

IMMUNOTOXICITY OF THE COMMERCIAL POLYBROMINATED DIPHENYL ETHER MIXTURE DE-71 IN RANCH MINK (*MUSTELA VISON*)

PAMELA A. MARTIN,*† GREG J. MAYNE,† STEVEN J. BURSIA,‡ GREGG TOMY,§ VINCE PALACE,§ CYNTHIA PEKARIK,† and JUDIT SMITS||

†Environment Canada, Canadian Wildlife Service, Burlington, Ontario L7R 4A6

‡Department of Animal Science, Michigan State University, East Lansing, Michigan 48824, USA

§Department of Fisheries and Oceans, Freshwater Institute, Winnipeg, Manitoba R3T 2N6, Canada

||Department of Veterinary Pathology, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada

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Abstract—Polybrominated diphenyl ethers (PBDEs) are persistent, bioaccumulative, organohalogen compounds that are increasing exponentially in the Great Lakes (Canada/USA) biota. The present study was undertaken to examine the immunological effects of a commercial PBDE mixture in ranch mink (*Mustela vison*). Twenty-week-old mink ($n = 10$ mink/group) were exposed to 0, 1, 5, or 10 ppm of DE-71 through their diet for eight weeks. The phytohemagglutinin-induced cutaneous reaction, and antibodies specific to keyhole limpet hemocyanin conjugated to dinitrophenol were measured. Liver microsomal ethoxyresorufin-*O*-deethylase (EROD) activity also was measured. Organs were weighed and spleens were examined histologically. No differences were found in the PHA-induced skin response in exposed mink; mink in the two highest treatments exhibited significant increases in antibody production over control mink. Systemic toxicity was apparent; significant body weight reductions were found in mink exposed to 5 and 10 ppm of DE-71. Exposed mink had significantly larger relative spleen, adrenal, and liver masses than control mink. Spleens of mink exposed to 10 ppm of DE-71 had significantly increased germinal center development and incidence of B-cell hyperplasia. The activity of EROD was induced in all treated mink relative to controls and was positively associated with the liver somatic index. Hematocrit in mink from the two highest exposure groups was significantly lower than control mink. Percentage neutrophils increased and percentage lymphocytes decreased significantly in mink from the higher two dosage groups. Our findings have direct relevance to wild mink in the Great Lakes ecosystem, because mink are top predators of the aquatic food web, providing evidence for the vulnerability of this species to the effects of environmental PBDE mixtures.

Keywords—Polybrominated diphenyl ethers Flame retardants Mink Immunotoxicology

INTRODUCTION

Recent studies have documented associations between persistent organic pollutants, such as polychlorinated biphenyls (PCBs) and metabolites of DDT, and modulation of the immune system in several marine mammals [1–3] and Great Lakes waterbirds [4,5]. In the Great Lakes basin ecosystem, concentrations of these compounds are decreasing in a variety of environmental media [6], thus hypothetically reducing the risk of organochlorine contaminant-induced immune modulation in wildlife. However, environmental concentrations of polybrominated diphenyl ethers (PBDEs) are increasing in the Great Lakes biota [7]. These compounds are produced in large quantities in North America and are incorporated into a variety of commercial products, such as fire retardants. Unfortunately, they find their way into the natural environment, where they persist and readily bioaccumulate in biota (for review, see de Wit [8]). Indeed, tissue analyses of lake trout (*Salvelinus namaycush*) and herring gull (*Larus argentatus*) eggs from the Great Lakes provide evidence for an exponential increase of PBDE contamination in fish and wildlife feeding at the top of the food chain [7,9]. Because PBDEs are similar in molecular structure and have many of the same chemical and physical properties as the immunotoxic PCBs and DDT metabolites, it is plausible that PBDE-induced immune modulation is occurring in sensitive Great Lakes wildlife populations.

Currently, the immunological effects of PBDEs on aquatic mammals feeding at the top of the Great Lakes food chain are unknown. Wild mink (*Mustela vison*) may be at particular risk, because approximately half of their diet is composed of fish [10]. Mink are widely distributed in temperate North America and are found in environments close to urbanization, further increasing their risk of exposure to PBDEs originating from sewage effluents. Mink are sensitive to the structurally related organohalogenated compounds, such as PCBs, dioxins, and furans [11–15]. Because of the above-mentioned characteristics and the fact that they are amenable to captive rearing, ranch mink serve as a useful model and a surrogate for their feral counterparts in testing for immunotoxicity of PBDE congeners and mixtures.

Evidence suggests that exposure to PBDE modulates immune system function and the structure of immune organs. Exposure of mice to the isomer BDE 47 or the technical formulation Bromkal 70-5DE suppressed the proliferation of lymphocytes and the production of antibodies [16,17]. Suppression of antibody production following sheep red blood cell challenge and reduced thymic weights also were reported in mice following subchronic treatment of 1,000 mg/kg body weight of the technical formulation DE-71 [18]. Laboratory experiments with rodents and, more recently, with fish have demonstrated that the lower-brominated PBDE congeners bioaccumulate readily and are stored in adipose tissue [19–21]. Bioaccumulation, however, is influenced by hepatic microsomal biotransformation enzymes. The commercial PBDE mixture

* To whom correspondence may be addressed (pamela.martin@ec.gc.ca).

Bromkal-70 was characterized as a mixed-type inducer, stimulating both ethoxyresorufin-*O*-deethylase (EROD) and benzphetamine-*N*-demethylase cytochrome P450 activities [22]. Fowles et al. [18] reported that the commercial mixture DE-71 induced EROD activity in mice.

The exponential increase in PBDE concentrations in the Great Lakes basin ecosystem as well as a need for PBDE immune toxicity data concerning aquatic mammals prompted the present study. As part of a series of studies addressing the effects of PBDEs on growth, development, and endocrine function in ranch mink, we investigated the possible immune effects of a commercial PBDE mixture, DE-71, on ranch mink. The primary antibody response to the T lymphocyte-dependent antigen, keyhole limpet hemocyanin (KLH), and the phytohemagglutinin (PHA)-induced skin test were used to examine immune function. The spleen also was evaluated using histological criteria. Additional health status indicators, such as growth and hematological parameters, were assessed.

MATERIALS AND METHODS

Animal husbandry

Forty first-year, natural dark, male mink from the Michigan State University (East Lansing, MI, USA) Experimental Fur Farm herd were randomly assigned to four treatment groups ($n = 10$ mink/group). Mink were housed individually in wire cages (length, 76 cm; width, 61 cm; height, 46 cm). A wooden nest box (length, 38 cm; width, 28 cm; height, 27 cm) bedded with aspen shavings and excelsior ("wood wool") was attached to the outside of each cage. Cages were suspended aboveground in an open-sided shed. Food and water were available ad libitum. Housing of animals exceeded guidelines specified in the Standard Guidelines for the Operation of Mink Farms in the United States (<http://www.furcommission.com/farming>).

