INTRAUTERINE PCB EXPOSURE ALTERS SEXUALLY DIMORPHIC BEHAVIOUR IN LAMBS (*Ovies aries*) IN A FEAR RELATED PARADIGM

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Introduction

The presence of chemicals in the environment with the potential to disrupt endocrine systems, so-called endocrine disrupting compounds (EDCs), has become a major focus of research during the last years, as both wildlife and humans may be affected.[1,2,3,4] EDCs such as PCBs were shown to cause alterations of sexually dimorphic behaviour in mammals and such effects may have wide implications for other exposed species including humans.[5,6,7,8]

The present study is a part of a larger study on "Endocrine disrupters: Risk assessment for food quality and animal health" with focus on effects on reproductive endpoints, immune function and behaviour in the offspring. The aim of this specific study was to determine whether intrauterine exposure to low levels of two known EDCs changes the behaviour of lambs (*Ovies aries*) in a light/dark (L/D) test that is the most commonly used behavioural test for anxiety in rodents.[9,10,11]

Animals, Materials and Methods

Fifty sheep of a Norwegian breed (Dala) were housed at the University farm in the Norwegian University of Life Sciences in Ås, Norway. Oestrus in the sheep was synchronized after which the sheep were mated twice a day with males until the last sheep came out of oestrus.

Experimental protocol of prenatal exposure

The sheep were allocated into three groups (2 groups of 17 and one of 16 sheep) using block randomization. The animals in the experimental groups were orally administered either PCB 153 (98 µg/kg body weight/day), PCB 118 (49 µg/kg body weight/day), or corn oil 3 times a week (Monday, Wednesday, Friday) throughout gestation until delivery that took place 146 days later. Animals were kept indoors in separate pens. The sheep were fed ad libitum on hay with an addition of concentrated diet. All sheep were inspected every day until lambing, and their weights were recorded once a week. Sheep delivered indoors and lambing was supervised in all cases. Gender, weight, condition and time of birth of the lambs were recorded. The mothers and lambs remained together until weaning (8 weeks post-partum).

Behavioural experiment: Emotional reactivity in the L/D test

At 24 to 28 days of age all lambs were transported individually into a different part of the stable where they had no contact with their mother, but where unfamiliar sheep were within sight and hearing distance. Lambs were placed into a box (2.50 m long, 1.25 m wide, 1 m high) with dark brown wooden walls and grey concrete floor that had a dark compartment, covered with a plastic ceiling and a light compartment that was open on top. This half of the compartment was additionally lighted by a 100 W bulb that was 1.2 m above the ground in a position that the ceiling produced a sharp border between the dark and the light compartment exactly under the edge of the ceiling further accentuating the two parts of the box (Figure 1). Lambs were placed into one of the compartments and number of vocalizations, time spent in the two compartments and number of position changes (counted once 3 legs passed the border line) was observed. Half the lambs started in the light and half in the dark compartment. The following day the experiment was repeated with the lambs starting in the opposite compartment.

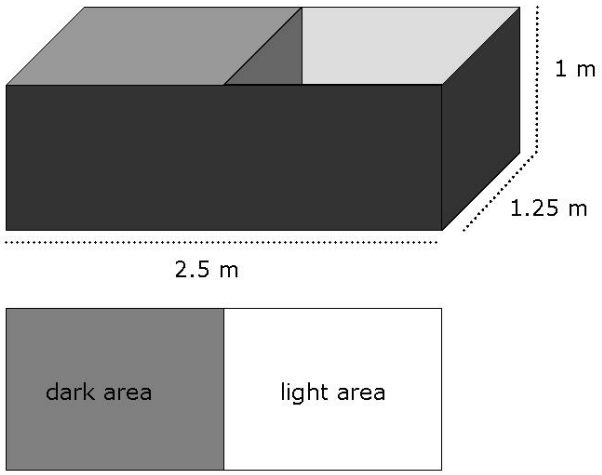

Figure 1: Scheme of the arena used. The light area was illuminated by a 100 W bulb from above in a way that produced a sharp contrast on the ground between the light and dark area

Chemicals
2,2',4,4',5,5'-hexachlorobiphenyl (IUPAC no. 153) and 2,3,4,4',5-pentachlorobiphenyl (IUPAC no. 118) in pure (99%) powder form were acquired from Accustandard (New Haven, CT, USA). PCB 153 was cleaned over charcoal to remove any traces of dioxins and furans that may be formed during synthesis of PCB 153. PCB 118 was not cleaned, as the synthesis path is different not leading to the formation of unwanted by-products.[12] PCBs were dissolved in acetone, and corn oil was added as a vehicle. The acetone was evaporated, and the PCB and the corn oil were mixed using an ultrasonification bath for 3 min. Actual concentrations of PCB 153 and PCB 118 were then determined by gas chromatography prior to use in the animal experiment to confirm actual concentrations. The solutions were stored at room temperature in order to remain homogeneous.

PCB analysis
Chemical analyses of PCBs 153 and 118 were performed at the Laboratory of Environmental Toxicology, Norwegian School of Veterinary Science, Oslo, Norway. Briefly, samples of tissue (~1-3 g) were weighed and internal standards PCB 29 and 112 added. The samples were extracted twice with cyclohexane and acetone using an ultrasonic homogenizer followed by centrifugation. The samples were cleaned up with ultra-pure sulfuric acid. Percent extractable fat was determined gravimetrically.

