TOXICOLOGICAL SCIENCES **127(1)**, 269–276 (2012)
doi:10.1093/toxsci/kfs076
Advance Access publication February 8, 2012

Early Exposure to 2,2′,4,4′,5-Pentabromodiphenyl Ether (BDE-99) Affects Mating Behavior of Zebra Finches

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Received November 8, 2011; accepted February 2, 2012

2,2′,4,4′,5-Pentabromodiphenyl ether (BDE-99) is a brominated flame retardant congener that has pervaded global food chains, being reported in avian egg and tissue samples throughout the world. Its effects on birds are not well known, but there is evidence in exposed mammals that it directly mediates and causes neurotoxicity, alters thyroid hormone homeostasis, and lowers sex steroid hormone concentrations. In birds, those processes could disrupt the song-control system and male mating behavior. In this study, the effects of nestling exposure to environmentally relevant levels of BDE-99 were assessed in a model songbird species, the zebra finch (*Taeniopygia guttata*). A tissue residue study in which zebra finch nestlings were orally exposed to 0, 2.5, 15.8, or 50.7 ng BDE-99/g body weight (bw) per day over the 21-day nesting period validated dosing methods and confirmed dose levels were environmentally relevant (332.7 ± 141.0 to 4450.2 ± 1396.2 ng/g plasma lipid). A full-scale study exposing nestlings to 0, 2.5, 15.8, 50.7, or 173.8 ng BDE-99/g bw/day was carried out to investigate long-term effects of BDE-99 on the adult song-control nuclei volumes, song quality, and male mating behavior. Early exposure to BDE-99 had significant effects on male mating behavior and the response of clean experienced females to exposed males. There was no effect on male song-control nuclei or song quality, and there were nondose-dependent effects on female song-control nuclei. The results demonstrate that early exposure to environmentally relevant levels of BDE-99 affects the behavior of zebra finches.

Key Words: **BDE-99; PBDEs; brain; song-control system; mating behavior; birds.**

Polybrominated diphenyl ethers (PBDEs), formulated as several technical mixtures, find wide commercial use as additive flame retardants in a variety of textiles, plastics, foams, and electronic circuitry. They have become ubiquitous in environmental, human, and wildlife samples (Darnerud *et al.*, 2001) and are consistently found in avian tissue and egg samples throughout the world (Chen and Hale, 2010). 2,2′,4,4′,5-Pentabromodiphenyl ether (BDE-99) is one of the most pervasive congeners (Chen and Hale, 2010; Darnerud *et al.*, 2001) and causes a range of toxicological effects in mammals; however, there is a lack of pertinent toxicological literature concerning its effects in birds. In mammals, exposure to BDE-99 causes a range of adverse effects, including decreased neurotransmitter receptor densities (Viberg *et al.*, 2004, 2005), aberrations in spontaneous behavior (Viberg *et al.*, 2004, 2005), disruption of thyroid hormone homeostasis (Hakk *et al.*, 2002; Kuriyama *et al.*, 2007), and lowered levels of sex steroid hormones (Alonso *et al.*, 2010; Lilienthal *et al.*, 2006). If BDE-99 has similar effects in birds, exposure could significantly alter, for example, the brain regions controlling the learning and production of bird song (song-control system) and male mating behavior.

The brain structures and neural pathways that make up the song-control system develop primarily in the first few months posthatch (Bottjer *et al.*, 1985; Mooney and Rao, 1994), making the song-control system sensitive to early environmental conditions. There is evidence in songbirds that exposure to halogenated organic contaminants (dichlorodiphenyltrichloroethane and its metabolites and polychlorinated biphenyls) at early life stages results in smaller song-control nuclei volumes (Hoogesteijn *et al.*, 2008; Iwaniuk *et al.*, 2006). Nuclei volumes may also increase following exposure to estrogen mimics (e.g., 17-β estradiol, dioctylphthalate, bisphenol A, and dibutylphthalate; Markman *et al.*, 2008). If BDE-99 lowers neurotransmitter receptor densities in birds, the song-control system may be directly affected, as concentrations of nicotinic and muscarinic cholinergic receptors are present within and at the boundaries of song-control nuclei (Ryan and Arnold, 1981; Watson *et al.*, 1988), and there is evidence of cholinergic input and modulation of the song-control circuits (Li and Sakaguchi, 1997; Salgado-Commissariat *et al.*, 2004; Shea *et al.*, 2010). The role of thyroid hormones in the development of the song-control system is not well known, but