Diet

Components of the basal diet were 29% XK-40 mink cereal (XK Mink Foods, Plymouth, WI, USA), 28% duck offal (United Feeds, Plymouth, WI, USA), 28% water, 6% fishmeal (Omega Protein, Hammond, LA, USA), 6% spray-dried egg (Van Eldern, Martin, MI, USA), 2% corn oil, 0.5% vitamin, and 0.5% mineral premixes (Michigan State University Feed Mill, East Lansing, MI, USA), with 0.12 mg D-biotin/kg feed (Archer Daniel Midland, Des Moines, IA, USA) and 0.15 ml Larvadex/kg feed (Novartis Animal Health, Greensboro, NC, USA).

For mink exposed to DE-71, an appropriate amount of DE-71 was dissolved in 20 ml of hexane, which was then added to 100 ml of corn oil and thoroughly mixed. The corn oil containing the dissolved PBDE was then added to 1 kg of commercial mink cereal, and the mixture was thoroughly blended. Plain corn oil was added to the diet of control animals. At the time of feed mixing, the cereal premix containing DE-71/corn oil was added to the other feed ingredients comprising the basal mink diet. Ingredients were mixed in a paddle mixer (capacity, 150 kg) for 10 min. Feed was packaged in 7.6-L plastic containers and stored frozen until 24 h before feeding.

Experimental design

Twenty-week-old mink were randomly assigned to four groups ($n = 10$ mink/group) and were given diets supplemented with 0, 1, 10, or 100 ppm of DE-71 for nine weeks. The concentrations of PBDEs in freshwater fish in North

America currently are on the order of 0.01 to 1 $\mu\text{g/g}$ wet weight but have undergone exponential increases over the past two decades [23–25]. With our dosing regime, we attempted to bracket current environmental concentrations (0–1 ppm) and those that might be expected in the next decade (10 ppm), and we also included a worst-case scenario (100 ppm). Animals were observed daily to assess health status, and body weights and food consumption were measured weekly. During the first week of the experiment, however, mink receiving the diet containing 100 ppm of DE-71 rejected their food and demonstrated precipitous declines in body mass. Mink from this group were then switched to a diet containing only 5 ppm of DE-71 for the duration of the treatment. At the end of the treatment period, mink were killed by CO_2 asphyxiation. At necropsy, organs were immediately weighed, then fixed in 10% buffered formalin or cryopreserved.

PHA skin response

The T lymphocyte-mediated immune response of 29-week-old mink was assessed by evaluating the skin reaction to stimulation by PHA, a mitogenic lectin from red kidney beans (*Phaseolus vulgaris*). In the left hind limb interdigital toe web between phalanges 2 and 3, 100 μg of PHA (Sigma-Aldrich Canada, Oakville, ON, Canada) in 100 μl of phosphate buffered saline (PBS) were injected subcutaneously with a 27-gauge needle. The same volume of PBS was injected into the other toe web to control for nonspecific inflammation. Each toe web was measured three times immediately before injection and again 24 h after the injection to within 0.01 mm using a caliper with a low-tension spring (model 304-196; Dyer Company, Lancaster, PA, USA). The caliper compressed the site until the skin showed the first sign of movement under the pressure. The PHA-induced response was calculated as the change in the thickness of the PHA-injected web minus the change in thickness of the PBS-injected web.

Antibody-mediated immunity

A vaccine was formulated using a hapten, dinitrophenol (DNP), conjugated to a carrier protein, KLH. A stock solution of 1 mg/ml of DNP-KLH (Calbiochem, Terochem Laboratories, Edmonton, AB, Canada) in 10 mM 3-[*N*-morpholino] propane-sulfonic acid buffer (pH 7.2) was prepared. Two milliliters of this DNP-KLH, 1,400 μl of carbonate buffer (pH 9.5), 1,200 μl of Rehydrigel HPA aluminum hydroxide gel (Reheis, Berkeley Heights, NJ, USA), 600 μl Tween 80, 240 μl of Span 80, and 1,000 μl of Freund's Incomplete Adjuvant (Sigma-Aldrich Canada) were emulsified.

Using 27-gauge, 1-cm needles and 1-ml heparinized syringes, blood samples (1 ml) were collected from mink on the first day of the trial. The mink were then vaccinated in the paralumbar region with 200 μl of vaccine. Mink received booster vaccinations with 200 μl of vaccine on days 25 and 47 after the initial immunization. Blood (1 ml) was collected by anterior vena caval puncture on mink anesthetized with an intramuscular injection of 18 to 20 mg/kg of ketamine HCl (Rogar, Montreal, QC, Canada) and 1 mg/kg of xylazine HCl (Bayvet, Etobicoke, ON, Canada) on days 0 (preimmune), 25, 47, and 59 after the initial immunization. Blood samples were immediately centrifuged at 900 g for 10 min at room temperature and plasma was flash-frozen in liquid nitrogen and stored at -80°C until the day of analysis.

Histological evaluation of the spleen

The spleens were sectioned through the midpoint and placed, cut-side down, in coded plastic cassettes to facilitate blind histological evaluation. Fixed spleens were embedded in paraffin wax, and two to three sections (thickness, 5 μm) were mounted on glass slides and routinely stained with eosin and hematoxylin (Histopathological Laboratory, University of Guelph, Guelph, ON, Canada). Spleen sections were evaluated by the same person as follows: Five random fields per section were assessed at $\times 400$ magnification. Three measurements were made of the degree of development of the T lymphocyte-dependent white pulp based on the occurrence of periarteriolar lymphatic sheaths: First, absent versus moderate to thick; second, the number of germinal centers in the B lymphocyte-dependent white pulp relative to the total number of germinal centers; and third, an overall assessment based on cell density of the white pulp, including the number of normal versus hyperplastic germinal centers. Two sections per spleen were examined.

Hepatic microsomal EROD activity

Microsomes were prepared from flash-frozen liver samples and analyzed for phase I activity, as EROD, using the methods described by Kennedy and Jones [26], except that microsomal protein was determined on a separate aliquot of the microsomal preparation as described by Shields and Eales [27].

Hematology

Hematocrit (%) was determined by measuring packed cell volume (PCV) as a percentage of blood volume after centrifugation in a microhematocrit tube at 10,000 g for 3 min. On the first and last sampling dates, two blood smears were made immediately subsequent to blood collection. Blood smears were stained with Wright Stain (Accustain Wright Stain Modified WS16, Sigma-Aldrich Canada). Slides were dipped in the Wright stain solution for approximately 15 s, then rinsed with deionized water and allowed to air-dry. Two hundred white blood cells from the feathered edge of the blood smear were identified and classified using oil immersion microscopy at $\times 1,000$ magnification.

