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A THREE-GENERATIONAL STUDY OF *IN OVO* EXPOSURE TO PBDE-99 IN THE ZEBRA FINCH

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Abstract—Based on a literature review of avian data for polybrominated diphenyl ethers (PBDEs), ecologically relevant doses, low (10 ng/egg), medium (100 ng/egg), and high (1,000 ng/egg) of the 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) congener along with dimethylsulfoxide (DMSO) control were injected into the yolk sac of un-incubated eggs of zebra finch, *Taeniopygia guttata*. Offspring development and adult phenotype were followed over three generations. No effects of in ovo PBDE exposure on hatching success, chick growth, thyroid hormone levels, or hematological traits were measured at sexual maturity (90 d posthatching). However, the authors did detect significant effects of BDE-99 treatment on adult phenotype of in ovo–exposed birds by breeding observations, in which clutch size was significantly smaller in all PBDE-dosed birds (low, medium, and high) compared with controls. A trend was also seen for longer laying intervals in PBDE-dosed birds (13–14 d) compared with control birds (8 d). In addition, a significant effect of PBDE was found on growth of the second-generation offspring of in ovo–treated females; body mass was significantly lower in the high-PBDE dosed birds compared with controls from hatch through to fledging (day 30). The authors found no evidence of effects over the longer term and in successive generations, whether in adult, reproductive phenotype of the second-generation offspring of in ovo–treated birds, or in the growth of their (third-generation) offspring. Their results suggest that egg levels as low as 10 ng/g BDE-99 may affect reproduction in small passerines by reducing clutch size. Environ. Toxicol. Chem. 2013;32:562–568. © 2012 SETAC

Keywords—Polybrominated diphenyl ethers-99 Zebra finch Reproduction Avian Clutch size

INTRODUCTION

Polybrominated diphenyl ether (PBDEs) compounds are persistent, bioaccumulative, and potentially toxic to humans and wildlife [1,2]. Polybrominated diphenyl ethers and other chemical flame retardants are added to high-impact polystyrene, electronic housings, furniture foams, and fabrics to increase the temperature of combustion [3]. Until recent restrictions on their usage, PBDEs belonged to the largest market group of flame retardants because of their low cost and effectiveness [1]. Being physically blended but not bonded chemically to polymeric materials, PBDEs can leach into nearby systems. Like polychlorinated biphenyls (PCBs) and organochlorine pesticides before them, PBDEs have now pervaded the global environment, being found far from industrial or urban centers of production and use. Over the past two decades, concentrations in environmental samples from many locations have increased rapidly [4–7], but at least the pentabrominated components may now be decreasing in some systems subsequent to decreased usage [8,9].

Of the 209 PBDE congeners, the most common reported in wildlife samples are BDE-47, -99 (also known as penta-PDE), and -153 [10,11]. In the present study we focused on a major component of the penta-BDE commercial mixture, a lower brominated PBDE congener, PBDE-99 (five bromines). The PBDE-99 is a viscous liquid used primarily in textiles as an additive (up to 30% of wt) to polyurethane foams [12]. Of particular concern, Eriksson et al. [13] and Alm et al. [14] reported developmental neurotoxicity in mice with this congener. Branchi et al. [15], studying the perinatal effect of PBDE-99 in mice, reported that a relatively high dosage of 6 mg/kg/d dose decreased the number of pups per litter, whereas the even higher dosage (30 mg/kg/d) led to a delay in sensory motor development. Viberg et al. [16] reported that neonatal exposure to PBDE-99 in rats can affect both spontaneous behavior and the cholinergic system in adult animals. Overall limited toxicological data are available for birds, especially on reproductive endpoints, and no multigeneration studies are available of embryo and chick development [2].

Here we used egg-injection [17] to study the effects of in ovo exposure to PBDE-99 on offspring development and adult phenotype over three generations in the zebra finch (*Taeniopygia guttata*). Based on a literature review of PBDEs in avian eggs worldwide (Supplemental Data, Table S1), we determined an ecologically relevant dosing range. The first (parental) generation was then exposed via egg injection to low, medium, and high doses of BDE-99. We then tested specific hypotheses related to effects on a range of biological endpoints, including embryonic development and egg fate (e.g., infertile, hatching, not hatching), chick growth during the nestling period, mass and size at fledging (30 d of age), and sexual maturity (90 d of age), female reproductive performance (breeding propensity, egg and clutch size, breeding success), and hematological and thyroid hormone measurements. Longer-term intergenerational effects of BDE-99 were followed by rearing chicks from the in ovo–dosed, first-generation female parents. We repeated assessments of embryo and chick growth, and adult mating and reproductive phenotype in the second (offspring) and third generation (grand-offspring) birds.

MATERIALS AND METHODS

Animal care

Zebra finches were maintained in controlled environmental conditions [17], with the same diet and supplementary egg food

All Supplemental Data may be found in the online version of this article.

