ether (PBDE 99), charge number VL02, was purchased from LGC Promochem GmbH (Germany) with a purity of 98%. Pregnant rats (control=16; PTU=16; 60µg PBDE/kg=21 and 300µg PBDE/kg=15) were treated orally by gavage with a single dose of 60 or 300 µg PBDE 99/kg or peanut oil (control) on day 6 of gestation. The volume of administration was 10 ml / kg BW. An additional group was administered the goitrogen, PTU (6-n-propyl-2-thiouracil, Sigma-Aldrich Chemicals GmbH, Steinheim, FRG), which served as reference control for thyroid hormone effects. PTU was given to the gravid dams by placing 5mg/L PTU in the drinking water on gestation days 7 through 21. The average PTU dose through tap water was calculated to be 916µg/kg per day. Dams were allowed to deliver and the litter size was not artificially altered. ***PND 1:*** On postnatal day (PND) 1, about half of the treated females (control= 8; PTU= 7; PBDE 60= 10; PBDE 300= 8) were killed by decapitation with their respective litter and liver and adipose tissue were weighed and frozen at –80°C. Body and brain weights were recorded from 1 male and 1 female per litter. Serum was collected from trunk blood, divided into five aliquots and stored at –20°C. Due to the small size of neonate pups, blood, liver and adipose tissue were pooled by gender on a litter basis and frozen. ***PND***

14: From the litters of the remaining lactating dams (control= 8; PTU= 9; PBDE 60= 11; PBDE 300= 7), two males and two females were randomly selected from each litter and sacrificed on PND 14 to collect blood, liver and adipose tissue. ***PND 22:*** At the end of lactation (PND 22), dams together with two males and two females per litter were sacrificed and the procedure from PND 1 was repeated. The experimental protocol has the approval of the National Animal Protection Law (Tierschutzgesetz BGBI. IS. 3082. Genehmigungsnummer: VB104 – G0012/00).

Table 1: Treatment scheme and groups used to assess the developmental effects of PBDE 99 in rat offspring.

Group	Substance	Dose	Treatment (gestation day)	N of litter
Control	Peanut oil (vehicle)	10 mL / kg BW	6	16
PTU	PTU	916µg/kg BW	7 – 21	16
PBDE 60	PBDE 99	60 µg/kg BW	6	21
PBDE 300	PBDE 99	300 µg/kg BW	6	15

2.1.2 THYROID HORMONE LEVELS

Thyroid hormone levels were measured in dam's serum at the beginning (PND 1) and the end of lactation (PND 22). On PND 1, approximately half of the pregnant females with their respective litters were sacrificed by decapitation and trunk blood was collected. Blood from offspring on PND 1 was collected with micro capillaries, centrifuged and the serum obtained after the centrifugation was pooled by gender on a litter basis. From the remaining litters, two males and two females per litter (randomly assigned) were sacrificed on PNDs 14 and 22 and trunk blood collected was pooled on a litter basis. After decapitation, blood was collected and allowed to clot on an ice bath (4°C) for 2 hours. Serum was obtained via centrifugation (2500 rpm for 15 min) of clotted samples and stored at –20°C for later analyses. Serum was frozen in five aliquots (one for each hormone), in order to avoid thawing-freezing effects of frozen samples. Total serum thyroxin (T4), free serum thyroxine (FT4), total serum triiodothyronine (T3), free serum triiodothyronine (FT3) and TSH were measured using the enzyme immunoassay (ELISA) kit purchased from DRG diagnostics –

GmbH, Germany, according to the manufacture instructions. Briefly, the assay is based on the competition principle and separation on a microtiter plate. An unknown amount of antigen present in the sample and a fixed amount of enzyme labeled antigen compete for the binding site of the antibodies coated onto the wells. After the incubation, the wells are washed to stop the competition reaction. The concentration of antigen is inversely proportional to the optical density (OD) measured. The measured ODs of the standard curve are used to construct a calibration curve against which the unknown samples are calculated.

2.1.3 TISSUE DISTRIBUTION OF PBDE 99

Tissue concentration of PBDE 99 in dams and offspring samples, were determined in liver and adipose tissue. During lactation, animals were sacrificed at different lactational periods (described in the paragraph 2.1.1) and tissue (adipose tissue and liver) was pooled based on gender and experimental group, collected from one animal per litter. Thus, PBDE 99 concentration was determined in one pool / group / day of sacrifice, and expressed on the basis of lipid content and per g of wet tissue.

