CHAPTER 3: RESULTS

3.1 EXPERIMENT I: EFFECTS OBSERVED DURING LACTATION

3.1.1 BODY WEIGHT AND ORGAN WEIGHTS

Dams: Exposure of dams at gestation day 6 to a single dose of PBDE 99 produces no significant effect on body weight gain during gestation (data not shown). After delivery, no gross abnormalities in the offspring were observed and sex ratio and litter weight were not significantly affected compared to control (data not shown). No significant changes in body and brain weights were observed during lactation, except for a slight increase in the absolute brain weight observed at the end of lactation (PND 22) in the 60µg PBDE 99 group (Table 4). However, exposing dams to a low dose of the goitrogen PTU resulted in a decrease in both absolute and relative (organ weight: body weight) liver weights at the end of lactation (PND 22) (Table 4).

Table 4: Body weight and organ weights from dams at the beginning (PND 1) and the end of lactation (PND 22).

	Parameter	Control	PTU	PBDE 60	PBDE 300
PND 1 &	Body (g)	233.5 ± 20.0	219.9 ± 18.3	238.8 ± 22.6	231.5 ± 17.7
	Brain (g)	1.76 ± 0.08	1.82 ± 0.07	1.78 ± 0.04	1.79 ± 0.07
	Brain (%)	0.76 ± 0.05	0.83 ± 0.05	0.75 ± 0.07	0.78 ± 0.07
	Liver (g)	9.7 ± 1.2	9.1 ± 1.4	9.6 ± 1.4	11.0 ± 1.43
	Liver (%)	4.1 ± 0.5	4.1 ± 0.3	4.1 ± 0.6	4.8 ± 0.5
PND 22 $	Body (g)	299.1 ± 14.9	298.0 ± 26.8	303.0 ± 25.6	291.7 ± 20.9
	Brain (g)	1.75 ± 0.07	1.80 ± 0.05	1.86 ± 0.09 *	1.81 ± 0.06
	Brain (%)	0.59 ± 0.03	0.61 ± 0.06	0.62 ± 0.05	0.62 ± 0.04
	Liver (g)	15.1 ± 1.8	12.5 ± 1.4 *	14.3 ± 2.0	13.8 ± 2.2
	Liver (%)	5.0 ± 0.5	4.2 ± 0.6 *	4.7 ± 0.3	4.7 ± 0.6

Values are mean ± SD. * $p<0.05$ by ANOVA followed by Dunnett t-test
& nº of dams: control=8; PTU=7; PBDE 60=10 and PBDE 300=8
$ nº of dams: control=8; PTU=9; PBDE 60=11 and PBDE 300=7
% - organ index mass related to body mass

Offspring: No differences in body and brain weights were observed in the offspring (Table 5). However, on PND 22 both absolute and relative liver weights were reduced in male offspring exposed pre- and postnatally (via milk) to 60µg PBDE 99 (Table 5).

Table 5: Body weight and organ weights from offspring at the beginning (PND 1) and the end of lactation (PND 22).

Parameter			Control	PTU	PBDE 60	PBDE 300
PND 1 [&]	Male	Body (g)	6.2 ± 0.8	6.1 ± 0.6	6.5 ± 0.4	6.4 ± 0.7
		Brain (g)	0.23 ± 0.03	0.23 ± 0.02	0.23 ± 0.01	0.25 ± 0.03
		Brain (%)	3.78 ± 0.50	3.74 ± 0.15	3.54 ± 0.30	3.95 ± 0.56
	Female	Body (g)	6.1 ± 0.6	5.6 ± 0.5	6.2 ± 0.4	6.3 ± 0.7
		Brain (g)	0.23 ± 0.02	0.21 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
		Brain (%)	3.85 ± 0.41	3.74 ± 0.29	3.51 ± 0.35	3.72 ± 0.38
PND 22 [$]	Male	Body (g)	42.4 ± 7.8	37.2 ± 8.8	40.7 ± 7.9	42.3 ± 3.5
		Brain (g)	1.38 ± 0.05	1.31 ± 0.12	1.36 ± 0.07	1.38 ± 0.05
		Brain (%)	3.09 ± 0.39	3.43 ± 0.67	3.33 ± 0.53	3.13 ± 0.14
		Liver (g)	1.68 ± 0.35	-	1.43 ± 0.35 *	1.70 ± 0.16
		Liver (%)	3.94 ± 0.23	-	3.50 ± 0.25 *	3.89 ± 0.27
	Female	Body (g)	38.0 ± 5.2	36.0 ± 8.8	40.6 ± 10.8	41.4 ± 4.0
		Brain (g)	1.33 ± 0.02	1.28 ± 0.10	1.28 ± 0.07	1.36 ± 0.06
		Brain (%)	3.35 ± 0.34	3.75 ± 0.96	3.37 ± 0.44	3.24 ± 0.51
		Liver (g)	1.48 ± 0.25	-	1.44 ± 0.40	1.61 ± 0.23
		Liver (%)	3.89 ± 0.25	-	3.57 ± 0.42	3.87 ± 0.28

Values are mean ± SD. * $p<0.05$ by ANOVA followed by Dunnett t-test
[&] nº of litter: control=8; PTU=7; PBDE 60=10 and PBDE 300=8
[$] nº of litter: control=8; PTU=9; PBDE 60=11 and PBDE 300=7
% - organ index mass related to body mass

3.1.2 THYROID HORMONE CONCENTRATIONS

Thyroid hormones play a crucial role during development. From womb to lactational period, the thyroid hormone signaling cascade is involved in many physiological processes

modulating neurodevelopment, sexual maturation and other hormonal systems. After a single dose of PBDE 99 at the beginning of the implantation period (GD 6), alterations in thyroid hormones were observed in dams and offspring. The main findings are reported below.

Table 6: Thyroid hormone concentration in maternal serum during lactation. Animals were exposed to a single dose of PBDE 99 by gavage on gestation day 6 or to the goitrogen, PTU on GD 7-21, via drinking water.

Parameters		Control	PTU	PBDE 60	PBDE 300
PND 1 $	T3 (ng/mL)	3.46 ± 0 .17	3.06 ± 0.15	2.71 ± 0.11*****	3.65 ± 0.20
	FT3 (pg/mL)	6.19 ± 0.38	6.07 ± 0.43	5.89 ± 0.38	5.30 ± 0.36
	T4 (ng/mL)	67.71 ± 6.56	41.04 ± 1.40*****	52.16 ± 2.13*****	45.29 ± 0.92*****
	FT4 (pg/mL)	57.49 ± 1.50	45.20 ± 1.45*****	53.44 ± 0.86	54.56 ± 1.09
	TSH (ng/mL)	47.41 ± 2.10	40.06 ± 1.77*****	34.04±1.52*****	41.71 ± 1.91
PND 22 §	T3 (ng/mL)	2.65 ± 0.20	2.40 ± 0.25	2.33 ± 0.22	2.45 ± 0.28
	FT3 (pg/mL)	5.31 ± 0.34	6.54 ± 0.37*****	5.45 ± 0.30	4.73 ± 0.41
	T4 (ng/mL)	56.20 ± 5.02	76.09 ± 8.87	45.91 ± 1.99	44.22 ± 3.93
	FT4 (pg/mL)	64.43 ± 1.07	64.89 ± 1.53	61.19 ± 1.37	61.07 ± 2.08
	TSH (ng/mL)	41.30 ± 1.93	36.91 ± 3.05	37.02 ± 2.69	40.28 ± 4.68

Values are mean ± standard deviation. Statistical analysis was performed per day using ANOVA followed by Dunnett t-test and significance (*) was confirmed when $p<0.05$.
$ (nº of animals): control = 8, PTU = 7, PBDE 60 = 10 and PBDE 300 = 8
§ (nº of animals): control = 8, PTU = 9, PBDE 60 = 11 and PBDE 300 = 6
% - organ index mass related to body mass

Dams: The thyroid hormone status in dams after PBDE 99 and PTU exposure is depicted in Table 6. Exposing dams to the goitrogen PTU produced severe hypothyroxinemia at the beginning of lactation (just after cessation of PTU treatment) (Table 6). Hypothyroxinemia was indicated by low total and free- thyroxin (T4) concentration in dams' serum. Interestingly, TSH levels were also reduced on PND1 after low dose exposure to PTU. The mechanism of action of PTU include (a) inhibition of iodination by competition between PTU and tyrosyl residues of thyroglobulin for oxidized iodine; (b) inhibition of the coupling reaction of diiodotyrosine by binding of PTU or one of its metabolites to

thyroglobulin resulting in a change of structure; and (c) inhibition of the deiodination of T4 to T_3, therefore, lowering the levels of T4 in treated animals [Harvey et al., 2002]. After 22 days (end of lactation), T4 and TSH levels were restored to normal levels as the exposure to

Table 7: Thyroid hormone levels in male offspring serum during lactation. Animals were exposed to a single dose of PBDE 99 by gavage on gestation day 6 or gestational exposure to the goitrogen PTU via drinking water.

Parameters		Control	PTU	PBDE 60	PBDE 300
PND 1 $	T3 (ng/mL)	1.53 ± 0.23	0.69 ± 0.05*****	1.05 ± 0.12	¥ 0.98 ± 0.12
	FT3 (pg/mL)	0.45 ± 0.03	0.49 ± 0.06	0.45 ± 0.03	¥ 0.56 ± 0.07
	T4 (ng/mL)	27.90 ± 1.47	20.67 ± 2.43*****	28.82 ± 1.30	28.98 ± 1.97
	FT4 (pg/mL)	1.90 ± 0.25	0.89 ± 0.20*****	1.30 ± 0.20	1.91 ± 0.19
	TSH (ng/mL)	31.80 ± 3.48	12.47± 3.42*****	20.78 ± 2.83*****	26.33 ± 3.12
PND 14 &	T3 (ng/mL)	2.66 ± 0.11	2.49 ± 0.16	2.58 ± 0.13	2.48 ± 0.16
	FT3 (pg/mL)	1.83 ± 0.15	1.67 ± 0.12	1.76 ± 0.15	1.75 ± 0.13
	T4 (ng/mL)	63.13 ± 3.05	48.29 ± 4.33*****	57.06 ± 3.91	55.02 ± 3.51
	FT4 (pg/mL)	2.93 ± 0.22	3.47 ± 0.76	2.52 ± 0.16	2.45 ± 0.04
	TSH (ng/mL)	26.11 ± 5.06	32.90 ± 7.77	27.18 ± 5.02	31.48 ± 6.91
PND 22 §	T3 (ng/mL)	2.84 ± 0.07	2.84 ± 0.04	2.62 ± 0.10	2.79 ± 0.08
	FT3 (pg/mL)	5.00 ± 0.19	4.66 ± 0.21	4.58 ± 0.19	4.68 ± 0.18
	T4 (ng/mL)	73.84 ± 3.49	76.35 ± 3.64	75.60 ± 3.42	60.12 ± 2.43*****
	FT4 (pg/mL)	17.16 ± 0.96	17.80 ± 1.49	20.43 ± 0.84	14.99 ± 1.15
	TSH (ng/mL)	21.59 ± 2.14	18.93 ± 2.52	22.01 ± 1.91	20.80 ± 1.59

Values are mean ± standard deviation. Statistical analysis was performed per day using ANOVA followed by Dunnett t-test and significance (*) was confirmed when $p<0.05$.
$ blood was pooled by litter on gender basis. (nº of litters):control = 8, PTU = 7, PBDE 60 = 10 and PBDE 300 = 8; **&** blood was pooled from two offspring per litter. (nº of litters):control = 8, PTU = 7, PBDE 60 = 9 and PBDE 300 = 6; **§** blood was pooled from two offspring per litter. (nº of litters):control = 8, PTU = 7, PBDE 60 = 10 and PBDE 300 = 6 **¥** measured only in 5 samples
% - organ index mass related to body mass

PTU was discontinued at the end of gestation. Nevertheless, a significant increase in FT3 levels was observed in this group.

