2009

Toxicity Of Polybrominated Diphenyl Ethers (De-71) In Chicken (*Gallus Gallus*), Mallard (*Anas Platyrhynchos*), And American Kestrel (*Falco Sparverius*) Embryos And Hatchlings

Moira A. McKernan
University of Maryland

Barnett A. Rattner
U.S. Geological Survey, brattner@usgs.gov

Robert C. Hales
Virginia Institute of Marine Science

Mary Ann Ottinger
University of Maryland

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 28, No. 5, pp. 1007–1017, 2009

Printed in the USA
0730-7268/09 $12.00 + .00

TOXICITY OF POLYBROMINATED DIPHENYL ETHERS (DE-71) IN CHICKEN (*GALLUS GALLUS*), MALLARD (*ANAS PLATYRHYNCHOS*), AND AMERICAN KESTREL (*FALCO SPARVERIUS*) EMBRYOS AND HATCHLINGS

MOIRA A. MCKERNAN,†‡ BARNETT A. RATTNER,*‡ ROBERT C. HALE,§ and MARY ANN OTTINGER†
†Marine, Estuarine, and Environmental Sciences and Department of Animal and Avian Sciences, University of Maryland, College Park, Maryland 20742, USA
‡U.S. Geological Survey, Patuxent Wildlife Research Center, BARC-East, Building 308, 10300 Baltimore Avenue, Beltsville, Maryland 20705
§Department of Environmental and Aquatic Animal Health, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062, USA

(*Received* 10 *July* 2008; *Accepted* 10 *November* 2008)

Abstract—Embryonic survival, pipping and hatching success, and sublethal biochemical, endocrine, and histological endpoints were examined in hatchling chickens (*Gallus gallus*), mallards (*Anas platyrhynchos*), and American kestrels (*Falco sparverius*) following air cell administration of a pentabrominated diphenyl ether (penta-BDE; DE-71) mixture (0.01–20 μg/g egg) or polychlorinated biphenyl (PCB) congener 126 (3,3′,4,4′,5-pentachlorobiphenyl; 0.002 μg/g egg). The penta-BDE decreased pipping and hatching success at concentrations of 10 and 20 μg/g egg in kestrels but had no effect on survival endpoints in chickens or mallards. Sublethal effects in hatchling chickens included ethoxyresorufin-*O*-dealkylase (EROD) induction and histological changes in the bursa, but these responses were not observed in other species. Polychlorinated biphenyl congener 126 (positive control) reduced survival endpoints in chicken and kestrel embryos and caused sublethal effects (EROD induction, reduced bursal mass and follicle size) in chickens. Mallards were clearly less sensitive than the other species to administered penta-BDE and PCB 126. In a second experiment, the absorption of penta-BDE (11.1 μg/g egg, air cell administered during early development) into the contents of chicken and kestrel eggs was determined at various intervals (24 h postinjection, midincubation, and pipping). By pipping, 29% of the penta-BDE administered dose was present in the egg contents in chickens, and 18% of the administered dose was present in kestrel egg contents. Based on uptake in kestrels, the lowest-observed-effect level on pipping and hatching success may be as low as 1.8 μg total penta-BDE/g egg, which approaches concentrations detected in eggs of free-ranging birds. Because some penta-BDE congeners are still increasing in the environment, the toxic effects observed in the present study are cause for concern in wildlife.

Keywords—Egg injection Lowest-observed-effect level Polybrominated diphenyl ether Species sensitivity Polychlorinated biphenyl congener 126

INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) have been commonly used as flame retardants in polymers, textiles, electronics, and other materials. These compounds bioaccumulate in aquatic and terrestrial organisms and biomagnify in food chains [1]. Monitoring studies indicate that PBDE concentrations in the environment have increased over the past 25 years. A retrospective study of archived herring gull (*Larus argentatus*) eggs from the Great Lakes, North America, demonstrated that individual congeners (BDEs 47, 99, and 100) found in the commercial penta-BDE mixture increased by one-and-a-half orders of magnitude between 1981 to 2000 (doubling time, 2.6–3.1 years) [2], and concentrations of these congeners in gull eggs have remained elevated [3]. On a wet-weight basis, concentrations of total PBDEs in avian eggs range up to 1.40 μg/g in herring gulls from the Great Lakes [2]; 0.928 μg/g in ospreys (*Pandion haliaetus*) from Chesapeake and Delaware bays, USA [4,5]; 4.24 μg/g in peregrine falcons (*Falco peregrinus*) from California, USA (K. Hooper, California Department of Environmental Protection Agency, Berkeley, CA, USA, personal communication); and 6.60 μg/g in peregrine falcons from the northeastern United States [6]. Interpretation of the significance of these concentrations in eggs is not possible, because adverse effect thresholds have yet to be adequately established in birds.

To our knowledge, only two studies in birds have examined developmental and reproductive effects of environmentally relevant concentrations of PBDEs [7–10]. In the initial study by Fernie et al. [7–9], American kestrel (*Falco sparverius*) eggs were injected with 18.7 μg of total PBDEs (BDEs 47, 99, 100, and 153) on day 19 of incubation, and then nestlings were gavaged daily with the same PBDE mixture at 15.6 ng/g body weight through day 29 posthatch. Using this combined egg injection/dietary exposure regimen, some evidence was found indicating increased growth (i.e., body wt, tarsometatarsus and feather length) [9] and structural changes in immune organs (i.e., fewer germinal centers in spleen, reduced apoptosis in bursa, and increased macrophages in thymus) [7]. As carcass concentrations of BDEs 47 and 183 increased, some alterations in immune function (i.e., greater phytohemagglutinin skin response and reduced antibody-mediated response) were detected [7]. The PBDE mixture also evoked oxidative stress (i.e., marginal increases in the ratio of oxidized to reduced glutathione, oxidized glutathione, and lipid peroxidation) in

* To whom correspondence may be addressed (brattner@usgs.gov).
Published on the Web 12/1/2008.
Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

kestrel nestlings. Additionally, in 29-d-old nestlings, plasma *thyroxine* (T_4), plasma retinol, and hepatic retinol were inversely related to carcass concentrations of BDEs 47 and 99 [8]. In a second study by Fernie et al. [10], reproductively active kestrels were fed concentrations of a commercial penta-BDE formulation (0.3 μg DE-71/g diet or 1.6 μg DE-71/g diet), and changes in reproductive behavior were noted (e.g., fewer bonding behaviors, less copulation, and less time spent in the nest box). Studies of PBDE toxicity in laboratory mice and rats, often using relatively high dosage levels, have demonstrated changes in behavior and memory, impaired immune function, decreased circulating concentrations of T_4, and induction of cytochrome P450–associated monooxygenases [11–15].

Considering the ubiquitous nature of PBDEs and their increasing concentrations in the environment, more complete ecotoxicological data are needed regarding these compounds. General toxicity studies should be conducted with species that may be at high risk of exposure. Developmental and reproductive effects are of particular concern because of their linkage to higher-order effects. More subtle responses also merit consideration as they may serve as early, sublethal warning signals of potential higher-order effects.

The present investigation examined the effects of penta-BDE exposure in avian embryos (domestic chicken, *Gallus gallus*; mallard, *Anas platyrhynchos*; and American kestrel) following air cell administration of a commercial PBDE mixture. Biological effects (e.g., embryonic survival and hatching success, as well as endpoints reflecting development and growth, histopathology, cytochrome P450 induction, and glandular T_4 content) are described in three well-characterized avian model species from several different feeding guilds (granivorous, omnivorous, and carnivorous). The null hypothesis being tested is that PBDE exposure does not affect survival or biochemical, endocrine, and histological endpoints in developing avian embryos. These data, in conjunction with findings on absorption of air cell–administered penta-BDE into chicken and kestrel egg contents (albumen, yolk, and embryo), will be useful in establishing effect thresholds and may be of value in ecological risk assessments of these flame retardants.

MATERIALS AND METHODS

Eggs and incubation

All animal procedures were approved by the Institutional Animal Care and Use Committees of the Patuxent Wildlife Research Center and the University of Maryland, College Park (MD, USA). Fertile white leghorn chicken eggs were obtained from CBT Farms, and mallard eggs were obtained from Whistling Wings. American kestrel eggs were collected fresh from the colony at the Patuxent Wildlife Research Center (Laurel, MD, USA). On arrival, all eggs were washed in a 40°C, 1% Betadine® solution (Purdue), then weighed and labeled with a number-two graphite pencil. Eggs were then stored in a cooler at 13°C for up to 3 d and were allowed to equilibrate to room temperature before placement into incubators. Eggs were artificially incubated (Kuhl Incubator Company) in trays that were adapted to turn horizontally oriented eggs 180° each hour. Incubators were set at the recommended incubation temperature: 37.6°C for chickens (21-d incubation), and 37.5°C for mallards and kestrels (27-d incubation). The relative humidity within the incubator was initially set at approximately 40% and was adjusted so that mean egg weight loss by the end of incubation was 14 to 16%. Eggs were incubated horizontally rather than vertically to mimic natural incubation more closely and, presumably, increase survival and hatching success of semidomesticated (mallard) and wild species (kestrel). Eggs were weighed at 3- to 4-d intervals during the course of incubation to determine weight loss. Eggs were candled at the time of weighing to confirm viability, and any unfertilized or dead eggs were removed.