there is evidence that many of the neurons that project into the song-control nuclei have thyroxine (T4) receptors, and exposure to abnormally elevated T4 levels increases cell death in song-control nuclei of zebra finches (Tekumalla *et al.*, 2002). Disruption of thyroid hormone homeostasis by BDE-99 could therefore alter the cell number and volume of the song-control nuclei. The song-control nuclei could also be affected by any changes in sex steroid hormones caused by BDE-99, as several of the song-control nuclei contain androgen and estrogen receptors, and decreased levels of sex steroids can result in smaller nuclei (Ball *et al.*, 2002). Despite evidence that BDE-99 disrupts many of the mechanisms that can affect the song-control system, there have been no studies investigating possible PBDE-induced effects on the song-control system of birds.

Reduction in song-control nuclei volume as a result of BDE-99 exposure could lead to lowered song quality and/or song perception, as the volumes of key song-control nuclei correlate with male song characteristics, such as song complexity and duration (Garamszegi and Eens, 2004), and the song-control nuclei can also play a role in song perception in females (Brenowitz, 1991). Singing is an important aspect of reproduction in birds, serving to define territories and attract females (e.g., Krebs *et al.*, 1978; Kroodsma, 1976; Searcy, 1992). Learned features of song can act as an honest signal of male quality (Nowicki *et al.*, 2002), and females of many species prefer males with higher song rate (Collins *et al.*, 1994) and more complex and longer songs (Clayton and Prove, 1989). Developmental conditions that result in smaller song-control nuclei, and reduced song quality in males, or altered song perception in females, could ultimately disrupt pair formation and lower reproductive success.

If BDE-99 lowers sex steroid hormones in birds as it does in mammals, then we would also expect exposed male birds to exhibit decreased mating behavior as androgens and estrogens play an important role in mediating sexual behaviors in birds (Ball and Balthazart, 2004). There are reports suggesting that penta-BDE technical mixtures containing BDE-99 may reduce reproductive behavior and circulating testosterone in male birds (Fernie *et al.*, 2008; Marteinson *et al.*, 2011), but no studies have specifically looked at the BDE-99 congener.

We investigated the effect of early developmental exposure to environmentally relevant levels of BDE-99 in birds, using the zebra finch (*Taeniopygia guttata*) as a model songbird species. The zebra finch is a well-established model species that has been extensively studied in avian neuroscience and endocrinology (e.g., Ball *et al.*, 2002) and has been successfully used in toxicological dosing studies (e.g., Hoogesteijn *et al.*, 2008). Our objectives were to first assess relationships between oral dosing levels of BDE-99 and plasma and lipid tissue residues and to then conduct a full-scale study to examine the effects of BDE-99 exposure on the song-control system and male mating behavior in sexually mature adult birds. We hypothesize that adult birds that had been exposed to BDE-99 early in development in the nest have (1) smaller song-control nuclei, (2) decreased song quality (i.e., song rate, song phrase duration, and syllable repertoire size), and (3) decreased mating behavior.

MATERIALS AND METHODS

Animals and husbandry. This study was conducted on a captive colony of zebra finches maintained at the Simon Fraser University Animal Care Facility located in Burnaby, British Columbia. Zebra finches were housed in a controlled environment (temperature 19°C–23°C; humidity 35–55%; photoperiod 14 h light to 10 h dark; lights on at 07:00). All birds were provided with mixed seed (panicum and white millet 1:2; 11.7% protein, 0.6% lipid, and 84.3% carbohydrate by dry mass), water, grit, and cuttlefish bone (calcium) *ad libitum* plus a multivitamin supplement in the drinking water once per week. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (657B-96) in accordance with guidelines from the Canadian Committee on Animal Care.

For all breeding and chick rearing, the same basic protocol was followed. Experienced adult zebra finches were randomly paired and housed in individual breeding cages (51 × 39 × 43 cm) equipped with an external nest box (14 × 14.5 × 20 cm). In addition to the *ad libitum* seed diet, breeding pairs were provided with an egg food supplement (20.3% protein:6.6% lipid) daily from pairing to clutch completion (2 days after the last egg was laid) and then again during the chick-rearing stage. Nest boxes were checked daily between 09:00 and 11:00 for egg laying, and new eggs were numbered in consecutive order and weighed (0.001 g). Breeding pairs that did not produce eggs within 15 days following pairing were separated and classified as "nonbreeders." Nest boxes were then checked again daily toward the end of the 12- to 14-day incubation period to determine hatching dates. Nestlings were weighed daily, and tarsus length was measured every 5 days using digital calipers (to the nearest 0.01 mm) to assess body condition.