Exposure to low dose PBDE 99 also produced changes in thyroid hormone concentrations observed during lactation. Although not as pronounced as in the PTU group, hypothyroxinemia was also detected in dams exposed to either 60µg or 300µg PBDE 99 on PND 1, displaying significant reductions in T4 concentrations (Table 6). Animals exposed to 60µg PBDE 99 exhibited lower total T_3 and TSH concentrations compared to controls (Table 6). At the end of lactation, no significant changes were observed in dams exposed to PBDE 99 in any thyroid hormones measured. However, the total and free T4 levels were about 80% of control, although the differences were not statistically significant. The subtle changes in thyroid hormone concentrations observed at the beginning of lactation were no longer apparent as the body burden of PBDE 99 decreased over the lactation. The rate of transfer of PBDE is very high into the milk, playing an important role in its elimination [Darnerud *et al.*, 2001].

Offspring: Thyroid hormone system is under development from middle gestation until late postnatal life. It has been shown that subtle changes in the thyroid signaling system may cause permanent deleterious effects in offspring [Poterfield, 1994; Poterfield, 2000]. Therefore, the measurement of thyroid hormone concentrations during development is a important tool to predict possible adverse effects late in life. Using a low dose of the goitrogen, PTU, significant decreases in T4 (total and free) and TSH in male and female offspring were seen on PND 1 (Tables 7 and 8). However, thyroid hormone concentrations returned to normal levels at the last half of the lactational period, probably because PTU and/or maternal thyroid hormone exposure ceased at the beginning of lactation (Table 7 and 8). Except for a significant reduction of total T4 levels in male offspring on PND 14, no pronounced alteration in thyroid hormone homeostasis was observed in PTU group on PNDs 14 and 22. PTU related changes in thyroid hormone concentrations are reversible in offspring as the concentrations returned to normal levels after cessation of PTU treatment.

Exposure to PBDE 99 during pre- and postnatal (via milk) periods caused slight changes in pups thyroid hormone status during lactation. On PND 1, males exposed to 60µg PBDE 99 exhibited lower TSH, while females from the same group displayed lower free T4 and TSH compared to controls (Table 7 and 8). No effect was seen in the 300µg group. Later, at the end of lactation (PND 22), males and females exhibited decreases in total T4 and free T4 (only females) (Tables 7 and 8). This data suggests that accumulation of PBDE 99 in offspring tissues (due to high intake from mother's milk) is involved in the lower T4

concentrations. These changes in humans, even being subtle, can translate into severe functional disorganization as has been demonstrated in some studies in humans [see review Colborn, 2004].

Table 8: Thyroid hormone concentrations in female offspring serum during lactation. Animals were exposed to a single dose of PBDE 99 by gavage on gestation day 6 or gestational exposure to the goitrogen PTU via drinking water.

Parameters		Control	PTU	PBDE 60	PBDE 300
PND 1 $	T3 (ng/mL)	0.98 ± 0.11	0.89 ± 0.06	1.07 ± 0.11	1.13 ± 0.19
	FT3 (pg/mL)	0.46 ± 0.07	0.47 ± 0.07	0.50 ± 0.06	0.57 ± 0.09
	T4 (ng/mL)	27.14 ± 0.97	20.69 ± 2.04*	26.87 ± 0.88	27.13 ± 1.68
	FT4 (pg/mL)	1.86 ± 0.33	0.87 ± 0.23*	0.67 ± 0.15*	1.76 ± 0.22
	TSH (ng/mL)	35.16 ± 5.02	16.19 ± 4.83*	17.33 ± 2.78*	40.80 ± 3.80
PND 14 &	T3 (ng/mL)	2.59 ± 0.20	2.17 ± 0.31	2.30 ± 0.14	2.40 ± 0.17
	FT3 (pg/mL)	1.93 ± 0.20	1.83 ± 0.19	2.06 ± 0.11	2.05 ± 0.18
	T4 (ng/mL)	62.88 ± 5.04	56.37 ± 4.31	60.20 ± 2.87	59.53 ± 3.63
	FT4 (pg/mL)	3.94 ± 0.89	2.63 ± 0.29	2.84 ± 0.36	3.43 ± 0.59
	TSH (ng/mL)	28.91 ± 5.22	29.18 ± 4.96	29.58 ± 4.64	31.48 ± 6.91
PND 22 §	T3 (ng/mL)	2.92 ± 0.12	2.75 ± 0.10	2.75 ± 0.08	2.82 ± 0.08
	FT3 (pg/mL)	5.07 ± 0.15	4.91 ± 0.20	4.92 ± 0.15	5.11 ± 0.17
	T4 (ng/mL)	73.25 ± 4.40	68.95 ± 3.43	61.64 ± 3.95	56.86 ± 2.67*
	FT4 (pg/mL)	19.93 ± 0.96	18.91 ± 1.81	20.66 ± 1.25	15.07 ± 1.05*
	TSH (ng/mL)	30.63 ± 0.84	27.64 ± 1.66	32.87 ± 1.12	29.56 ± 1.08

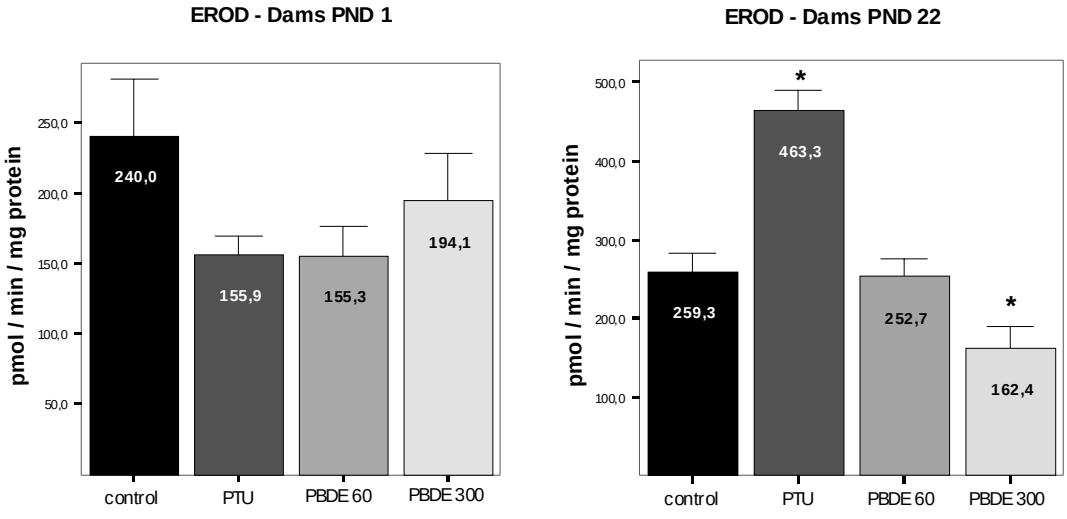
Values are mean ± standard deviation. Statistical analysis was performed per day using ANOVA followed by Dunnett t-test and significance (*) was confirmed when $p<0.05$.
$ blood was pooled by litter on a gender basis. (nº of litters):control = 8, PTU = 7, PBDE 60 = 10 and PBDE 300 = 7; **&** blood was pooled from two offspring per litter. (nº of litters):control = 8, PTU = 7, PBDE 60 = 9 and PBDE 300 = 6; **§** blood was pooled from two offspring per litter. (N of litters):control = 8, PTU = 7, PBDE 60 = 10 and PBDE 300 = 6
% - organ index mass related to body mass

3.1.3 EFFECTS ON HEPATIC ENZYME ACTIVITIES

Activities of hepatic EROD, being indicative of specific induction of CYP1A, and uridinediphosphate glucuronosyltransferase (UDPGT), the main enzyme from the phase II metabolism, in dams and offspring are given in Figures 8, 9 and 10. Monooxygenases from the family CYP1A are involved in xenobiotic clearance and activation, being inducible by several environmental pollutants, including 2,3,7,8-tetrachlorodibenzyl-*p*-dioxin (TCDD) and PCBs [WHO, 2002]. Since this CYP family is responsible for biotransformation of several xenobiotics and it is postulated that the toxic mechanism is mediated via the Ah receptor, EROD activity has been widely used as a biomarker for induction of this enzyme family. UDPGT activity was measured because experimental studies have shown that PBDEs alter UDPGT activity in rodents. Moreover, since UDPGT is involved in T4 metabolism, induction of this enzyme could lead to increased elimination of T4.

Enzyme activity in dams: Exposing pregnant females to a low dose of PBDE 99 produced slight changes in hepatic enzyme levels. At the beginning of lactation (PND 1) EROD activity was normal in dams from all groups. At the end of lactation (PND 22), dams exposed to the goitrogen, PTU, displayed an increased EROD activity (almost twice higher than control), while dams exposed to 300µg PBDE 99 had a significant reduction in EROD activity, about 40% reduced from control (Figure 8). The same tendency was also seen for

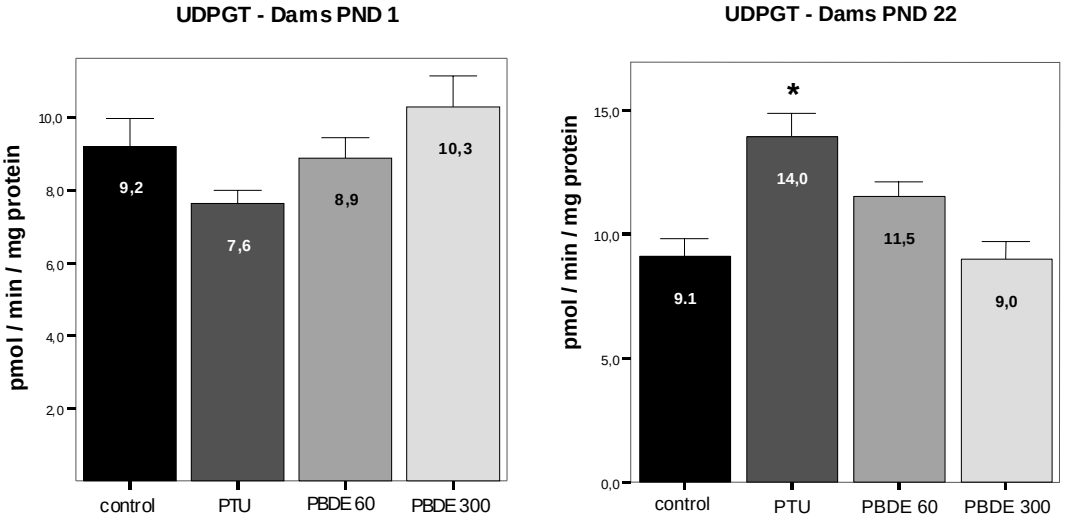
Figure 8: Ethoxyresorufin-O-deethylase (EROD) activities in dams at the beginning (PND 1) and at the end (PND 22) of lactation.