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schedule. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit no. 864B-08 in accordance with guidelines from the Canadian Committee on Animal Care.

Dosing solution

For the egg injection, we used three different concentrations of PBDE-99 (CAS No. 60348-60-9) dissolved in 5 μl dimethylsulfoxide (DMSO). Doses were based on environmentally relevant PBDE-99 concentrations (Supplemental Data, Table S1) for small passerine birds [18–20]. The nominal concentrations of the three dosing solutions were 2 ng/μl, or 10 ng/egg (low dose), 20 ng/μl, or 100 ng/egg (medium) and 200 ng/μl, or 1,000 ng/egg (high) dose levels. Chemical analysis in the laboratory of Dr. R. Letcher confirmed the actual concentrations of these dosing solutions as 1.9 ng/μl, 18.8 ng/μl, and 229.3 ng/μl for the low, medium, and high solutions, respectively, by methods described in Winter et al [17]. Control birds received DMSO injection only. The BDE-99 (2,2′,4,4′,5-PentaBDE) was obtained from Cambridge Isotope Laboratories, dissolved in nonane at a concentration of 50 μg/ml, 1.2 ml per vial.

Breeding and egg-injection protocol

Fifty pairs of birds were randomly assigned to breeding with initial measurements and breeding conditions described previously [17]. Nest boxes were checked daily between 9:00 AM and 12:00 noon Pacific Standard Time, and all new eggs were weighed (±0.001 g) and numbered to obtain data on egg size, clutch size, and laying interval (the time between pairing and laying of the first egg). Newly laid eggs were randomly assigned to either control DMSO ($n = 57$) or low ($n = 57$), medium ($n = 59$), and high ($n = 59$) PBDE dose injection, so that each clutch contained eggs with all four different treatments. All eggs were injected on the day of laying (day 0).

Eggs were injected as described in detail previously [17]. Briefly, a hole was made with a sterile needle, and DMSO or a DMSO-containing chemical was injected 7 mm deep into the yolk sac. A constant volume (5 μl/egg) of DMSO was injected into the yolk using a sterile Hamilton syringe. Our injection volume represents approximately 0.5% of egg volume (1 g = 1 ml). The hole was then sealed with Vetbond.

In total, 232 eggs were injected (control DMSO [$n = 57$] or low [$n = 57$], medium [$n = 59$], and high [$n = 59$]), but 10 eggs were destroyed soon after treatment, and we excluded them from initial statistics (hatching success and so forth). We therefore had 222 eggs (control = 54, low = 53, medium = 54, 54, high = 59); of these, 99 chicks hatched. However, only 79 chicks survived to the sexual maturity stage, and therefore, only 79 participated in the final growth and reproduction test studies.

Nest boxes were monitored daily to record missing or broken eggs. Before hatching, nest boxes were checked three times per day (9:00 AM, 1:00 PM, 5:00 PM Pacific Standard Time) to determine hatching success, and the exact duration of incubation, of each egg. All newly hatched chicks were weighed within 24 h of hatching and were individually marked by clipping down feathers from specific feather tracts using a unique combination to identify each chick within a brood. At day 8 posthatching, all chicks were permanently banded using numbered metal bands. Hatching success per treatment was recorded, and all chicks were weighed at days 0, 7 (rapid growth phase), 30 (= age of independence), and 90 (= sexual maturity) posthatching. At 30 and 90 d, blood samples were obtained for hematocrit and hemoglobin analysis.

Measurements of reproductive phenotype and second-generation effect

Chicks were separated from their parents at 30 d of age, and maintained in nonbreeding groups until they could be sexed with appearance of sexually dimorphic plumage, and thereafter were kept in single-sex groups. At sexual maturity (90 d of age), we then assessed reproductive phenotype or individual quality of these second-generation offspring of the in ovo treated females by breeding.

Females were bred at 90 d of age with an experienced (successfully bred), clean, unrelated adult male, and laying interval, clutch size, and egg mass were recorded. If no eggs were laid within 14 d of pairing, the male was replaced, and females were given a second trial with a new mate. Chicks were individually marked and maintained as described previously, and weighed at days 0, 7, 30, and 90.

After completion of breeding, all birds from the first, parental, in ovo treated generation were killed, and blood samples were taken for thyroid hormone analysis.

Measurement of reproductive phenotype and third-generation effect

At sexual maturity (90 d of age), we assessed reproductive phenotype or individual quality of the third-generation, grandoffspring of the in ovo treated females using mating trials in males, and by breeding in females, as described. Chicks from this third generation were individually marked and maintained as described, and weighed at days 0, 7, 30, and 90. No birds from the second or third generation were blood sampled or killed.