The analytical method employed for PBDE 99 quantification as well as its validation are described in detail in the thesis from Schütz [Schütz, 2004]. Briefly, the method employed was conducted as follows: ***Extraction:*** Prior to extraction, adipose tissue was mixed with a surplus of water-free Na_2SO_4 and powdered in a mortar, and liver was freeze-dried. Then, dried samples were extracted by accelerated solvent extraction (ASE) with toluene (140 bar, 175° C, 5 static cycles at 5 min.). Internal ^{13}C-PBDE standards were added before extraction. The extraction cells (33 ml) were filled up with silica. A blank sample was added to each series of extraction. For this purpose, the extraction cell was filled with silica or, in case of adipose tissue, with silica and Na_2SO_4. ***Clean up:*** Extracts were first purified on a column with silica / 44% concentration sulphuric acid and eluted with n-heptane. Then, in the second clean up, extracts were purified in the column containing 2.5 g alumina B super I, eluted with 25 ml n-hexane : dichloromethane (DCM) (98:2) and 25 ml n-hexane : DCM (1:1), when the PBDEs are found. ***Separation and detection:*** Before GC/MS measurement, recovery standard was added. Separation was performed by GC (gas chromatography) on a DB-5MS capillary column (15 m, 0.25 mm ID, 0.1 μm film thickness) and detection was performed with EI-MS (electron impact ionization – mass spectrometry) in the single ion monitoring (SIM) mode.

2.1.4 ENZYME ASSAY: SAMPLES AND MICROSOME FRACTION PREPARATION

EROD and UDPGT activity were measured in dams and offspring on PND 1 and 22. Immediately after sacrifice, livers were quickly removed, weighed, shock-frozen in liquid N_2 and stored at –80°C. For the liver microsome preparation, hepatic tissue was homogenized in 0.25M sucrose (4 mL/g wet liver weight) using a Potter-Elvehjem glass Teflon homogenizer and centrifuged at 9000 *g* for 20 min to remove the cell debris, nuclei and mitochondria. The post-mitochondrial fraction was then centrifuged at 100,000 *g* for 60 min in a Sorvall® Combi Plus ultracentrifuge. The pellet obtained by ultracentrifugation was suspended in buffer (0.1 M Tris-HCl, 0.15 M KCl, pH 7.4) and centrifuged again at 100,000 *g* for 60 min. The microsomal pellet of the second ultracentrifugation was suspended in a phosphate buffer solution (0.1 M, pH 7.4) containing glycerol (20% v/v) and EDTA (1 mM) and stored in liquid nitrogen until use. Protein concentration in the microsomal fraction was determined by a colorimetric method using Coomassie brilliant blue G dye (Bradford Reagent, Sigma Chemical Co., St. Louis, MO, USA) and bovine serum albumin (Sigma) as standard.

2.1.5 EROD ASSAY

A number of fenaxazone ethers (*e.g.* metoxyresorufin, etoxyresorufin and pentoxyresorufin) work as substrates for *O*-dealkylation reactions mediated by cytochrome p450 (CYP450) enzymes. These substrates are metabolized preferentially by different CYP450 isoforms (*e.g.* etoxyresorufin is metabolized by CYP450 subfamily 1A and pentoxyresorufin metabolized by CYP450 subfamily 2B) but have in common, as a final step, the hydroxylation of the fenaxazone ring which generates hydroxifenaxazone or resorufin. Ethoxyresorufin-*O*-deethylase (EROD) activities were determined essentially as reported by Burke et al. (1985) [Burke *et al.*, 1985] except for the use of an NADPH regenerating system which consisted of 0.25 mM β-NADP, 2.5 mM $MgCl_2$, 5 mM glycose-6-phosphate and 0.5 units of glucose-6-phosphate-dehydrogenase per ml of incubation mixture. Reactions were carried out in quartz fluorimeter cuvettes in a total volume of 2 mL. In the cuvette the following was added: the buffer K_2HPO_4 100mM, pH 7.8 in a volume necessary to complete 2mL, 10µL of the substrate (ethoxyresorufin 1mM in DMSO), performing a final concentration of 5µm in the cuvette and the microsomal protein (the volume depends on the activity expected). The mixture containing microsomes, substrate and potassium phosphate

buffer was equilibrated for 2 min at 37°C and the reaction was started by the addition of 50 µL NADPH-regenerating system. The reaction was carried out for 90 seconds and the increasing fluorescence caused by the accumulation of resorufin was registered in a spectrofluorimeter (Kontron SFM-25) using excitation and emission wavelengths of 550 nm and 582 nm, respectively, with a 5-nm band slit width. The activities (pmol of resorufin / min / mg of microsomal protein) were calculated automatically using special software (Kontron), plotting the resorufin products on the standard resorufin curve. All reactions were carried out in triplicates with a coefficient of variation below 10%.