* ANOVA followed by Dunnett t-test. Differences were considered significant when $p<0.05$. Values are mean ± standard deviation

UDPGT levels when the activities were measured during lactation. At the beginning (PND 1), no significant changes were observed in UDPGT levels from animals exposed to either PTU or PBDE 99 (Figure 9). However, at the end of lactation (PND 22), UDPGT activity was elevated in dams exposed to the goitrogen PTU compared to control (the same profile observed in EROD activity), being approximately 50% higher in this group (Figure 9). No effect was seen in any enzyme measured 22 days post partiturition in dams exposed to PBDE 99 (Figure 9).

Figure 9: Uridine diphosphoglucuronosyl transferase (UDPGT) activities in dams at the beginning (PND 1) and at the end (PND 22) of lactation.

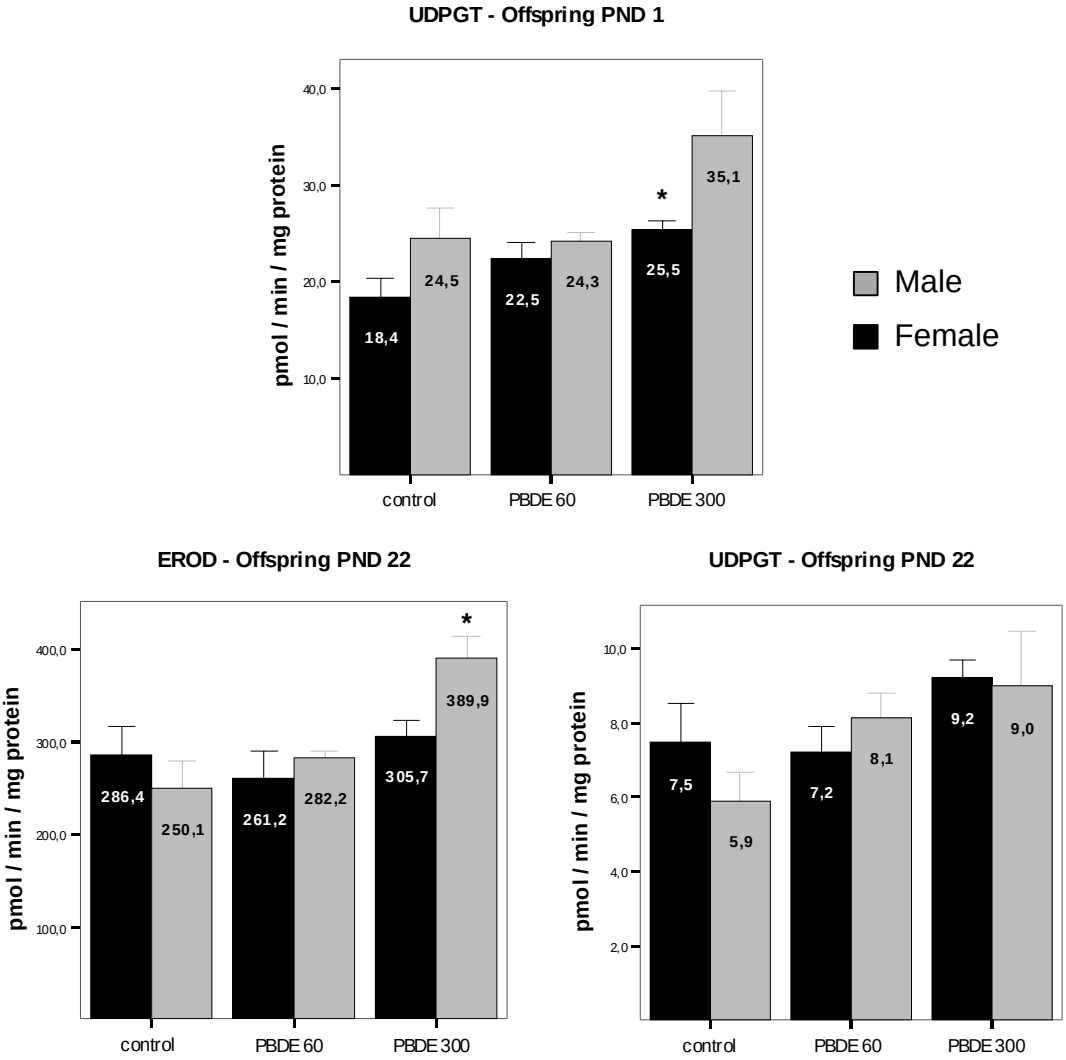


* ANOVA followed by Dunnett t-test. Differences were considered significant when $p<0.05$. Values are mean ± standard deviation

Enzyme activity in offspring: Hepatic EROD and UDPGT activities from offspring are depicted in Figure 10. Using the fluorimetric assay that allows measurement of EROD activity in a 2 mL final reaction volume, EROD activity was below the detection limit in offspring on PND 1. Increasing the concentration of microsomal protein loaded in the cuvette, did not allow reliable measurements in the detection range of the assay. Therefore, we do not report the data for EROD activity at the beginning of lactation. In general, in utero/lactational exposure to low dose PBDE 99 produced no major changes in offspring enzymatic levels. In the 300µg PBDE 99 group, males and females had higher levels of UDPGT on PND 1, although these changes were only statistically significant in females (Figure 10). It is worth to mention that, the post-hoc analysis revealed that the differences between male control and 300µg PBDE groups were close to the margin of significance

(P=0.08). At the end of lactation (PND 22), no statistical differences in the UDPGT activity were apparent, although UDPGT activities tended to be higher in offspring exposed to PBDE 99 (Figure 10). A sex-specific effect was noted for EROD activity on PND 22 with males from 300µg PBDE group exhibiting increased activity (~50% higher than control), while no effect was observed for females.

Figure 10: Uridine diphosphoglucuronosyl transferase (UDPGT) and ethoxyresorufin-*O*-deethylase activities in dams at the beginning (PND 1) and at the end (PND 22) of lactation.



* ANOVA followed by Dunnett t-test. Differences were considered significant when $p<0.05$. Values are mean ± standard deviation

3.1.4 PBDE 99 TISSUE CONCENTRATION

Toxicokinetic data are essential for the design and interpretation of toxicological studies, particularly for the problem of species differences, and for attempts to extrapolate experimental findings to humans [Nau H, 1992]. We determined the concentrations of PBDE 99 in different tissues in both dams and offspring. ***Concentration in dams:*** The five-fold difference between the two doses (60µg and 300µg) was confirmed when the concentration of PBDE 99 was determined in adipose tissue and in liver from dams on PND 1 (Table 9). This result indicates that the preparation and administration of the solutions were reliable. PBDE 99 accumulates more in adipose tissue than in liver as seen by comparing the concentrations based on fat weight and wet tissue weight (Table 9). In dams the concentration of the parent compound (absolute concentration) did not change over the lactation (Table 9). However, the PBDE 99 body burden is much less at the end of lactation as the amount of adipose tissue is drastically reduced by that time. In liver, a clear reduction in PBDE 99 concentration was observed at the end of lactation and it appears that the rate of elimination (in this organ) differs according to the dose (Table 9). The PBDE 99 concentration decreased in a factor of 3 in the 300µg PBDE 99 / kg group, while a reduction by a factor of 12 was observed in the 60µg PBDE group. From PND 1 to 22, the liver concentration of PBDE 99 expressed by wet tissue weight (w/w) allows us to estimate the amount of the original dose present in this organ. The percentage of the original dose found in liver was calculated according to the following equation:

$$\% \text{ of original dose} = \frac{(C_{PBDE\ 99} \text{ liver [ng/g (w/w)]}}{(C_{dosis} \text{ [ng/g]}} \times \frac{\text{m liver (g)}}{\text{m body (g)}} \times 100$$

where: $C_{PBDE\ 99}$ liver = PBDE 99 concentration in liver (ng/g) wet weight;

C_{dosis} = dose administered (ng/g)

m = mass (g)

This calculation reveals that 0.27% from the original dose was detected in the liver from dams at the beginning of lactation (PND 1) for both doses administered. However, at the end of lactation (PND 22) 0.024% of the original dose was present in dam's liver from the

60µg group, while 0.11% was still present in the liver of 300µg group. No significant concentration of PBDE 99 was found in the control group.

Table 9: PBDE 99 concentration in lactating dams after a single gestational (day 6) exposure to low dose PBDE 99. Values are reported in ng/g based on lipid weight and fresh tissue weight.

PND		Adipose tissue				Liver			
		Control	PBDE 60	PBDE 300	Lipid content (%)	Control	PBDE 60	PBDE 300	Lipid content (%)
1	**lipid weight**	3.6	306	1994	76	6.0	230	1173	1.7
22		4.2	421	2307	60	2.0	19	431	1.6
1	**wet tissue weight**	2.8	221	1538	-	0.1	4	20	-
22		2.3	259	1487	-	0.03	0.3	7	-

% – percentage of lipid content in tissue sample

Concentrations in offspring: Lipids were extracted from the samples in order to determine the concentration of the parent compound in this matrix. The mean lipid content in different samples and time points during lactation are reported in Tables 9 and 10. On PND 1, the lipid content of the liver was two-fold higher in offspring compared to other days (PND 14 and 22) (Table 10). Due to technical problems (small size of pups and paucity of abdominal fat on PND 1), PBDE 99 concentration in the adipose tissue from offspring on PND 1 was not measured. The high levels of the parent compound detected in liver on PND 1 (day of birth) suggest that placental transfer plays a significant role for developmental exposure (Table 10). On PND 1, the PBDE 99 concentration per g lipid is 2.5 fold higher in the liver of the offspring compared to the dam (Table 10). Adipose tissue on PND 1 is nearly absent and, therefore, it seems that PBDE 99 accumulated preferentially in liver. This notion is supported by the higher lipid content in liver from offspring on PND 1 compared to PNDs 14 and 21 (approx. 2-fold higher).

Table 10: PBDE 99 concentration in offspring after a single gestational (day 6) exposure to low dose PBDE 99. PBDE 99. Values are reported in ng/g based on lipid weight and fresh tissue weight. Mean lipid content are also reported according to treatment day.