Test solutions and administration

Corn oil (Sigma-Aldrich) was used as the vehicle, because many studies have demonstrated that low volumes cause little mortality in avian embryos during early development [16–18]. A penta-BDE mixture (DE-71; LGC Promochem) was chosen for the present study, because it contains congeners that are commonly detected in North American bird eggs [2,4,5].

Injection solutions were prepared by dissolving neat penta-BDE or polychlorinated biphenyl (PCB) congener 126 (3,3′,4,4′,5-pentachlorobiphenyl congener; AccuStandard) in acetone before mixing with corn oil. Solutions were stirred for 3 h. Each dosing solution, including the corn oil control, contained 1% acetone by volume.

Before injection, the blunt end of each egg was cleaned with an alcohol swab. A hole was drilled (Dremel) into the blunt end of the egg. A constant volume (0.5 μl/g egg) of vehicle, penta-BDE, or PCB 126 was injected into the air cell with an Eppendorf repeat pipettor [17,18]. The hole was then sealed with ethylene vinyl acetate adhesive using a hot glue gun. Injected and uninjected eggs were kept in a vertical position (blunt end up) for 30 min postinjection outside the incubator to allow the oil to spread over the air cell membrane. Eggs were then placed in trays in a horizontal position and returned to the incubator.

Experiment 1: Biological effects of penta-BDE

The penta-BDE doses for chickens and mallards (0.01, 0.1, 1, 10, and 20 μg/g egg) were arranged around environmentally relevant concentrations detected in herring gull, osprey, and peregrine falcon eggs. Because of the limited availability of eggs from the kestrel colony, doses for kestrels ranged from 0.1 to 20 μg/g egg. Nominal concentrations of penta-BDE dosing solutions were verified analytically by gas chromatography/mass spectrometry (GC/MS; Varian 3400 and Varian 4D ion trap). Corn oil test solutions were diluted in hexane. Congener peak areas were compared to that of an internal standard (*p*-terphenyl). Congener response factors were determined using authentic PBDE standards (AccuStandard). Detection was in the electron-ionization mode. The GC was equipped with a DB-5 column (length, 60 m; inner diameter, 0.32 mm; film thickness, 0.25 μm; J&W Scientific), and the carrier gas was He. Injections were made in the splitless mode. Identification was achieved by MS in the full-scan, electron-ionization mode. Quantification was performed by comparison of the sum of the areas of the three major ions of each PBDE congener (BDEs 17, 28, 47, 49, 66, 85, 99, 100, 153, and 154) in DE-71 versus that of the internal standard. For the 1 μg/g egg dosage, penta-BDE was analytically verified to be from 134 to 152% of the nominal concentration, and for the 10 μg/g egg dosage, penta-BDE was analytically verified to be from 96 to 104% of the nominal concentration.

The well-studied and highly toxic PCB congener 126 was used as a positive control. The PCB 126 dose chosen in the present study (0.002 μg/g egg) may seem high compared to that in other studies; however, the toxicity of air cell–admin-

istered PCB 126 on day 4 of incubation is much lower in horizontally incubated eggs [18] compared to that in vertically incubated eggs (complete failure to hatch at 0.0005 μg/g egg) [19,20]. Concentrations of PCB 126 in the dosing solution were verified as described above. Analytically verified concentrations of PCB 126 were from 45 to 51% of the nominal concentration.

Chicken eggs were incubated for 4 d, and mallard and kestrel eggs were incubated for 5 d (i.e., developmental equivalent of a 4-d-old chicken embryo), at which point they were candled to confirm fertility. Any infertile, nonviable, or cracked eggs were discarded. Eggs were then randomly assigned to uninjected control, vehicle control, PCB 126, or penta-BDE groups (chicken, $n = 22$–42 eggs/treatment; mallard, $n = 26$–27 eggs/treatment; kestrel, $n = 18$–20 eggs/treatment), and vehicle or test compounds were administered on this day.

Monitoring survival and sample collection. Embryo viability during incubation was monitored at 3- to 4-d intervals by candling or with a viability detection instrument (Buddy; Vetronic). Embryos that died during development or failed to pip were removed from the eggshell and evaluated for stage of development and presence of abnormalities. Survival through 90% of the incubation period, incidence of pipping, and hatching success were determined.

Day-old hatchlings were examined for evidence of edema and teratogenicity (e.g., eye, foot, and bill deformities). Each hatchling was weighed and then killed via decapitation. Immediately, the liver (minus the gallbladder) and the yolk sac were removed and weighed. A small piece of the liver was fixed in formalin for histopathogical examination, and the remaining tissue was placed in a cryovial, snap-frozen, and stored at −80°C for assay of hepatic microsomal P450–associated monooxygenase activity. Sex was determined by examination of the gonads. The weight of paired thyroid glands in chickens and mallards and of the left thyroid in kestrels was measured, after which they were frozen for subsequent hormone analysis. The weights of the bursa of Fabricius, spleen, and thymus also were determined.

Skeletal preparations and histopathology. After euthanasia and sample collection, the remaining carcass was labeled and stored in 70% ethanol. Carcasses were cleared, feathers removed, and skeletons stained using the method described by Karnofsky [21]. Crown-rump, humerus, radius-ulna, femur, tibiotarsus, and metatarsus lengths were measured to the nearest millimeter.

Formalin-fixed livers, bursae of Fabricius, and other tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxlyin and eosin (American HistoLabs). Liver and bursa (two step sections) were examined by light microscopy for 10 or more individuals for control and dosage groups (except groups with poor hatching success). Hepatocyte density (number of hepatocytes per 10 μm of length at three locations), incidence of enlarged hepatocytes (narrowing of sinusoids), vacuolation, and other lesions were noted.

Because of qualitative changes in the bursae of chicken hatchlings (but not those of mallards or kestrels), morphometric measurements (two sections/hatchling) were conducted on the number of follicles and their size. The number of follicles per section was determined by averaging those observed at two locations for each bursa. Images of follicles were digitized using IPLab for Windows (Scanalytics), and average size was determined by measuring 10 follicles per bursa.

Cytochrome P450. Liver samples were thawed and homogenized (1:4 w/v) in ice-cold, 1.15% KCl in 0.01 M sodium/potassium phosphate buffer at pH 7.4. The homogenate was centrifuged at 9,000 *g* for 20 min at 4°C; the supernatant was then centrifuged at 100,000 *g* for 1 h. The microsomal pellet was resuspended in 0.05 M sodium/potassium phosphate buffer at pH 7.6 containing 0.001 M disodium ethylenediaminetetra-acetate at 3 to 5 mg protein/ml.

Ethoxyresorufin-*O*-dealkylase (EROD) was assayed in triplicate on a fluorescence 96-microwell plate scanner (Fluroroscan II; ICN Flow Laboratories) [22]. The assay used 1.25 μM ethoxyresorufin (Sigma-Aldrich Chemical) substrate, 0.125 mM nicotinamide adenine dinucleotide phosphate (Sigma-Aldrich Chemical), and microsomal protein and was brought to a constant volume with 66 mM Tris buffer. Assays were run in a total volume of 260 μl at 37°C. Microsomal protein (5–30 μg/well) was optimized for each species to obtain linear reaction rates. Reference mallard microsomes were included with each plate. The change in fluorescence units over time was converted to the rate of product formation with the use of a seven-point standard curve (0.01–0.4 μM). Protein was determined using the BCA Protein Assay kit (Pierce Chemical Company). Ethoxyresorufin-*O*-dealkylase activity was calculated as pmol product formed/min/mg microsomal protein. The coefficient of variation for hatchling samples ($n = 253$) run in triplicate averaged 15%. Each species' assay was run over a period of 8 d, and the average interassay coefficient of variation for mallard reference microsomes ($n = 12$ assays) averaged 9.56%.

Thyroid hormone. Glandular hormone content was measured using the method described by McNabb and Cheng [23]. Briefly, thyroid tissue was digested in capped microcentrifuge tubes containing 25 mg of Pronase (Sigma-Aldrich Chemical) in 350 μl of distilled water at 37°C for 24 h. One milliliter of ethanol was added to the digested sample, which was then vortexed. This mixture was held at −20°C for 24 h, and then tubes were centrifuged at 13,500 *g* for 5 min. The supernatant was removed and stored at −20°C for T_4 analysis. Thyroxine concentrations in the extract were determined in duplicate by radioimmunoassay using a Coat-A-Count Canine Total T_4 assay kit (Diagnostic Products). After sample (25 μl) incubation, the bound and free fractions were separated, and bound radioactivity remaining in each tube was counted for 1 min using a Wallac 1470 Wizard gamma counter (PerkinElmer). Average counts for duplicate tubes were log-transformed, and then total T_4 was estimated from a six-point (0–15 ng/ml) standard curve. Thyroxine assays were validated for each species before running samples by testing various dilutions of extract samples against the standard curve for parallelism and by spiking sample extracts with known concentration of T_4 standard (3 μg/dL) prepared in ethanol. Mean T_4 spike recoveries from chicken, mallard, and kestrel extracts ranged from 81 to 99%. Assay precision (coefficient of variation for duplicate determinations) was 9.93% ($n = 284$). Values are expressed as ng total T_4/mg thyroid tissue.