Hematological assay

Hematocrit (%) was measured as packed cell volume using a centrifuge (3 min at 13,000 *g*). Hemoglobin (Hb; g/dl) was measured by the cyanmethemoglobin method [20] with a microplate spectrophotometer using 5 μl whole blood diluted in 1.25 ml Drabkin's reagent (D5941 Sigma Aldrich Canada) with absorbance measured at 540 nm. Intra-assay and interassay coefficients were 1.53 to 2.25% and 4.38%, respectively.

Thyroid hormone assay

Plasma total (TT4 and TT3) and free (fT4 and fT3) concentrations of thyroxin (T4) and triiodothyronine (T3) hormones were measured using Accu-Bind ELISA Microwells test system (225–300 Monobind), following the manufacturer's standard protocol. This method is based on a competition reaction between the enzyme conjugate and thyroid hormones for a limited number of antibody-combining sites immobilized on the microplate well. We validated kits for parallelism and recovery using zebra finch plasma. In each plate we included hen plasma from a plasma pool to assess reproducibility and intra-assay precision, as well as quality control standards (Monobind ML-300) that we verified were in the expected range.

All samples were randomly distributed and analyzed within two assay kits for each type of assay. The detection limits for the assay were 0.15 μg/dl for TT4, 0.03 ng/ml for TT3, 0.03 ng/dl for fT4, and 0.44 pg/ml for fT3. We did not have sufficient plasma to measure both hormones, bound and free, for all individuals, but to maximize sample sizes and statistical power we used data for all measured individuals in analysis of treatment effect for each hormone (so sample sizes differ among hormones, bound and free; see Table 1). Average coefficients of

Table 1. Thyroid hormone levels in in ovo-treated birds measured after mating and reproduction trials as sexually mature adults (>90 d posthatching)[a]

Treatment	Total T4 (μg/dl)	Total T3 (ng/ml)	Free T4 (ng/dl)	Free T3 (pg/ml)
DMSO (5M:10F)	0.76 ± 0.13	0.64 ± 0.14	0.59 ± 0.10	3.16 ± 0.46
Low (9M:11F)	0.63 ± 0.10	0.62 ± 0.10	0.60 ± 0.10	3.43 ± 0.52
Medium (10M:11F)	0.76 ± 0.11	0.67 ± 0.11	0.76 ± 0.10	3.88 ± 0.44
High (9M:14F)	0.64 ± 0.10	0.81 ± 0.09	0.74 ± 0.08	3.78 ± 0.41

[a] Sexes have been pooled (see *Results*). Values are means ± standard error with sample sizes for males and females indicated in parentheses.
DMSO = dimethylsulfoxide.

variation based on two quality standards across each type of assay were 1.85 to 4.14% for TT4, 5.48 to 1.96% for TT3, 3.70 to 7.59% for fT4, and 1.10 to 4.35% for fT3. Intra-assay coefficients calculated as an average coefficient of variation based on samples from the same plate were 6.97 to 8.66% for TT4, 6.46 to 7.08% for TT3, 5.50 to 6.23% for fT4, and 5.22 to 5.85% for fT3. All sample tests were done in duplicates. Readings with a coefficient of variation greater than 15% were excluded from the final data set [21].

Statistical analysis

All statistical analyses were conducted using the SAS statistical computing system, Version 9.1.3 (SAS Institute 2003). Effect of treatment on egg fate and hatchability was tested using χ^2 (proc Freq), with the Fisher's exact test for small sample sizes. Treatment effects on chick growth were analyzed using generalized mixed linear models (proc MIXED) to compare chicks from DMSO, oil, sham-injected, and control eggs, with brood as a random factor, and with other covariates included in the model as necessary (e.g., egg mass, number of chicks per brood; see *Results*). We then used post hoc multiple comparisons with Bonferroni correction to identify specific pairwise differences among treatments. Values are presented as least square means ± standard error of the mean unless otherwise stated.

RESULTS

Parental, in ovo–treated, birds (generation 1)

There was no difference in mean egg mass for eggs subsequently assigned to control (1.035 ± 0.022 g) or low (1.049 ± 0.022 g), medium (1.051 ± 0.022 g), or high PBDE treatments (1.043 ± 0.021 g; $F_{3,179} = 0.63$, $p > 0.59$). Similarly, mean egg laying sequence of injected eggs did not differ by treatment (control, 3.4 ± 0.3; low, 3.6 ± 0.3; medium, 3.8 ± 0.3; high, 3.7 ± 0.3; $F_{3,179} = 0.36$, $p > 0.78$). In other words, eggs were randomly assigned to either DMSO or PBDE-99 treatment with respect to mass and laying sequence.

Egg/embryo fate and hatching success. There was no effect of treatment on the distribution of different egg/embryo fates ($\chi^2 = 8.75$, $df = 12$, $p > 0.72$). Because cell sample sizes were small for some fates, we pooled data to compare hatched versus all not hatched eggs. No effect of treatment on overall hatching success was seen ($\chi^2 = 1.67$, $df = 3$, $p > 0.64$), and pooled hatching success for all treatments was 45%. No effect of treatment was seen on offspring sex ratio ($\chi^2 = 0.89$, $df = 3$, $p > 0.82$), overall 33 (41.7%) males and 46 females (58.2%) hatched.