Statistical analyses
All data are reported as means ± standard error of the mean (SEM). Differences between means were tested with one-way ANOVA. The acceptance level was set at $P<0.05$. All statistical analyses were performed using SPSS/PC+, version 10.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion
Due to erroneous handling of animals, single events of cross contamination occurred resulting in a mixed exposure scenario in all groups rather than the planned exposure to single compounds. The mean PCB 153 concentration in adipose tissue at 60 days of age in the PCB 153 group (group 3) was 51 900 ng/g lipid weight (range 45 000 – 58 700), in the PCB 118 group (group 2) 7 420 ng/g (range 6 240 – 8 610) and in the control group (group 1) 892 ng/g (range 778 – 1 010). The mean PCB 118 concentrations were 1 310 ng/g (range 1150 – 1 480), 8 760 ng/g (range 7 230 – 10 300) and 47 ng/g (range 41 - 54) in the three groups respectively. The group planned to represent a control group has therefore be considered as to be low contaminated.

There was no significant overall effect of treatment on any of the behaviours recorded in this experiment although female animals treated with mainly PCB 118 (group 2) had a trend to a longer time spent in the dark part of the arena compared with control females when the experiment started in the dark (52±6 vs 23±3 sec, one-way ANOVA, $P<0.1$) (Table 1).
The dark part of the test chamber was the less preferred, the lambs spending on average 30 % of the test time in it. If this preference was based partly on anxiety, we have to assume that it was a low level of anxiety, since 30% of the times spend in the dark area, does not indicate a high level of avoidance.
When control males started the test in the dark (less preferred) box, they spent significantly more time in the dark part of the pen than control females (44±10 vs 23±3 sec, one-way ANOVA, $P<0.05$). Females from group 2 mainly exposed to PCB 118 had a trend to spent more time in the dark part of the pen compared with the experiment starting in the light (52±6 vs 30±7 sec, one-way ANOVA, $P<0.1$) (Figure 2).
Contrary to the situation when the experiment was started in the dark part of the pen no significant differences were observed when animals started the test in the light area (Figure 2).

Table 1: Behaviour of lambs when placed first in the dark part of the pen at the start of the 120 seconds observation period (mean ± SEM)

males		n	time spent in dark seconds	changes n	vocalization n
control	group 1	7	44 ± 10[a*]	5.8 ± 1.8	22.9 ± 2.1
PCB 118	group 2	9	43 ± 5	7.8 ± 1.5	22.3 ± 3.2
PCB 153	group 3	9	39 ± 10	5.7 ± 1.1	25.2 ± 3.4
females					
control	group 1	15	23 ± 3	4.1 ± 2.7	24.7 ± 1.3
PCB 118	group 2	13	52 ± 6[b(*)]	7.1 ± 4.4	22.8 ± 2.1
PCB 153	group 3	12	31 ± 7	3.1 ± 3.2	20.5 ± 2.5

Statistical evaluation between genders (a) and treatments (b) was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Student-Newman-Keuls test that controlled Type I experimentwise error, * $P<0.05$; (*) $P<0.1$

Table 2: Behaviour of lambs when placed first in the light part of the pen at the start of the 120 seconds observation period (mean ± SEM)

males		n	time spent in dark seconds	changes n	vocalization n
control	group 1	7	20 ± 4	6.6 ± 1.8	27.4 ± 1.9
PCB 118	group 2	9	44 ± 12	5.1 ± 1.3	20.7 ± 3.1
PCB 153	group 3	9	36 ± 14	3.6 ± 1.1	22.0 ± 3.3
females					
control	group 1	15	26 ± 5	6.8 ± 1.4	25.7 ± 1.8
PCB 118	group 2	13	30 ± 7	5.4 ± 1.1	21.9 ± 1.9
PCB 153	group 3	12	36 ± 7	5.5 ± 0.7	23.3 ± 2.3

Statistical evaluation between genders and treatments was made by one-way ANOVA, and pairwise comparisons of means within significant treatments were made using Student-Newman-Keuls test that controlled Type I experiment wise error

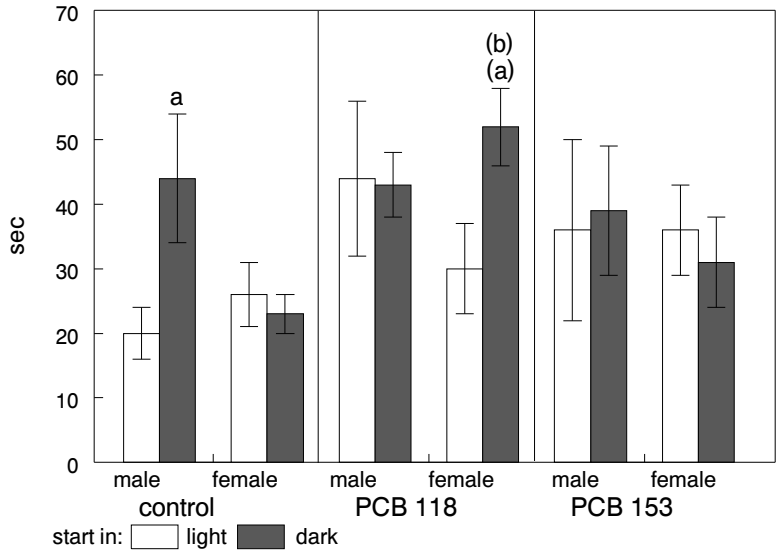

Figure 2: Time spent in the dark part of the pen (seconds) depending on whether the experiment was started in the dark or the light part of the pen

Based on these results we suggest that the experimental paradigm, provided the test animals start the test in the less preferred of the two areas, reveals a sex-difference, i.e. results in a higher level of avoidance (anxiety?) shown by females as expected. It, therefore, allows the study of the effect of EDCs on the differential development of male and female lambs.
The results support the hypothesis that intrauterine exposure to widely distributed EDCs such as PCBs can alter sexually dimorphic behaviour of offspring later in life including an important aspect as anxiety response. Under the tested conditions PCB 118 exposure resulted in a masculinisation of female behaviour.

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