Experiment 2: Absorption of air cell–administered penta-BDE

As part of a larger PBDE uptake and metabolism study in eggs of several avian species, corn oil vehicle or penta-BDE (analytically determined to be 11.1 μg/g egg) was administered into the air cell of chicken ($n = 9$) and kestrel ($n = 10$) eggs at a volume of 0.5 μl/g egg on day 4 and day 5, respectively, of incubation. Eggs were randomly sampled 24 h postinjection,

Table 1. Effects of polychlorinated biphenyl (PCB) congener 126 and pentabrominated diphenyl ether mixture (penta-BDE; DE-71) on chicken *(Gallus gallus)*, mallard *(Anas platyrhynchos)*, and American kestrel *(Falco sparverius)* embryos through hatching[a]

	Dose (μg/g egg)						
		PCB 126	Penta-BDE				
	Combined control	0.002	0.01	0.1	1	10	20
Chicken							
Survival to day 18	65/71 (91.5%)	17/30 (56.7%)*	25/30 (83.3%)	32/38 (84.2%)	19/22 (86.4%)	24/30 (80.0%)	26/30 (86.7%)
Pipped	61/71 (85.9%)	8/30 (26.7%)*	23/30 (76.7%)	28/38 (73.7%)	16/22 (72.7%)	23/30 (76.7%)	22/30 (73.3%)
Hatched	59/71 (83.1%)	6/30 (20.0%)*	22/30 (73.3%)	28/38 (73.7%)	16/22 (72.7%)	20/30 (66.7%)	22/30 (73.3%)
Edema/failed to hatch	2/12	9/24	2/8	1/10	1/6	1/10	3/8
Mallard							
Survival to day 24	49/54 (90.7%)	24/28 (85.7%)	22/27 (81.5%)	27/27 (100%)	23/27 (85.2%)	25/27 (92.6%)	21/27 (77.8%)
Pipped	36/54 (66.7%)	17/28 (60.7%)	13/27 (48.1%)	15/27 (55.6%)	20/27 (74.1%)	15/27 (55.6%)	13/27 (48.1%)
Hatched	35/54 (64.8%)	17/28 (60.7%)	12/27 (44.4%)	15/27 (55.6%)	19/27 (70.4%)	14/27 (51.9%)	13/27 (48.1%)
Edema/failed to hatch	9/19	6/9	10/15	9/12	3/8	12/13	8/14
Kestrel							
Survival to day 24	37/40 (92.5%)	13/20 (65.0%)*		18/18 (100%)	18/20 (90.0%)	16/20 (80.0%)	16/20 (80.0%)
Pipped	34/40 (85.0%)	9/20 (45.0%)*		16/18 (88.9%)	13/20 (65.0%)	11/20 (55.0%)*	11/20 (55.0%)*
Hatched	32/40 (80.0%)	9/20 (45.0%)*		14/18 (77.8%)	12/20 (60.0%)	9/20 (45.0%)*	9/20 (45.0%)*
Edema/failed to hatch	2/8	1/11		4/4	5/8	7/11	6/11

[a] Values are presented as the response/*n* (%). An asterisk indicates a significant difference ($p < 0.05$) from the combined control for a given species.

midway through incubation, and at pipping. Eggs were removed from the incubator and weighed, and the apex end of the egg was gently cut away so that the contents could be poured into a chemically clean jar. Care was taken not to allow the inner shell membrane to be included with the sample. The embryo was sacrificed, and samples were frozen at −20°C until analysis for total PBDE content.

Egg contents were analyzed, following the methods described by Hale et al. [24] and Rattner et al. [4], at the Virginia Institute of Marine Science in Gloucester Point (VA, USA). Briefly, eggs were lyophilized, and subsamples were spiked with surrogate standard PCB 204 (Ultra Scientific). Blanks were run along with samples to evaluate possible laboratory contamination. Egg samples were subjected to enhanced solvent extraction (Dionex ASE 200) with methylene chloride. Large-molecular-weight compounds were separated from the PBDEs in the extracts on an Envirosep size-exclusion chromatography column (length, 350 mm; diameter, 21.2 mm; guard column, 60 m × 21.1 mm; Phenomenex). The PBDE-containing fraction was then purified on a 2,000-mg, silica gel, solid-phase extraction glass column (Enviroprep; Burdick and Jackson). The PBDEs in the purified extracts were separated by GC/MS as previously described. Data were corrected based on the recovery of surrogate standard PCB 204 in each sample. Mean recovery of surrogate PCB 204 from the eggs was 72.9%. The limit of quantification was 100 pg/g wet weight.

Data analyses

Data were analyzed using SAS® (SAS Institute). For each species, survival through 90% of incubation, pipping, and hatching success were compared using contingency analysis with Bonferroni correction. Uninjected and vehicle-injected controls were initially compared, and if no statistically significant differences ($p > 0.05$) were found, these groups were combined as a single control.

Continuously distributed variables (experiment 1: body and organ weight, organ to body weight ratio, bone length, histological measurements, EROD activity, and glandular T_4 concentration; experiment 2: PBDE concentration in eggs) were examined for homogeneity of variance and tested for normality with the Shapiro–Wilk (*W*) statistic. Uninjected and vehicle-injected controls were compared using a Student's *t* test, and if no differences were found, these groups were combined as a single control group. Differences among measurement endpoints were determined using one-way analysis of variance and Tukey's honestly significant difference method of multiple comparison.

RESULTS

Experiment 1: Survival, pipping, and hatching success

Embryonic survival through 90% of incubation as well as pipping and hatching success of uninjected and vehicle-injected controls were within an acceptable range (chicken: 90–93, 79–91, and 79–86%, respectively; mallard: 86–96, 68 and 68, 63–68%, respectively; kestrel: 90–95, 75–95, and 75–85%, respectively). These endpoints did not differ between uninjected and vehicle-injected groups ($p = 0.08$–1) and, thus, were combined into a single control group. Administration of PCB 126 (positive control) elicited mortality resulting in reduced survival, pipping, and hatching success in chickens and kestrels (Table 1). Mallards were less sensitive, because 0.002 μg PCB 126/g egg did not affect these endpoints. At doses up to 20 μg/g egg, penta-BDE did not affect embryonic survival, pipping, or hatching success in chickens or mallards. In kestrels, doses of penta-BDE up to 20 μg/g egg had no effect on survival through 90% of incubation; however, dose-dependent decreases in pipping and hatching success were apparent at 1, 10, and 20 μg/g egg, with significant differences ($p < 0.05$) occurring at 10 and 20 μg/g egg.

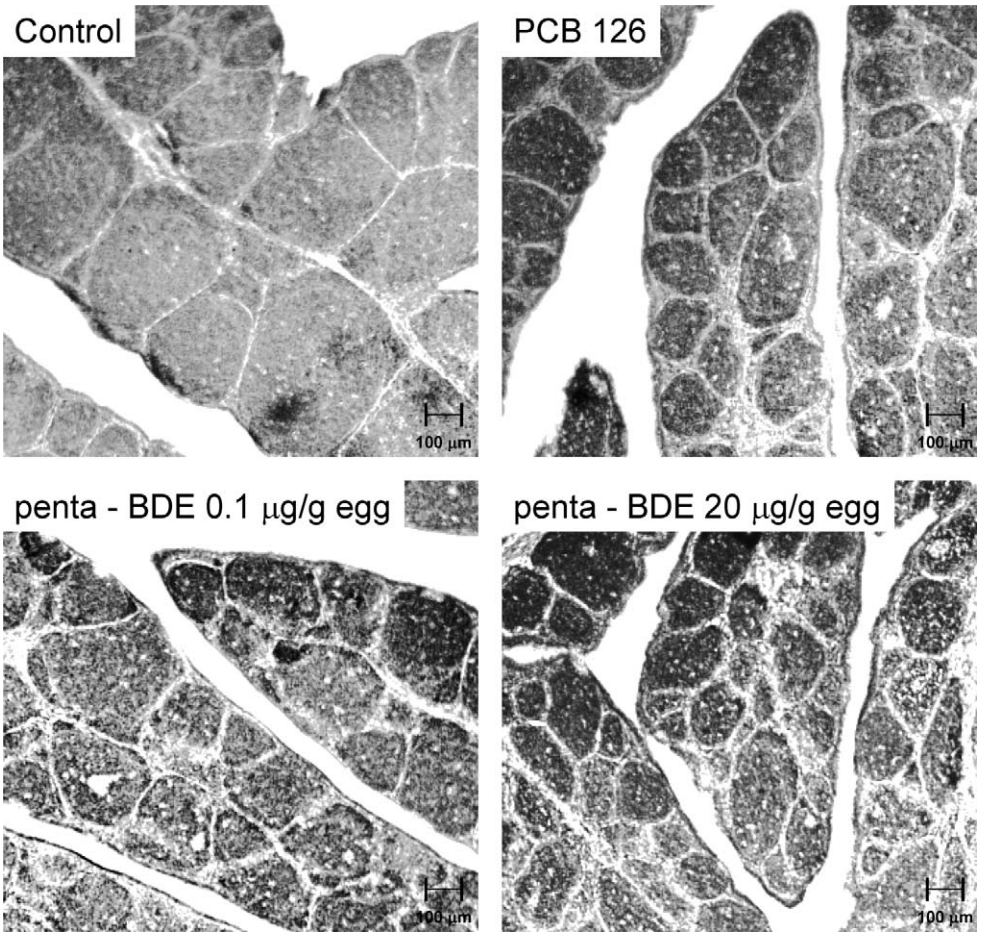

Fig. 1. Low-magnification photomicrographs of bursa of Fabricius demonstrating smaller follicle size of chicken (*Gallus gallus*) hatchlings that had been treated with polychlorinated biphenyl 126 and pentabrominated diphenyl ether mixture (DE-71) in ovo.

