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REPRODUCTIVE TOXICITY IN MINK (*MUSTELA VISON*) CHRONICALLY EXPOSED TO ENVIRONMENTALLY RELEVANT POLYCHLORINATED BIPHENYL CONCENTRATIONS

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Abstract—Female mink were exposed to a technical polychlorinated biphenyl (PCB) preparation (Clophen A50 [A50]; 0.1 or 0.3 mg/animal/d), one fraction of A50 containing the non- and mono-*ortho*-chlorinated congeners (0–1-*ortho*-chlorobiphenyls [CBs]), another fraction of A50 containing the congeners with two to four *ortho*-chlorines (2–4-*ortho*-CBs), or an organic extract from Baltic gray seal blubber. The animals were exposed for 18 months, including two reproduction seasons. Among the animals given the highest dose of A50, the whelping frequency was reduced in the second reproductive season, and all kits died within 24 h of birth. Reproduction was also impaired by the lower dose of A50. Daily exposure to the 0–1-*ortho*-CBs separated from 0.3 mg A50 severely reduced kit survival. Reproduction was not significantly impaired by daily exposure to the 2–4-*ortho*-CBs separated from 0.3 mg A50 or by exposure to the blubber extract. We conclude that the reproductive toxicity in chronically PCB-exposed mink is caused by the aryl hydrocarbon (Ah) receptor agonists. The lowest-observed-effect level for reproductive impairment was 2.4 ng 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TEQs) per kilogram body weight and day (22 pg TEQs/g feed). Ethoxyresorufin-*O*-dealkylase (EROD) was strongly induced by the 0–1-*ortho*-CBs and pentoxyresorufin-*O*-dealkylase by the 2–4-*ortho*-CBs. High EROD activity was correlated with low kit production, and consequently EROD may serve as a marker for reproductive toxicity by Ah receptor agonists in mink.

Keywords—Mink Reproductive toxicity Ethoxyresorufin-*O*-dealkylase Polychlorinated biphenyls 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin equivalents

INTRODUCTION

Piscivorous birds and mammals living in contaminated environments such as the Baltic region accumulate persistent lipophilic environmental contaminants to concentrations that can have adverse health effects [1]. The Baltic populations of gray seal (*Halichoerus grypus*), ringed seal (*Phoca hispida*), and harbor seal (*Phoca vitulina*) are all under threat by environmental contaminants [1]. Furthermore, the population density of the European otter (*Lutra lutra*) declined in many regions in Sweden in the 1970s and 1980s, probably partly because of polychlorinated biphenyl (PCB) contamination of their diet [2–4]. As endangered species are generally not available for experimental studies, model species have to be studied. Mink (*Mustela vison*) are farmed for their pelt, and ranch mink are available for toxicological research. Moreover, this mustelid is a candidate species for use when monitoring exposure of piscivorous wildlife to environmental contaminants. Mink are opportunistic predators, and a large proportion of their diet consists of fish. It was reported by Erlinge [5] that the diet of mink along a Swedish river consisted of 60% fish, 24% waterfowl, and 9% mammals. To identify suitable endpoints for use in biomonitoring, the responses of mink to various environmental contaminants have to be studied experimentally.

Mink is not an endangered species in Sweden, but hunting data indicated diminishing mink populations in the 1960s, followed by increases since the late 1970s [1]. In feeding studies, fish taken from the Great Lakes in North America have had adverse effects on reproduction in mink [6–8]. A hazard assessment was conducted by Giesy et al. [9] to determine the potential for adverse effects on mink consuming fish from three rivers that flow into the Great Lakes. It was concluded that consumption of fish below dams in the rivers would pose a risk to mink, mainly because of the high PCB levels in the fish.

It has been known for many years that mink are susceptible to PCB and that effects on their reproduction occur at relatively low exposure levels [6,10–12]. In most mink studies, the animals were treated with technical PCB for a few months, including a single reproduction season. Kihlström et al. [13] exposed 10 female mink to a daily dose of 2 mg Clophen A50 (A50) for three months during the reproduction season, giving a total dose of about 170 mg PCB/animal at parturition. The 10 females produced all together only one kit. Whether a lower dose over a substantially longer period would cause similar dramatic effects on reproduction has scarcely been examined. In a recent study, female mink were exposed to PCB-contaminated carp from Saginaw Bay (Lake Huron, USA) for up to 18 months [14]. Fewer kits survived, and body weights of the kits were reduced at a concentration of 500 μg PCB/kg feed (about 0.1 mg PCB/mink/d). In addition to PCB, the carp also contained polychlorinated dibenzo-*p*-dioxins/furans (PCDDs/Fs) and pesticides.

Various commercial products of technical PCB may differ

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in chlorine content, and the mix of individual chlorinated biphenyls in technical PCB includes more than 100 different congeners. Certain of these congeners are aryl hydrocarbon (Ah) receptor agonists, therefore having toxic effects similar to those of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other PCDDs/Fs. Highly toxic to adult mink, TCDD has a median lethal dose (LD50) value of about 47 ng/kg/d in females exposed for 125 d [15]. Another Ah receptor agonist, 3,3′,4,4′,5,5′-hexachlorobiphenyl, caused 50% mortality in mink exposed for three months via the feed to a concentration of 100 μg/kg feed, corresponding to a daily dose of 15 μg per animal [16].

The objective of the present study was to examine the effects of long-term exposure to relatively low dietary concentrations of various PCB preparations on the reproductive performance of mink. An organic extract of blubber from Baltic gray seal was also studied to ascertain what effects, if any, weathered PCB and other contaminants accumulated in seal blubber might have.

MATERIAL AND METHODS

Chemicals

A commercial PCB brand, Clophen A50, produced by Bayer (Leverkusen, Germany), was donated by Dr. Lars Förlin (Department of Zoophysiology, University of Gothenburg). 2,3,3′,4,4′,5,5′-Heptachlorobiphenyl (CB-189) (PCB congener numbers according to Ballschmiter et al. [17]), 2,3,3′,4,5,5′-hexachlorobiphenyl (CB-159), and 2,2′,3,6′-tetrachlorbiphenyl (CB-46) were synthesized as described by Sundström [18]. 2,2-Bis(phenyl)-1,1-dichloroethene (ϕ-DDE) was prepared via sodium hydroxide–promoted dehydrochlorination of 2,2-bis(phenyl)-1,1,1-trichloroethane (ϕ-DDT), prepared by a method similar to the synthesis of 2,2-bis(4-fluorophenyl)-1,1,1-trichloroethane [19]. A $^{13}C_{12}$-labeled standard mixture of 3,3′,4,4′-tetrachloro-[$^{13}C_{12}$]-biphenyl, 3,3′,4,4′,5-pentachloro-[$^{13}C_{12}$]-biphenyl, and 3,3′,4,4′,5,5′-hexachloro-[$^{13}C_{12}$]-biphenyl (5.0 ppm of each CB in nonane, 99% purity) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

All solvents used were of pro analysi quality or higher. The sodium chloride (Baker reagent) was obtained from J.T. Baker Chemicals B.V. (Deventer, The Netherlands). The silica gel (Silica gel 60, 70–230 mesh ASTM) was purchased from Macherey-Nagel (Düren, Germany) and heated overnight at 280°C before use.