PND		Adipose tissue				Liver			
		Control	PBDE 60	PBDE 300	Lipid content (%)	Control	PBDE 60	PBDE 300	Lipid content (%)
1	lipid weight	-	-	-	-	9.7	571	2964	5.5
14		6.4	323	3592	45	6.4	291	1885	2.2
22		8.9	170	1584	64	22.1	169	1256	2.4
1	wet tissue weight	-	-	-	-	0.5	32	162	-
14		2.6	169	1547	-	0.1	6	46	-
22		5.8	99	1074	-	0.5	5	30	-

% – percentage of lipid content in tissue sample

Then, a progressive decline occurs in the PBDE 99 concentration in the liver during lactation suggesting that PBDE 99 redistributes to the adipose tissue, as the accumulation of fat deposits increases postnatally (Table 10). PBDE 99 accumulates preferentially in adipose tissue, presumably via significant transfer of PBDE 99 to pups via the milk. Despite the decline in PBDE 99 concentration throughout lactation in offspring tissue, it is reasonable to assume that the pup's body burden is very high due to the cumulative placental + milk exposure. The decline in PBDE 99 concentrations might be explained as a "dilution effect" since body mass increases extremely fast until weaning and the absolute levels may, therefore, be very high. At weaning (PND 22), the concentration of the parent compound was still high indicating that exposure continues after lactation. The ratio adipose tissue / liver PBDE 99 concentration in the offspring indicates that the accumulation of PBDE 99 in the two compartments varies with the dose. For example, in animals exposed to 60µg PBDE 99, the ratio was 1.10 and 1.0 on PNDs 14 and 22, respectively. However, when animals were

exposed to 300µg PBDE 99 the ratio changes to 1.90 and 1.26 on PNDs 14 and 22, respectively.

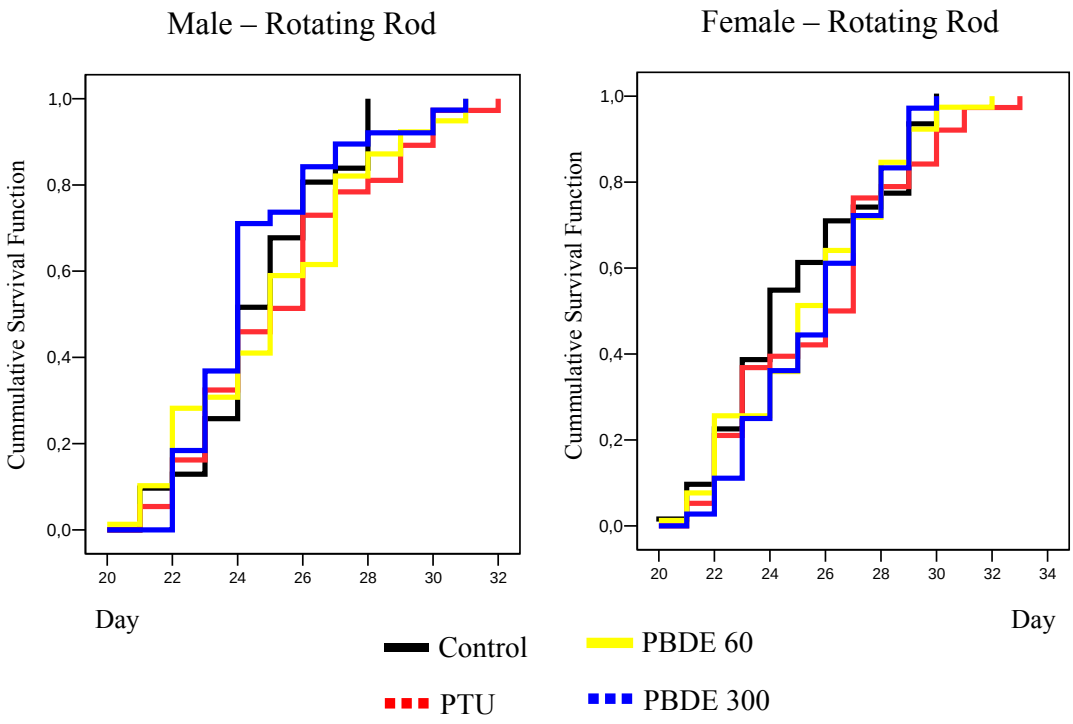
3.2 EXPERIMENT II: ADVERSE EFFECTS OBSERVED POST-WEANING TO ADULTHOOD

In this section, the results obtained from the experiment II will be reported. The experimental procedure (dose regimen, animals, etc...) was the same as experiment I, but the endpoints investigated here focuses on neurobehavior and reproductive effects observed post-weaning. In order to economise space, plots of survival functions are presented in two ways: when there was no significant effects, all doses are plotted together (*e.g.*, Figure 11). However, for relevant data showing statistical significance, plots are presented individually for better clarity (*e.g.* Figure 12).

3.2.1 DEVELOPMENTAL LANDMARKS AND SPONTANEOUS REFLEX

Developmental landmarks (*i.e.* the time of bilateral eye opening, fur development, eruption of incisors and testes descent) and spontaneous postnatal reflexes (*i.e.* cliff-drop aversion and rotating rod test) are parameters used as indicators of sensory-motor function. The cliff-drop aversion reflex, *i.e.*, the ability to avoid the cliff, requires the function of

Figure 11: Cumulative survival function of the rotating rod test. Line represents the cumulative (%) number of animal achieving the development of the reflex.



different brain centers and their interaction. The perception of a dangerous depth is a prerequisite for the reaction to occur [Fox, 1964]. The rotating rod test relies on the ability of the experimental animal to remain on a rotary bar for a certain time, requiring the development of high brain structures, in particular the cerebellum.

No effect on the ability of the animals to stay on a rotating rod for 3 min was observed for any of the treatments. In other words, the age at which the treated animals could remain in the rod was the same that for the control group (Figure 11). However, a sex-specific modulation of the cliff-drop aversion reflex was seen in offspring exposed to PBDE 99. Males exposed to 300µg PBDE 99 exhibited a delay in achieving the cliff-drop aversion reflex, while females did not. The goitrogen, PTU, which induces hypothyroidism, also delayed the acquisition of the cliff-drop aversion reflex in both males and females (Figure 12).

There were not statistically significant differences between the sexes in terms of age at which developmental landmarks were acquired and, therefore data from male and female offspring were pooled together. The postnatal landmarks evaluated in this study revealed that exposure to low dose PBDE 99 did not causes significant changes, except for time required for the eruption of incisors in the 300µg PBDE 99 group which was delayed (Figure 13). However, the goitrogen, PTU, altered the time required for bilateral eye opening and the eruption of incisors (Figure 13 A). Precocious eye opening occurred in the young pups exposed PTU, while the eruption of incisors was delayed as it was in animals exposed to 300µg PBDE 99 (Figure 13 A). Both substances did not affect the development of fur development and the time for testes descent (Figure 13 B).

Figure 12: Cumulative survival function of the cliff-drop aversion reflex. Line represents the cumulative (%) number of animal achieving the development of the reflex.

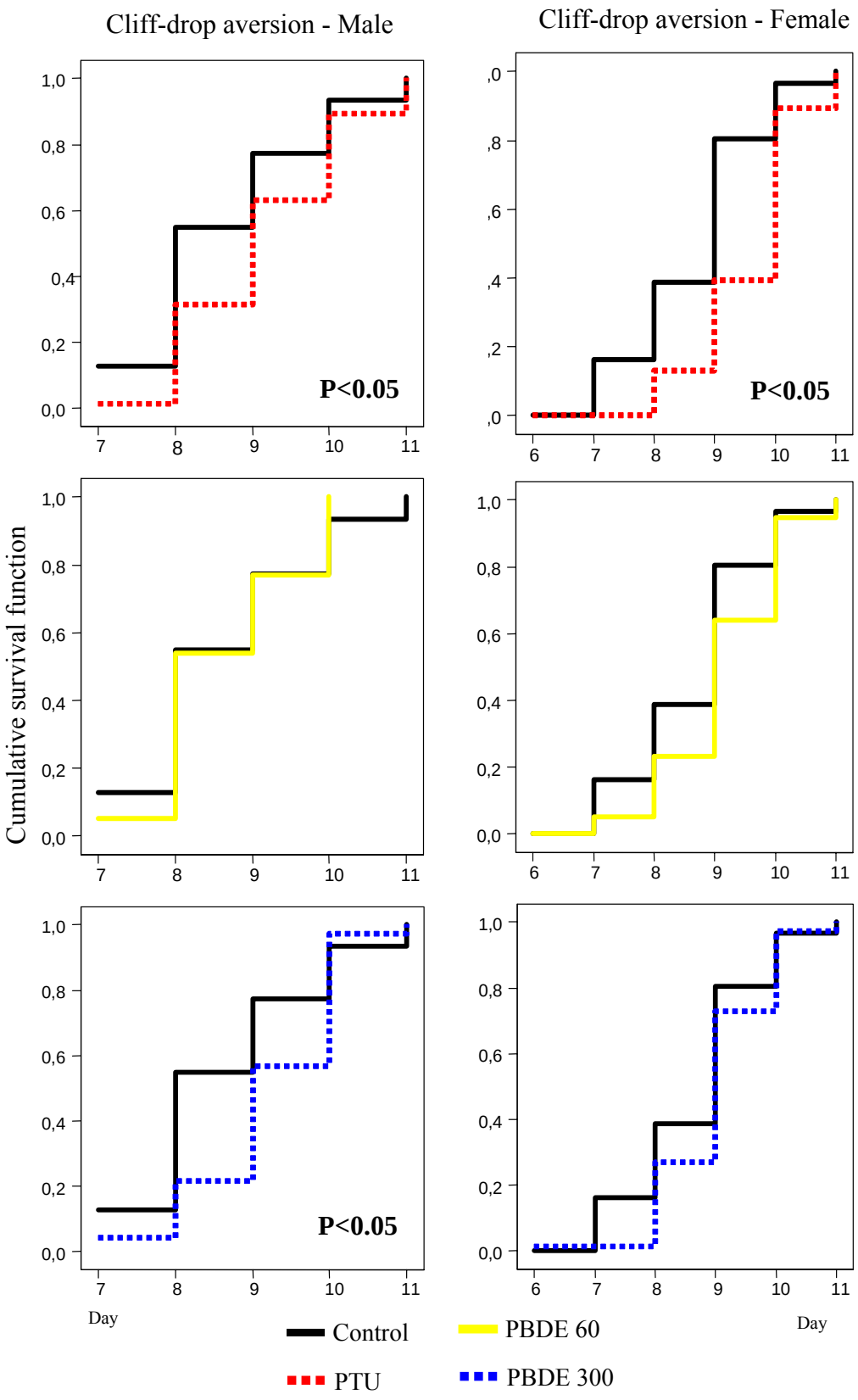


Figure 13A: Developmental landmarks, eye opening and eruption of incisor, observed during lactation in offspring exposed to PBDE 99 or PTU.