Chick growth. We compared patterns of chick growth from 0 to 90 d using chick mass as the dependent variable, age, sex, and treatment as main effects, and brood and band number as random factors. Not surprisingly, highly significant effect of age ($F_{3,213} = 4160.3$, $p < 0.001$) and egg mass ($F_{1,54.2} = 8.06$, $p < 0.007$) on chick mass were seen. Treatment*sex*age ($F_{12,213} = 1.51$, $p > 0.13$) and treatment*age interactions ($F_{9,213} = 1.22$, $p > 0.29$) were not significant, and no overall effect of treatment ($F_{3,59.2} = 0.31$, $p > 0.82$) was seen.

A significant effect of sex was found in the model ($F_{1,69.7} = 6.00$, $p < 0.017$). On average, females had higher body mass than males. We therefore compared the effect of treatment on chick mass with age for each sex separately, controlling for effects of egg mass and brood size at hatching. However, no significant effect of treatment on chick mass from hatching day to day 90 was seen in either females ($F_{3,151} = 2.12$, $p > 0.1$) or males ($F_{3,22.8} = 0.92$, $p > 0.45$). Similarly, no effect of treatment was seen on tarsus length measured at 90 d of age ($F_{3,59.3} = 1.00$, $p > 0.40$). We excluded sex from the model and found no treatment effect on hatching mass ($F_{3,69} = 0.50$, $p > 0.68$), 7-d mass ($F_{3,71} = 1.33$, $p > 0.27$), 30-d mass ($F_{3,67} = 0.17$, $p > 0.91$), and 90-d mass ($F_{3,74} = 0.32$, $p > 0.80$).

Hematocrit and plasma hemoglobin. No significant effect of treatment on hematocrit at 90 d of age was seen ($F_{3,61.3} = 0.49$, $p > 0.69$): control, 54.2 ± 2.1%; low PBDE, 54.6 ± 1.7%; medium PBDE, 53.1 ± 1.7%; and high PBDE, 52.5 ± 1.8%. Similarly, there was no effect of treatment on plasma hemoglobin at 30 d and at 90 d of age ($F_{3,64.3} = 0.08$, $p > 0.97$): control, 17.11 ± 0.86 g/dl; low, 16.813 ± 0.708 g/dl; medium, 16.705 ± 0.703 g/dl; and high, 17.040 ± 0.741 g/dl.

Plasma thyroid hormones. In in ovo–treated birds sampled at older than 90 d of age, no treatment effect was found for total T4 level ($F_{3,55} = 0.48$, $p > 0.6$), total T3 level ($F_{3,52.8} = 0.84$, $p > 0.4$), free T4 level ($F_{3,51} = 0.90$, $p > 0.4$), and free T3 level ($F_{3,40} = 0.54$, $p > 0.6$; Table 1). We also did not observe any significant sex ($p > 0.30$) or treatment*sex interaction effect ($p > 0.10$) for plasma levels of either thyroid hormone, bound or free.

Adult female reproductive phenotype and reproductive success of in ovo–treated birds. Sample sizes for in ovo–treated females that reproduced when paired at 90 d of age were DMSO, $n = 6$; low PBDE, $n = 7$; medium PBDE, $n = 10$; and high PBDE, $n = 9$. No effect of treatment on body mass at pairing ($F_{3,31} = 0.11$, $p > 0.90$), mean egg mass ($F_{3,32.9} = 0.35$, $p > 0.75$), or mean duration of the incubation period ($F_{3,27} = 0.18$, $p > 0.90$; Table 2) was seen. No significant treatment effect was seen on laying interval ($F_{3,31} = 1.33$, $p > 0.25$; Table 2), likely because of the large variance in this trait, but mean laying interval was 8 d in DMSO-treated females and 14 d in each PBDE-treated dose group (Table 2). Controlling for variation in laying interval, a significant effect of treatment was seen on mean clutch size ($F_{3,31} = 3.03$, $p < 0.05$; Table 2): clutch size was smaller in the low ($p = 0.027$) and medium ($p = 0.007$) PBDE groups, and marginally significantly smaller in the high PBDE group ($p = 0.068$), compared with DMSO females.