Dosing procedure. All dosing was done with technical grade BDE-99 (> 98% purity, Cambridge Isotope Labs, Andover, MA). BDE-99 was analyzed via gas chromatography (GC)-mass spectrometry (MS) (electron capture negative ionization mode [ECNI]) (see Chemical Analysis section below), and the only bromide ion that was quantifiable was BDE-99. The BDE-99 was dissolved in safflower oil (Spectrum Organics, Boulder, CO), and the microliter amounts of BDE-99 dissolving solvent (nonane) were evaporated off using a steady stream of purified nitrogen gas. There were four dose levels (2.5, 15.8, 50.7, and 173.8 ng BDE-99/g body weight [bw] per day) and a safflower oil-only control group. Within 24 h of hatching, individual chicks within each nest were marked with feather tract removal for identification, and dose levels were randomly assigned within the nest to account for any heritable effects. Nestlings were orally dosed daily from 24 h after hatching (day 1) until fledging (day 21), using a micropipette. Doses were adjusted daily according to chick mass, with the dose volume being 10 µl/g bw. All surviving nestlings were banded at 10 days of age. Nestlings were returned to the nests immediately after handling and dosing. There were no observed adverse effects with the dosing approach on chick growth or survival.

Tissue residue study. An initial study was conducted to assess relationships between oral dosing levels of BDE-99 and plasma and lipid tissue residues. Twenty nestlings ($n = 5$ per dose group) were dosed with 0, 2.5, 15.8, or 50.7 ng/g bw/day for 21 days. At 30 days of age, birds were euthanized via anesthetic (50:50 ketamine:rompun) followed by exsanguination. Plasma and adipose tissue samples were collected. All plasma samples ($n = 20$), a subset of adipose tissue samples ($n = 6$) and dosing solutions ($n = 4$), were analyzed for BDE-99 and six less brominated PBDE congeners (see "Chemical Analysis" subsection).

Full-scale study. Following the tissue residue study, a full-scale study was carried out using 48 male and 29 female nestlings and 5 dose groups (male control: $n = 13$, 2.5 ng/g $n = 9$, 15.8 ng/g $n = 9$, 50.7 ng/g $n = 9$, 173.8 ng/g $n = 8$; female control: $n = 6$, 2.5 ng/g $n = 6$, 15.8 ng/g $n = 5$,

50.7 ng/g $n = 6$, 173.8 ng/g $n = 6$). Birds were orally dosed between day 1–21 posthatching. Once young were independent from parents (day 30), they were placed into cages (102 × 39 × 43 cm) as juvenile groups and separated by sex once adult plumage and bill color started to form. Two adult male song tutors were placed in each juvenile cage containing males, and birds were not visually or acoustically isolated from birds in adjacent cages. Blood samples were collected at 30 and 90 days of age.

Once birds reached sexual maturity (day 90), male mating trials were conducted. For each exposed male, two mating trials were conducted over two separate days. At the start of each mating trial, an experienced clean wild-type female was randomly chosen from a pool of 60 females and placed in a cage for 5 min to acclimate alone. Different females that were novel to the experimental male were chosen for each male and trial. The cage contained two perches, grit, a cuttlefish bone, but no water or food inside. A microphone was positioned in the upper right corner of the cage. For each trial, an experimental male was placed in the cage with the experienced female, and the behaviors of both the male and the female were recorded for 10 min by an observer blind to treatment. All of the courtship trials were performed between 09:00 and 12:00 h. The following typical male courtship behaviors (described in Zann, 1996) were recorded during the trial: invitation (Y or N), bill wiping (number of wipes against perch), head or tail twisting (scored per left to right cycle), following (number of times the male followed the female), number of copulation attempts, number of successful copulations, and time in seconds to initial copulation attempt. The female response to the male was also recorded (scored 1–5; 1 = no response, 5 = solicitation of copulation). All songs were digitally recorded during the male mating trials using a Sennheiser ME62 microphone with a K6 power module, connected to a laptop computer.