Fractionation of A50

Using a slightly modified version of a previously described procedure [20], the A50 (4.0 g) was separated into one fraction containing the CBs that lack or have only one chlorine atom in an *ortho* position (0–1-*ortho*-CBs) and one fraction containing CBs with two to four chlorine atoms in *ortho* positions of the biphenyl structure (2–4-*ortho*-CBs). The PCB mixture was dissolved in heptane:benzene (95:5, v/v, 200 ml) and slowly added (10 ml/h) to a charcoal column (56 g). Heptane: benzene (95:5, v/v, 2,250 ml) was passed through the column, followed by toluene (180 ml). The 1,200 ml of solvent eluting first contained only 2–4-*ortho*-CBs (2.92 g). The material that eluted thereafter was pooled and the solvent evaporated. The PCB residue (510 mg), containing a mixture of PCB congeners with zero to four *ortho*-chlorine atoms, was dissolved in heptane:benzene (95:5, v/v, 30 ml) and added to a fresh charcoal column (5.6 g), through which heptane:benzene (95:5, v/v, 200 ml) was passed, followed by 100 ml of toluene. From a first fraction (110 ml), 288 mg of 2–4-*ortho*-CBs was isolated and pooled with the 2.92 g of 2–4-ortho-CBs initially isolated (compare above). In a second fraction from the second charcoal column, 221 mg of 0–1-*ortho*-CBs were isolated. However, the bulk of the 0–1-*ortho*-CBs present did not elute from the first charcoal column but was recovered by Soxhlet extraction (20 cycles with toluene) of the charcoal [20]. In this way, 620 mg of 0–1-*ortho*-CBs were obtained, and thus all together 841 mg of 0–1-*ortho*-CBs were isolated. Of the PCB congeners obtained from 4.0 g of A50, 80% were 2–4-*ortho* (3.2 g) and 20% were 0–1-*ortho* (0.8 g).

Isolation of persistent organic pollutants from gray seal blubber

Anthropogenic compounds in gray seal blubber were isolated and used for dosing one group of mink. Blubber from three gray seals, kindly provided by Dr. M. Olsson (Swedish Museum of Natural History, Stockholm), was used for industrial cold-press isolation of oil (blubber pressed by Sildoljeand Sildemelindustrins Research Institute, Bergen, Norway). The isolated oil contained a mixture of lipophilic anthropogenic compounds accumulated in the seal blubber. The persistent organic pollutants in the seal blubber oil (4.2 kg) were isolated by partitioning between the oil and acetonitrile in a cyclic procedure as described by Jensen et al. [21]. The procedure was repeated once. The aryl methyl sulfones were removed by dimethyl sulfoxide partitioning [22].

Animals and treatments

Female mink (*Mustela vison,* standard breed, six months old) were divided into six groups of 20 individuals each. The body weights of the females on commencing the study were similar (mean = 1.2–1.3 kg) in the various groups. The females were housed individually outdoors in sheltered cages and had free access to tap water. All animals were tested for plasmacytosis and proved negative. Twice a day (morning and afternoon) they were fed a basic diet containing cereals (10%), fish heads and backbones (40%), slaughter offal (50%), and a mixture of vitamins and trace elements (Rovemix, Hoffman LaRoche, Stockholm, Sweden, <1%). The daily feed ration was about 130 g.

Pollutant exposure started on November 24, 1992, when the females were six months old, and ended just over 18 months later (May/June 1994). The compounds studied were dissolved in oil prepared from capelin (*Mallotus villosus;* Norsildmel, Minde, Norway) captured in the north Atlantic. The concentrations of various pollutants in the oil were low (0.15 μg PCB/g oil, 0.06 μg total DDT [1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane + 1,1-dichloro-2,2-bis[4-chlorophenyl]ethene + 1,1-dichloro-2,2-bis[4-chlorophenyl]ethane]/g oil, 0.03 μg hexachlorobenzene/g oil, and 0.03 μg lindane/g oil, lipid weight). Three times a week, 1 ml of capelin oil solution was added to the morning feed ration. The capelin oil solution was palatable to the animals. Designation of the treatment groups and exposure of the mink were as follows:

A50 high: Females exposed to 0.7 mg A50 three times a week, corresponding to 0.3 mg/d.

A50 low: Females exposed to 0.24 mg A50 three times a week, corresponding to 0.1 mg/d.

0–1-*ortho*: Females exposed to a fraction of A50 containing 0–1-*ortho*-CBs and any dicyclic aromatic contaminants such as PCDFs; the daily dose corresponded to the amounts of these

congeners separated from 0.3 mg of A50 (the daily dose of A50 in A50 high group).

2–4-*ortho*: Females exposed to a fraction of A50 containing 2–4-*ortho*-CBs; the daily dose corresponded to the amounts of these congeners separated from 0.3 mg of A50 (the daily dose of A50 in A50 high group).

Blubber extract: Females exposed to an extract of Baltic gray seal blubber containing organochlorine pollutants but without aryl methyl sulfones; the daily dose of the extract contained 0.064 mg total PCB and 0.021 mg total DDT.

Control: Females given 1 ml of the fish oil vehicle three times a week.

The level of PCB in the basic diet was analyzed in aliquots sampled on two different occasions during the experiment. The highest total PCB and total DDT concentrations in the feed were 0.011 and 0.004 μg/g (wet-wt basis), respectively.

During the mating periods (last three weeks in March), 30 nonexposed males were used. In general, each female was given two opportunities to mate, and the mating day(s) was noted. The frequency of successful mating (number of mated females/total number of females) was high (0.8–1.0) both years in all groups.