Chemical analysis

Sample extraction and cleanup. Tissue samples were frozen, dry-ice homogenized, and weighed before extraction with ASE (accelerated solvent extraction) 300 (Dionex Canada, Oakville, ON, Canada). Weighed samples were mixed with heat-treated (600°C for 6 h), pelleted diatomaceous earth and added to 100 ml of ASE cells. Chlorinated diphenylether surrogates 17, 99, and 170 were added to the mixture, and heat-treated (600°C for 6 h) Ottawa sand (20–30 mesh; Fisher Scientific, Nepean, ON, Canada) was used to fill any voids. The following ASE parameters were used: Solvent, 50:50 dichloromethane:hexane; temperature, 125°C; pressure, 1,500 psi; heat-up time, 6 min; static time, 5 min; flush volume, 30%; purge time, 120 s; and two static cycles. The organic extracts were then dried over heat-treated (600°C for 6 h) anhydrous sodium sulfate (10–60 mesh), reduced in volume, and filtered (1- μm polytetrafluoroethylene syringe filters; Gelman Laboratory Ann Arbor, MI, USA). An aliquot of each extract was evaporated to dryness, and lipid weights were determined gravimetrically. Lipid was removed from the remaining extract using gel-permeation chromatography [18]. The gel-permeation chromatographic columns (length, 400 mm; inner di-

ameter, 29.5 mm) were packed with 60 g (dry wt) of 200 to 400 mesh S-X3 Envirobeads (ABC Instruments, Joplin, MO, USA) that had been soaked in dichloromethane/hexane (1:1) overnight. Further purification was achieved on a column (length, 300 mm; inner diameter, 10.5 mm) of reagent-grade Florisil (1.2% [w/w] deactivated, 8 g, 60–100 mesh; Fisher Scientific) before gas chromatography.

Gas chromatography/electron-capture detector analysis

All analyses were performed on a Varian Star 3600 Cx gas chromatograph (63Ni) electron-capture detector (ECD) fitted with a DB-5 capillary column (length, 60 m; inner diameter, 0.25 mm; film thickness, 0.25 μm ; J&W Scientific, Folsom, CA, USA) with the exception of BDE 209, for which analysis was performed using a DB-1 capillary column (length, 10 m; inner diameter, 0.25 mm; film thickness, 0.25 μm ; J&W Scientific). Hydrogen was used as the carrier gas and nitrogen as the makeup gas. Splitless injections of 2 μl were made by a Varian 8200 Cx autosampler (J&W Scientific) with the injector temperature set isothermally at 260°C. The initial oven temperature was set at 90°C with no hold time, ramped at 20°C/min to 250°C with no hold time, and then ramped at 5°C/min to 300°C and held for 22 min. The ECD detector was held isothermally at 300°C. For BDE 209 analysis, splitless injections of 2 μl were made with the injector temperature set isothermally at 280°C. To minimize degradation of BDE 209, a fast temperature program was employed to reduce the residence time on the column. The initial oven temperature was set at 90°C with no hold time, ramped at 25°C/min to 300°C, and then held for 10 min. The ECD detector was held isothermally at 300°C. All samples were quantified using the BDE standard from Cambridge Isotope Laboratory (Andover, MA, USA).

Gas chromatography/mass spectrometry analysis. All analyses were performed on an Agilent 5973 gas chromatography with a mass-selective detector fitted with a DB-5 capillary column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm ; J&W Scientific). The ultrahigh-purity helium was used as the carrier gas. Splitless injections of 2 μl were made by a 7683 Agilent autosampler (J&W Scientific) with the injector set isothermally at 260°C. The initial oven temperature was set at 90°C with no hold time, ramped at 20°C/min to 250°C with no hold time, and ramped at 5°C/min to 300°C and then held for 22 min. Confirmation of penta- and hexa-BDE unknowns was done in the electron-ionization mode under selected-ion monitoring conditions using two ions in the [M-2Br]⁺-cluster: Penta-BDEs (m/z 404/406), and hexa-BDEs (m/z 484/486). Quantitation was achieved by comparing the integrated response of each unknown to the response of the nearest eluting BDE from the Cambridge Isotope Laboratory external standard.

Statistical analysis

Statistical analysis was performed using STATISTICA for Windows (Statsoft, Tulsa, OK, USA). All data were examined for normal distributions, and Levene's tests were performed to check for homogeneity of variance. Data that met these criteria were analyzed using parametric analysis, and results were considered to be significant at $p < 0.05$ [28]. Before statistical analysis, the percentages of eosinophils, monocytes, basophils, lymphocytes, and neutrophils were transformed using the arcsine transformation of the square root, and the neutrophil to lymphocyte ratio was transformed with the logarithm

Table 1. Concentrations of brominated diphenyl ether (BDE) congeners measured in livers of male mink (*Mustela vison*) exposed to the commercial polybrominated diphenyl ether (PBDE) mixture DE-71^a

BDE congener	Control (n = 10)	1 ppm (n = 10)	5 ppm (n = 8)	10 ppm (n = 10)
17/25	0.002 (0.004)	0.065 (0.040)	0.119 (0.063)	0.109 (0.059)
28/33	0.012 (0.008)	0.786 (0.369)	0.905 (0.458)	0.986 (0.688)
138/66	0.017 (0.015)	3.262 (1.267)	5.044 (2.680)	7.126 (6.221)
47	0.546 (0.442)	86.418 (35.567)	186.459 (104.162)	146.309 (91.384)
66	0.004 (0.007)	0.904 (0.438)	1.494 (1.071)	1.223 (1.048)
85	0.126 (0.140)	12.798 (6.603)	40.363 (21.719)	44.506 (47.472)
99	0.173 (0.176)	45.009 (19.062)	48.515 (27.541)	60.378 (34.975)
100	0.039 (0.031)	8.648 (3.662)	9.256 (5.980)	12.323 (8.310)
140	0	0.595 (0.414)	1.246 (0.991)	1.485 (0.988)
153	0.365 (0.280)	56.124 (27.656)	112.022 (54.886)	120.956 (118.792)
154	0.033 (0.020)	0.963 (0.525)	0.730 (0.541)	1.109 (0.873)
155	0.002 (0.006)	0.252 (0.130)	0.251 (0.275)	0.257 (0.217)
181	0.007 (0.007)	0.164 (0.084)	0.055 (0.113)	0.136 (0.127)
209	1.996 (0.797)	1.606 (0.549)	0.435 (0.251)	0.379 (0.381)
Unknown	0	0.630 (0.630)	1.093 (0.664)	1.180 (0.962)
Unknown	0.027 (0.025)	4.807 (2.101)	8.438 (4.193)	11.486 (11.079)
ΣPBDEs (wet wt)	3.348 (1.62)	223.03 (90.11)	416.43 (208.79)	410.00 (304.52)
ΣPBDEs (lipid corrected)	58.82 (26.85)	5,067.09 (1,077.23)	18,505.33 (8,221.748)	27,909.02 (8,737.41)
% Lipid	5.74	4.53	2.52	1.59

^a Values are presented as the mean (standard deviation). The 5 ppm dose group initially received 100 ppm of DE-71 for the first week, then were switched to 5 ppm of DE-71 for the remainder of the trial because of food avoidance and weight loss concerns at the higher dose. ΣPBDEs = sum of PBDE congener concentrations.