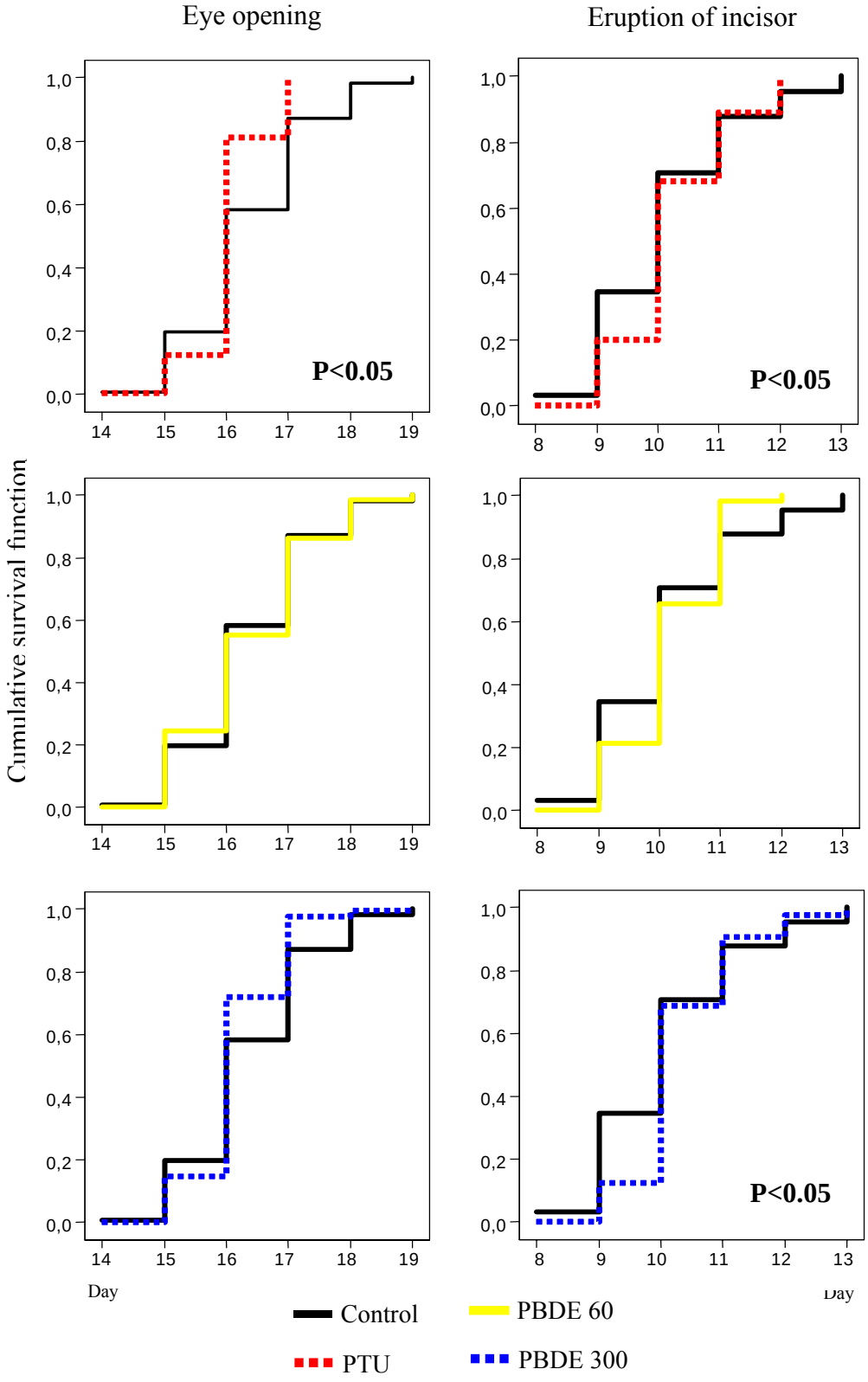
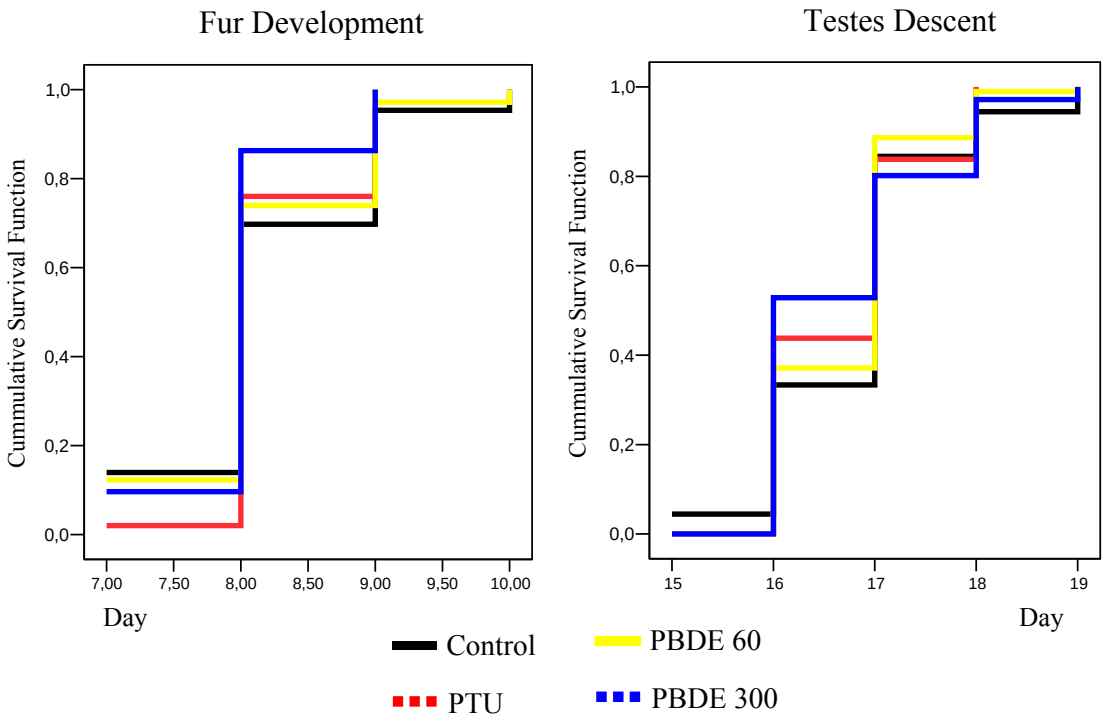


Figure 13B: Developmental landmarks, fur development and testes descent, observed during lactation in offspring exposed to PBDE 99 or PTU.



3.2.2 EFFECTS ON LOCOMOTOR ACTIVITY

Measurement of locomotor activity in experimental animals is an important tool to assess neurotoxic effects. The Mobilitron® has proved to be reliable to quantify and qualify basal locomotor activity in rats and mice [Thiel *et al.*, 1989]. The main advantages of this method include: 1 - the possibility to perform up to 48 individual measurements simultaneously; 2 – monitoring the rat movement in their usual housing environment; and 3 –

Table 11: Body weight from offspring before and after Mobilitron test. The locomotor activity was measured on PNDs 36 and 71 and body weight was recorded before and after each session.

Group	Session I		Session II	
	PND 35	PND 37	PND 70	PND 71
Control	46.4 ± 11.4	51.5 ± 12.6	173.7 ± 31.4	178.7 ± 32.4
PTU	52.3 ± 12.6	58.8 ± 13.2	184.3 ± 32.9	195.1 ± 33.2
PBDE 60	44.7 ± 10.4	48.3 ± 11.3	175.4 ± 31.7	177.6 ± 33.3
PBDE 300	46.7 ± 8.9	49.7 ± 10.9	176.8 ± 31.3	179.3 ± 29.9

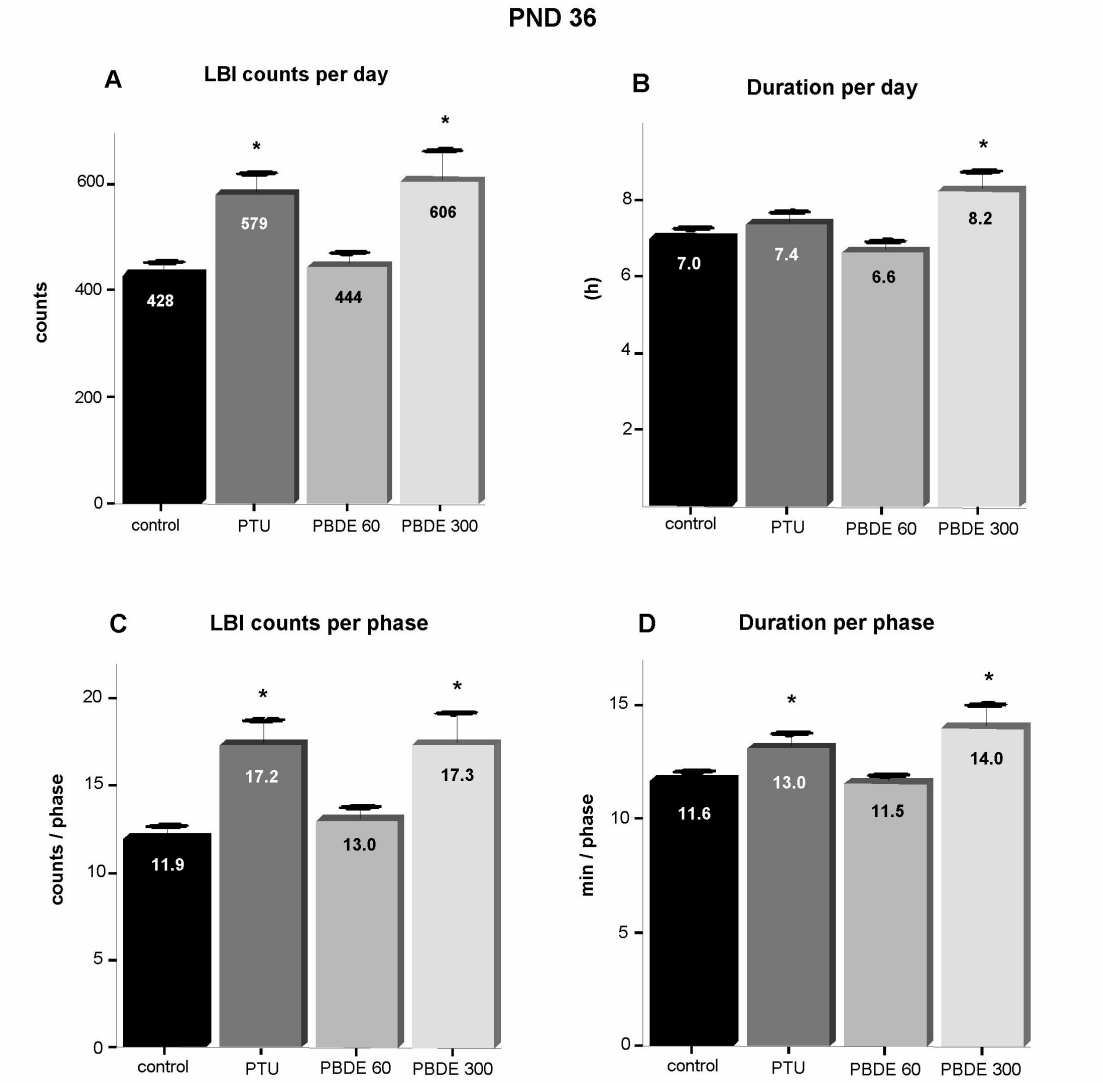
Values are mean ± SD.

the ability for long-term measurements. In this study, individual locomotion of rats was measured over 24 hours in juvenile offspring. Individual body weight was recorded in offspring one day before and one day after the test. As shown in Table 12, no statistically significant difference in body weight was observed among the groups, suggesting a minor role of this variable on locomotor activity changes.