2.1.6 UDPGT ASSAY

The p-nitrophenol glucuronidation is the reaction that quantifies the activity of uridine-diphosphoglucuronosyl transferase (UDPGT), the enzyme that catalyzes the conjugation reactions of the glucuronic acid with hydroxyl, carboxyl or amine groups from the substrate. The UDPGT is the main enzyme from the phase II metabolism. In general, the glucuronosyl transferases display an enzymatic latency in the microsomal membrane; therefore, the assay is conducted in the presence of a detergent (Triton X-100). The UDP-glucuronosyltransferase (UDPGT) activity was determined essentially as reported by Bock et al. (1983) [Bock *et al.*, 1983] with modifications made by Martin & Black (1994) [Martin *et al.*, 1994]. UDPGT activity was measured in a spectrophotometric assay using UDP-glucuronic acid (UDPGA) (Sigma Aldrich) and p-nitrophenol (Merck) as substrates. Briefly, liver microsomes (0.1 mg) were incubated at 37 ºC in a pre-incubation mixture containing Tris (100 µM, pH 7.4), $MgCl_2$ (5mM); Triton X-100 (0.1% w/v), p-nitrophenol (0.5mM) and water in a final volume of 0.5 mL. All samples were incubated in triplicates and a reference tube was prepared containing all reagents, including p-nitrophenol. After 2 min pre-incubation, the reaction was started adding UDPGA (3mM) and stopped after 30 min with 1 mL trichloracetic acid 5% (w/v). All samples were centrifuged during 30 min at 2500 rpm to precipitate the glucuronidated products. One mL of the supernatant was carefully transferred to a spectrophotometric cuvette containing 250 µl NaOH 2M and read in a multiwell spectrophotometer (O.D. at 412 nm). The activity was calculated by subtracting the p-nitrophenol concentration between the reference tube vs. the sample tube. The extinction coefficient for p-nitrophenol at pH > 10 is 14,9 x $10^3 M^{-1}$ cm^{-1} and the activities were expressed as pmol of p-nitrophenol glucoronidated per min and per mg of protein.

2.1.7 STATISTICAL ANALYSES

The statistical analyses were performed with SPSS software, version 11.5 for Windows. Data from hormone levels, body and organ weights as well as enzyme activity were analyzed by ANOVA (analysis of variance) followed by Dunnett-T test to evaluate the differences between groups. Differences were considered statistically significant when $p<0.05$.

2.2 EXPERIMENT II

For comparative terms, the same experimental protocol (*i.e.* dose levels, animal strain, day of exposure, etc.) from the experiment I was used. However, in the experiment II the endpoints were focused to assess pre-pubertal to adulthood effects in offspring.

2.2.1 ANIMALS AND TREATMENT

Acclimatization of Wistar rats (HsdCpb:WU; Fa. Harlan-Winkelmann, Borchen, FRG) weighing 200 ± 15g was performed for two weeks. The rats were exposed to constant light/dark periods of 12 hours each, a temperature of 21 ± 1°C and 50 ± 5% relative humidity. Rodent chow (Altromin 1324) obtained from Altromin GmbH (Lage, FRG) and tap water were available *ad libitum*. The mating procedure was performed as proposed by Chahoud and Kwasigroch (1977). Two nongravid females were placed with one male for three hours per day and the day of sperm detection in the vaginal smear was considered as day 0 of gestation. The gravid females were randomly assigned among the four groups and housed individually in Type III macrolon cages with stainless steel covers and wood shavings obtained from Altromin GmbH. 2,2´,4,4´,5-pentabromodiphenyl ether (PBDE 99), charge number VL02, was purchased from LGC Promochem GmbH (Germany) with a purity of 98%. Pregnant rats (control=16; PTU=19; 60µg PBDE/kg=20 and 300µg PBDE/kg=19) were treated orally by gavage with a single dose of 60 or 300 µg PBDE 99/kg or peanut oil (control) on day 6 of gestation. The administration volume was 10 ml / kg BW. An additional group was administered the goitrogen, PTU (6-n-propyl-2-thiouracil, Sigma-Aldrich Chemicals GmbH, Steinheim, FRG), which served as reference control for thyroid hormone effects. PTU was given to the gravid dams by adding 5mg/L PTU in the drinking water on gestation days 7 through 21. The average PTU dose through tap water was calculated to be 916µg/kg per day. Dams were allowed to deliver and the litter size was not artificially altered. The experimental protocol has the approval of the National Animal Protection Law (Tierschutzgesetz BGBI. IS. 3082. Genehmigungsnummer: VB104 – G0012/00).