Following the completion of breeding and mating trials, birds were anesthetized and then exsanguinated. Brains were immediately dissected from the cranium, weighed (0.001 g), and fixed by immersing in buffered 4% paraformaldehyde (pH 8.5) for 2 weeks. After fixation, the brains were cryoprotected in 30% sucrose for 24 h, then frozen on pulverized dry ice, and stored at −80°C until further processing.

Song analysis. Digital song recordings were measured using Syrinx-PC software (J. Burt, Seattle, WA). For each mating trial, we measured three variables of song quality: (1) the song rate (number of song phrases per hour), (2) the song phrase duration, and (3) the syllable repertoire size (number of different syllables types). Songs were analyzed based on methods in Airey and DeVoogd (2000). Duration and repertoire were estimated based on 10 song phrases for each recording period, and measures were averaged over both recordings. Birds that did not sing during either recording attempt ($N = 21$) were not included in the song analysis. All mating behaviors and song measurements were collected and analyzed blind to the treatment group of the males.

Tissue processing and neuroanatomical measurements. Using a cryostat (−20°C), we sectioned brains into 40-μm coronal sections and collected these in 0.1M PBS (pH 7.5). Tissue sections were then mounted onto Superfrost Plus microscope slides (VWR). Sections were Nissl stained with thionin, serially dehydrated in ethanol, cleared in solvent, and then protected with coverslips affixed with Permount (Fisher Scientific). An observer (M.L.E.) blind to subject and treatment group then made all measurements. Slides were examined with a bright field microscope (Leici DM5500 B) equipped with a digital microscope camera (DFC420 C). Images of sections of the song-control regions, HVC (proper name, not an acronym), area X, and RA (robust nucleus of the arcopallium), were captured (Fig. 1). The song nuclei were all readily defined by darkly stained cells relative to surrounding tissue. Telencephalon images were captured using a high-resolution (2400 dpi) flatbed scanner. The software ImageJ (version 1.42q, National Institutes of Health) was used to trace the outlines of these regions and measure their area. We then combined these areas using the formula for the volume of a cone frustum to estimate the total volume of each structure. Volume estimates were based on the areas of every 10th section for the telencephalon (400 μm intervals) and every second section for the song-control regions (80 μm intervals). Poor staining or tissue damage prevented volume reconstruction of RA and area X in three male brains and HVC and RA in three female brains.

Chemical analysis. All PBDE standards (BDE-17, -28/33, -47, -49, -66, -85, and -99) were purchased from Wellington Laboratories (Guelph, ON, Canada). Plasma, adipose tissue, and dosing solutions were analyzed for BDE-17, -28/33, -47, -49, -66, -85, and -99.

Plasma samples (0.14–0.37 g), adipose tissue samples (0.05–0.17 g) and dosing solutions (150 μl) were accurately weighed, and neutral fractions were extracted and cleaned up using established methodologies (Gauthier *et al*., 2008; Verreault *et al*., 2005). In brief, plasma samples were spiked with 50 ng of each of the internal standards (BDE-30 and -156), acidified, denatured, and liquid-liquid extracted with 50% (vol/vol) methyl tert-butyl ether (MtBE)/hexane. The organic phase layer containing the PBDEs was separated and collected. Lipid content of plasma was determined colorimetrically using olive oil as the calibration standard (Frings *et al*., 1972). Adipose tissue samples were ground with ~25 g of anhydrous sodium sulfate and extracted with 50% dichloromethane (DCM)/hexane using an accelerated solvent extraction (ASE) system (Dionex ASE 200). The extraction columns were spiked with 20 ng of each internal standard. The column extraction eluent was concentrated to 10 ml and a 10% portion was removed for gravimetric lipid determination. The remaining extracts were cleaned by gel permeation chromatography (GPC) and eluted from the GPC column with 50% DCM/hexane. The first fraction (140 ml) containing lipids and biogenic material was discarded, and the second fraction (200 ml) containing PBDEs was concentrated to a volume of ~4 ml. Dosing solution samples were initially processed with GPC and did not go through ASE extraction. The dosing solutions were spiked with 20 ng of each internal standard.

All samples were cleaned up using a silica solid phase extraction column (J.T. Baker). The column was conditioned with successive washes of 10% (vol/vol) methanol (6 ml) in DCM and then 8 ml of 5% DCM in hexane. The sample was then loaded onto the cartridge and eluted with 8 ml of 5% DCM/hexane. The eluent was then concentrated and solvent exchanged with isooctane to a final volume of approximately 175 μl. The exact mass of each sample was recorded, and the final volume determined by dividing by the density of 2,2,4-trimethylpentane (0.69 g/ml).