Statistical analysis revealed no gender difference for all groups tested and, therefore, the data from the males and females are presented together. Two weeks after weaning, one male and one female per litter (from every litter) were tested in the Mobilitron® device,

Figure 14: Locomotor activity in offspring rat on PND 36.

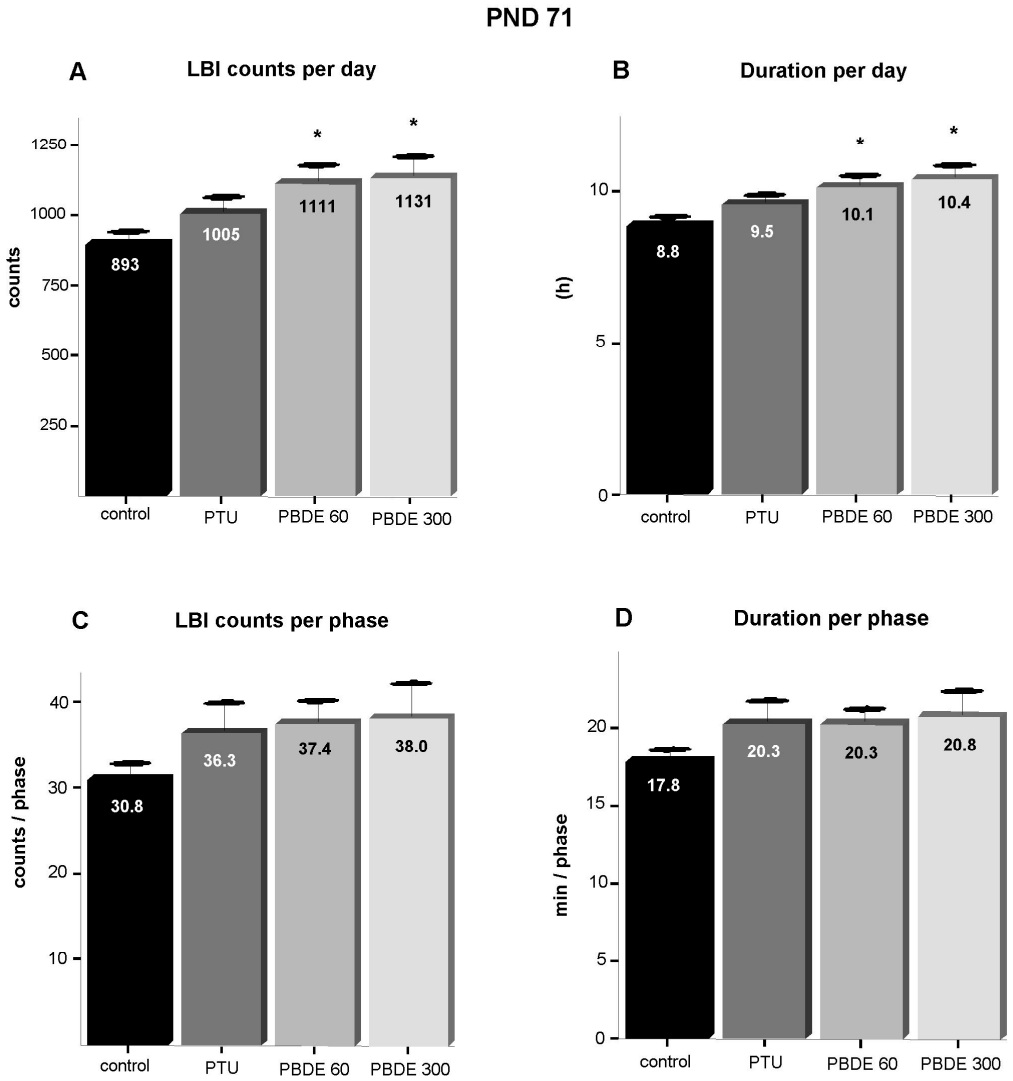
Bars represent mean ± SEM and (*) significance was detected by ANOVA followed by Dunnett t-test when $p<0.05$.



except for the 300µg PBDE group where the activity was measured in 15 litters out of 19 litters. As shown in Figure 14, total light beam interruption (LBI) count per day was significantly greater in the PTU and 300µg PBDE groups than the control group. The number of active hours per day was longer in the PBDE 300 group, an effect not seen in the PTU group (Figure 14 A and B). The qualitative analysis or characterization of the phases (*i.e.* 1- LBI count / phase and 2- duration of activity / phase) on the same day (PND 36) confirms what was observed in the quantitative analysis. Both the PTU and 300µg PBDE/kg groups were more active during the active phases compared to control and the duration of the active

Figure 15: Locomotor activity in rat offspring on PND 71.

Bars represent mean ± SEM and (*) significance was detected by ANOVA followed by Dunnett t-test when p<0.05.



phases was also longer (Figure 14 C and D). An active phase is defined when the animal begins to move (associated with light beam interruptions) until a pause (a period of no light beam interruption) is observed. On PND 36, no statistical differences were seen in the 60µg PBDE/kg group compared to control. The number of active phases was not different among groups.

The neurotoxicity-induced by PBDE 99 as revealed by changes in animal's locomotion was still apparent at puberty. On PND 71, the quantitative analysis revealed that the two PBDE groups were hyperactive (Figure 15 A and B). In other words, both the LBI counts and duration of activity per day were significantly increased in the 60µg PBDE/kg and 300µg PBDE/kg groups. At this time, the hyperactivity-induced by PTU disappeared suggesting that the effect was transient. It is interesting to note that animals exposed to the lower dose of PBDE 99 (60µg/kg BW) showed normal patterns of movement on PND 36, but were hyperactive at puberty. No statistically significant qualitative differences were observed among the groups on PND 71 (Figure 15 C and D).

3.2.3 MALE REPRODUCTIVE HEALTH

Using different methodology, general reproductive performance of male offspring was assessed in this study. Male reproductive health was impaired after low dose exposure to the flame retardant PBDE 99. Following, according to the endpoint investigated, the results will be listed in sections.

3.2.4 ORGAN WEIGHT AND STEROID HORMONE LEVELS

Assessment of general clinical signs of toxicity normally involves the evaluation of organ weight. To avoid confounding differences due to body mass, relative organ weight (*i.e.,* representation of organ mass in relation to the body mass) is used as a reliable variable. Statistically significant changes in organ weights were observed in animals exposed pre- and postnatally (via milk) to PBDE 99. Although final body weights were similar on PND 140, absolute weight of the spleen was greater in both PTU and PBDE 99 exposed animals (Table 12). The relative weight (organ weight in relation to body weight), however, was greater only in the 60µg PBDE 99 group (Table 12).

Table 12: Absolute and relative (% body weight) organ weights from adult male offspring (PND 140) exposed pre- and postnatally (via milk) to PBDE 99.

Parameters	Control N=12	PTU N=12	PBDE 60µg/kg N=12	PBDE 300µg/kg N=12
Body Weight (g)	311.7 ± 28.8	335.9 ± 34.5	320.5 ± 20.5	334.9 ± 29.9
Liver weight (g)	10.43 ± 1.63	11.17 ± 1.83	10.82 ± 0.73	11.26 ± 1.38
(%)	3.35 ± 0.25	3.31 ± 0.24	3.38 ± 0.13	3.36 ± 0.23
Thymus weight (g)	0.34 ± 0.07	0.34 ± 0.12	0.36 ± 0.07	0.32 ± 0.09
(%)	0.11 ± 0.02	0.10 ± 0.03	0.11 ± 0.02	0.10 ± 0.02
Spleen weight (g)	0.55 ± 0.05	0.63 ± 0.10 *	0.60 ± 0.07 *	0.60 ± 0.07 *
(%)	0.17 ± 0.01	0.19 ± 0.02	0.19 ± 0.02 *	0.18 ± 0.02

Absolute and relative organ weights were analyzed using Student t-test. Values are mean ± standard deviation and significance was confirmed when $p<0.05$.
% - organ index mass related to body mass

The exposure to the goitrogen, PTU, and to PBDE 99 also affects reproductive organ weights. These differences were subtle, but statistically significant. Comparing absolute testis and epididymis weights among groups, no difference was observed. However, when the organ weights are expressed as a percentage of body weight (relative weight), the PTU and PBDE 300 groups had smaller testes, while the epididymis relative weights were decreased in all three treatment groups compared to control (Table 13). Even though the effect was not severe, changes in testicular and epididymal weights may affect normal spermatogenesis. Accessory sexual organs (prostate and seminal vesicle) weights (absolute and relative) were normal, suggesting that PBDE 99 at low dose level possess no androgenic / anti-androgenic activity (Table 13). Steroid hormone levels were within the control range revealed by normal concentrations of testosterone and LH in PTU and PBDE 99 exposed groups (Table 13).

Table 13: Reproductive organ weights and hormone concentrations in adult offspring (PND 140) exposed pre- and postnatally (via milk) to PBDE 99.

Parameters	Control N=12	PTU N=12	PBDE 60µg/kg N=12	PBDE 300µg/kg N=12
Testis weight (g)	1.57 ± 0.23	1.47 ± 0.34	1.58 ± 0.11	1.53 ± 0.13
(%)	0.51 ± 0.07	0.44 ± 0.11 *	0.49 ± 0.04	0.46 ± 0.05 *
Epididymis weight (g)	0.58 ± 0.07	0.55 ± 0.08	0.56 ± 0.03	0.58 ± 0.07
(%)	0.19 ± 0.02	0.17 ± 0.02 *	0.18 ± 0.01 *	0.17 ± 0.02 *
Seminal Vesicle weight empty (g)	0.99 ± 0.14	1.11 ± 0.14	1.00 ± 0.10	1.04 ± 0.16
(%)	0.32 ± 0.04	0.33 ± 0.04	0.31 ± 0.03	0.31 ± 0.04
Prostate (g)	0.38 ± 0.11	0.40 ± 0.08	0.40 ± 0.04	0.43 ± 0.11
(%)	0.12 ± 0.04	0.12 ± 0.03	0.12 ± 0.01	0.13 ± 0.03
LH (ng/mL)	10.8 ± 7.8	12.4 ± 4.6	14.4 ± 7.4	10.3 ± 3.8
Testosterone (ng/mL)	8.7 ± 4.2	10.0 ± 4.9	7.5 ± 3.4	8.4 ± 4.7