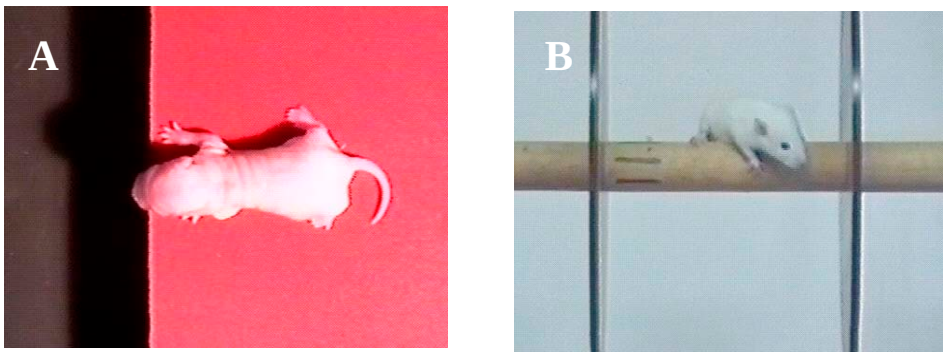
Table 2: Treatment scheme and groups used to assess the developmental effects of PBDE 99 in rat offspring.

Group	Substance	Dose	Treatment (gestation day)	N of litter
Control	Peanut oil (vehicle)	10 mL / kg BW	6	16
PTU	PTU	916µg/kg BW	7 –21	19
PBDE 60	PBDE 99	60 µg/kg BW	6	20
PBDE 300	PBDE 99	300 µg/kg BW	6	19

2.2.2 POSTNATAL REFLEX AND DEVELOPMENTAL LANDMARKS

Developmental landmarks (*i.e.* the day of eruption of incisors, fur development, bilateral eye opening and testes descent) and postnatal reflexes were evaluated in all pups (control=163; PTU=200; 60µg PBDE=218 and 300µg PBDE=200). Starting on postnatal day 3, the offspring were monitored for the development of the spontaneous reflexes righting on surface, forelimbs grasp, cliff-drop aversion and rotating rod test. A brief explanation of each reflex test will be given. Cliff-drop aversion: the pup was placed on the edge of a cliff, the forepaws and the head over the edge. It turned and crawled away from the cliff (Figure 5A). Rotating rod test: the ability to stay on a rotating rod for 3 minutes at 7 rpm was examined beginning on PND 18 (Figure 5B).

Figure 5: Postnatal reflexes : A: Cliff-drop aversion and B: rotating rod test



2.2.3 LOCOMOTOR ACTIVITY (MOBILITRON)

Measurement of circadian motility in rodents has been widely used as sensitive indicator of neurodevelopmental derangements. Some authors have traced a parallel between hyperactivity-induced by xenobiotics in experimental animals with some features of the ADHD in humans [Holene *et al.*, 1998; Rice, 2000]. Therefore, the measurement of locomotor activity in rodents is a sensitive and validated endpoint to assess neurobehavioral toxicity. Circadian motility was measured over 24-h periods on PND 36 and PND 71 in individual offspring using an automated device (Mobilitron®). A more detailed description of the method and experimental procedure will be given below.

Mobilitron® apparatus: The device allows us to monitor up to 48 animals simultaneously over a period of 24 h. Every individual cage is placed between two electronic circuit board, containing 3 infrared light beam barriers which monitor the horizontal movements of the rat. The light beams are situated on the longitudinal axis of the cage and are connected in such way that an impulse is only given when the animal leaves one light barrier and reaches another. Thus, no impulse is registered by a sleeping/resting animal. All 48 cages are connected to an interface (Mobilitron®) which "translates" the information generated to a personal computer. The duration of cycles/phases (1 min to 30 min interval) and measurements (30 min to months) can be adjusted according to the experimental protocol. In this study, we have used the standard protocol consisting of 5-min intervals over 24-h period measurement. The following parameters were analyzed at the end of an experiment: *Intensitiy of activity per day:* the number of light beam interruptions (LBI) was counted per day; *Mean intensity of activity per phase:* total light beam interruption was divided by the number of activity phases. *Total duration (h) of activity per day:* the sum of duration of all active phases. *Mean duration (min) of activity per phase:* total duration divided by the number of active phases. *Frequency:* the number of active phases per day. The method has been described in detail by Thiel et al. (1989).