No effect of treatment was seen on egg or embryo fate of eggs laid by these in ovo–treated females ($\chi^2 = 10.0$, $df = 12$, $p > 0.60$, $n = 159$ eggs total; however, sample sizes were small

Table 2. Reproductive data for in ovo–treated females (generation 1) and daughters of in ovo–treated females (generation 2)[a]

Generation	Trait	DMSO	Low PBDE-99	Med. PBDE-99	High PBDE-99
In ovo–treated females (generation 1)	Body mass (g)	15.1 ± 0.6	15.6 ± 0.6	15.3 ± 0.5	15.5 ± 0.5
	Mean egg mass (g)	1.072 ± 0.031	1.066 ± 0.030	1.047 ± 0.025	1.085 ± 0.028
	Laying Interval (d)	8.1 ± 2.6	14.0 ± 2.4	14.1 ± 2.0	13.7 ± 2.1
	Clutch size	6.5 ± 0.6	4.6 ± 0.5	4.3 ± 0.4	5.0 ± 0.5
	Incubation duration (d)	12.8 ± 0.5	12.7 ± 0.5	12.8 ± 0.4	13.1 ± 0.5
	Brood size (fledging)	3.3 ± 0.8	2.7 ± 0.7	2.7 ± 0.6	3.1 ± 0.6
Daughters of in ovo–treated females (generation 2)	Body mass (g)	16.6 ± 0.7	15.5 ± 1.3	15.6 ± 0.9	15.9 ± 0.8
	Mean egg mass (g)	1.080 ± 0.021	1.020 ± 0,037	1.074 ± 0.024	1.026 ± 0.026
	Laying interval (d)	8.0 ± 1.1	6.0 ± 2.0	7.5 ± 1.4	10.3 ± 1.3
	Clutch size	5.0 ±0.5	7.2 ± 0.9	5.2 ± 0.6	5.3 ± 0.6
	Incubation duration (d)	12.1 ± 0.5	12.5 ± 0.8	12.2 ± 0.7	12.4 ± 0.6
	Brood size (fledging)	2.2 ± 0.7	4.3 ± 1.1	3.1 ± 0.8	2.3 ± 0.8

[a] Values are means ± standard error except for clutch size, which is least square mean ± standard error, controlling for laying interval.
DMSO = dimethylsulfoxide; PBDEs = polybrominated diphenyl ethers.

for some cells). We pooled data to compare hatched versus all not hatched eggs, but no significant effect of treatment was found on overall hatching success ($\chi^2 = 0.37$, $df = 3$, $p > 0.90$). Pooling all treatments, overall hatching success was 61.7%. No effect was seen of treatment on offspring sex ratio ($\chi^2 = 3.15$, $df = 3$, $p > 0.35$). Overall, 44 males (51.2%) and 42 females (48.8%) were hatched. Finally, no effect was seen of maternal treatment on brood size at hatching ($F_{3,31} = 0.22$, $p > 0.80$) or brood size at fledging ($F_{3,31} = 0.19$, $p > 0.85$; Table 2).

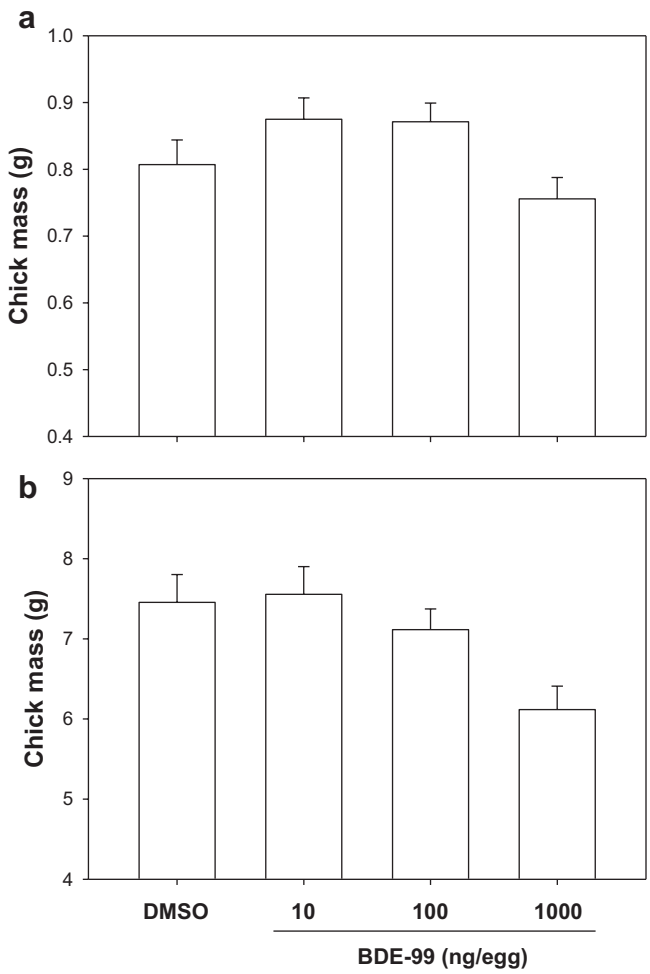

Fig. 1. Effect of maternal in ovo brominated diphenyl ether (BDE)-99 exposure on body mass of second-generation male offspring at (**a**) hatching, and (**b**) 7 d posthatching. Values are least square means ± SE.