Measurements and sampling

At parturition the first reproductive season (April–May 1993), the total number of kits (dead and alive) in each litter was recorded. The kits were removed from the dams within 24 h after birth, sexed, and inspected for gross malformations. The live kits were weighed (±0.01 g) and killed by decapitation. Many of the dead kits were dehydrated, and therefore only live kits were weighed. Immediately after decapitation, blood was collected in heparinized glass capillaries from blood vessels in the neck. After centrifugation, the plasma samples were pooled separately for each sex and litter and frozen (−20°C). Thymus was excised and weighed (±0.0001 g). Various organs were excised and frozen in liquid nitrogen or on dry ice.

At parturition the second reproductive season (April–May 1994), the total number of kits (dead and alive) in each litter was recorded. Within 24 h of birth, the kits were sexed and inspected for gross malformations, and the live kits were weighed (±0.01 g). The kits were weighed at two and five weeks of age, and immediately after the last weighing they were anesthetized by electric shock and killed by decapitation. During the anesthesia, the jugular veins and carotid arteries were opened by an incision in the neck. Blood from these vessels was collected in heparinized glass tubes, and after centrifugation the plasma was transferred to plastic vials. Various organs were excised and weighed (±0.001 g). Samples were frozen in liquid nitrogen or on dry ice or were fixed in formalin or glutaric aldehyde for subsequent histopathological examination.

The adult females were weighed (±0.1 g) at the start of the study, after 12 months of exposure, and finally at the end of the 18-month exposure period. One to two weeks after removing the kits, the dams were killed by cervical dislocation during anesthesia provoked by electric shock. While the animals were anesthetized, blood was collected in heparinized vacutainers by heart puncture. After centrifugation, the plasma was transferred to plastic vials and frozen (−20°C). The nonreproducing dams were killed one to two weeks after expected parturition. Tissues from all dams were excised and treated in the same way as tissues from the five-week-old kits.

Results from analysis of part of the samples collected are presented in this paper. The studies were approved by the Local Ethics Committee for Research on Animals.

Enzyme assays

Frozen pieces (~3 g) of the maternal livers were thawed in ice-cold buffer (0.15 M KCl in 0.05 M Tris-HCl, pH 7.5) and homogenized in a Potter-Elvehjem homogenizer (Kontes Glass, Vineland, NJ, USA). Microsomes were prepared by differential centrifugation as previously described [23]. Ethoxyresorufin-*O*-dealkylase (EROD) activity in the microsomal fractions was determined using a slightly modified version of the method presented by Pohl and Fouts [24] as previously described [25]. Pentoxyresorufin-*O*-dealkylase (PROD) was determined using essentially the method described by Lubet et al. [26]. Microsomal protein concentration was determined by the method of Lowry et al. [27] using bovine serum albumin as a standard.

Instruments for chemical analysis

All tissue samples were homogenized with a DISP 25 homogenizer (InterMed, Roskilde, Denmark) equipped with a scissors rod (8-mm i.d.). A vacuum centrifuge (Genevac SF 50, Sales Development, Ipswich, England) was used for solvent evaporations. Gas chromatography was performed on a Varian 3400 gas chromatograph, equipped with a Varian 8200 autosampler and an electron-capture detector. The column used was a DB-5 fused silica capillary column (30-m × 0.25-mm i.d., 0.25-μm film thickness) manufactured by J&W Scientific (Folsom, CA, USA). Hydrogen was used as a carrier gas and nitrogen as a makeup gas. The column temperature was held at 80°C for 2 min and then increased by 10°C/min to 300°C, which was held for 10 min. The split/splitless injector in splitless mode was held at 250°C and the electron-capture detector at 360°C. The data system EldsPro from Chromatography Data Systems AB (Kungshög, Sweden) was used to collect and process the data.

High-performance liquid chromatography was performed on a module consisting of a Varian 9012 programmable solvent delivery system, a Varian 9050 variable wavelength ultraviolet-visible light detector (Varian, Walnut Creek, CA, USA), a Valco injector (Vici AG, Schenkon, Switzerland) equipped with a 100-μl loop, and two 150 × 4.6-mm-i.d. comsosil 5-PYE columns (2-[1-pyrenyl]ethyldimethylsilylated silica gel, particle size 5 μm, Nacalai Tesque, Kyoto, Japan) coupled in series and equipped with a column cooler (Jones Chromatography, Lakewood, CO, USA).

Gas chromatography/mass spectrometry (GC/MS) was performed on a TSQ 700 (Finnigan MAT, Bremen, Germany) equipped with a DB-5 column (30-m × 0.25-mm i.d., 0.25-μm film thickness). Helium was used as carrier gas at a head pressure of 12 psi. The mass spectrometer was operated in electron ionization mode with an electron energy of 70 eV. The injector and transfer line temperature was 280°C, and the temperature in the ion source was 150°C. The column temperature was kept at 60°C for 4 min, followed by a linear temperature increase of 30°C/min up to 180°C and then a linear temperature increase of 3°C/min up to a final temperature of 300°C that was held for 3 min. The instrument was run in selective ion monitoring detection mode. The mass of the two most intense ions in each molecular ion cluster was measured by the isotope dilution technique.

Table 1. Concentrations of major non- and mono-*ortho*-chlorobiphenyls (CBs) assigned a toxic equivalency factor (TEF) value [28] in the various capelin oil solutions of PCB used to dose the mink[a]

Treatment group	PCB congener concn. (ng/ml)						
	CB-77	CB-126	CB-169	CB-105	CB-118	CB-156	CB-157
Control[b]	0.87	≤0.36	≤0.91	4.6	7.4	1.6	0.73
A50 low	69	20	1.3	9.2×10^3	22×10^3	2.2×10^3	0.57×10^3
A50 high	0.20×10^3	61	2.0	25×10^3	61×10^3	6.5×10^3	1.8×10^3
0–1-*ortho*	69	17	1.2	24×10^3	52×10^3	6.0×10^3	1.8×10^3
2–4-*ortho*	≤3.0	≤0.86	0.81	0.63×10^3	6.0×10^3	0.35×10^3	49
Blubber extract	1.1	0.85	1.0	89	0.17×10^3	0.23×10^3	43

[a] CB-123 and CB-167 were detected in the oils A50 low, A50 high, and 0–1-*ortho*, but values for them are not given in the table, as they coeluted with other congeners. Their concentrations were estimated on the basis of relative congener concentrations in Clophen A50 [30]. Calculations from the estimated concentrations and the congener-specific TEFs showed that their contribution to total 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations was of a minor degree (<4%).
[b] The concentrations found in the capelin oil.