Experimental conditions: One day before the beginning of the test, one male and one female per litter were transferred to the Mobilitron room in order to allow the animals to adjust to their new environment and the solitary accommodation before measurements were taken. Since we were interested to evaluate the effects of pre- and postnatal exposure to PBDE 99 on the basal locomotor activity, the habituation time was extremely necessary to avoid the exploratory behavior which is common in rodents when placed into a new environment. The rats were exposed to constant light/dark periods of 12 hours each, a

temperature of 21 ± 1°C and 50 ± 5% relative humidity. Rodent chow (Altromin 1324) obtained from Altromin GmbH (Lage, FRG) and tap water were available *ad libitum*. The animals were randomly assigned to the Mobilitron® device to avoid confounding factors. Individual measurements were registered in 5 min intervals over 24-h (*i.e.* the sum of light beams interruptions in every 5-min interval was added during one day - 288 cicles/interval per day) on PNDs 36 and 71 in male and female offspring.

2.2.4 REPRODUCTIVE ASSESSMENT OF MALE OFFSPRING

A general description of male offspring reproductive assessment is presented in Table 3. At adulthood (± PND 110), testes from approximately 6 animals per group from different litters were collected for testicular morphometric analysis and Sertoli cell count.

Later on PND 140, 12 males per group (from different litters) were killed by decapitation for spermatogenesis analysis. From these animals, trunk blood was collected for hormone analysis and organ weights (thymus, spleen, liver, testis, epididymis, seminal vesicle and ventral prostate) were recorded. The relative weights were also calculated according to the following formula: (organ weight/body weight) X 100. The right testis and caudal epididymis were kept in saline buffer for spermatid and sperm counts, respectively, while the left testis was evaluated for testicular cell population (flow cytometry). Additionally, littermates (about 20 per group) were assessed for reproductive performance and sexual behavior at approximately PND 160. Detailed description of each endpoint will be presented in separate topics.

Table 3: Reproductive assessment of male offspring.

PND	N of animals / group	Endpoint
110	5- 6	Right testis: Testicular Morphology and Sertoli cell count
140	12	Right testis and epididymis: spermatid and sperm count and sperm morphology Left testis: testicular cell population (flow cytometry) Blood: testosterone and LH levels Organ weight
158-162	16-20	Male reproductive performance and sexual behavior

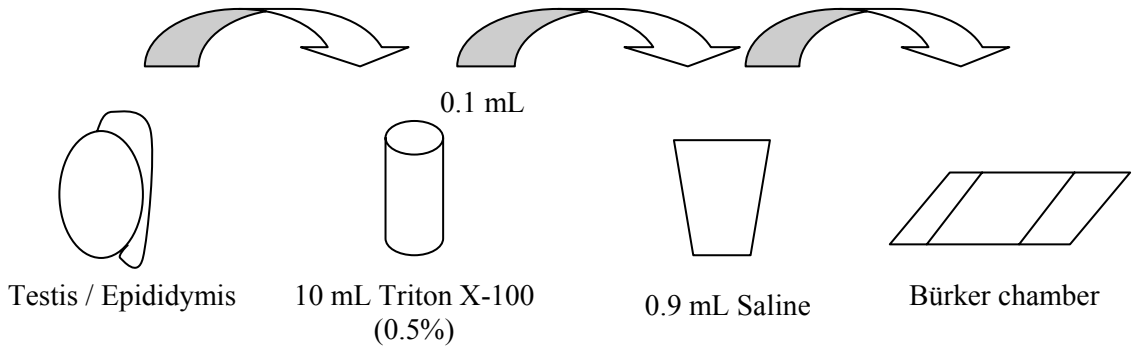
2.2.5 SPERMATID COUNT AND DAILY SPERM PRODUCTION (DSP)

After removing the tunica albuginea, the testis was minced and homogenized for one minute in 10 mL 0.9% NaCl containing 0.5% Triton X-100 at medium speed in an IKA-RW 15 tissuemizer (Janke and Kunkel, Staufen in Breisgau, Germany). The homogenate was diluted 1:10 in saline and the number of homogenization-resistant spermatids (stage 17 to 19) was counted in a hemocytometer (Buerker) (Figure 6). DSP was calculated, dividing the number of homogenization-resistant spermatids by 6.1 [Robb et al. 1978]. The factor of 6.1 corresponds to the number of days that the spermatids on stages 17 to 19 are present in the seminiferum epithelium.

2.2.6 SPERM COUNT AND MORPHOLOGY

Cauda epididymis was minced and homogenized for one minute in 10 mL 0.9% NaCl containing 0.5% Triton X-100 at medium speed in an IKA-RW 15 tissuemizer (Janke and Kunkel, Staufen in Breisgau, Germany). The number of homogenization-resistant sperm was counted in a hemocytometer (Buerker) (Figure 6). For sperm morphology, the ductus deferens was rinsed with 1 mL 0.9% NaCl and the obtained sperm suspension was stained with 2% eosin. Two hundred sperm per animal were analyzed and the number of sperm displaying tail or head abnormalities was registered.