Experiment 1: Edema, deformities, organ weights, and bone lengths

Head and neck edema were observed in many embryos that failed to hatch (Table 1). Subcutaneous edema in the rump area was observed in one control kestrel and one mallard that received 1 μg penta-BDE/g egg. In chickens, edema frequently was observed in PCB 126–treated eggs that failed to hatch, but this was not the case with penta-BDE–treated embryos. Overall, the incidence of edema in embryos that failed to hatch was greater in mallards ($p = 0.0048$) than in chickens or kestrels. One unhatched control mallard embryo exhibited exencephaly, and three hatchlings had splayed legs (a chicken that received 20 μg penta-BDE/g egg, a control mallard, and a kestrel that received 0.1 μg penta-BDE/g egg). Many of the kestrel embryos that died were developmentally stunted and physically small relative to their incubation age, but this tendency was not observed in chicken or mallard embryos.

Crown-rump length, body weight (without yolk sac), and liver to body weight ratio did not differ between uninjected and vehicle-injected groups for chickens, mallards, and kestrels ($p > 0.15$). Liver to body weight ratio, however, differed between the mallard uninjected and vehicle-injected control groups ($p < 0.001$). With minor exceptions, no differences were found in crown-rump length, body weight, liver to body weight ratio, thyroid to body weight ratio, and bone lengths in hatchling chickens, mallards, or kestrels that had been treated with PCB 126 or penta-BDE. Compared to controls, femur length appeared to be shorter in PCB 126–treated birds, but this was only significant ($p = 0.0086$, Student's t test) for the right femur in kestrels (mean ± standard error, 9.49 ± 0.11 vs 8.74 ± 0.31 mm).

The bursa to body weight ratio of the six surviving chicken hatchlings treated with PCB 126 in ovo was markedly smaller (52%; $p = 0.002$) compared to controls, but weights of other immune organs were unaffected. No differences were found in the organ to body weight ratios of spleen, bursa, or right thymus in penta-BDE–exposed chickens, mallards, or kestrel hatchlings.

Experiment 1: Histopathology

Hepatic lipidosis, associated with yolk assimilation, was observed in all species. No lesions were seen in liver sections of chicken hatchlings that had been treated in ovo with PCB 126 or penta-BDE. Because of some qualitative observations suggesting changes in hepatocyte cellularity in chickens (but not mallards or kestrels), the number of hepatocytes per 10 μm of length was determined, but this number did not differ among treatments ($p > 0.3$). Liver samples from a small number of kestrel and mallard hatchlings exhibited focal necrosis in control, PCB, and penta-BDE treatment groups. The incidence of lesions in treated groups, however, was not different from controls ($p > 0.3$).

Bursal follicle size was smaller ($p < 0.02$) in PCB 126–exposed chickens compared to controls. Bursal follicle size of

chicken hatchlings in all penta-BDE treatment groups, including the lowest doses (0.01 and 0.1 μg/g), seemed consistently smaller than in controls (Fig. 1). Based on this observation and preliminary statistical findings, additional chicken bursa samples at all dose levels were processed. Bursal follicle size of chicken hatchlings was consistently smaller in all penta-BDE treatment groups compared to controls (24–42%; p = 0.057–0.001). Mallard and kestrel bursa and follicle size did not differ among PCB 126, 20 μg penta-BDE/g, and the combined control groups ($p > 0.3$).

Experiment 1: Hepatic microsomal EROD activity

For mallard and kestrel hatchlings, hepatic EROD activity of uninjected and vehicle-injected controls did not differ within species (kestrel: $p = 0.60$; mallard: $p = 0.64$). The EROD activity in chickens, however, was significantly different between uninjected and vehicle-injected controls ($p = 0.0379$); therefore, only the vehicle-injected group was used for comparisons with PCB and penta-BDE treatment groups. In chicken hatchlings, log-transformed EROD activity was induced more than 35-fold in PCB 126-exposed embryos, fivefold at 1 μg penta-BDE/g, 21-fold at 10 μg penta-BDE/g, and 22-fold at 20 μg penta-BDE/g ($p < 0.0001$) (Fig. 2). Activity of EROD was not induced by PCB 126 or penta-BDEs in kestrels and mallards.

Experiment 1: Glandular T_4 content

Because of heterogeneity of variance, thyroid T_4 content data (ng/mg thyroid and ng/thyroid) were log-transformed before comparisons among treatments. Thyroxine content did not differ among PCB 126 and penta-BDE treatments in hatchling chickens ($p > 0.2$) or mallards ($p > 0.7$). Both total glandular T_4 content and T_4 concentration per milligram of thyroid were lower ($p < 0.001$) in kestrel hatchlings exposed to PCB 126 but were unaffected by penta-BDE (Table 2).

Experiment 2: Penta-BDE absorption into chicken and kestrel eggs

The administered dose of penta-BDE (analytically verified to be 11.1 μg/g egg) was gradually absorbed through the air cell membrane during the exposure period in chickens (Table 3), as indicated by an increase in total PBDE concentration in contents of eggs ($p < 0.048$) over the course of incubation. Of the dose administered in chicken eggs, 0.64% was absorbed after 24 h, 7.71% after 6 d, and 29.6% by the time of pipping (i.e., 17 d after administration). On a lipid-weight basis, PBDE absorbed into chicken eggs was 0.855 ± 0.459, 10.0 ± 0.78, and 47.0 ± 6.12 μg/g lipid on day 5, 10, and 21 of incubation, respectively.

In kestrels, a significant ($p < 0.008$) increase in PBDE uptake was found between injection and midway through incubation (1.64% was absorbed after 24 h and 18.4% after 8 d). Uptake rate seemingly was not sustained through the last half of incubation (18.6% at pipping, day 26 of incubation), although the sample size was small ($n = 4$) and the uptake variable (13.3–24.8%). On a lipid-weight basis, PBDE absorbed into kestrel eggs was 3.10 ± 1.64, 37.2 ± 6.91 and 54.4 ± 10.6 μg/g lipid on day 6, 13, and 26 of incubation, respectively.

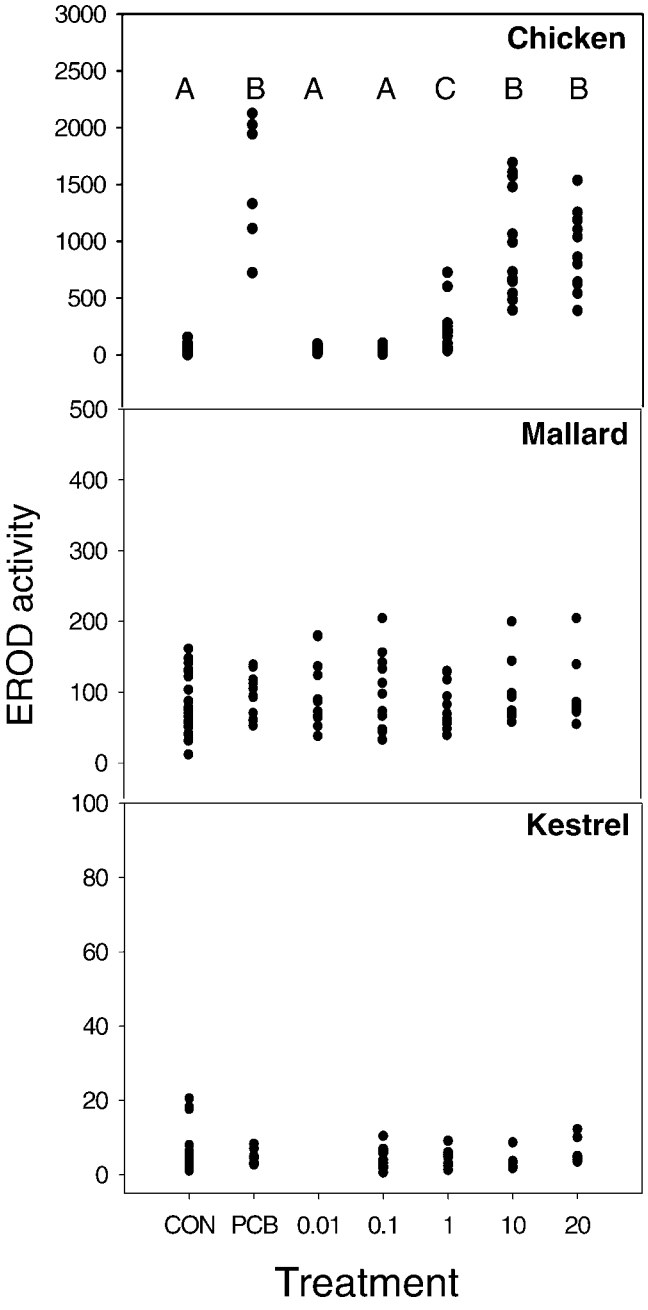

Fig. 2. Hepatic ethoxyresorufin-*O*-dealkylase activity (EROD; pmol product/min/mg microsomal protein) of chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel (*Falco sparverius*) control (CON) hatchlings or hatchlings that had been treated with 0.002 μg polychlorinated biphenyl (PCB) 126/g egg and pentabrominated diphenyl ether mixture (0.01–20 μg DE-71/g egg) in ovo. For chickens, groups with different capital letters are significantly different ($p < 0.05$).