(base 10) [28]. Nonparametric statistical tests were used for data that were not normally distributed.

A single-factor analysis of variance (ANOVA), using Tukey's tests as a post hoc test, was used to determine whether body masses, organ masses, somatic indices, PCV, end points of quantitative histology, and immune parameters differed among dosing groups. If body mass was correlated with immune parameters, it was incorporated as a covariate. A two-factor repeated-measures ANOVA was used to detect the effects of PBDE exposure (dose) and time of sampling (age, the repeated measures component) on changes in body mass, antibody titer, PCV, and white blood cell counts. Post hoc comparisons among treatment groups were done with Tukey's honestly significant difference test for unequal sample sizes [28]. For qualitative histological assessments, chi-square tests were used to test differences among treatments.

Spearman rank correlation coefficients were used to identify specific associations between liver burdens of PBDE congener concentrations in mink and immune and histological end points. All statistical analyses were considered to be significant at $p < 0.05$.

RESULTS

Hepatic PBDE concentrations

The sum PBDE concentrations (ΣPBDEs) in mink livers did not increase with increasing DE-71 exposure when examined on a wet-weight basis. The mean ΣPBDEs was almost identical in mink dosed with 5 ppm of DE-71 compared with those exposed to 10 ppm. These were followed by mink exposed to 1 ppm and then by control mink, with very low concentrations (Table 1). A significant difference was found in the mean percentage lipid content in livers of mink ($p < 0.0001$) (Table 1). Therefore, the ΣPBDEs were examined on a lipid-normalized basis. The lipid-normalized ΣPBDEs for each group corresponded with each group's dietary exposure to DE-71 (Table 1).

Of the 16 BDE congeners that were detected in mink livers, 14 were positively identified, and two were unknown (Table

1). In the control mink, the seven congeners that constituted approximately 99% of the ΣPBDEs were, from highest to lowest, BDEs 209, 47, 153, 99, 85, 100, and 154. The congener BDE 209 contributed 62% to the ΣPBDEs (Fig. 1). In contrast, the dominant congeners in livers of the mink exposed to DE-71 were BDEs 47, 153, 99, 85, 100, an unknown congener, and 138/66. These congeners contributed approximately 98% to the ΣPBDEs. The percentage contribution of BDE 47 to the ΣPBDEs ranged between 38.7 and 43.7% (Fig. 1).

Food consumption and estimated daily intake of DE-71

Mink differed significantly in their average daily food consumption among the treatment groups ($p = 0.004$) and over the course of the experiment ($p < 0.001$). A significant dose × age interactive effect on food consumption also was found ($p < 0.001$). As described in *Materials and Methods*, after the first week of the experiment, mink exposed to 100 ppm of DE-71 consumed significantly less food and demonstrated a substantial decline in body mass (Fig. 2). Consequently, the dietary concentration of DE-71 for these mink was decreased to 5 ppm for the duration of the experiment. This concentration, which was intermediate between the 1 and 10 ppm groups,

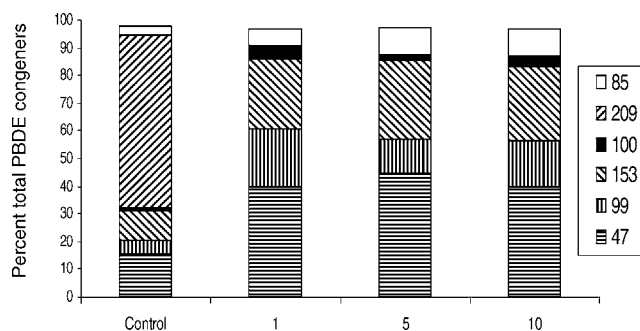


Fig. 1. The percentage congener contribution of the dominant congeners to the sum of polybrominated diphenyl ethers (PBDE) congeners detected in the livers of male mink fed 1, 5, and 10 ppm of DE-71.

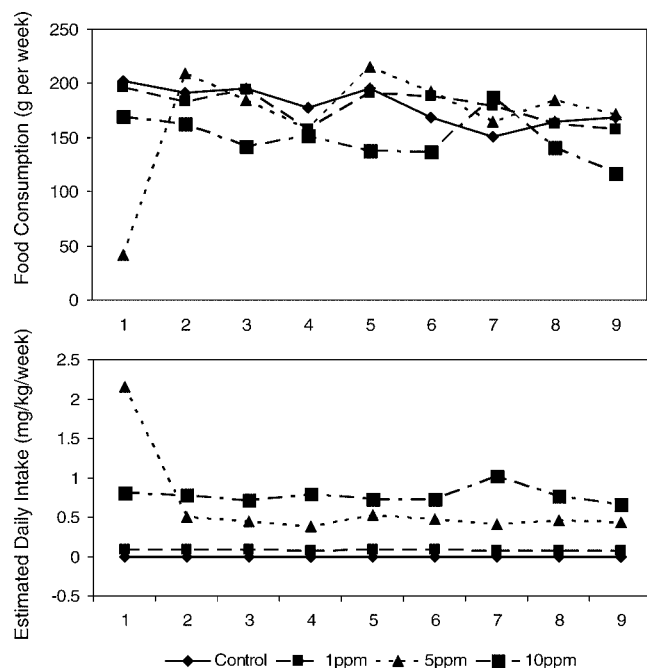


Fig. 2. Mean weekly food consumption and estimated daily intake of polybrominated diphenyl ethers (PBDE) by male ranch mink (*Mustela vison*) exposed to the commercial PBDE mixture DE-71. The 5 ppm dose group initially received 100 ppm of DE-71 for the first week, then were switched to 5 ppm of DE-71 for the remainder of the trial because of food avoidance and weight loss concerns at the higher dose.

was chosen because animals in the 10 ppm group also were experiencing slight declines in food consumption and body mass, and these were not end points we were intending to induce. Food consumption in mink from this group (5 ppm) then increased substantially by week 2, surpassing both the 1 and 10 ppm groups and remaining the highest from week 5 to the end of the experiment. Whereas mink from the control and 1 ppm groups consumed similar quantities of food over the course of the experiment, mink exposed to 10 ppm of DE-71 generally consumed less food relative to other groups.