Figure 6: Sperm and spermatid calculation



$$\frac{\text{N° of counted cells}}{\text{Y}} = \frac{5{,}76 \times 10^{-4}\ \text{mL (chamber volume)}}{1\ \text{mL}}$$

(Y) x 100 (Factor of dilution) = N° of cell per animal

2.2.7 FLOW CYTOMETRY ANALYSIS

Testicular cell population (12 animals per group) on the basis of their DNA ploidy was investigated in the left testis of the same animals which had been evaluated for spermatid and sperm count. That provided us complementary information for the sperm and spermatid counts performed in the other testis. Following, a brief description of the method will be presented. *Testicular cell preparation:* The tunica albuginea was removed and testes were cut and incubated for 30 min at 37°C in a medium containing PBS, 0.1% glucose, 25 µg/mL deoxyribonuclease I supplemented with 0.25% trypsin. Cells were centrifuged and incubated again in the same medium supplemented with 1 mg/mL collagenase. The pellet was suspended in the medium containing 0.1% bovine serum albumine and filtered through 200 µm nylon mesh to discard the debris. After the last centrifugation of the cell filtrates, the cells were fixed in 70% ethanol and stored at -20°C. *Staining of cells for flow cytometry:* For staining procedure, approximately 10 X 10^6 fixed cells were thawed and washed twice with PBS. DNA staining was performed by suspending the cells in PBS with 100µg/mL ribonuclease A and 50 µg/mL propidium iodide and incubated in the dark room for 30 min. Propidium iodide fluorescence was measured at a wavelength of 620 nm in an

spectrofluorimeter (Shimadzu) and according to cellular DNA contents cells were differentiated as mature haploid, immature haploid and diploid.

2.2.8 TESTICULAR MORPHOMETRY AND SERTOLI CELL COUNT

On PND 110, animals (6 per group) were sacrificed by decapitation and the right testis was removed and fixed in Bouin's solution (66.7% picric acid; 26.7% formalin; 6.6% acetic acid) for 24 h and than dehydrated in increasing concentrations of ethanol and xylol before paraffin embedding for histological preparation. Tissue was cut in 5 μm sections and stained with hematoxilin / eosin. *Determination of Sertoli cell number per tubule cross section:* Sertoli cells nucleoli are counted in 25 round or nearly round seminiferous tubule cross-sections, chosen at random for each animal at 1000 x magnification (100 x oil immersion objective). These counts are corrected for section thickness and the nucleolar diameter as described in equation A. Twenty-five Sertoli cell nucleoli diameter are measured for each animal at 1000 x magnification in a light microscope fitted with a 3CCD Sony camera. The nucleolar diameter is measured in the captured images using the Scion image analyzer program previously calibrated with a stage micrometer (100 μm in 2 μm) [Russell et al., 1990; Hess et al., 1993; Neves et al., 2002]. *Determination of the volume density and absolute volume of the seminiferous tubules:* The volume density of the seminiferous tubules is determined by point counting. The testis is observed at 25 x magnification in a light microscope fitted with a 3CCD Sony camera using the Scion image analyzer program. Photomicrographs of ten randomly chosen fields per testis are saved in a CD for later analysis. The photomicrographs are analyzed using the Irfanview graphic viewer, where a 140-intersection grid is superimposed on the images. Cross sections falling over the seminiferous tubules (comprising tunica albuginea, epithelium and lumen) are scored and the volume density expressed as the ratio of the number of points over the seminiferous tubules to total points analyzed (1400 points) (**equation B**). The absolute volume of the seminiferous tubules is obtained by multiplying the volume density by the testis volume. For this calculation the specific gravity of the testis is assumed to be 1.0, so that the testis weight (g) is used as equivalent to testis volume (cm^3) (**equation C**). *Determination of the diameter of the seminiferous tubules:* In each testis, the smallest diameter of 30 round or nearly round tubular profiles chosen at random are measured at 100 x magnification in a light microscope fitted with a 3CCD Sony camera. The diameter is measured in the captured images using the Scion image analyzer program previously calibrated with a stage micrometer (1000 μm in 10

μm). Sertoli cells nucleoli were counted in 25 rounds or nearly round seminiferous tubule cross-sections, chosen at random, for each animal. These counts were corrected for section thickness and the smallest recognizable nucleolar profile (cap section) as described previously [Russel et al., 1990; Hess et al., 1993; Neves et al., 2002]. Twenty-five Sertoli cell nucleoli diameter were measured for each animal. The results were expressed as Sertoli cell number/ seminiferous tubule cross section. All measurements were made blind by the same analyst. *Determination of the total length of the seminiferous tubule:* The total length of the seminiferous tubule is calculated from the absolute volume and the mean diameter of the seminiferous tubule as described in **equation D**. *Total number of Sertoli cells per testis***:** The number of Sertoli cells per testis is obtained from the Sertoli cell number per tubule cross section and the total tubule length as described in **equation E**.