PBDEs in the isolated chemical fractions were analyzed using gas chromatography-mass spectrometry working in electron capture negative ionization mode (GC/ECNI-MS). Analytes were separated and quantified on an Agilent 6890 series GC equipped with a 5973 quadrupole MS detector

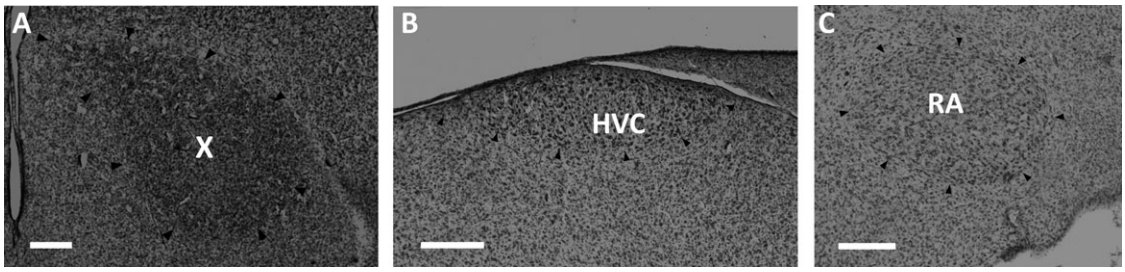

FIG. 1. Photomicrographs showing the three song nuclei measured, area X (A), HVC (B), and the robust nucleus of the arcopallium (C). The solid triangles indicate the borders delineating each region. X-area X. Scale bars = 300 μm.

(Agilent technologies, Palo Alto, CA). The analytical column was a 15 m × 0.25 mm × 0.10 μm DB-5HT fused-silica column (J & W Scientific, Brockville, ON, Canada). Helium and methane were used as the carrier and reagent gases, respectively. A sample volume of 1 μl was introduced to the injector operating in pulsed splitless mode (injection pulse at 25.0 psi until 0.50 min; purge flow to split vent of 96.4 ml/min to 2.0 min; gas save flow of 20 ml/min at 2.0 min), with the injector held at 240°C. The GC oven ramping temperature program was as follows: initial 100°C for 4.0 min, 25°C/min until 260°C, 2.5°C/min until 280°C for 10.0 min, 25°C/min until 325°C, and hold for a final 7.0 min. The GC to MS transfer line was held at 280°C, ion source temperature was 200°C, and the quadrupole temperature was 150°C.

PBDE congeners were monitored using the bromine anions of *m/z* 79 and 81. Analytes were identified by comparison of retention times and ECNI mass spectra to those of the authentic standards.

Mean internal standard recoveries for the BDE-30 was 97 ± 3% SE for plasma analysis and 73 ± 2% for the adipose tissue and dosing solution analysis. Analytes were quantified using an internal standard approach; thus, all reported values were inherently recovery corrected. The method limits of quantification (MLOQ) for BDE-99, based on a signal-to-noise ratio of 10, was 0.05 ng/g wet weight (w.w.) for plasma analysis and 1.1 ng/g w.w. for adipose and dosing solution analysis. Method blanks ($n = 4$) were included for each sample batch to assess background interference and possible contamination, and a blank subtraction was done for BDE-28/33, -47, -49, -66, and -99. BDE-17, -28/33, -47, -49, -66, and -85 were generally below the MLOQ or at sub ng/g w.w. levels and thus essentially not present in the plasma or adipose samples. Duplicate analysis of the samples was not possible as all plasma and adipose tissue were consumed to ensure quantifiable analyte levels. In-house standard reference material (polar bear [*Ursus maritimus*] plasma for plasma analysis and double-crested cormorant [*Phalacrocorax auritus*] egg for adipose and dosing solution analysis) was also included in each sample batch to ensure consistency of data acquisition (within two SD of in-house mean).