DISCUSSION

In ovo administration of penta-BDE in chickens reduced the number and size of bursal follicles at administered doses as low as 0.01 μg/g egg and induced hepatic EROD activity starting at doses of 1 μg/g egg. No effects of penta-BDE on embryonic survival or pipping and hatching success were observed in chickens. Pipping and hatching success in kestrels, however, appeared to be lower (not statistically significant, p = 0.12) at an administered dose of 1 μg/g egg, and adverse reproductive effects were statistically significant at 10 and 20 μg/g egg. The reproductive effects of penta-BDE observed in kestrels, but not in chickens, are in contrast to the extreme sensitivity of the chicken embryo to coplanar PCBs (present

Table 2. Glandular thyroxine (T_4) content in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel *(Falco sparverius)* exposed to polychlorinated biphenyl (PCB) congener 126 and pentabrominated diphenyl ether mixture (penta-BDE; DE-71) in ovo[a]

		Dose (μg/g egg)					
		PCB 126	Penta-BDE				
	Combined control	0.002	0.01	0.1	1	10	20
Chicken							
n	30	6	13	14	12	14	12
Paired thyroid wt (mg)	6.39 ± 0.22	7.83 ± 0.79	5.82 ± 0.37	5.88 ± 0.38	6.00 ± 0.32	5.81 ± 0.34	6.66 ± 0.26
T_4 (ng/mg thyroid)	440 ± 29.7	484 ± 52.8	645 ± 64.2	459 ± 69.5	491 ± 84.4	448 ± 46.4	538 ± 75.8
T_4 (ng/paired thyroids)	2,758 ± 171	3,619 ± 212	3,493 ± 186	2,551 ± 317	2,649 ± 374	2,540 ± 250	2,515 ± 391
Mallard							
n	23	12	11	11	12	12	11
Paired thyroid wt (mg)	6.45 ± 0.29	6.39 ± 0.27	7.77 ± 0.42	7.58 ± 0.40	7.16 ± 0.34	6.51 ± 0.33	6.99 ± 0.55
T_4 (ng/mg thyroid)	317 ± 28.0	313 ± 68.0	212 ± 19.9	283 ± 29.4	232 ± 25.3	284 ± 24.8	298 ± 28.7
T_4 (ng/paired thyroids)	2,023 ± 200	1,933 ± 359	1,701 ± 240	2,133 ± 233	1,628 ± 225	1,898 ± 240	2,060 ± 159
Kestrel							
n	30	9		13	9	8	9
Left thyroid wt (mg)	1.19 ± 0.09	1.39 ± 0.21	—	1.30 ± 0.12	1.04 ± 0.14	1.32 ± 0.15	1.28 ± 0.19
T_4 (ng/mg thyroid)	464 ± 64.4	144 ± 48.2*	—	333 ± 70.4	378 ± 54.8	371 ± 64.9	405 ± 80.3
T_4 (ng/left thyroid)	438 ± 29.3	159 ± 23.8*	—	350 ± 35.3	351 ± 48.9	443 ± 53.8	403 ± 29.1

[a] Untransformed values are presented as the mean ± standard error and *n.* An asterisk indicates a significant difference ($p < 0.001$) difference from the corresponding control.

study) and [19,25,26]. Although PBDEs and PCBs have some structural similarities, their toxicity (histology of the bursa, cytochrome P450 induction, and lethality) appears to be markedly different in chickens and kestrels.

Survival, pipping, and hatching success

Administration of PCB 126 (positive control) impaired embryonic survival, pipping, and hatching success in chickens and kestrels. Clearly, PCB 126 was most toxic in chickens; only 20% of injected eggs hatched. Mallard embryos appeared to be far more tolerant of PCB 126 than chickens, as previously noted for this congener and other coplanar PCBs [25,27,28]. Decreased embryonic survival and hatching success has been reported in chickens receiving air cell–administered PCB 126 at doses as low as 0.00025 μg/g egg (i.e., 250 pg/g) [19,20], and the estimated median lethal dose of this congener may be as high as 0.0031 μg/g egg (i.e., 3,100 pg/g) when injected on day 7 of incubation [26]. The dose used in the present study (0.002 μg/g egg) is high compared to previously reported toxicity thresholds for chickens [19,20,29]. Air cell administration of PCB 126 [18] and, presumably, other compounds (e.g., methylmercury) [17], however, is considerably less embryotoxic in eggs incubated horizontally compared to those incubated vertically [19,20,29]. In vertically incubated eggs, the embryo is situated directly under the air cell, and because of its proximity to the injection site, the embryo may be more directly exposed to administered PCB 126 [18].

Pipping and hatching success were only affected in kestrels, with decreases in these endpoints seemingly starting at a dose of 1 μg/g egg and becoming definitive at 10 and 20 μg/g egg. The metabolism of lipid-soluble xenobiotic compounds depends, in part, on the action of phase I enzymes, and the activity of some of these (e.g., cytochrome P450–associated monooxygenases) are lower in fish-eating birds and raptors than in other groups of birds and mammals [30]. The reduced ability of kestrels to metabolize persistent organic pollutants, such as PBDEs, may contribute to sustained exposure and, thus, greater toxicity. In addition, kestrels are semialtricial,

Table 3. Uptake of air cell–administered pentabrominated diphenyl ether mixture (penta-BDE; DE-71) during incubation in chicken and kestrel eggs[a]

	Sampling day		
	24 h Postinjection	Midincubation	Pipped
Chicken			
Vehicle injected (μg total penta-BDE/g egg wet wt)	ND	ND	0.00042 ± 0.0002
Penta-BDE injected (μg total penta-BDE/g egg wet wt)[b]	0.084 ± 0.0459A	1.03 ± 0.111B	4.93 ± 0.994C
Uptake of analytically verified penta-BDE dose (%)[c]	0.64 ± 0.356	7.71 ± 0.857	29.6 ± 4.56
Kestrel			
Vehicle injected (μg total penta-BDE/g egg wet wt)	0.00043 ± 0.000216	ND to 0.00232	0.00038 ± 0.000198
Penta-BDE injected (μg total penta-BDE/g egg wet wt)[b]	0.208 ± 0.1129A	2.43 ± 0.452B	2.80 ± 0.498B
Uptake of analytically verified penta-BDE dose (%)[c]	1.64 ± 0.871	18.4 ± 3.32	18.8 ± 3.04

[a] The penta-BDE was administered at an analytically verified dose of 11.1 μg/g egg. Values are presented as the mean ± standard error (chickens, $n = 3$ per sampling day; kestrels, $n = 3$–4 in penta-BDE–treated eggs/sampling day and $n = 2$–3 in vehicle-treated eggs/sampling day). ND = below detection limit.

[b] Groups with different uppercase letters are significantly different ($p < 0.05$).

[c] Total quantity of penta-BDE in egg contents/total quantity of penta-BDE administered to whole egg.

and the structural and metabolic capabilities of the liver and kidneys are relatively less developed during incubation and at hatch compared with those of precocial species (chicken and mallard).

In a study by Fernie et al. [9], hatching success after an air cell–administered dose of 1.5 μg PBDE/g to kestrel eggs on day 19 of incubation did not differ from that of vehicle-injected controls. Their treatment regimen, however, only permitted an 8-d exposure period before pipping, unlike our 22-d exposure period. In addition, hatching success of their control group was quite low compared to the present study (i.e., 53.6% vs 80.0%). The 1.5 μg PBDE/g dose approaches environmentally relevant wet-weight concentrations in eggs of fish-eating birds, including herring gulls (1.4 μg/g) [2] and ospreys (0.928 μg/g) [4,5]. Wet-weight concentrations of PBDEs in eggs of peregrine falcons, a terrestrial predatory species, have been reported to average 0.23 μg/g in northern Sweden [31]. A recent study involving analysis of 114 unhatched peregrine falcon eggs from the northeastern United States revealed an average of 0.59 μg PBDE/g wet weight, with 8.8% of the samples exceeding 1 μg/g and values ranging up to 6.6 μg/g [6]. These values in eggs from the once-endangered peregrine falcon are within the range of the lowest-observed-effect levels (LOELs) for pipping and hatching success in kestrels. These concentrations are of concern, because PBDE values in bird eggs have increased for 20 years [2], although values appear to have leveled off, possibly because of a decrease in production of penta-BDE and octa-BDE formulations [3].