The estimated daily intake (EDI) of DE-71 for mink from the 1 ppm group remained relatively constant, with a maximum EDI of 0.0909 mg/kg body wt/d in the first week, which declined to 0.06892 mg/kg/d by trial termination (Fig. 2). Although mink exposed initially to 100 ppm of DE-71 consumed just 42 g food/d during the first week, their EDI was 2.16 mg DE-71/kg/d, a 24-fold increase over the mink exposed to 1 ppm and an approximately threefold increase over mink exposed to 10 ppm of DE-71. Their EDIs decreased substantially to between 0.3843 to 0.5298 mg DE-71/kg/d for the duration of the experiment after being placed on the 5 ppm of DE-71 diet. Mink exposed to 10 ppm of DE-71 had an average EDI of 0.777 mg DE-71/kg/d.

Body mass and organ weights

On day 0 of the experiment, body masses were similar between dosing groups. Body masses had decreased significantly with increasing dose of DE-71 by days 25 ($p = 0.012$) and 47 ($p = 0.001$) and at trial termination ($p = < 0.001$) (Table 2). A significant interactive effect of dose \times age on weight gain also was found (dose \times age: $F_{9,102} = 11.43$, $p < 0.0001$) (Table 2). Whereas mink dosed with 0 and 1 ppm of

Table 2. Body and organ masses, somatic indices, ethoxyresorufin-*O*-deethylase (EROD), phytohemagglutinin (PHA) skin test, packed cell volume, and humoral immune responses in ranch mink (*Mustela vison*) exposed to the commercial polybrominated diphenyl ether (PBDE) congener mixture DE-71^a

	Control	1 ppm	5 ppm	10 ppm
Body mass (g)				
Day 0	2,182.80 \pm 99.34	2,178.20 \pm 99.34	2,223.13 \pm 111.06	2,125.30 \pm 99.34
Day 25	2,297.70 \pm 89.89 A	2,287.80 \pm 89.89 A	2,020.37 \pm 100.50 AB	1,930.50 \pm 89.89 B
Day 47	2,382.70 \pm 100.48 A	2,340.90 \pm 100.48 A	1,996.12 \pm 112.34 AB	1,838.90 \pm 100.48 B
Day 59	2,352.45 \pm 104.74 A	2,296.35 \pm 104.74 AB	1,865.25 \pm 117.10 BC	1,705.50 \pm 104.74 C
Spleen mass (g)				
Spleen mass (g)	4.86 \pm 0.29 A	4.93 \pm 0.27 A	4.71 \pm 0.33 A	4.54 \pm 0.26 A
Spleen somatic index				
Spleen somatic index	0.0021 \pm 0.0020 A	0.0022 \pm 0.00013 AB	0.0025 \pm 0.00014 AB	0.0027 \pm 0.00013 B
Adrenal mass (g)				
Adrenal mass (g)	0.127 \pm 0.006 A	0.129 \pm 0.006 A	0.152 \pm 0.006 AB	0.159 \pm 0.006 B
Adrenal somatic index				
Adrenal somatic index	5.7 E-5 \pm 6.0 E-6 A	5.7 E-5 \pm 5.0 E-6 A	8.3 E-5 \pm 5.0 E-6 B	9.5 E-5 \pm 5.0 E-6 B
Liver mass (g)				
Liver mass (g)	78.47 \pm 4.75 A	93.51 \pm 4.75 AB	101.44 \pm 5.31 B	88.6 \pm 4.75 AB
Liver somatic index				
Liver somatic index	0.034 \pm 0.002 A	0.041 \pm 0.002 B	0.054 \pm 0.002 C	0.052 \pm 0.002 C
PHA index				
PHA index	0.237 \pm 0.046 A	0.322 \pm 0.043 A	0.366 \pm 0.051 A	0.249 \pm 0.046 A
Antibody production (% increase over preimmunization with DNP-KLH^b)				
Day 25	38.70 \pm 12.67 A	49.12 \pm 15.13 A	111.12 \pm 14.14 B	70.70 \pm 12.66 AB
Day 47	131.90 \pm 34.98 A	116.70 \pm 34.98 A	325.50 \pm 39.11 B	181.20 \pm 34.98 AB
Day 59	158.40 \pm 42.94 A	99.60 \pm 42.94 A	420.00 \pm 48.00 B	228.60 \pm 42.94 A
EROD (pg/min/mg protein)				
EROD (pg/min/mg protein)	0.37 \pm 1.61 A	7.35 \pm 1.34 B	8.21 \pm 1.51 B	5.37 \pm 1.42 AB
Spleen histology				
Germinal centers (<i>n</i>)	12.8 \pm 0.88 A	15.9 \pm 0.88 AB	16.5 \pm 0.99 AB	17.4 \pm 0.88 B
Hyperplasia (%)	0 A	0 A	25 B	40 B
Hematocrit (%)				
Day 0	49.92 \pm 1.10 A	46.97 \pm 1.00 A	47.81 \pm 1.14 A	46.24 \pm 1.02 A
Day 59	49.79 \pm 0.99 A	49.07 \pm 1.05 A	43.54 \pm 1.05 B	43.73 \pm 0.99 B

^a Values are presented as the mean (standard error). Means sharing a common uppercase letter do not differ at $p < 0.05$ according to Tukey's honestly significant difference test.

^b DNP-KLH = dinitrophenol conjugated to a carrier protein, keyhole limpet hemocyanin.

DE-71 demonstrated slight increases in mass over time, the mink from the groups dosed with 5 and 10 ppm had significant reductions in growth rate.

Body organ somatic indices differed between exposure groups (Table 2). No significant difference was observed in spleen mass; however, mink exposed to 10 ppm of DE-71 had a significantly greater mean relative spleen mass than control mink ($p = 0.014$). Across all treatment groups, spleen somatic indices correlated positively with individual Σ PBDEs in liver ($p = 0.012$, $r = 0.40$). Significant differences were found in the absolute and relative adrenal gland mass with increasing DE-71 exposure. Mink receiving the highest dose of PBDE had significantly greater absolute adrenal masses ($p < 0.001$) and adrenal somatic indices ($p < 0.001$) relative to controls. The absolute adrenal gland mass ($p = 0.001$, $r = 0.50$) and the adrenal somatic index ($p < 0.001$, $r = 0.72$) correlated positively with individual Σ PBDEs in liver. The ratio of liver mass to body mass was significantly greater in mink exposed to 5 and 10 ppm of DE-71 relative to controls ($p < 0.0001$) and was positively associated with hepatic Σ PBDEs ($p < 0.001$, $r = 0.83$).