Growth and reproductive phenotype of offspring of in ovo*–treated females (generation 2)*

Chick growth. In an overall analysis of chick growth in relation to maternal treatment, with sex, age, and treatment as main factors and brood and band number as random effects, a significant effect of sex ($F_{1,74.4} = 5.52$, $p = 0.021$), a treatment*age interaction ($F_{9,231} = 2.03$, $p = 0.037$), and a marginally significant treatment*sex*age interaction ($F_{12,231} = 1.76$, $p = 0.057$). We therefore analyzed each sex separately for an effect of maternal treatment on chick growth.

In female offspring no effect was seen of maternal treatment of growth ($F_{3,16} = 0.83$, $p > 0.4$) and no treatment*age interaction ($F_{9,111} = 0.63$, $p > 0.7$), although not surprisingly a strong effect of age was found ($F_{3,111} = 1267.68$, $p < 0.001$). In contrast, in male offspring, a highly significant maternal treatment*age interaction was found ($F_{9,120} = 4.08$, $p < 0.001$) and a marginally significant main effect of treatment ($F_{3,15.6} = 2.80$, $p = 0.074$), as well as an effect of age ($F_{3,120} = 2860.17$, $p < 0.001$). Therefore, in male offspring we compared chick mass by maternal treatment for each age separately.

In male offspring, a significant effect was found of maternal treatment on hatching mass ($F_{3,39} = 3.27$, $p = 0.031$, controlling for egg mass). Although chicks from high-PBDE mothers were not different from control chicks, they were lighter than low- and medium-PBDE chicks ($p < 0.05$ in both cases, Fig. 1A). Similarly, a significant effect of maternal treatment on male chick mass was seen at day 7 ($F_{3,16} = 4.56$, $p = 0.017$); chicks from high-PBDE–treated mothers were lighter than control chicks ($p = 0.009$), and were also lighter than low-PBDE ($p = 0.006$) and medium-PBDE chicks ($p = 0.020$, Fig. 1B). The effect of treatment on male chick mass was marginally significant at day 30 ($F_{3,16.1} = 2.61$, $p = 0.087$), but no effect of treatment on chick mass or tarsus length was seen at day 90 posthatching ($p > 0.20$).

Adult female reproductive phenotype in daughters of in ovo*–treated females*

Sample sizes for second-generation female offspring of in ovo–treated females that reproduced when paired at 90 d of age were DMSO, $n = 12$; low PBDE, $n = 4$; medium PBDE, $n = 8$; and high PBDE, $n = 9$. No effect was seen of maternal treatment on their daughter's body mass at pairing ($F_{3,32} = 0.35$, $p > 0.75$), mean egg mass ($F_{3,37.9} = 1.30$, $p > 0.25$), laying interval ($F_{3,32} = 1.33$, $p > 0.25$), clutch size ($F_{3,32} = 1.58$, $p > 0.20$, controlling for laying interval), or incubation duration ($F_{3,25} = 0.09$, $p > 0.90$; Table 2).

No effect was found of maternal treatment on egg or embryo fate of eggs laid by female offspring of in ovo–treated females ($\chi^2 = 8.78.0$, $df = 9$, $p > 0.40$, $n = 176$ eggs total; although samples sizes were small for some cells). We pooled data to compare hatched versus all not hatched eggs, but no significant effect of treatment on overall hatching success was found ($\chi^2 = 2.38$, $df = 3$, $p > 0.40$). Pooling all treatments, overall hatching success was 52.6%. No effect was seen of maternal treatment on the sex ratio of their offspring's chicks ($\chi^2 = 0.52$, $df = 3$, $p > 0.90$): overall 48 males (57.8%) and 35 females (42.2%) were hatched. Finally, no effect of maternal treatment on their female offspring's breeding success was found: brood size at hatching ($F_{3,32} = 0.73$, $p > 0.50$) or brood size at fledging ($F_{3,32} = 1.02$, $p > 0.40$; Table 2).

Growth of grand-offspring of in ovo*–treated females (generation 3)*

In an overall analysis of chick growth in third-generation offspring in relation to grand-maternal treatment, with sex, age, and treatment as main factors and brood and band number as random effects, a significant effect of age was found ($F_{3,279} = 126.62$, $p < 0.001$) but no effect of treatment ($F_{3,18.9} = 1.11$, $p > 0.30$) or sex ($F_{1,291} = 0.98$, $p > 0.30$). Furthermore, none of the two- or three-way interactions between treatment, sex, and age were significant ($p > 0.20$ in all cases).