(A) Sertoli cell number per tubule cross section

$$MSN \times \frac{ST}{ST + MND}$$

Where:

MSN = Mean Sertoli cell nucleolar number per tubule

ST = Section thickness

MND = Mean nucleolar diameter

(B) Volume density (VS) of the seminiferous tubule

$$\frac{\text{Points over seminiferous tubules}}{\text{Total points}}$$

(C) Absolute volume of seminiferous tubule (AVS) (cm^3)

VS x Testis volume (= Testis weight (g)) *

* The testis weight is used assuming that the specific gravity of the testis is 1.0

(D) Total length of seminiferous tubule (cm)

$$\frac{AVS}{\pi\ (3{,}1416) \times \frac{[MDST\ (cm)]^2}{2}}$$

Where:
AVS = Absolute volume of seminiferous tubule
MDST = Mean diameter of seminiferous tubule

(E) Sertoli cell number per testis
Sertoli Cell number per tubule cross section (A) x Total length of seminiferous tubule (D)

2.2.9 TESTOSTERONE AND LH LEVELS

After decapitation, trunk blood was collected and allowed to clot on an ice bath (4°C) for 2 hours. Serum was collected via centrifugation of clotted samples (2500 rpm for 15 min) and stored at –20°C for later analyses. Serum testosterone and LH were measured using the enzyme immunoassay (ELISA) kit purchased from DRG diagnostics – GmbH, Germany. Testosterone was measured in crude rat serum which is reliable for comparison among groups. Briefly, the assay is based on the competition principle and microtiter plate separation. An unknown amount of antigen present in the sample and fixed amount of enzyme labelled antigen compete for the binding site of the antibodies coated onto the wells. After the incubation the wells are washed to stop the competition reaction. The concentration of antigen is inversely proportional to the optical density measured. The measured ODs of the standard curve are used to construct a calibration curve against which the unknown samples are calculated. The testosterone / LH concentrations in the sample are determined plotting the result in a standard curve.

2.2.10 MALE REPRODUCTIVE PERFORMANCE

To evaluate the fertility and paternal mediated effects, adult male offspring (158-162 days old, N=16-20 animals/group), representing all litters, were mated with untreated females (1:1) to determine whether the males were fertile and could sire normal offspring. The mating occurred daily for 14 days and the day when sperm were found in the vaginal smear was recorded. Vaginal smears were collected daily and examined for the presence of sperm. The day of sperm detection in vaginal smears was considered as day 0. The dams were sacrificed on gestation day 21 and the uterus was excised. The uterine and fetal weights and the number of implantations, resorptions and fetuses were determined. The fetuses were examined for external anomalies and sexed.

2.2.11 MALE SEXUAL BEHAVIOR

One week after the end of mating, the same rats were assessed to check whether the exposure to PBDE 99 during development would affect their sexual behavior. Briefly, about 20 males per group (representing all litters) were mated with untreated females in estrus (1:1) and the sexual behavior of each mating was recorded for 20 min under blue light illumination (black light, 75W; Osram, Germany) using a video camcorder (Hi8 Handycam CCD-V800E, Sony). The recorded videos, which provide a permanent record and the opportunity for replay, were evaluated by a trained observer in a blind way. The phase of the estrous cycle of the untreated females was predetermined by examining vaginal smears. The Figure 7 illustrates the mating stage in rats, from the introduction of the female until ejaculation. The following endpoints were evaluated in every 20 min mating period:

a) Mounting latency: time lapse (sec) from the introduction of the female in the male's cage until the first mount (with or without penetration);

b) Intromission latency: time lapse (sec) from the introduction of the female in the male's cage until the first penetration;

c) Ejaculatory latency: time lapse (sec) from the first penetration until the first ejaculation;

d) Number of penetrations until the first ejaculation

e) Intromission frequency: number of penetrations (min) until the first ejaculation