Edema, deformities, hatchling organ weights, and bone lengths

As previously noted [19,20,29,32], air cell administration of PCB 126 resulted in stunted growth and edema in many chicken and kestrel embryos that failed to hatch. Greater concentrations of PCB 126, however, were required to evoke these responses, presumably because of the horizontal position of incubating eggs [18]. In the present study, neither body weight nor liver to body weight ratio was affected by PCB 126 in day-old hatchlings. This is similar to observations in chickens that received air cell–administered PCBs, but it is in contrast to findings in kestrels (decreased body and liver wt) [19]. Some evidence of reduced growth (i.e., shorter femur length) was observed in kestrel hatchlings following administration of PCB 126, which is similar to the findings of Hoffman et al. [19]. Notably, yolk sac administration of higher doses of PCB 126 (0.032 μg/g egg) have been reported to result in lower body weight of hatchlings and to increase the ratios of brain, heart, and liver to body weight (but not absolute weights of these organs) [33].

Stunted growth and edema were observed in most penta-BDE–treated kestrel embryos that failed to hatch. Most of the mallard embryos that failed to hatch, including both controls and penta-BDE groups, exhibited edema (but not stunted growth). This is attributed to technical difficulties in the artificial incubation of mallard eggs, perhaps associated with retention of the waxy eggshell cuticle and apparent retention of fluid (i.e., difficulties in attaining 14–16% moisture loss throughout incubation). Body weight, crown-rump length, liver to body weight ratio, and bone lengths were not affected in any of the species in the present study. In the study by Fernie et al. [9], growth and tarsometatarsus bone length were marginally greater in PBDE-treated nestling and fledgling kestrels. Larger liver weights have been reported in mice, rats, and mink exposed to PBDEs [34,35]. These effects, however, occurred in animals that were exposed repeatedly to much higher doses of PBDEs.

Liver EROD activity and histopathology

Toxic effects of dioxins and dioxin-like compounds are principally mediated through binding to a cytosolic aryl hydrocarbon receptor (AhR), and this ligand-activated factor increases or decreases transcription of mRNAs and translation of proteins. Induction of cytochrome P450 1A (CYP1A), which is mediated by AhR, is a well-characterized response to dioxin-like compounds. The induction of CYP1A-associated monooxygenases, specifically EROD activity, has been used extensively as an exposure biomarker of dioxin-like compounds (e.g., coplanar PCB congeners) in birds [19,25,36,37]. In agreement with other studies, our findings (Fig. 2) illustrate that EROD activity is induced by PCB 126 in chickens. In contrast, doses at least 50-fold greater are required to induce EROD in mallards and kestrels [19,26]. This greater sensitivity in the chicken is apparently caused by the presence of two amino acids (isoleucine-325 and serine-381) in the ligand-binding domain of the AhR in chickens, for which substitutions exist in some avian species (less sensitive species, alanine-381; insensitive species, valine-325 and alanine-381) [38,39].

Polybrominated diphenyl ethers are structurally similar to polyhalogenated aromatic hydrocarbons that bind to AhR and induce EROD activity, although PBDE mixtures and individual congeners are less potent (10^{-2} to 10^{-5}) than dioxin [14]. There have been conflicting reports about the ability of PBDEs to induce EROD activity in mammals and mammalian hepatocyte cultures [14,35,40–42]. Induction in some studies may have been caused by dioxin and dibenzofuran impurities in the technical mixture. Martin et al. [35] analyzed DE-71, the technical mixture used in the present study, and those authors did not detect dioxins or dibenzofurans (detection limit, <30 pg/g). The failure to detect dioxins or dibenzofurans would suggest that the observed EROD induction in chickens during the present study may not have been caused by impurities. Despite reproductive effects in kestrels, EROD activity was not induced. Based on this observation, the toxic effects of PBDEs in kestrels may not be principally mediated through the AhR.

Despite the induction of EROD activity in chicken hatchlings following the administration of PCB 126, no evidence of liver lesions was found in survivors. The absence of liver pathology may be caused by a survivor effect. In other words, by the time of hatching, any embryos that might have had hepatic lesions had died, whereas the least-affected chicken embryos hatched. Notably, Rifkind et al. [43] described narrowing of hepatic sinusoids in day-18 chicken embryos 24 h after administration of PCB 77 at approximately 0.0225 μg/g egg (22,500 pg/g egg). This congener has half the toxic potency of PCB 126 in birds [44]. Thus, the dose used in the present study (0.002 μg PCB 126/g egg) was less than one-fifth the LOEL observed in the study by Rifkind et al. [43] (~0.01125 μg/g egg). Administration of PCB 126 in kestrel eggs evoked mortality, but no histopathological lesions were observed. Hoffman et al. [45] reported multifocal necrosis in liver of kestrel hatchlings exposed repeatedly to PCB 126 from day 1 to day 10 posthatch, receiving a total administered dose of approximately 87 μg. In the present study, kestrel eggs were injected once and received a total of approximately 0.03 μg. Again, the effect level observed by Hoffman et al. resulted from administration of a much greater dose. Fox et al. [46]

observed periportal hepatitis in livers of adult herring gulls from the Great Lakes. Concentrations of PCBs in liver tissue averaged 13.4 μg/g. Clearly, the single early exposure regimen in the present study was well below the threshold eliciting microscopic effects in liver tissue of surviving embryos that hatched.

Thyroid gland and hormones

Alterations to the thyroid system can affect metabolism, growth, and thermoregulation. In the present study, exposure to PCB 126 resulted in lower glandular T_4 content in kestrel hatchlings but not in the other species tested. As previously discussed, American kestrels are semialtricial and are less developed at hatch compared with precocial species. Based on the number and size of follicles, colloid staining characteristics, and T_4 content, the thyroid is less developed in altricial embryos and hatchlings compared to precocial species [47,48]. The difference in development in kestrels may render the thyroid gland more sensitive to in ovo exposure to thyroid-disrupting chemicals, such as PCBs. Notably, pipping herring gull embryos and chicks (semiprecocial species) from PCB-contaminated sites in the Great Lakes have been reported to have lower glandular T_4 content than gulls from reference sites [49].

Compared to circulating concentrations of T_4 and triiodothyronine, and to thyroid weight, glandular T_4 content has been reported to be a more sensitive indicator of decreased thyroid function in studies of PCB and perchlorate toxicity in birds [49–51]. Toxic effects of PBDEs on thyroid function are incompletely known. In laboratory studies of mice and rats, PBDE exposure decreases plasma T_4 concentrations [12,15,52], may alter plasma transport of T_4 through competitive binding mechanisms [53], and induces enzymatic degradation of thyroid hormones through induction of hepatic T_4-glucuronidation activity [15,54]. Fernie et al. [8] suggested that PBDE exposure results in slightly lower plasma T_4 concentrations in kestrels, although no alterations in thyroid histology were observed. In the present study, the absence of effects on thyroid weight and glandular T_4 content in chicken, mallard, and kestrel embryos suggests that thyroid function may not be altered by in ovo exposure to PBDEs at the doses tested.

Immune organs and histology

In birds, the thymus, bursa of Fabricius, and spleen are all recognized as integral parts of the immune system. The bursa is a primary lymphoid organ, which is unique to birds, and is necessary for normal development of the humoral immune system. In the present study, bursal weights were significantly lower in PCB 126–treated chicken hatchlings. This and other AhR active congeners have been reported previously to induce atrophy of the bursa in chickens exposed in ovo [20,29,55]. Bursal somatic index decreased with increasing concentrations of BDE 47 in kestrel hatchlings exposed in ovo and posthatching [7]. Nonetheless, this change was not seen in penta-BDE–treated chickens, mallards, and kestrels exposed in ovo. The number of follicles per bursa and the follicle size, however, were consistently lower in chickens treated with PCB 126 and all doses of penta-BDE. Fernie et al. [7] reported reduced antibody-mediated response in kestrels exposed to PBDEs in ovo and posthatching. Thus, results from both of these studies indicate that the bursa may be sensitive to embryonic PBDE exposure. It also has been demonstrated that immune organ cellularity may be a more sensitive indicator of PCB-induced atrophy than organ mass [20,55]. Lavoie et al. [56] suggest that the immune system may recover from in ovo exposure to PCBs, and this could be the case for PBDEs. Therefore, more studies of the immune system with multiple endpoints in birds chronically exposed to PCBs and PBDEs seem warranted.

Penta-BDE absorption into the egg

Artificial treatment of eggs by air cell or yolk sac injection is believed to approximate the toxicity of maternally deposited compounds. Embryotoxic responses (e.g., cytochrome P450, edema, deformities, and mortality) evoked by egg injection of PCBs compare favorably to those observed following natural exposure [57]. At equal concentrations, however, air cell–injected methylmercury seems to be more toxic than naturally incorporated methylmercury [58]. Air cell–injected compounds must cross the air cell membrane into the albumen, blood vessels, and yolk to reach the embryo. It is unknown if air cell–administered or yolk-injected compounds mimic the distribution of naturally deposited contaminants in eggs, but these techniques frequently are employed to circumvent the difficulties and expense of studying such effects in feeding trials. With these caveats in mind, actual concentrations absorbed into egg contents were determined and can be related to embryotoxic effects.