DISCUSSION

We tested for effects of single in ovo PBDE exposure, with an environmentally relevant dose, to approximate natural exposure through maternal transfer of contaminants to eggs. We chose early egg injection to ensure PBDE is present, provided it is not metabolized [17], during the initial stages of embryo development. If early embryo development is critical and particularly sensitive to PBDEs, the potential existed for observing later life stage effects from early exposure of the embryo. In addition, the egg injection method can overcome difficulties with feeding trials [22] and costs of exposing a large number of adult healthy breeders. However, it brings uncertainty in the rate of uptake, absorption, and chemical metabolism [23]. To address that issue, we previously analyzed the content of injected eggs at day 3 of incubation, body carcasses at hatching, and livers of adult birds, at day 150, after reproductive phenotype tests [17]. That study confirmed in ovo dosing as a reliable method for studies of embryotoxicity of xenobiotics in small songbirds such as the zebra finch, which is increasingly being used as an avian model in toxicological studies.

In the present investigation, no effects were seen of in ovo PBDE exposure on hatching success, chick growth, thyroid hormone levels, or hematological traits measured at sexual maturity (90 d posthatching). However, we did observe effects of BDE-99 treatment on adult phenotype of in ovo–exposed bird: clutch size was significantly smaller in all PBDE-dosed birds (low, medium and high) compared with controls. A trend also was seen for longer laying intervals in PBDE-dosed birds (13–14 d) compared with control birds (8 d). In addition, a significant effect of PBDE on growth of the second-generation offspring of in ovo–treated females was seen; body mass was significantly lower in the high-PBDE dosed birds compared with controls from hatch through to fledging (day 30). However, we found no evidence of longer-term multigenerational effects in either adults, reproductive phenotype of the second-generation offspring of in ovo–treated birds, or the growth of their (third-generation) offspring.

Previous studies that have evaluated developmental and reproductive effects of environmentally relevant concentrations of PBDE-mixtures using in ovo exposure via egg injection were all conducted in relatively large avian species with larger egg sizes (15–60 g). Fernie et al. [24,25] injected American kestrel (*Falco sparverius*) eggs into the air sac with a PBDE mixture of BDE-47, -99, -100, and -153 dissolved in safflower oil (2.1 μg/μl, or 16–27 μl per egg) relatively late in incubation, day 19, and then fed nestlings daily with the same PBDE mixture through day 29 posthatch, with the mean daily dose equivalent to 15.6 ng/g/d. No significant effect was seen on hatching and fledgling success, although that study design confounds early pre-hatching effects with posthatching effects. Fernie et al. [24,25] did report that PBDE-exposed kestrels were heavier at day 21 and at fledging (day 27), and they gained weight more quickly than control birds, although effects were only marginally significant. McKernan et al. [26] examined the effects of penta-BDE exposure in avian embryos of domestic chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel through air cell administration of a commercial PBDE mixture on day 4 (chicken eggs) and day 5 (mallard and kestrel eggs) of artificial incubation and found that 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. They suggested that a lowest observed adverse effect level (LOAEL) for impaired pipping and hatching success could be as low as 1.8 μg/g egg wet weight [26]. Finally, Marteinson et al. [27] examined the effect of embryonic exposure of DE-71 (a commercial PBDE mixture) on reproduction in male American kestrels via maternal transfer by feeding mothers. They reported lower reproductive success in pairs with high-exposed males, and female partners of high-exposed males laid smaller clutches, and produced smaller eggs with reduced fertility.

We observed no significant differences between the control and BDE-99–exposed groups for any of the plasma thyroid hormones we measured. Our plasma values for T3 and T4 fell within the known range for passerine birds [28], and they correspond to other results from our laboratory (M. Eng, Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada). Van den Steen et al. [29] studied the effect of environmentally relevant PBDE-mixture dose on European starlings (*Sturnus vulgaris*), where adult female birds had received an implantation dose of approximately 150 μg (~1,740 μg/kg bodyweight). Plasma concentrations of triiodothyronine (T3) and thyroxine (T4) were measured before and after implantation (14 d, two months, and six months after implantation) with no significant differences between the control and exposed groups (although a trend was seen for T3 concentrations to be lower in the exposed group compared with the control group 14 d after implantation). Exposure of American kestrels before and after hatching to different PBDE congeners resulted in no significant differences in any thyroid parameter between treatment or control birds, but some weakly negative associations between plasma T4 and body burden of various congeners, including BDE-99 [30]. The latter study was done on fledgling kestrels, whereas in the present study and that of van der Steen et al. [29], only reproductively matured adults were sampled. Cesh et al. [31] reported a significant negative relation between plasma PCBs, but not PBDEs, and T4 in bald eagle (*Haliaeetus leucocephalus*) nestlings but not adults. Because of the small size of a zebra finch, and the relatively low numbers per group, we did not collect any blood samples for thyroid hormone assay before the end of the reproductive phenotype test

for the exposed generation. Therefore, we do not know whether thyroid hormone levels in the exposed group were affected at an earlier developmental stage. Future studies should consider possible interactions between BDE-99 and bird reproductive hormones, such as estradiol and prolactin, because many of thyroid-active environmental chemicals do not solely target the thyroid system [32–34].