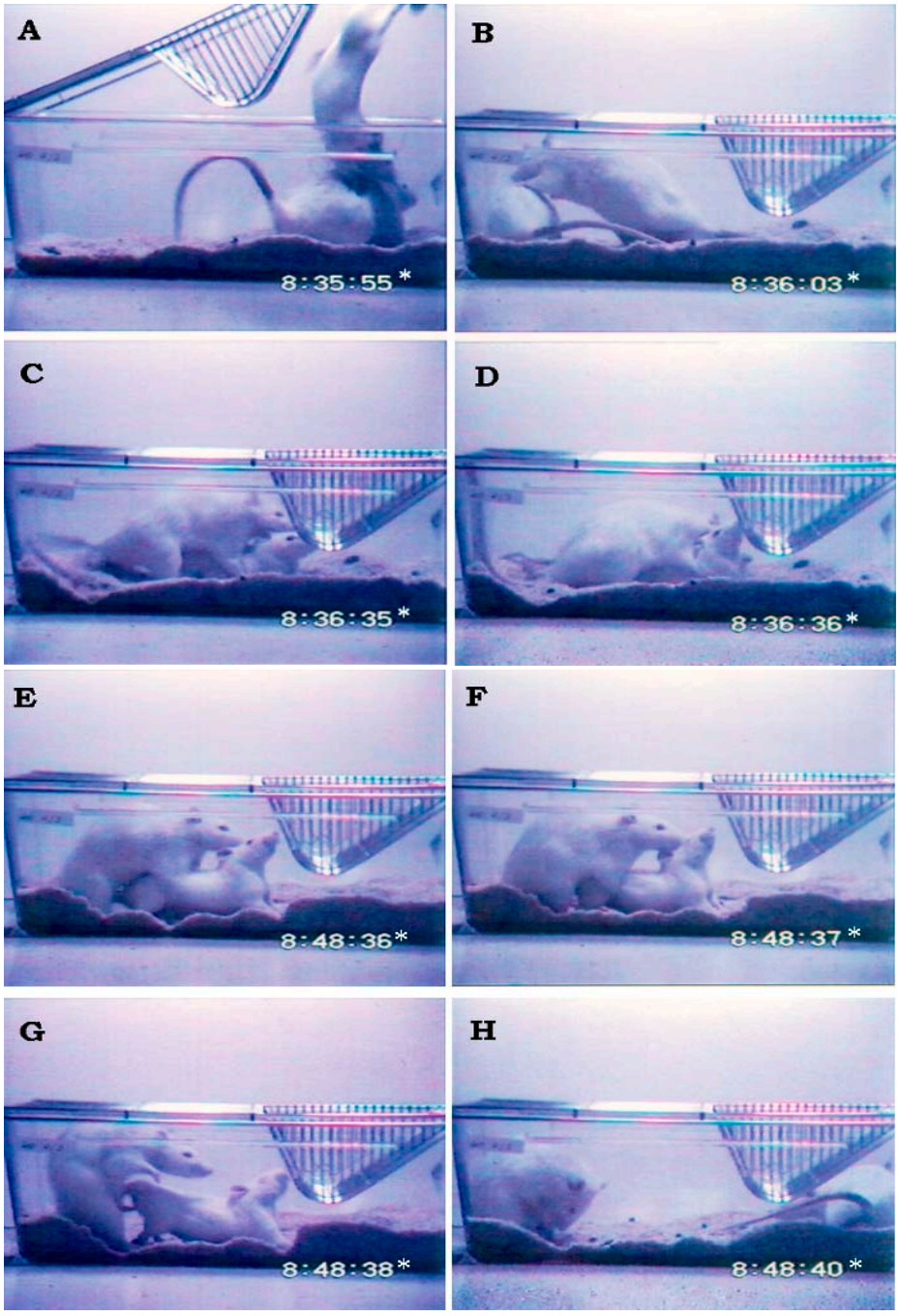
f) Number of animals with two or more ejaculations in 20 min mating.

The method was previously described in detail by Chahoud and Faqi (1998).

2.2.12 STATISTICAL ANALYSES

The statistical analyses were performed with SPSS software, version 11.5 for Windows. Male and female data within each group were tested by the Student t-test. Data with normal distribution were analyzed by ANOVA (analysis of variance) followed by Dunnett-T test. The equality of survival distributions was performed using Kruskal-Wallis test followed by Mann-Whitney U test. Proportions were analyzed by the Fisher's exact test and statistical differences were considered significant when $p<0.05$.

Figure 7: Sexual behavior in rats



From DALSENTER, P. R. **Reproduktionstoxikologische und toxikokinetische Untersuchungen an männlichen Ratten, die gegenuber γ-Hexachlororcyclohexan (lindan) während der Laktationsperiode exponiert waren.** Berlin, 1996. PhD thesis in veterinary and toxicology). Institut für Klinishe Pharmakologie und Toxikologie - Freien Universität Berlin.
(A) Introduction of the female in the male's cage; (B) first mounting; (C-D) first mounting followed by penetration; (E) mounting with penetration; (F-G) ejaculation; (H) penis cleaning.