De Roode and van den Brink [59] injected PCBs into the yolk of chicken eggs before incubation and measured an exponential increase in uptake by the embryo, with 18% of the administered dose absorbed into the embryo by day 19. Maervoet et al. [60] noted a similar exponential uptake after yolk injection of PCBs 77, 153, and 180 into the embryo during the last week of incubation. The penta-BDEs injected into the air cell of chicken eggs were gradually absorbed over the 17-d exposure period, with an apparent increase in uptake rate into egg contents between day 10 and pipping, perhaps suggesting an exponential absorption relationship. This increase in absorption rate could be the result of increased size and density of vitelline blood vessels and the vast growth of the blood vessels of the chorioallantoic network under the inner shell membrane. Compared to chickens, penta-BDE appeared to be absorbed at a greater rate through midincubation in kestrels (i.e., 18.4% vs 7.71% of administered dose). Thereafter, PBDE absorption in kestrels appeared to level off, although sample size was small.

Based on the concentrations of total PBDEs absorbed into chicken and kestrel eggs (Table 3), effects in the present study are occurring at substantially lower concentrations compared with those in dosing solutions administered into the air cell. We observed up to 29.6% of the analytically verified dose of 11.1 μg penta-BDE/g egg in the chicken egg by pipping. Sublethal effects were noted in chicken hatchlings in the present study, but from an ecological perspective, the most important effects were on pipping and hatching in kestrels, a toxicological model species often used in risk assessments for raptorial birds. During the first half of incubation, more than twice as much PBDE was absorbed in kestrel eggs compared to chicken eggs. This could have influenced mortality rate; however, the majority of mortality occurred at the end of incubation. By the end of incubation, findings in experiment 2 indicate that 18% of the administered dose was absorbed in kestrels. Adverse reproductive effects were observed in kestrels receiving 10 μg/g egg of this technical mixture into the air cell, and at 18% uptake, the LOEL associated with impaired pipping and hatching success could be as low as 1.8 μg/g egg wet weight.

This exposure is only approximately twofold greater than the total PBDE concentrations reported in osprey eggs [4,5] and is well within the range of total PBDE concentrations detected in peregrine falcon eggs from the northeastern United States [6]. On a lipid-weight basis, the LOEL for impaired pipping and hatching success would be approximately 32 μg PBDE/g egg lipid weight.

CONCLUSION

The present study demonstrates that air cell administration of environmentally realistic concentrations of penta-BDE induced EROD activity and reduced bursal follicle size and number in chicken hatchlings. Survival endpoints also were affected in kestrel embryos. As previously demonstrated, PCB 126 reduced survival endpoints in both chicken and kestrel embryos. Mallards were less sensitive than chickens or kestrels to both PCB 126 and penta-BDEs. The observed effects are cause for concern in free-ranging avian predators and other wildlife exposed to PBDEs. Although the concentrations of penta-BDE congeners commonly detected in the environment seem to have plateaued in herring gull eggs in the Great Lakes, levels of higher-brominated congeners are still increasing [3]. Further effect studies with higher-brominated congeners in top predators are suggested.

Acknowledgement—We thank B.K. Ackerson, M.E.B. Bohannon, K.R. Stebbins, and S.D. Sifleet for help with egg management and sampling; M.J. LaGuardia, D. Chen, G. Mears, and E. Harvey for assistance with PBDE analyses; E.T. Lavoie and K. Whitehouse for guidance with T_4 assay validation; P.N. Klein and R.J. Montali for reviewing histopathological findings; and D.J. Hoffman and G.H. Heinz for technical advice and reviewing the manuscript.

REFERENCES

1. de Wit CA. 2002. An overview of brominated flame retardants in the environment. *Chemosphere* 46:583–624.
2. Norstrom RJ, Simon M, Moisey J, Wakeford B, Weseloh DV. 2002. Geographical distribution (2000) and temporal trends (1981–2000) of brominated diphenyl ethers in Great Lakes herring gull eggs. *Environ Sci Technol* 36:4783–4789.
3. Gauthier L, Hebert CE, Weseloh DVC, Letcher RJ. 2008. Dramatic changes in the temporal trends of polybrominated diphenyl ethers (PBDEs) in herring gull eggs from the Laurentian Great Lakes: 1982–2006. *Environ Sci Technol* 42:1524–1530.
4. Rattner BA, McGowan PC, Golden NH, Hatfield JS, Toschik PC, Lukei RF, Hale RC, Schmitz-Afonso I, Rice CP. 2004. Contaminant exposure and reproductive success of ospreys (*Pandion haliaetus*) nesting in Chesapeake Bay regions of concern. *Arch Environ Contam Toxicol* 47:126–140.
5. Toschik PC, Rattner BA, McGowan PC, Christman MC, Carter DB, Hale RC, Matson CW, Ottinger MA. 2005. Environmental contaminant exposure and reproduction of ospreys nesting in the Delaware River and Bay. *Environ Toxicol Chem* 24:617–628.
6. Chen D, LaGuardia MJ, Harvey E, Amaral M, Wohlfort K, Hale RC. 2008. Polybrominated diphenyl ethers in peregrine falcon (*Falco peregrinus*) eggs from the northeastern U.S. *Environ Sci Technol* 42:7594–7600.
7. Fernie KJ, Mayne G, Shutt LJ, Pekarick C, Grasman KA, Letcher RJ, Drouillard KG. 2005. Evidence of immunomodulation in nestling American kestrels (*Falco sparverius*) exposed to environmentally relevant PBDEs. *Environ Pollut* 138:485–493.
8. Fernie KJ, Shutt LJ, Mayne G, Hoffman DJ, Letcher RJ, Drouillard KG, Richie IJ. 2005. Exposure to polybrominated diphenyl ethers (PBDEs): Changes in thyroid, vitamin A, glutathione homeostasis, and oxidative stress in American kestrels (*Falco sparverius*). *Toxicol Sci* 88:375–383.
9. Fernie KJ, Shutt LJ, Ritchie JL, Letcher RJ, Drouillard KG, Bird DM. 2006. Changes in the growth, but not the survival, of American kestrels (*Falco sparverious*) exposed to environmentally relevant polybrominated diphenyl ethers. *J Toxicol Environ Health A* 69:1541–1554.
10. Fernie KJ, Shutt JL, Letcher RJ, Ritchie JI, Sullivan K, Bird DM. 2008. Changes in reproductive courtship behaviors of adult American kestrels (*Falco sparverius*) exposed to environmentally relevant levels of the polybrominated diphenyl ether mixture, DE-71. *Toxicol Sci* 102:171–178.
11. von Meyerinck L, Hufnagel B, Schmoldt A, Benthe HF. 1990. Induction of rat liver microsomal cytochrome P450 by the pentabromo diphenyl ether Bromkal 70 and half-lives of its components in the adipose tissue. *Toxicology* 61:259–274.
12. Fowles JR, Fairbrother A, Baecher-Steppan L, Kerkvliet NI. 1994. Immunologic and endocrine effects of the flame-retardant pentabromodiphenyl ether (DE-71) in C57BL/6J mice. *Toxicology* 86:49–61.
13. Eriksson P, Jakobsson E, Fredriksson A. 2001. Brominated flame retardants: A novel class of developmental neurotoxicants in our environment? *Environ Health Perspect* 109:903–908.
14. Chen G, Konstantinov AD, Chittim GG, Joyce EM, Bols NC, Bunce NJ. 2001. Synthesis of polybrominated diphenyl ethers and their capacity to induce CYP1A by the Ah receptor–mediated pathway. *Environ Sci Technol* 35:3749–3756.
15. Zhou T, Ross DG, de Vito MJ, Crofton KM. 2001. Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* 61:76–82.
16. de Witt JC, Meyer EB, Henshel DS. 2005. Environmental toxicity studies using chickens as surrogates for wildlife: Effects of vehicle volume. *Arch Environ Contam Toxicol* 48:260–269.
17. Heinz GH, Hoffman DJ, Kondrad SL, Erwin CA. 2006. Factors affecting the toxicity of methylmercury injected into eggs. *Arch Environ Contam Toxicol* 50:264–279.
18. McKernan MA, Rattner BA, Hale RC, Ottinger MA. 2007. Egg orientation affects toxicity of air cell administered polychlorinated biphenyl 126 (3,3′,4,4′,5-pentachlorobiphenyl) in chicken (*Gallus gallus*) embryos. *Environ Toxicol Chem* 26:2724–2727.
19. Hoffman DJ, Melancon MJ, Klein PN, Eisemann JD, Spann JW. 1998. Comparative and developmental toxicity of planar polychlorinated biphenyl congeners in chickens, American kestrels, and common terns. *Environ Toxicol Chem* 17:747–757.
20. Lavoie ET, Grasman KA. 2007. Effects of in ovo exposure to PCBs 126 and 77 on mortality, deformities and posthatch immune function in chickens. *J Toxicol Environ Health A* 70:547–558.
21. Karnofsky DA. 1965. The chick embryo in drug screening: Survey of teratological effects observed in the 4-day-old chick embryo. In Wilson JG, Warkany JK, eds, *Teratology: Principles and Techniques.* University of Chicago Press, Chicago, IL, USA, pp 194–213.
22. Melancon MJ. 1996. Development of cytochromes P450 in avian species as a biomarker for environmental contaminant exposure and effect. Procedures and baseline values. In Bengston DA, Henshel DS, eds, *Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment,* Vol 5. STP 1306. American Society for Testing and Materials, West Conshohocken, PA, pp 95–108.
23. McNabb FMA, Cheng MF. 1985. Thyroid development in altricial ring doves, *Streptopelia risoria*. *Gen Comp Endocrinol* 58:243–251.
24. Hale RC, La Guardia MJ, Harvey E, Mainor TM, Duff WH, Gaylor MO. 2001. Polybrominated diphenyl ether flame retardants in Virginia freshwater fishes (USA). *Environ Sci Technol* 35:4585–4591.
25. Brunström B. 1988. Sensitivity of embryos from duck, goose, herring gull, and various chicken breeds to 3,3′,4,4′-tetrachlorobiphenyl. *Poult Sci* 67:52–57.
26. Brunström B, Halldin K. 1998. EROD induction by environmental contaminants in avian embryo livers. *Comp Biochem Physiol C Toxicol Pharmacol* 121:213–219.
27. Brunström B, Reutergardh L. 1986. Differences in sensitivity of some avian species to the embryotoxicity of a PCB, 3,3′,4,4′-tetrachlorobiphenyl, injected into the eggs. *Environ Pollut Ser A* 42:37–45.
28. Jin X, Kennedy SW, Di Muccio T, Moon TW. 2001. Role of oxidative stress and antioxidant defense in 3,3′,4,4′,5-pentachlorobiphenyl–induced toxicity and species-differential sensitivity in chicken and duck embryos. *Toxicol Appl Pharmocol* 172:241–248.
29. Fox LL, Grasman KA. 1999. Effects of PCB 126 on primary