Statistical analysis. Before analysis, all nonparametric continuous variables were natural log transformed to more closely approximate normal distributions and to homogenize variance. An index of body condition of the tissue residue study birds was estimated as the residuals from a linear regression of body mass on tarsus length (Schulte-Hostedde *et al.*, 2005). Male and female brains were analyzed separately because of prominent sexual differences in anatomy (Nottebohm and Arnold, 1976) and because in females the borders of area X are poorly defined and were not measured. Left and right brain hemispheres were compared for HVC, RA, area X, and telencephalon using paired *t*-tests, and no evidence of anatomical lateralization was found, so all the analyses were performed on summed left and right volumes. Allometric effects were tested for using linear regression on the volume of each nucleus against the volume of the telencephalon. Male area X and female RA were significantly related to telencephalon volume ($F_{1,39} = 6.01$, $p = 0.019$ and $F_{1,27} = 10.05$, $p = 0.004$, respectively), so the residuals from the regression were used to represent the relative size of those nuclei. The effect of dose was assessed using generalized linear models for continuous variables, and *post hoc* tests for differences between means were adjusted for multiple comparisons following the Tukey-Kramer method. Fisher's exact probability test was used to assess effects of dose for categorical variables. To determine the repeatability of mating behaviors across the two mating trials, nested ANOVA was used following Lessells and Boag (1987). Correlations were measured by Pearson's correlation coefficient. All statistical analyses were done using SAS 9.1.3 (SAS Institute, 2003).

RESULTS

Tissue Residue Study

We measured PBDE concentrations in 20 zebra finch plasma samples ($n = 5$ per dose level) and 6 adipose samples. There was a strong dose-dependent relationship for plasma BDE-99 levels at 30 days of age among the control and dose groups, with concentrations ranging from 332.7 ± 141.0 to 4450.2 ± 1396.2 ng/g lipid weight (l.w.) (Fig. 2). In addition, adipose tissue concentrations were significantly correlated with plasma levels (for lipid normalized values, $r = 0.981$, $p = 0.0005$) and were related to each other according to the equation ([adipose BDE-99 l.w. concentration] = 1.16 × [plasma BDE-99 l.w. concentration] + 86.98). The BDE-99 dose levels in the initial tissue residue study had no effect on body condition ($F_{3,37} = 0.14$, $p = 0.934$) or survival ($p = 0.255$, Fisher's exact test, FET), and so we added an additional dose group of 173.8 ng BDE-99/g bw/day in the full-scale study. Using the significant relationship between dose group and known plasma concentrations ($r = 0.724$, $p = 0.0003$; plasma concentration = 78.38 × dose + 453.57), it was estimated that the additional 173.8 ng/g dose group would result in plasma concentrations of approximately 14079.7 ng/g l.w.

Full-Scale Study

In male zebra finches, we found no effect of dose on the size of any of the song-control nuclei or on total brain mass (Table 1). In females, HVC volume was significantly larger in the control group than the 2.5 and 50.7 ng/g dose groups ($p = 0.016$, Table 1). Female RA volume and brain mass were not significantly different across dose groups (Table 1).

Of the song characteristics, duration and repertoire size were repeatable within individual males across mating trails, with individual explaining 64.8% of the variation in phrase duration ($F_{16,17} = 4.68$, $p = 0.001$) and 76.9% of the variation in repertoire size ($F_{16,17} = 0.53$, $p < 0.0001$). Song rate was not

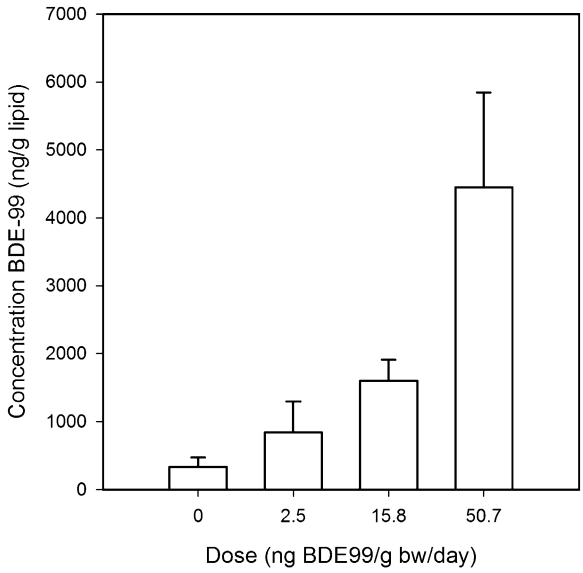

FIG. 2. 2,2′,4,4′,5-Pentabromodiphenyl ether (BDE-99) concentrations measured in zebra finch plasma on day 30 of the tissue residue study, following oral exposure to BDE-99 over the 21-day nesting period. Values represent the mean and SE of five replicates.