Cell-mediated and humoral immune responses

The skin response to PHA challenge did not differ significantly among groups, nor were any significant correlations with the Σ PBDEs for liver or any individual congeners found (Table 2). The total serum antibody production in response to sensitization with DNP-KLH was significantly different among exposure groups for the primary antibody response on day 25 ($p = 0.004$) and for the secondary antibody responses on days 47 ($p = 0.001$) and 59 ($p < 0.001$). Relative to control mink, total antibody titers in mink exposed to 5 ppm of DE-71 were 2.5- to 2.9-fold higher for both primary and secondary responses. A significant dose \times age interaction effect on antibody production also was found, as measured by percentage increase over preimmunization (i.e., nonspecific background) antibody titers (dose \times age: $F_{6,62} = 4.15$, $p = 0.001$) (Table 2). Mink from all exposure groups increased their antibody production with increasing age, except for the mink exposed to 1 ppm of DE-71, which had a rate of increase lower than those of all other groups between days 59 and 75. The greatest percentage increase in antibody production over preimmunization titers was observed in the 5 ppm group, followed by mink dosed with 10 ppm.

Histology of the spleen

Exposure of mink to DE-71 changed the appearance of spleen morphology with respect to development of the T lymphocyte-dependent and B lymphocyte-dependent white pulp (Table 2). Sections from control animals and mink exposed to 1 ppm of DE-71 had a moderate degree of periarteriolar lymphatic sheath development and occasional germinal centers scattered throughout the spleen, but mink exposed to 10 ppm of DE-71 had significantly more germinal centers ($p = 0.006$). Splens from 25 and 40% of mink exposed to 5 and 10 ppm, respectively, were characterized as having B-cell hyperplasia. The majority of the spleen section in these mink consisted of well-populated periarteriolar lymphatic sheath and many well-formed germinal centers. The number of germinal centers was positively associated with increasing Σ PBDEs in liver (Spearman correlation: $p < 0.001$; $r = 0.53$).

Hepatic EROD enzyme activity

Exposure of mink to DE-71 resulted in significant induction of hepatic EROD activity (Kruskal-Wallis ANOVA: $p = 0.006$) (Table 2). Induction of EROD enzyme activity was highest in mink exposed to 5 ppm of DE-71, with a 22-fold increase over control mink. Even at the lowest dose, EROD was induced approximately 20-fold above control mink. Induction of EROD activity in mink exposed to DE-71 was lowest in the 10 ppm group, and the wide variation in activity accounted for the nonsignificant difference relative to controls. Across all treatments, a significant and positive association existed between EROD activity and hepatic Σ PBDEs on both a wet-weight (Spearman correlation: $p = 0.002$, $r = 0.51$) and a percentage lipid-corrected (Spearman correlation: $p = 0.033$, $r = 0.37$) basis as well as with the liver somatic index ($p = 0.033$, $r = 0.38$).

Hematology

No difference was found in the PCV at trial initiation; however, at trial termination, mink from the two highest exposure groups had a significantly ($p = 0.001$) lower ($\sim 12\%$) hematocrit than control mink and the 1 ppm group (Table 2). A significant dose \times age effect on the PCV (dose \times age: $F_{3,30} = 3.62$, $p = 0.024$). Whereas the control and 1 ppm exposure groups had constant or slightly increased PCV over time, mink from the two highest dose groups had up to a 10% reduction in PCV, which on day 75 was negatively correlated with Σ PBDEs in liver (Spearman correlation: $p < 0.001$, $r = -0.65$). Apart from congeners BDEs 181 and 209, negative correlations also were found with all individual PBDE congeners ($p < 0.004$, $r = -0.48$ to -0.68).

Most of the ranges in the differential white blood cell counts fell within that expected for male mink of this age [29], and no pretreatment differences were found among treatment groups. Percentages of neutrophils increased significantly, and percentages of lymphocytes decreased significantly, at the postdose measurements for animals in the 5 and 10 ppm treatment groups (Fig. 3). The percentage of neutrophils showed a strong age effect in that a consistent, increasing pattern from the predose to the postdose measurement was found for all treatment groups (Fig. 3). Post hoc tests showed a significant increase in the neutrophil to lymphocyte ratios at the postdose measurement for the 5 and 10 ppm treatment groups, which was not seen in the control and 1 ppm treatment groups (Fig. 3). Percentage of monocytes showed a consistent but nonsignificant pattern of decline from the predose to the postdose measurement for all treatment groups; however, this consistency was not seen for eosinophils or basophils. For eosinophils and basophils, the control and 1 ppm treatment groups showed nonsignificant increases between the predose and postdose measurements; however, for the two high treatment groups, the percentages decreased nonsignificantly or remained the same between the two time periods.

DISCUSSION

The Σ PBDEs in livers of mink corresponded with increasing dietary DE-71 exposure when examined on a percentage lipid-content basis. Of particular interest is the inconsistency in the dominant PBDE congener profile in livers of controls and of mink exposed to DE-71. Although the greatest contribution to Σ PBDEs in livers of control mink was made by BDE 209, mink exposed to DE-71 had livers containing mostly the

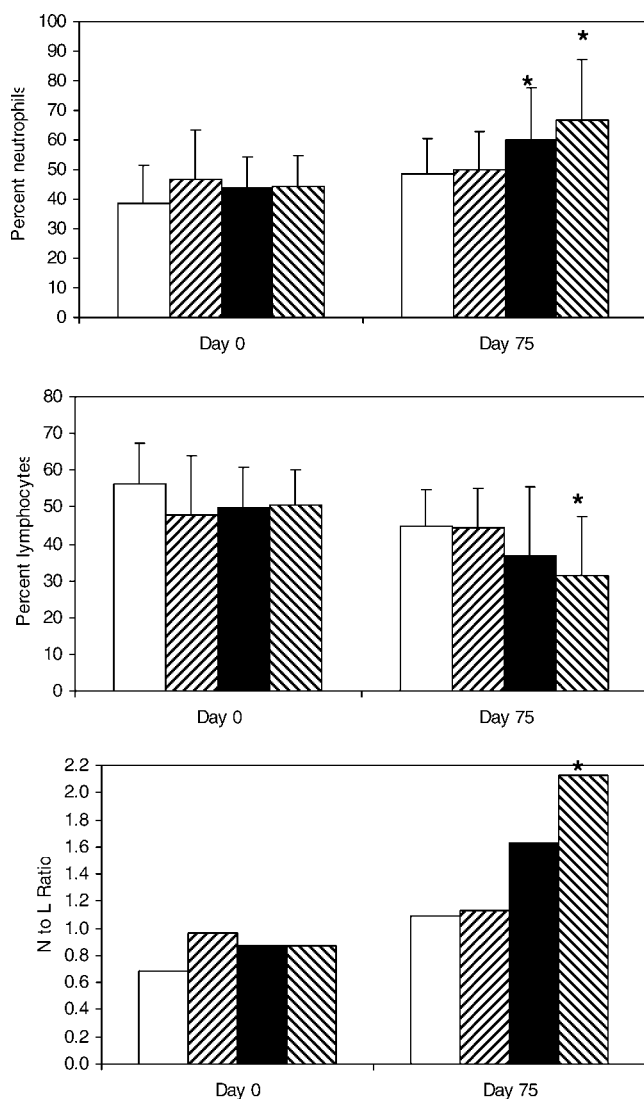


Fig. 3. Differential white blood cell counts (%; mean \pm standard deviation) in mink (*Mustela vison*) exposed to the commercial polybrominated diphenyl ethers (PBDE) mixture DE-71. A dose group mean marked by an asterisk is significantly different from other means within that treatment day. Open bars = control dose group; right hatch = 1 ppm dose group; solid bar = 5 ppm dose group; left hatch = 10 ppm dose group.