immune organ development in chicken embryos. *J Toxicol Environ Health* 58:233–244.
30. Walker CH. 1980. Species variations in some hepatic microsomal enzymes that metabolize xenobiotics. In Bridges JW, Chasseaud LF, eds, *Progress in Drug Metabolism,* Vol 5. John Wiley, West Sussex, UK, pp 113–163.
31. Lindberg P, Sellstrom U, Haggberg L, de Wit CA. 2004. Higher brominated diphenyl ethers and hexabromocyclododecane found in eggs of peregrine falcons (*Falco peregrinus*) breeding in Sweden. *Environ Sci Technol* 38:93–96.
32. Brunström B, Andersson L. 1988. Toxicity of 7-ethoxyresorufin *O*-deethylase–inducing potency of coplanar polychlorinated biphenyls (PCBs) in chick embryos. *Arch Environ Contam Toxicol* 62:263–266.
33. Powell DC, Aulerich RJ, Meadows JC, Tillitt DE, Giesy JP, Stromborg KL, Bursian SJ. 1996. Effects of 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) injected into the yolks of chicken (*Gallus domesticus*) eggs prior to incubation. *Arch Environ Contam Toxicol* 31:404–409.
34. International Program on Chemical Safety. 1994. Brominated diphenyl ethers. Environmental Health Criteria 162. World Health Organization, Geneva, Switzerland.
35. Martin PA, Mayne GJ, Bursian SJ, Tomy G, Palace V, Pekarik C, Smits J. 2007. Immunotoxicity of the commercial polybrominated diphenyl ether mixture DE-71 in ranch mink (*Mustela vison*). *Environ Toxicol Chem* 26:988–997.
36. Bosveld ATC, Gradener J, van Kampen M, Murk AJ, Evers EHG, Van den Berg M. 1993. Occurrence and effects of PCBs, PCDDs, and PCDFs in hatchlings of the common tern (*Sterna hirundo*). *Chemosphere* 27:419–427.
37. Rattner BA, Hatfield JS, Melancon MJ, Custer TW, Tillitt DE. 1994. Relation among cytochrome P450, Ah-active PCB congeners and dioxin equivalents in pipping black-crowned night-heron embryos. *Environ Toxicol Chem* 13:1805–1812.
38. Karchner SI, Franks DG, Kennedy SW, Hahn ME. 2006. The molecular basis for differential dioxin sensitivity in birds: Role of aryl hydrocarbon receptor. *Proc Natl Acad Sci U S A* 103: 6252–6257.
39. Head JA, Hahn ME, Kennedy SW. 2008. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. *Environ Sci Technol* 42:7535–7541.
40. Chen G, Bunce NJ. 2003. Polybrominated diphenyl ethers as Ah receptor agonists and antagonists. *Toxicol Sci* 76:310–320.
41. Peters AK, van Londen K, Bergman A, Bohonowych J, Denison MS, van den Berg M, Sanderson JT. 2004. Effects of polybrominated diphenyl ethers on basal and TCDD-induced ethoxyresorufin activity and cytochrome P450-1A1 expression in MCF-7, HepG2, and H411E cells. *Toxicol Sci* 82:488–496.
42. Peters AK, Nijmeijer S, Gradin K, Backlund M, Bergman A, Poellinger L, Denison MS, van den Berg M. 2006. Interactions of polybrominated diphenyl ethers with the aryl hydrocarbon receptor pathway. *Toxicol Sci* 92:133–142.
43. Rifkind AB, Firpo A Jr, Alonso DR. 1984. Coordinate induction of cytochrome P-448 mediated mixed function oxidases and histopathologic changes produced acutely in chick embryo liver by polychlorinated biphenyl congeners. *Toxicol Appl Pharmocol* 72: 343–354.
44. Van den Berg M, Birnbaum L, Bosveld ATC, Brunström B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FXR, Liem AKD, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, Zacharewski T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ Health Perspect* 106:775–792.
45. Hoffman DJ, Melancon MJ, Klein PN, Rice CP, Eisemann JD, Hines RK, Spann JW, Pendleton GW. 1996. Developmental toxicity of PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl) in nestling American kestrels (*Falco sparverius*). *Fund Appl Toxicol* 34: 188–200.
46. Fox GA, Grasman KA, Campbell GD. 2007. Health of herring gulls (*Larus argentatus*) in relation to breeding location in the early 1990s. II. Cellular and histopathological measures. *J Toxicol Environ Health A* 70:1471–1491.
47. McNabb FMA, McNabb RA. 1977. Thyroid development in precocial and altricial avian embryos. *Auk* 94:736–742.
48. McNabb FMA, King DB. 1992. Thyroid hormone effects on growth, development and metabolism. In Schreibman MP, Scanes CG, Pang PKT, eds, *The Endocrinology of Growth, Development and Metabolism in Vertebrates.* Academic, San Diego, CA, USA, pp 393–417.
49. McNabb FMA, Fox GA. 2003. Avian thyroid development in chemically contaminated environments: Is there evidence of alterations in thyroid function and development? *Evol Dev* 5:76–82.
50. McNabb FMA, Jang DA, Larsen CT. 2004. Does thyroid function in developing birds adapt to sustained ammonium perchlorate exposure? *Toxicol Sci* 82:106–113.
51. McNabb FMA, Larsen CT, Pooler PS. 2004. Ammonium perchlorate effects on thyroid function and growth in bobwhite quail chicks. *Environ Toxicol Chem* 23:997–1003.
52. Hallgren S, Sinjari T, Hakansson H. 2001. Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Arch Toxicol* 75:200–2008.
53. Hallgren S, Darnerud PO. 2002. Polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and chlorinated paraffins (CPs) in rats—Testing interactions and mechanisms for thyroid hormone effects. *Toxicology* 177:227–243.
54. Richardson VM, Staskal DF, Ross DG, Dilberto JJ, DeVito MJ, Birnbaum LS. 2008. Possible mechanisms of thyroid hormone disruption in mice by BDE-47, a major polybrominated diphenyl ether congener. *Toxicol Appl Pharmocol* 226:244–250.
55. Goff KF, Hull BE, Grasman KA. 2005. Effects of PCB 126 on primary immune organs and thymocyte apoptosis in chicken embryos. *J Toxicol Environ Health A* 68:485–500.
56. Lavoie ET, Wiley F, Grasman KA, Tillit DE, Sikarskie JG, Bowerman WW. 2007. Effect of in ovo exposure to an organochlorine mixture extracted from double-crested cormorant eggs (*Phalacrocorax auritus*) and PCB 126 on immune function of juvenile chickens. *Arch Environ Contam Toxicol* 53:655–661.
57. Hoffman DJ, Rice CP, Kubiak TJ. 1996. PCBs and dioxins in birds. In Beyer WN, Heinz GH, Redmon-Norwood AW, eds, *Environmental Contaminants in Wildlife: Interpreting Tissue Concentrations.* CRC, Boca Raton, FL, USA, pp 165–208.
58. Heinz GH, Hoffman DJ, Klimstra JD, Stebbins KR, Kondrad SL, Erwin CA. 2009. Species differences in the sensitivity of avian embryos to methylmercury. *Arch Environ Contam Toxicol* 56: 129–138.
59. de Roode DF, van den Brink NW. 2002. Uptake of injected PCBs from the yolk by the developing embryo. *Chemosphere* 48:195–199.
60. Maervoet J, Beck V, Roelens SA, Covaci A, Voorspoels S, Geuns JMC, Darras VM, Schepens P. 2005. Uptake and tissue-specific distribution of selected polychlorinated biphenyls in developing chicken embryos. *Environ Toxicol Chem* 24:597–602.