Analysis of PCB in oil preparations used in the experiment

Two samples (1 ml) of each oil preparation were analyzed for 0-*ortho*-CBs and 1-*ortho*-CBs. Each oil sample was dissolved in hexane (10 ml) and spiked with 25.5 μg of the $^{13}C_{12}$-labeled internal standard mixture for the 0-*ortho*-CBs and with 0.01 (control) to 14 μg (A50 high) CB-159 for the 1-*ortho*-CBs. The samples were then treated with concentrated sulfuric acid (5 g). The tubes were carefully inverted 20 times and then centrifuged, and the hexane was removed. The procedure was repeated once. The volume of the pooled hexane was reduced to 0.5 ml under a gentle stream of nitrogen. This solution was applied to a sulfuric acid:silica gel column (1:2, w/w, 1.2 g), and hexane (10 ml) was used as the mobile phase. The hexane was evaporated to leave a residue of about 50 μl, and the sample was then analyzed in the high-performance liquid chromatography system described previously. The two serially coupled PYE columns were kept at 5°C, and the mobile phase was kept at 0°C. The fraction borders were chosen according to the results obtained from a separation of a standard mixture of CB-77, CB-118, CB-157, and CB-169. Three fractions (fractions 1–3) were collected: fraction 1: 4.0 to 7.3 min (2–4-*ortho*-CBs); fraction 2: 7.3 to 9.8 min (1-*ortho*-CBs); and fraction 3: 9.8 to 16.3 min (0-*ortho*-CBs). The column was retroflushed for 40 min to avoid any contamination of subsequent samples. The solvent volume in fraction 3 (0-*ortho*-CBs) was reduced by evaporation, and the samples were then transferred to GC vials and the volume adjusted to 100 μl prior to analysis by GC/MS. Φ-DDE (18.9 ng) was added as injection standard. The 0-*ortho*-CBs were quantified by using a multilevel calibration curve and the internal standard method. For analysis of the 1-*ortho*-CBs, CB-189 was added as injection standard. The analyses were carried out by GC/electron-capture detection. The recovery of the internal standard (CB-159) was 93% ± 3%. The 2–4-*ortho*-CBs were analyzed as described in the following for the analysis of these congeners in muscle.

The TCDD-equivalent (TEQ) exposure of the mink in the various groups was calculated using the toxic equivalency factors (TEFs) recently suggested by a World Health Organization (Paris, France) working group [28] for CB-77, -105, -118, -123, -126, -156, -157, -167, and -169.

Analysis of PCB in mink muscle

All tissue samples were extracted using a slightly modified version of a general method for PCB analysis [29]. The tissue was cut in small pieces and homogenized in hexane:acetone (2:5, v/v). Following centrifugation, the supernatant was transferred to a test tube containing sodium chloride (0.9%) and phosphoric acid (0.1 M) for extraction. The tissue residue was extracted twice with methyl *tert*-butyl ether:hexane (1:9, v/v), and after centrifugation the solvent was transferred to the extraction tube containing sodium chloride/phosphoric acid solution. The phases were mixed by rocking the tube for a few minutes. Following centrifugation, the organic phase was transferred to a preweighed tube for subsequent gravimetric determination of lipid weight. The aqueous phase was re-extracted with hexane, and after centrifugation the hexane was transferred to the preweighed tube. The bulk of organic solvent was removed by means of the vapor vacuum pump, and any solvent residue was evaporated in a heating block under a gentle stream of nitrogen. The lipid weight of each sample was determined gravimetrically. The residue was dissolved in a small amount of hexane, and the lipids were removed on a sulfuric acid–impregnated silica gel column (1:2, w/w, 1.0 g). The CBs were eluted with hexane as the mobile phase and were collected in 10 ml.

To facilitate recovery evaluation, an injection standard (CB-46) was added to each sample prior to GC analysis. Recovery was 98% ± 5%. The individual CBs were quantified using A50 as a standard for PCB. The relative amounts were calculated according to Schultz et al. [30].

Statistics

Mean values of the treated mink were compared with the control values using Dunnett's *t* test. Log-transformed values were used when variances differed significantly (Bartlett's test, $p < 0.05$). Medians were compared using the Mann–Whitney test, and frequencies were compared by using Fisher's exact probability test. In the statistical treatment of the kit data, the mean value for each litter was used as the representative value for the corresponding female.

RESULTS

The PCB analysis of the oil preparations used to dose the mink in the groups A50 high and A50 low confirmed that the animals in these groups were exposed to amounts corresponding to 0.3 and 0.1 mg PCB/d, respectively. Congener-specific analysis of the oil preparations (Table 1) showed that the fractionation was not entirely successful, especially with regard to the 0-*ortho*-CBs. The concentrations of CB-77 and CB-126 in the 0–1-*ortho*-CB fraction were only about 30% of those in A50 high, and the remainder was not recovered in the 2–

Table 2. The various treatments of female mink and body weights during the exposure period of 18 months

Treatment group	Average daily exposure of each animal	TEQ exposure (ng/d)[a]	Body weight (g)[b]		
			At start	After one year	After 18 months
Control	<1.5 μg PCB	0.02	1,198 ± 188 (n = 20)	1,297 ± 230 (n = 18)	1,064 ± 178 (n = 18)
A50 low	0.1 mg Clophen A50	2.9	1,265 ± 194 (n = 20)	1,295 ± 161 (n = 19)	1,133 ± 189 (n = 19)
A50 high	0.3 mg Clophen A50	8.4	1,197 ± 148 (n = 20)	1,178 ± 167 (n = 20)	995 ± 170 (n = 19)
0–1-*ortho*	Amounts in 0.3 mg Clophen A50[c]	5.8	1,300 ± 155 (n = 20)	1,303 ± 151 (n = 19)	1,131 ± 174 (n = 19)
2–4-*ortho*	Amounts in 0.3 mg Clophen A50	0.41	1,209 ± 170 (n = 20)	1,165 ± 167 (n = 20)	1,105 ± 172 (n = 20)
Blubber extract	0.064 mg PCB + others	0.11	1,276 ± 153 (n = 20)	1,227 ± 121 (n = 19)	1,107 ± 160 (n = 19)

[a] 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin equivalent (TEQ) exposure was calculated from congener-specific analysis of the various oils and the toxic equivalency factors suggested by van den Berg et al. [28]. The TEQ concentration in the feed is not included but was deemed to be a minor contributor to total TEQ exposure, except in the control group.
[b] Values are presented as mean ± standard deviation.
[c] Only about 30% of the non-*ortho*-chlorinated congeners was recovered.