lower-brominated congeners (BDEs 47, 99, 153, and 85). Our results corroborate the earlier finding of Bursian et al. [30], in which BDE 209 also occurred at the same concentrations in the livers of four treatment groups of mink fed increasing levels of Lake Michigan carp. In that study, as the proportion of carp increased, the concentrations of BDE 47 increased substantially, whereas those of BDE 209 remained constant, suggesting the contamination was in the commercial diet mix, which was the same as that used in the present study, rather than in the environmental samples of fish. This finding also contradicts the theory that the molecular size of BDE 209 makes it too large to be bioavailable and, hence, not easily assimilated [31]. The presence of very-low-brominated congeners (i.e., BDEs 17/25 and 28/33) in the livers of mink when having been exposed to a mixture of predominantly pentabrominated congeners suggests that biotransformation, presumably debromination, resulted in the loss of some PBDE congeners in the diet and in the formation of other PBDE

congeners. Previous studies have demonstrated that biotransformation of PBDEs occurs in other vertebrates, such as lake trout [19] and carp (*Cyprinus carpio*) [32].

The intradermal swelling of the toe web in mink indicated that T lymphocytes were sensitive to the mitogenic stimulation of PHA. However, mink exposed to DE-71 did not differ significantly in their response to PHA relative to control mink, and the PHA-induced response was not associated with individual liver PBDE congener concentrations. The lack of effect of DE-71 exposure on the early T-cell response is surprising given the sensitivity of mink to organohalogenated compounds [15], the high doses used in the present experiment, and the fact that this test has been found to be a sensitive method in detecting immunomodulatory effects in other contaminant-exposed wildlife [33,34]. The PHA test depends on an early, clonal proliferative response of T cells that have not yet reached functional maturity. These young T cells may be less sensitive to contaminant exposure than more mature, differentiated T cells that are involved in more complex immunological responses, such as the delayed-type hypersensitivity response [35]. Unfortunately, little published information is available regarding comparisons of cell-mediated immune data in mink exposed to PBDEs. Thymotoxic effects (decreased weight of thymus) have occurred in mice exposed to 1,000 mg DE-71/kg body weight [18], a dose that far exceeded the exposure in the present experiment. Studies using captive mink and seals have revealed significant cell-mediated immune suppression following consumption of fish contaminated with organohalogenated compounds having molecular structures similar to those of PBDEs. For instance, captive mink fed fish from rivers receiving bleached kraft mill effluent demonstrated a reduced delayed-type hypersensitivity response relative to control mink [35]. Harbor seals (*Phoca vitulina*) given a diet of fish containing high levels of PCBs for two years demonstrated suppression in the delayed-type hypersensitivity relative to control animals [36]. Unlike the present study, in which the PHA skin test was used to determine a primary response on first contact with PHA, delayed-type hypersensitivity integrates antigen processing and presentation as well as the T-helper-cell response, which coordinates a secondary response in the skin reaction. This is a more integrative test of T cell-mediated immunity, and its utility as an immune end point merits consideration in future exposure studies with mink.

We demonstrated that immunization and subsequent challenge with DNP-KLH led to significant differences in the primary antibody responses in mink exposed to DE-71. Mink given diets with 5 ppm of DE-71 generally had the greatest increase in anti-DNP-KLH antibodies over preimmunization titers, a pattern that was sustained following each sensitization during the course of the experiment. The exaggerated humoral immune response produced by mink from the 5 ppm exposure group may be a reflection of the change in dietary exposure to DE-71. Recall that mink from this group initially were exposed to 100 ppm of DE-71 in their diet. This was decreased to 5 ppm after the first week of the experiment, because the animals became anorexic and clinically sick. Despite the short duration of exposure to 100 ppm, the average cumulative EDI of DE-71 was greatest in these mink up until the seventh week, after which the mink exposed to 10 ppm of DE-71 had the highest EDI.

Our findings of a stimulated antibody response are inconsistent with earlier rodent studies in which humoral immunity tends to be suppressed by PBDEs. For example, exposure of

mice to the very high dosage of 1,000 mg DE-71/kg body weight for 14 d reduced antibody production to sheep red blood cells [18], and 36 mg tetrabromodiphenyl ether/kg body weight reduced the number of splenocytes in mice following 14 d of exposure [17]. In the present study, at much lower, chronic levels of exposure, we characterized the overzealous antibody production and spleen enlargement as being PBDE-induced immunomodulation. Similar findings of enhanced humoral immune responses following exposure to other organohalogen compounds have been reported. For instance, serum antibodies to tetanus toxoid increased, and the spleen and lymph nodes were enlarged in weanling rats fed 0, 500, 1,000, and 2,000 mg hexachlorobenzene/kg body weight for three weeks [37]. Total serum immunoglobulin M levels were significantly increased in rats exposed to 450 and 900 mg hexachlorobenzene/kg body weight for 30 d [38]. Mink exposed to pulp mill effluent also had enhanced antibody responses compared with control animals [39]. In avian studies, adult female American kestrels (*Falco sparverius*) exposed daily to an Aroclor mixture at 7 mg/kg body weight for 120 d had a higher KLH-specific antibody response in comparison to control birds [33], and Caspian terns in PCB-contaminated Great Lake sites showed an increase in antibody response [5].