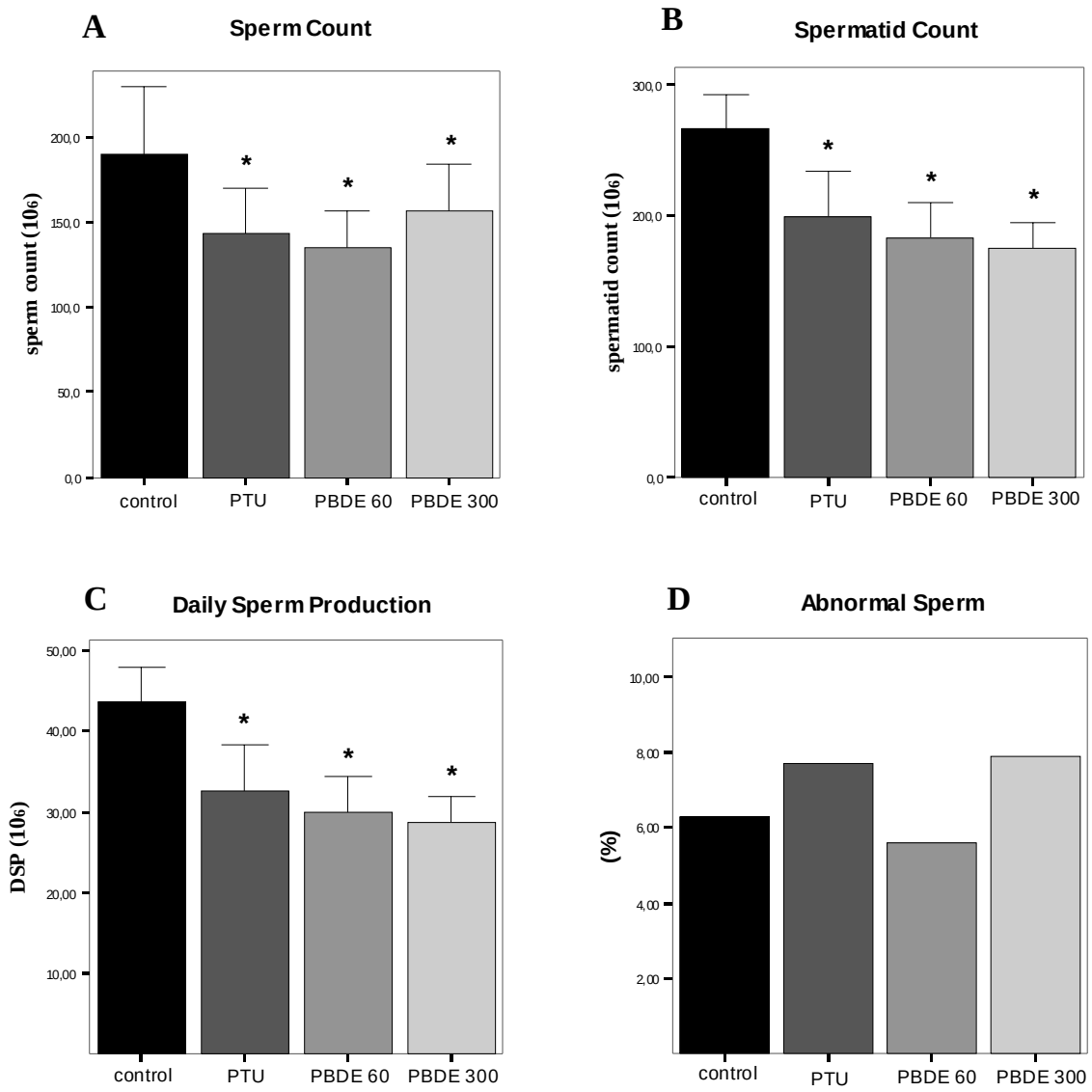
Absolute and relative organ weights were analyzed using Student t-test. Values are mean ± standard deviation and significance was confirmed when $p<0.05$.
% - organ index mass related to body mass.

3.2.4 DAILY SPERM PRODUCTION, SPERM COUNT AND MORPHOLOGY

In rats, sperm count, daily sperm production and sperm morphology are reliable endpoints to assess spermatogenesis [Amann, 1986]. The decrease in testis and epididymis weights was accompanied by reductions in sperm and spermatid counts, as well as daily sperm production (DSP) (Figure 16 A, B and C). Reductions in testicular spermatid count and sperm count from the caudal epididymis were observed in all treatment groups (Table 2).

Figure 16: Daily sperm production, sperm count and sperm morphology in adult rat offspring.

Bars represent mean ± SD and (*) significance was detected by ANOVA followed by Dunnett t-test when $p<0.05$.



For example, for sperm count a reduction of 25%; 29% and 18% were found in PTU, 60µg PBDE and 300µg PBDE groups, respectively. In a similar way, daily sperm production was also reduced 24%; 31% and 35% in PTU, 60µg PBDE and 300µg PBDE groups, respectively. The decrease in sperm production was not associated with poor sperm quality as the percent of abnormal sperm was within normal range in all groups (Figure 16 D). Testosterone and LH were within normal limits indicating that steroid hormones play a minor role for in the impairment of sperm production.

3.2.5 TESTICULAR MORPHOMETRY AND SERTOLI CELL COUNT

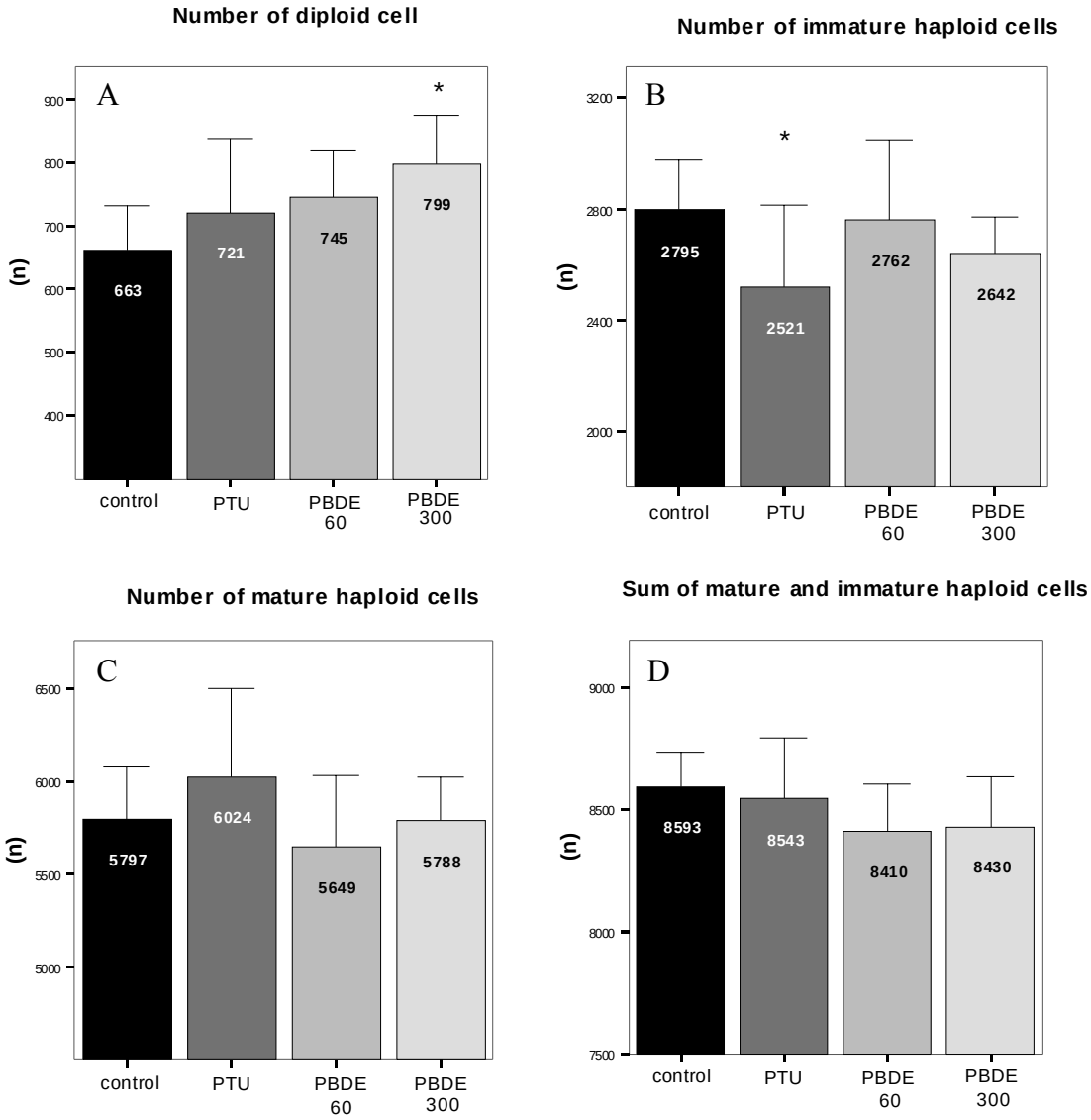
In order to investigate a possible mode of action by which PBDE 99 and PTU impair rat spermatogenesis, Sertoli cell number and testicular morphometric analyses were performed in male offspring. Testicular seminiferous tubule integrity is essential for normal spermatogenesis process; therefore, we employed a quantitative analysis of seminiferous tubule. Moreover, sperm production is related to the number of Sertoli cells in the seminiferous tubules, as these cells support a finite number of germ cells [Russel *et al.*, 1990]. Therefore, we evaluated the number of Sertoli cells to investigate whether the impaired spermatogenesis was a consequence of reduced Sertoli cell number.

Table 14: Testicular morphometry and Sertoli cell number in adult rat offspring exposed to PBDE 99 or PTU. Values are mean ± SD.

Parameter	Control (N=4)	PTU (N=6)	PBDE 60 µg/kg (N=5)	PBDE 300 µg/kg (N=6)
Absolute density of seminiferous tubule (cm^3)	1.27 ± 0.06	1.36 ± 0.12	1.23 ± 0.09	1.34 ± 0.05
Relative density of seminiferous tubule (%)	83 ± 4	83 ± 3	82 ± 4	84 ± 2
Total length of seminiferous tubule (m)	22.1 ± 2.9	22.1 ± 3.6	18.9 ± 2.1	23.1 ± 2.4
Mean diameter of seminiferous tubule (µm)	271 ± 16	281 ± 11	289 ± 19	273 ± 14
Mean diameter of Sertoli cell nucleoli (µm)	2.0 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.1
Number of Sertoli cell per testis ($x10^6$)	22.9 ± 4.5	22.1 ± 4.4	19.7 ± 2.3	23.5 ± 3.7
Number of Sertoli cell per g of testis ($x10^6$)	15.1 ± 3.2	13.5 ± 2.3	13.3 ± 2.1	14.8 ± 2.4

Based on the morphometric endpoints, it seems that treatment with either PTU or PBDE 99 did not affect seminiferous tubule (SeT) integrity (Table 14). Morphometric scores for testicular morphology such as, relative density of SeT (total area in % of SeT in the slide) and absolute density of SeT (relative density corrected by testis weight) were comparable to that of the control group (Table 14). Also the Sertoli cell number was similar to the control group suggesting that a difference in Sertoli cell number is not the mechanism of impaired spermatogenesis.

Figure 17: Flow cytometry analysis of testicular cell population on the basis of their DNA ploidy. Adult rats (PND 140) were exposed in utero to low dose PBDE 99.