4-*ortho*-CB fraction. The recoveries of the 1-*ortho*-CBs on fractionation were high. Eighty-six percent of CB-118 was obtained in the 0–1-*ortho*-CB fraction, representing the lowest recovery of the 1-*ortho*-CBs analyzed. The small portion of 1-*ortho*-CBs not recovered in the 0–1-*ortho*-CB fraction was present in the 2–4-*ortho*-CB fraction. The TEQ exposures of the mink in the various groups, as calculated from the chemically determined concentrations of Ah receptor agonists, are shown in Table 2.

In the PCB-exposed adult females, no signs of toxicity were observed, judging by the general appearance of the animals and the lack of effects on body weight (Table 2). In the first reproductive season, after six months of exposure of the dams, a decreased birth weight (18% lower than in controls) was observed in group A50 high (0.3 mg A50/animal/d), whereas whelping frequency and litter size were not significantly affected (Table 3). Thymus weights were decreased in the kits in groups A50 high and 0–1-*ortho* (Table 4). The kits in A50 high also appeared to have a reduced viability, judging by their feeble efforts to search for milk and their weak vocal calls.

In the second season, the effects on reproduction were more pronounced and clearly dose dependent (Table 5). In group A50 high, only 39% of the dams produced offspring, compared with 93% in the control group. The newborn kits in group A50 high weighed 25% less than the control kits, and they all died within 24 h. Reproduction was impaired also in group A50 low (0.1 mg A50/mink/d). Kit production, measured as the number of two-week-old kits per mated female, was reduced (Fig. 1), and the two-week survival frequency of the kits born (including stillborn kits) was 36%, whereas survival was 73% in the control group. The body weight of kits in group A50 low was lower than in the control kits, at both two and five weeks of age (Fig. 2). In both seasons, the frequency of abnormalities (mainly edemas) among the stillborn kits in group A50 high (29% the first season and 24% the second season) exceeded that in the control group (0 and 10%).

Treatment with the 0–1-*ortho*-CB fraction strongly reduced (by two postnatal weeks) the number of kits per mated female (Fig. 1), and the two-week survival frequency in this group was only 7%. The growth rate of the kits was substantially

Table 3. Reproductive performance of female mink after six-month exposure to PCBs in the feed

Treatment group	% Mated females whelping[a]	Litter size, live kits[b,c]	Litter size, dead and live kits[b,c]	Birth weight (g)[b,d]
Control	93	4.0 ± 2.7 (n = 25)	4.9 ± 2.4 (n = 25)	9.6 ± 2.0 (n = 22)
A50 low	89	5.2 ± 2.1 (n = 17)	5.9 ± 2.1 (n = 17)	9.5 ± 1.9 (n = 16)
A50 high	90	3.8 ± 2.3 (n = 17)	5.1 ± 2.3 (n = 17)	7.9 ± 1.2* (n = 13)
0–1-*ortho*	78	3.6 ± 2.3 (n = 12)	4.8 ± 2.4 (n = 12)	9.1 ± 1.7 (n = 12)
2–4-*ortho*	79	4.2 ± 2.2 (n = 15)	5.3 ± 2.1 (n = 15)	9.9 ± 2.1 (n = 14)
Blubber extract	89	4.9 ± 2.2 (n = 17)	5.4 ± 1.8 (n = 17)	9.2 ± 1.6 (n = 16)

[a] There were 27 mated females in the control group and 18 to 20 in the other groups.
[b] Values are presented as mean ± standard deviation.
[c] Nonwhelping females not included.
[d] Only live kits were weighed; values given are means of the litter means in each group.
* Significantly different from control value (Dunnett's t test, $p < 0.05$).

Table 4. Kit organ weights (as % or ‰ of body wt) at autopsy; values given are means of the litter means in each group[a]

Treatment group	Newborn kits, first season	Five-week-old kits, second season		
	Thymus (‰ of body wt)	Thymus (% of body wt)	Liver (% of body wt)	Adrenal (‰ of body wt)
Control	0.97 ± 0.25 (n = 19)	0.35 ± 0.11 (n = 12)	3.98 ± 0.25 (n = 12)	0.102 ± 0.01 (n = 12)
A50 low	0.91 ± 0.20 (n = 15)	0.34 ± 0.05 (n = 10)	3.68 ± 0.44 (n = 10)	0.112 ± 0.018 (n = 10)
A50 high	0.56 ± 0.18** (n = 13)	—	—	—
0–1-*ortho*	0.78 ± 0.13* (n = 12)	0.27 ± 0.11 (n = 4)	3.72 ± 0.66 (n = 4)	0.117 ± 0.015 (n = 4)
2–4-*ortho*	0.93 ± 0.18 (n = 14)	0.35 ± 0.09 (n = 15)	3.64 ± 0.40 (n = 15)	0.109 ± 0.035 (n = 15)
Blubber extract	0.85 ± 0.14 (n = 16)	0.33 ± 0.09 (n = 12)	3.72 ± 0.50 (n = 12)	0.096 ± 0.014 (n = 12)

[a] Values are presented as mean ± standard deviation.
* Significantly different from control value (Dunnett's t test; $p < 0.05$).
** Significantly different from control value (Dunnett's t test, $p < 0.01$).

reduced by the 0–1-*ortho*-CBs (Fig. 2), whereas the 2–4-*ortho*-CB fraction affected neither kit production (Fig. 1) nor their growth rate (Fig. 2). However, a numerically lower two-week survival frequency (50%) than in the control group (73%) was observed also for the kits in group 2–4-*ortho*. In the group exposed to the seal blubber extract, no adverse effects on reproduction were noted.

No significant effects on thymus, liver, or adrenal weights (relative to the body wt) were observed in any group in the five-week-old kits at autopsy (Table 4).

The hepatic cytochrome P450–dependent enzyme activities PROD and EROD were induced in a dose-dependent manner in the female adults exposed to A50 (Figs. 3 and 4). We observed a similar PROD induction by the highest dose of A50 and the 2–4-*ortho*-CB fraction and a lack of PROD induction in animals exposed to the 0–1-*ortho*-CB fraction (Fig. 3). Both PCB fractions induced EROD, although the 0–1-*ortho*-CB fraction was much more potent (Fig. 4). The contaminants extracted from Baltic gray seal blubber strongly induced PROD (Fig. 3), whereas EROD was only slightly induced in this group (Fig. 4).

Reduced kit production occurred concomitantly with increased hepatic EROD activity in the adult females. The negative correlation between TEQ exposure and kit production and the positive correlation between TEQ exposure and EROD activity are shown in Figure 5. Exposure to TEQs was calculated from the chemically analyzed levels of Ah receptor agonists in the oils. The TEQ concentration in the basal feed was not included in this calculation.