As demonstrated by validation studies on mink and rodents, KLH elicits a strong T lymphocyte-dependent humoral immune response [40,41]. This test evaluates the integrative response of macrophages, T helper cells, cytokines, and B lymphocytes. The finding that the primary and secondary antibody responses of mink from the two highest exposure groups exceeded that of control mink suggests an alteration, possibly an up-regulation, in one or more aspects of the antibody response. There also was an increase in the spleen somatic index and greater germinal center development in spleens of mink exposed to DE-71. No mechanistic literature is available to elucidate what provoked these changes. Perhaps exposure to DE-71 modulated mink immune function by enhancing the T-helper-cell function. Cytokine release from activated T helper cells directs antigen-stimulated B lymphocytes in the spleen to differentiate into antibody-producing plasma cells [42]. It is possible that the combination of KLH and dietary exposure to DE-71 induced this response, because rats treated with KLH either alone or in combination with the calcineurin inhibitor, FK506, also exhibited significant germinal center development [41]. Although spleen enlargement has been related to an expansion of the marginal zones and B lymphocyte-containing follicles in rats treated with hexachlorobenzene [37], we did not find an association between the number of germinal centers and Σ PBDEs in liver.

Measurement of hepatic microsomal EROD activity is a widely used biomarker of exposure to environmental cytochrome P450 inducers, and its induction is thought to be mediated through the binding to a cytosolic aryl hydrocarbon receptor. Conflicting reports exist regarding the ability of PBDE congeners to induce EROD or other phase I enzyme activities mediated through the aryl hydrocarbon receptor. It generally is accepted that PBDEs are several orders of magnitude less potent than PCBs, dioxins, or furans and that impurities in technical mixtures of PBDEs may have contributed to induction in some previous studies (for review, see Tomy et al. [21]). In the present study, we had a sample of the DE-71 technical material analyzed for contamination with brominated dioxins and furans by a commercial analytical laboratory (AXYS Analytical Services, Victoria, BC, Canada). All

dioxin and furan isomers were nondetectable or nonquantifiable (detection limits of most congeners, <0.03 ppb), indicating that the presence of dioxin-like impurities in the technical mixture probably is not a confounding factor in the induction of EROD activity or any other effects. We found positive associations between Σ PBDEs and EROD activity, and EROD induction correlated with the liver somatic index.

Indirect factors associated with DE-71 exposure also may have contributed to the stimulated immune response observed in treated mink. On the basis of the significantly enlarged relative adrenal weights in mink with increasing Σ PBDEs in liver, we speculate that stress-related neuroendocrine and immune responses may be interacting to elicit the augmentation in antibody production. This theory is consistent with reports of elevated corticosterone levels in mice with increasing dosage of DE-71 [18]. It is widely accepted that glucocorticoids can alter immune responsiveness by inhibiting interleukin-2 and interferon production by a subset of T helper cells (T_H1) responsible for cell-mediated immunity [43]. Glucocorticoids, however, are not necessarily antagonistic to immunity: Basal blood levels are essential to antibody formation, and specific doses can improve resistance to *Escherichia coli* [44]. Glucocorticoids may act synergistically with other soluble factors (interleukin-4) on a separate subset of T cells, which may lead to an amplification of B lymphocyte-mediated responses [45]. Alternatively, it also is possible that the increase in adrenal somatic index might be unrelated to PBDE exposure, because antigens also are known to stimulate glucocorticoid secretion in vertebrates, such as birds [44].

The mean hematocrit decreased significantly in mink from the highest dose group over the course of the trial. A decline in hematocrit during the weeks following birth is typical of mammals and generally is explained by plasma volume expansion with growth [46]. Clearly however, this is not the case in the present experiment, because mink from the highest dose groups experienced a significant weight loss from trial initiation to completion. The significant decline in mean hematocrit with increasing Σ PBDEs in liver indicates a contaminant-induced effect. Previous studies have reported that organochlorine contaminants can decrease hematocrit. Toxic equivalents and metabolites of DDT concentrations in adult herring gulls from the Great Lakes also were negatively correlated with hematocrit [34]. The decline in PCV could be a sign of anemia in mink. Other conditions that can result in a low hematocrit include vitamin or mineral deficiencies, renal disease, recent blood loss, and cirrhosis of the liver. General toxicity in the mink treated with 5 and 10 ppm of DE-71, as indicated by loss in body weight, was similar to the wasting syndrome described for mink intoxicated with PCBs.

The decrease in eosinophils observed in the postdose measurements for the two highest treatment groups may have been associated with the effects of PBDE exposure. Similar results have been reported in animals such as goats (*Capra hircus*), mink, and Japanese quail (*Coturnix coturnix japonica*) exposed to organochlorine compounds and corticosteroids [14,47,48]. However, the decrease in eosinophils is most likely a normal expression of the so-called stress leukogram, in which both eosinophils and lymphocytes are decreased in stressed animals but neutrophils are increased [49].

Lymphocyte ratios for the two highest treatment groups had significant increases in the neutrophil. Other studies have reported similar changes in the counts and/or relative percentages of these cell types for mink exposed to crude oil, PCBs, and

2,3,7,8-tetrachlorodibenzo-*p*-dioxin as well as Japanese quail exposed to dexamethasone [14,47]. In harbor seals, increased leukocyte counts and immature neutrophils were positively associated with PBDE concentrations [50]. Although in the present study the relative decrease in lymphocytes cannot be directly attributed to dosing with PBDEs, the fact that all treatment groups were subjected to the same amount of housing and handling stress indicates that PBDE dosing at the two highest concentrations was an additional source of stress to the mink.

CONCLUSION

The present findings of immunomodulation in mink are important given the complete lack of PBDE-induced immune toxicity data for animals other than laboratory rodents. Effects are evident at the two highest exposure concentrations of 10 and 100 ppm switched to 5 ppm in the diet, but not at the lowest dietary concentration of 1 ppm. Currently, the higher dietary concentrations are above those seen in wild fish in North America; however, recent rates of increasing PBDE concentrations in biota suggest that these levels may be commonplace in the near future. It would be extremely useful to obtain information regarding levels of contamination in wild mink, against which concentrations measured in the mink in the present study could be compared, allowing more realistic speculation as to the potential for effects in wild populations. The immune enhancement reported in the present study could be considered costly in terms of the reallocation of energy and nutrients for body maintenance and growth to immune function, particularly in wild animals, and may result in inappropriate immunological responses. Immune stimulation can be as harmful as suppression in immune system function, because autoimmunity and hypersensitivity are possible outcomes of chronic stimulation of immune system activity. The present study was conducted on juvenile animals for a relatively short period of time; future work could focus on longer-term exposure to more sensitive stages of development, including in utero exposure to fetuses and exposure of young kits during lactation.

The present findings also have direct relevance to free-ranging mink in the Great Lakes basin ecosystem and emphasize the need for monitoring and research. Mink are top predators of the aquatic food web and bioaccumulate significant body burdens of persistent organic pollutants [51]. The present results provide evidence for the vulnerability of this species to the immune effects of bioaccumulative PBDE congeners. If environmental levels of PBDEs continue their exponential increase in the Great Lakes basin ecosystem, mink inhabiting PBDE-contaminated bodies of water may experience immune dysfunction, lowering resistance to environmental pathogens and disease.

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