We found no effect of in ovo PBDE exposure on hematocrit or on hemoglobin, which is consistent with van der Steen et al.'s [29] study on European starlings but in contrast with some previous studies on mammals. Leijs et al. [35] reported a positive correlation between serum levels of hemoglobin and serum concentrations of PBDEs. Hematocrit in ranch mink (*Mustela vison*) exposed to a PBDE mixture was reported to be significantly lower compared with control mink [36]. Neale et al. [37] found erythrocyte level to be inversely correlated to PBDE concentrations in harbor seal (*Phoca vitulina*). Based on those contradictory results for birds and mammals, we suggest that the blood system of a bird may be less sensitive to the effects of PBDE exposure, possibly because of hematological differences between those taxa [29].

A principle aim was to investigate possible longer-term, intergenerational effects of embryonic exposure to BDE-99. We found an effect of BDE-99 exposure on adult reproductive phenotype, as well as an effect on chick growth in the second-generation offspring of PBDE-exposed females. All treatment groups, regardless of the BDE-99 dose, showed significantly lower average clutch size (1.8–1.2 eggs less) than the control group. Clutch size and laying interval are negatively correlated in zebra finches: the longer the time between pairing and laying of the first egg, the smaller the clutch size [38]. The mean laying interval within our control group of approximately 8 to 9 d corresponds to the typical laying interval for a three-month inexperienced bird [39], whereas mean laying intervals for all PBDE-exposed groups fell into the range of 13 to 14 d. The typical clutch size for an inexperienced three-month zebra finch female is 5.7 eggs [40], close to the control group clutch in our experiment, and one egg larger on average than those of PBDE-exposed birds. The reduced clutch size effect was robust when we controlled for laying interval. At the same time, a trend was seen for delayed laying interval in all exposed groups. We believe that the reduced clutch size in our experiment may be related to a delay in egg laying, and that a shorter laying interval is also a possible treatment effect. Possibly PBDE-dosed birds were less sensitive to male courtship behavior or needed a longer time to develop their reproductive system in response to male courtship. Consistent with that idea, Eng et al. [40] found that early exposure of zebra finch chicks to BDE-99 had significant effects on male mating behavior and the response of clean experienced females to exposed males.

Blight [41] reported a significant decline in clutch size of the glaucous-winged gull (*Larus glaucescens*) at colonies in the Salish Sea region of the Pacific coast of Canada. Declines in the availability and quality of prey were thought to be the primary cause. Contaminants, including PBDEs, were judged not to be a factor because of the lack of coincidence in timing of an increase in PBDE residues and the decline in clutch size. However, that study's data indicate that the greatest decrease in clutch size occurred between the 1980s and 2010, a period over which PBDEs increased exponentially in eggs of fish-eating birds in that region [6]. Given the concurrence of those two phenomena and the data from the present study showing an effect of BDE-99 on clutch size, further examination of clutch size in birds from PBDE-exposed populations is perhaps warranted.

We report an intergenerational effect of BDE-99 exposure; chicks from the parents given the highest dose of PBDE-99 had lower body mass than control and low-dosed birds at day 7 posthatching, which represents the most rapid growth stage in zebra finches. That growth difference may be caused by several factors, such as stress on the embryo of the injection, or DMSO as a vehicle, or even selection pressure on the following generations. Indeed, only good female breeders from exposed groups could produce offspring, and only good female breeders from the second generation gave rise to the third one. The body mass difference within the same generation may be attributed to a treatment effect. By age of sexual maturity, that effect disappeared.

Multigenerational studies are important in toxicology because reproductive effects may carry over to future generations. The smaller body mass within second-generation offspring may be attributed to the altered behavior of their affected parents. Highly exposed females possibly did not participate in feeding or other type of parental care at the same rate as other females. To test that hypothesis, we would recommend either a cross-fostering experiment, or monitoring of feeding rates. The short period of zebra finch maturation provides an opportunity to complete the multigenerational studies relatively quickly, at least for two generations. Third-generation analyses could be more difficult, because of increased selective pressure and reduced genetic variability. Thus, only successful female breeders could lay eggs for further reproductive testing. In addition, while comparing chicks from different broods in offspring and grand-offspring, we have to take into account inter-brood variations.

In summary, our results suggest that PBDE-99 may affect reproduction in small passerines, where wet weight concentration in the egg is as low as 10 ng/g, by costing an adult breeding pair one or two eggs per clutch. Although exposure to PBDE-99 in ovo may not influence chick survival and growth directly, the population dynamic may be changed because of smaller clutch and, therefore, lower brood size in following generations. Furthermore, PBDE-99 single exposure in ovo can have multigenerational effects. Unlike the parental group, the chicks from high dosed females showed smaller body mass at hatching and early stage of bird life. Additional research is required to confirm and explore these effects on clutch size, and to investigate potential mechanisms of PBDE interaction with reproductive hormones and neurodevelopment.

SUPPLEMENTAL DATA

Table S1. (103 KB DOC).

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