The concentration of total PCB in muscle of the adult females after the experiment is shown in Figure 6. The mean concentration (n = 7) of total PCB in A50 high was 54 μg/g (lipid wt), whereas the concentrations in the other exposed groups were much lower and in the same range (10.6–14.4 μg/g), even though different types of PCB were given. The lipid content in muscle was 2.4 ± 0.4%. The bioaccumulation factors (BAFs), defined as the muscle concentrations (lipid wt) of the various CB congeners, divided by feed concentrations

Table 5. Reproductive performance of female mink after 18-month exposure to PCBs in the feed

Treatment group	% Mated females whelping[a]	Litter size, live kits[b,c]	Litter size, dead and live kits[b,c]	Birth weight (g)[b,d]
Control	93	4.4 ± 2.5 (n = 14)	5.1 ± 2.4 (n = 14)	8.9 ± 1.8 (n = 14)
A50 low	88	5.9 ± 2.4 (n = 14)	6.2 ± 2.3 (n = 14)	8.0 ± 1.0 (n = 14)
A50 high	39**	2.0 ± 1.7*[e] (n = 7)	4.1 ± 1.8 (n = 7)	6.7 ± 2.2* (n = 5)
0–1-*ortho*	72	4.0 ± 2.3 (n = 13)	5.0 ± 2.2 (n = 13)	8.1 ± 1.2 (n = 12)
2–4-*ortho*	95	4.9 ± 2.1 (n = 18)	6.0 ± 2.3 (n = 18)	9.2 ± 1.5 (n = 18)
Blubber extract	93	5.1 ± 1.8 (n = 14)	5.7 ± 1.9 (n = 14)	8.9 ± 1.7 (n = 14)

[a] The numbers of mated females were 15 to 19.
[b] Values are presented as mean ± standard deviation.
[c] Nonwhelping females not included.
[d] Only live kits were weighed; values given are means of the litter means in each group.
[e] All kits died within 24 h.
* Significantly different from control value ($p < 0.05$; litter size: Mann–Whitney test; birth weight: Dunnett's t test).
** Significantly different from control value (Fisher's exact probability test, $p < 0.01$).

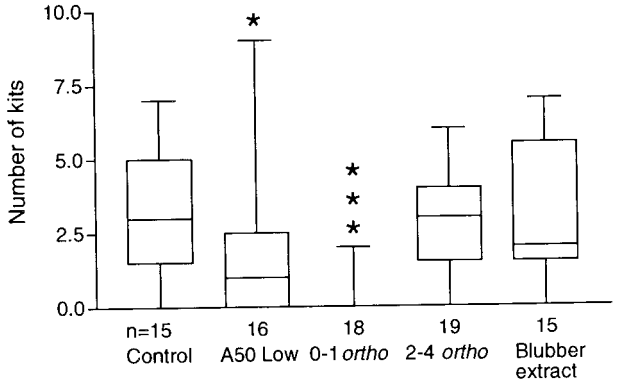

Fig. 1. Numbers of two-week-old kits per mated female mink following exposure to polychlorinated biphenyl (PCB) for 18 months. Boxes extend from the 25th to the 75th percentile, with a horizontal line at the median. The whiskers show the range of the data among mated females in each group. The numbers of litters in each group are shown below boxes and whiskers. Asterisks denote values that differ significantly from the control value (*$p < 0.05$, ***$p < 0.001$, Mann–Whitney test, one tailed).

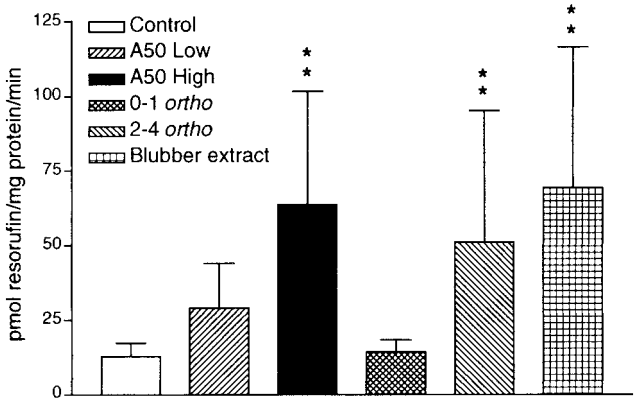

Fig. 3. Hepatic pentoxyresorufin-*O*-dealkylase (PROD) activities in adult female mink exposed to various polychlorinated biphenyl (PCB) preparations for 18 months. Values shown are means and standard deviations for seven animals in each group. Asterisks denote values that differ significantly from the control value (**$p < 0.01$, Dunnett's *t* test).

(lipid wt), differed in the various groups given different CB mixtures. The BAFs were 2.1, 1.3, 2.3, 0.7, and 1.9 for the groups A50 high, A50 low, 0–1-*ortho*, 2–4-*ortho*, and blubber extract, respectively.

DISCUSSION

In this paper we show that long-term exposure to low doses of PCB impairs reproduction in mink, manifested as fetal deaths, abnormalities, decreased kit survival, and decreased kit growth. The doses of A50 given (0.1 and 0.3 mg/mink/d) are relevant in terms of wildlife exposure to PCB via the diet in contaminated areas. Mean PCB concentrations in samples of fish from Swedish coastal waters and lakes in 1991–1992 were as follows: in sea trout (*Salmo trutta*) from the Gulf of Bothnia, 331 μg/kg fresh weight; in salmon (*Salmo salar*) from the Baltic Sea, 430 μg/kg; and in arctic char (*Salvelinus alpinus*) from Lake Vättern, Sweden, 797 μg/kg [31]. If the diet of fish-eating mammalian wildlife, such as otters, consisted solely of fish with a PCB concentration of 500 μg/kg fresh weight, then the animals would be exposed to about 0.1 mg PCB/kg body weight/d (food consumption assumed to be 200 g/kg body wt/d). We found a marked effect on kit survival and growth in the group exposed to this dose of PCB (A50 low), a dose that is slightly lower than the lowest-observed-adverse-effect level (LOAEL) of 0.16 mg PCB/mink/d as reported for mink fed carp from Saginaw Bay, Lake Huron, Michigan, USA [8]. A comparison of the LOAEL values for PCB obtained in our study and in that carried out by Heaton and coworkers [8] is hampered by several discrepancies in design of the two studies. We exposed the mink to a technical PCB preparation for 18 months, whereas the mink in the study by Heaton and coworkers were exposed for six months to the weathered PCB present in the carp. The carp also contained compounds other than PCBs, such as PCDDs/Fs, which probably contributed to the effects observed in that study (see the following comparison of TEQ exposure). In our study, the concentration in the feed was the same during the two reproduction seasons, resulting in a reduced frequency of whelping females in the second season only. This finding suggests that