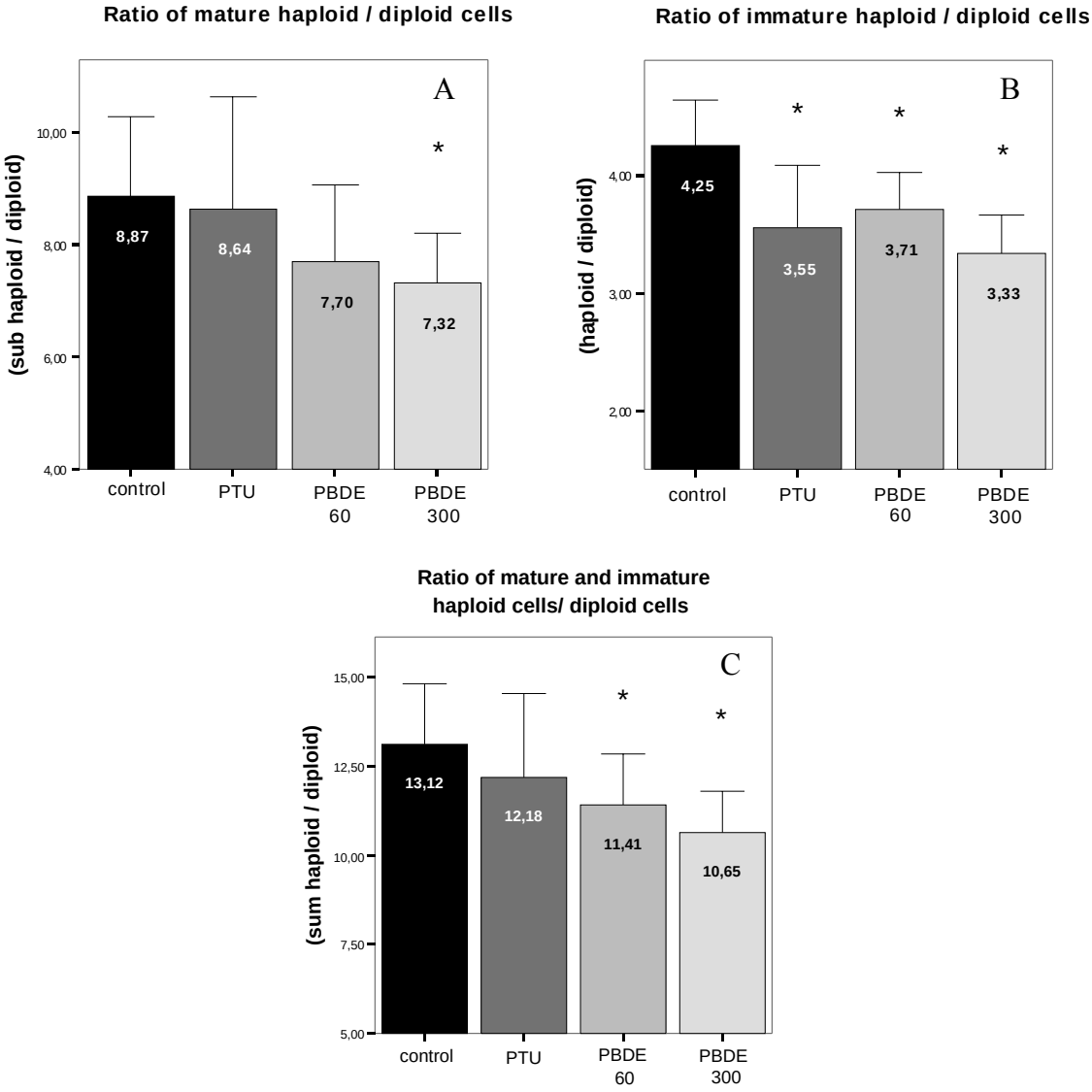
Bars represent mean ± SD and differences were detected by ANOVA followed by Dunnett t-tes when $p<0.05$.

3.2.6 FLOW CYTOMETRY ANALYSIS

Flow cytometry method (FCM) has been shown to be useful for analysis of semen quality as it provides quantitative evaluation of different cell types on the basis of their DNA ploidy/level of staining. Three major phases including mature haploid cells (elongated spermatid), immature haploid cells (round/elongating spermatid) and diploid cells (spermatogonia, spermatocyte, Sertoli cells, and Leydig cells) were distinguished by comparing fluorescent properties of propidium iodide-stained testicular cells.

Analysis of the testicular cell population on the basis of their DNA ploidy/level of staining from male offspring exposed to either PTU or PBDE 99 corroborates with the observed impaired spermatogenesis. In Figure 17, three populations of testicular cell counts are presented: diploid cell (mainly spermatogonia, spermatocyte, Sertoli cell, Leydig cell and somatic cell); mature haploid cell (mostly elongated spermatid); and immature haploid cell (round/elongating spermatid). A clear effect was seen in the number of diploid cells in the 300µg PBDE group, being higher than control. The same tendency was observed for 60µg PBDE and PTU group, but statistical significance was not achieved (Figure 17 A). In the PTU group, a significant decrease in the number of immature haploid cell was observed, and although not significant, the same trend was observed in the 300µg PBDE animals (Figure 17 B). The number of mature haploid and the sum of haploid cells were not statistically different, even though a tendency for reduction was seen in the PBDE 99 treated animals (Figure 17 C and D). However, a clear effect was observed by evaluating the number of haploid (mature and immature) over the number of diploid cell. Reduced spermatogenesis is indicated by an increasing number of diploid cell population with concomitant reduction in the haploid cell. In this analysis, the ratio of mature haploid / diploid cells shows that animals exposed to 300µg PBDE 99 had a lower ratio suggesting a decline in spermatogenesis (Figure 18 A). A more pronounced effect was seen by the ratio of immature haploid / diploid where all treated groups had lower ratios than the control group (Figure 18 B). The sum of haploid cells, *i.e.* all spermatid cell undergoing final steps of differentiation, divided by the number of diploid cells also indicated that the treatment with PBDE 99 affected offspring spermatogenesis (Figure 18 C).

Figure 18: Flow cytometry analysis of testicular cell population on the basis of their DNA ploidy. Haploid vs. diploid ratio. Adult rats (PND 140) were exposed in utero to low dose PBDE 99.



Bars represent mean ± SD and differences were detected by ANOVA followed by Dunnett t-test when $p<0.05$.

3.2.6 MALE REPRODUCTIVE PERFORMANCE AND SEXUAL BEHAVIOR

As part of the general reproductive health assessment, male reproductive performance and sexual behavior were monitored in adult male offspring. **Male reproductive performance:** mating the litter mates of animals analyzed for sperm counts with untreated females revealed that exposed males could sire offspring similar to the control group (Table 15). Uterus weight, litter size and number of implantations, resorptions and viable fetuses were within the normal range (Table 15). However, a significant increase in offspring (F2) body weight was observed in both groups exposed to PBDE 99 when the single fetus was

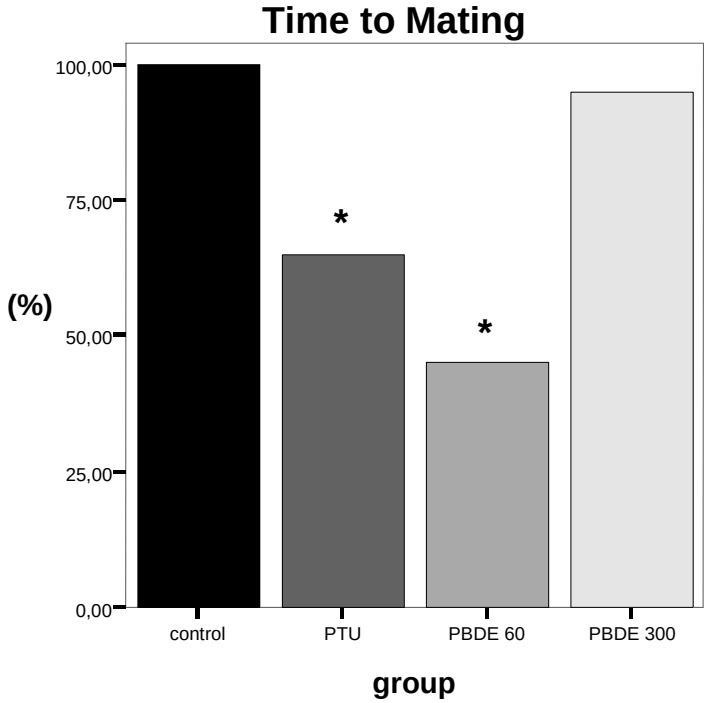
Table 15: Reproductive performance of adult male rats after mating with non-exposed females. Male rats were pre- and postnatally exposed to a low dose (60µg or 300µg/kg) of PBDE 99. Values are mean ± SD and asterisk indicates $p<0.05$.

Parameter	Control	PTU	PBDE 60µg/kg	PBDE 300µg/kg
Number of dams	19	18	15	19
Dams body weight gain (%)	49.3	46.3	47.5	50.9
Uterus weight	73.6 ± 18.6	77.8 ± 7.2	71.2 ± 10.3	71.1 ± 14.7
Implantation (*n*)	214	203	161	217
Implantation/litter	11.3 ± 2.7	11.3 ± 1.5	10.7 ± 1.9	11.4 ± 1.8
Viable fetus/litter	10.8 ± 2.7	10.9 ± 1.6	10.1 ± 1.8	10.3 ± 2.4
Resorptions total (%)	9 (4)	6 (3)	10 (6)	21 (10)
Fetus weight (g)	4.70 ± 0.53	4.63 ± 0.41	4.91 ± 0.59 *	4.81 ± 0.42 *
Fetus weight /litter (g)	4.70 ± 0.43	4.66 ± 0.25	4.96 ± 0.50	4.85 ± 0.32
Sex ratio (M/F)	47.3 / 52.7	47.4 / 52.6	46.4 / 53.6	42.9 / 57.1

used as the statistical unit. When fetal body weight was compared per litter, *i.e.* the mean fetus weight within a litter was used as the statistical unit, this difference disappears (Table 15). The resorptions rate was slightly increased in treated animal, however, a resorption rat up to 10% is within normal limits for our strain. Another endpoint used to assess reproductive performance is the determination of the number of days necessary for the rat to mate. After 5

consecutive days of mating, the number of sperm positive smear in the control group was 100% (Figure 19). Similar performance was also observed in males exposed to 300µg PBDE, while PTU and 60µg PBDE males were significantly delayed compared to controls (Figure 19). After 5 days, only 50% and 60% of males succeeded in mating in the 60µg PBDE and PTU groups, respectively (Figure 19).

Figure 19: Fertility index of adult male offspring mated with untreated females after pre- and postnatal exposure to low dose PBDE 99 (60 and 300 µg/kg b.w.). Time to mating: number of sperm positive females after 5 days mating. * p<0.05



Sexual behavior: Pre- and postnatal exposure to either dose of PBDE 99 or PTU did not impair sexual behavior of the adult male offspring. Ejaculatory and mounting latencies, intromission frequency and latency and number of penetrations were normal when all groups were compared to controls (Figure 20). However, the number of animals which had two or more ejaculations during 20 minutes of mating was significantly lower in the PBDE exposed animals. Approximately 50% of controls had a second ejaculation while only 39% and 21% of the males from the 60µg PBDE / kg and 300µg PBDE / kg groups, respectively, achieved a second ejaculation (Figure 20 F).

Figure 20: Sexual behaviour of adult male offspring. A: Intromission latency; B: Intromission frequency; C: Mounting latency; D: Ejaculatory latency; E: Number of penetration and F: N of animals with two or more ejaculations. Bars are mean ± SD and differences were detected by ANOVA followed by Dunnett t-test when $p<0.05$.