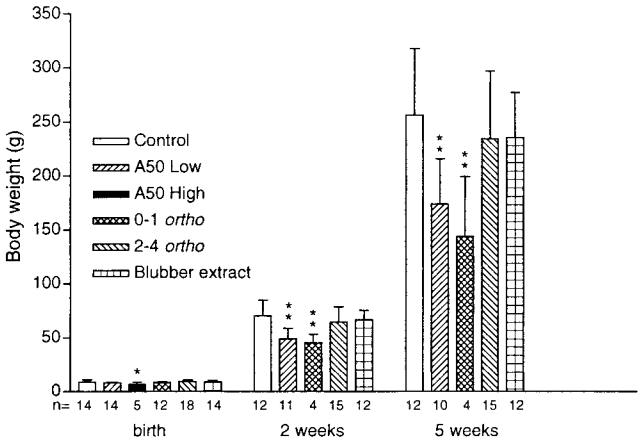

Fig. 2. Weights at birth and at two and five weeks of age in kits of female mink exposed to polychlorinated biphenyl (PCB) for 18 months. Values shown are means and standard deviations of the litter means in each group. The number of litters in each group is shown below the bars. Note that there were no kits by two and five weeks in A50 high. Asterisks denote values that differ significantly from the control value (*$p < 0.05$, **$p < 0.01$, Dunnett's *t* test).

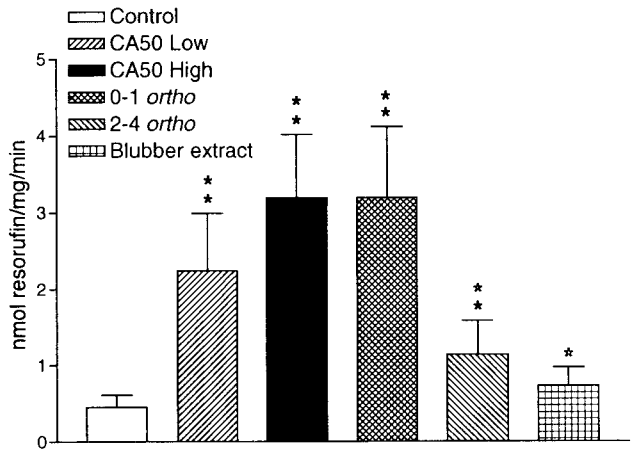

Fig. 4. Hepatic ethoxyresorufin-*O*-dealkylase (EROD) activities in adult female mink exposed to various polychlorinated biphenyl (PCB) preparations for 18 months. Values shown are means and standard deviations for seven animals in each group. Asterisks denote values that differ significantly from the control value (*$p < 0.05$, **$p < 0.01$, Dunnett's *t* test).

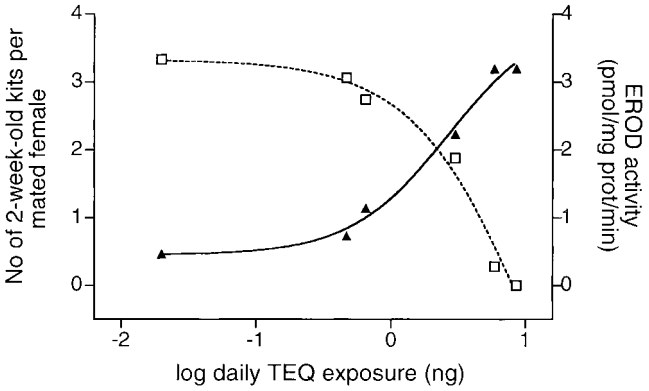

Fig. 5. Production of two-week-old kits per mated female mink and hepatic 7-ethoxyresorufin-*O*-dealkylase (EROD) activity in the adult females as functions of daily 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent (TEQ) exposure for 18 months. □ = number of kits, ▲ = EROD activity.

the PCB concentration in the animals increased from the first to the second reproduction season, showing the relevance of long-term exposure for estimation of a LOAEL. In a recent multigenerational study by Restum et al. [14], mink were exposed to PCBs and other contaminants present in carp from Saginaw Bay. At a feed concentration of 500 μg PCB/kg (0.1 mg PCB/mink/d, assuming a feed consumption of 200 g/d), litters had greater mortality and lower body weights than controls.

We found a pronounced reproductive toxicity without any obvious toxic effects in the dams, confirming that impaired reproduction is a very sensitive marker for PCB-induced toxicity in the mink. The lack of obvious toxic effects in the dams might be explained by the relatively low levels of PCB found in the animals, 10 to 50 μg PCB/g in muscle (lipid wt). Using experimental data on PCB levels and reproduction outcome in female mink, Leonards et al. [32] estimated the median effect level (EC50) to be 1.2 μg total PCB/g wet weight. Assuming a lipid concentration of 2 to 3% in muscle, this value corresponds to a concentration of 40 to 60 μg PCB/g (lipid wt). The data from groups A50 high and A50 low in our study suggest a lower EC50 with regard to reproductive impairment when animals are chronically exposed. The PCBs in European otter (*Lutra lutra*) were studied by Smit et al. [33], who reported that concentrations above 50 μg/g lipid weight are found in many European countries. For instance, Mason and

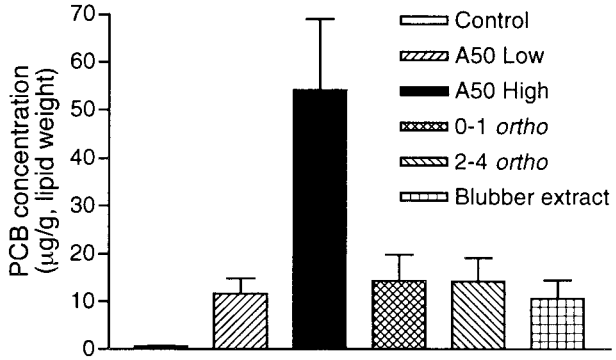

Fig. 6. Total polychlorinated biphenyl (PCB) concentrations (μg/g, lipid wt) in muscle from adult female mink exposed to various PCB preparations for 18 months. Values shown are means and standard deviations for seven animals in each group.

Madsen [34] reported that PCB concentrations exceeded 50 μg/g in 19%, 30 μg/g in 30%, and 10 μg/g in 70% of tissue samples (71 liver, two muscle) from 73 otters found dead in Denmark between 1980 and 1990. Median PCB concentrations (μg/g, lipid wt) in otters traumatically killed in 1990 to 1994 were 7.7 (range 0.6–75, $n = 29$) in northern Sweden and 29 (range 7–149, $n = 10$) in southern Sweden [35].

Our results show the decisive role played by the dioxin-like PCB congeners in the PCB-induced impairment of reproduction in mink. Congener-specific PCB analysis of the various oils made it possible to estimate their TEQ concentrations and to assess TEQ exposures in the mink. A negative correlation between TEQ exposure and overall reproductive performance was found. The PCB congeners lacking affinity for the Ah receptor had a very limited effect (if any) on mink reproduction at the doses given. The finding that the reproductive toxicity is due solely to the dioxin-like PCBs differs from the conclusions by Kihlström et al. [13], who suggested that the congeners lacking Ah receptor affinity also contribute to the reproductive toxicity of PCB. Adult mink are known to be highly susceptible to TCDD, and Hochstein et al. [36] found a single oral 28-d LD50 of 4.2 μg/kg body weight for males. In female mink, LD50 values of 264 and 47 ng/kg/d were estimated for 28- and 125-d exposure periods, respectively [15]. Daily intraperitoneal injections of 0.1 μg TCDD/kg body weight in newborn kits for 12 consecutive d significantly reduced body weights and produced over 50% mortality within 10 weeks [37]. In our experiment, the exposure of the mink in groups A50 high and A50 low gave 8.4 and 2.9 ng TEQ/d, respectively, corresponding to 65 and 22 pg TEQ/g feed (fresh wt) and a total exposure over the 18 months of approximately 4.6 and 1.6 μg TEQ/kg, respectively.

The LOAEL in our study was 2.9 ng TEQ/animal/d (about 2.4 ng/kg body wt/d) or 22 pg TEQ/g feed (fresh wt) because we noted effects on kit production and kit growth in group A50 low. According to Tillitt et al. [38], the mink in the group receiving the lowest dose in the study by Heaton et al. [8] (10% carp in the feed) were exposed to 21.2 pg TEQ/g feed. The daily feed consumption in that group was reported to be 218 g/mink, resulting in an exposure of 4.6 ng TEQ/animal/d. Kit body weights and survival at three and six weeks of age were significantly reduced in the animals exposed to 10% carp in the feed. Thus, a similarity exists in the effects on reproduction between our study and that carried out by Heaton and coworkers, even though the animals were exposed to PCB from different sources (technical and weathered) and the exposure times differed. We found no significant adverse effect on reproduction in the mink exposed to the 2–4-*ortho*-CB fraction. The TEQ exposure in that group was 0.41 ng TEQ/animal/d (about 0.35 ng/kg body wt/d) or 3.2 pg/g feed (fresh wt, TEQ concentration in the basal feed not included), which represents no-observed-adverse-effect levels in terms of reproductive effects in our study. However, both EROD and PROD activities were induced in that group, and hence a biological effect existed also in those animals.

The BAFs differed for the CB mixtures given to the various groups. The mink in group A50 high were exposed to a threefold higher PCB dose (0.3 mg/d) than the animals in group A50 low (0.1 mg/d). The greater-than-threefold difference in total PCB levels between females from these groups (54 vs 12 μg/g, BAFs: 2.1 and 1.3, respectively) most probably shows that the animals in A50 high did not rid any PCB via the milk, whereas the dams in A50 low had one to five kits that suckled

for five weeks. The animals treated with the 2–4-*ortho*-CB-fraction were exposed to more total PCB than those given the 0–1-*ortho*-CBs or the low dose of A50. By the end of the study, the residual levels of PCB were similar in these groups, and the estimated BAFs were 2.3 (0–1-*ortho*-CBs), 1.3 (A50 low), and 0.7 (2–4-*ortho*-CBs). These differences in accumulation probably reflect partly a greater persistence of the 0–1-*ortho*-CBs than of the 2–4-*ortho*-CBs in mink and were partly a consequence of only a few kits suckling from the females exposed to the 0–1-*ortho*-CBs. It has been shown previously that many 2–4-*ortho*-CBs, notably those with unsubstituted 3,4 positions, are readily metabolized by mink [22]. The Baltic gray seal blubber contained low relative concentrations of both 0- and 1-*ortho*-CBs (compare Table 1), indicating a high capacity of the gray seal to metabolize these CBs. There might consequently be considerable differences between mink and seals with respect to PCB metabolism. The BAF for total CBs in the blubber extract was fairly high (1.9), reflecting the high persistence of the 2–4-*ortho*-CBs that had accumulated in the blubber.

Induction of EROD proved to be a sensitive marker for dioxin-like biological effects in the mink, and the non- and mono-*ortho*-chlorinated CBs were potent inducers. The fraction that consisted mainly of 2–4-*ortho*-CBs and the blubber extract also caused significant induction, showing that these fractions contained Ah receptor agonists in concentrations high enough to cause a dioxin-like biological effect. The inverse relationship between EROD activity and production of kits shown in Figure 5 supports EROD as a biomarker predicting toxic effects in the mink, and it also shows that reproductive impairment and EROD are induced by fairly similar doses of Ah receptor agonists. In mink exposed to PCB-contaminated carp, a dose-dependent EROD induction was noted, and EROD was suggested as a potential biomarker for PCB exposure in mink [39]. Another marker for dioxin-like effects is a reduced thymus weight. We noted such a reduction in newborn kits of mothers exposed to the two highest TEQ concentrations in the feed (groups A50 high and 0–1-*ortho*) for six months, but the lack of effect in the five-week-old kits in the second season suggests that thymus weight is not a useful biomarker in juvenile mink. The PROD activity was strongly induced in the group treated with the 2–4-*ortho*-CBs but not in the group exposed to the 0–1-*ortho*-CBs. This is in accordance with PROD as being induced by noncoplanar PCB congeners, and induction of this enzyme may consequently be used as an indicator of PCB exposure.

Several conclusions may be drawn from this study. Long-term exposure of mink to PCB impairs reproduction when PCB residue levels in the mothers are considerably below 50 μg/g, a value often referred to as a threshold for reproductive toxicity in mink. The PCB congeners that are Ah receptor agonists were responsible for the reproductive dysfunction of the animals in this study. Furthermore, kit survival and kit growth were sensitive endpoints for toxicity caused by the Ah receptor agonists. Induction of EROD in adult female mink appears to be a good marker for reproductive effects, as reproductive performance decreased concomitant with an increase in enzyme activity. In the otter, coplanar PCB congeners bioaccumulate to a high degree [40], and exposure to these compounds is probably one important factor behind declines of otter populations.

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