TABLE 1
Brain Region Volumes (Mean mm^3 ± SE) and Mass (Mean g ± SE) for Reproductively Mature Zebra Finches Exposed to 2,2′,4,4′,5-Pentabromodiphenyl Ether (BDE-99) During the Nestling Period

	Dose (ng BDE-99/g body weight/day)						
	0	2.5	15.8	50.7	173.8	F	p
Male							
HVC	0.682 ± 0.041	0.733 ± 0.026	0.734 ± 0.042	0.824 ± 0.058	0.690 ± 0.043	$F_{4,43} = 1.70$	0.167
RA	0.467 ± 0.023	0.477 ± 0.030	0.473 ± 0.023	0.528 ± 0.024	0.473 ± 0.020	$F_{4,40} = 1.08$	0.379
Relative area X	−0.110 ± 0.093	−0.146 ± 0.163	0.031 ± 0.210	0.432 ± 0.164	−0.211 ± 0.233	$F_{4,36} = 2.21$	0.088
Mass	0.444 ± 0.008	0.446 ± 0.006	0.452 ± 0.007	0.454 ± 0.008	0.454 ± 0.012	$F_{4,43} = 0.39$	0.813
Female							
HVC	0.077 ± 0.01[a]	0.036 ± 0.008[b]	0.055 ± 0.01[ab]	0.044 ± 0.006[b]	0.063 ± 0.007[ab]	$F_{4,21} = 3.88$	0.016
Relative RA	0.008 ± 0.007	−0.004 ± 0.003	0.000 ± 0.003	−0.007 ± 0.003	0.001 ± 0.005	$F_{4,21} = 1.26$	0.316
Mass	0.418 ± 0.013	0.416 ± 0.013	0.422 ± 0.014	0.402 ± 0.012	0.404 ± 0.007	$F_{4,24} = 0.56$	0.697

Note. Significant difference between groups are indicated by different lower case letters (superscript a and b) ($p < 0.05$).

repeatable ($F_{16,17} = 0.53$, $p = 0.897$, 0%) and so was not considered in further analysis. BDE-99 dosing had no effect on phrase duration ($F_{4,22} = 0.75$, $p = 0.568$) or repertoire size ($F_{4,22} = 1.06$, $p = 0.398$).

Male behaviors that were repeatable across mating trials include the number of copulation attempts ($F_{47,48} = 3.46$, $p < 0.0001$, 55.2%), the number of bill wipes ($F_{47,48} = 5.58$, $p < 0.0001$, 69.6%), whether the male invited the female ($F_{47,48} = 3.89$, $p < 0.0001$, 59.1%), and whether a male sang ($F_{47,48} = 3.85$, $p < 0.0001$, 58.7%). The response of different clean experienced females to the same male was also repeatable ($F_{47,48} = 3.10$, $p < 0.001$, 51.1%). Other mating behaviors were not repeatable and not considered in subsequent analysis.

BDE-99 exposure affected whether males engaged in courtship behavior ($p = 0.013$, FET), with significantly fewer males in the 173.8 ng/g dose group inviting females than expected (Fig. 3A). Whether a male sang or not was also significantly affected by BDE-99 exposure ($p = 0.023$, FET), with the 50.7 and 173.8 ng/g dose groups having a lower than expected proportion of birds that sang (Fig. 3B). Female response to exposed males was significantly reduced in the highest dose group (Fig. 3C; $F_{4,43} = 3.35$, $p = 0.018$). The number of bill wipes and copulation attempts were not significantly affected by BDE-99 exposure ($F_{4,43} = 1.65$, $p = 0.179$ and $F_{4,43} = 1.22$, $p = 0.315$, respectively). Within each dose group, there was no effect of female used on any of the mating trial variables ($p > 0.760$ for all variables).

DISCUSSION

In this study, we found that early exposure to BDE-99 caused significant effects in female HVC volume, male mating behavior, and the effectiveness of male courtship (as assessed by female response to exposed males), at environmentally relevant concentrations. However, there were no significant effects of BDE-99 on the size of song-control regions of the brain or on the song quality of male birds.

In our tissue residue study, we demonstrated that birds were being exposed in a dose-dependent manner, and that oral exposure to BDE-99 served as a valid dosing method. Control birds had detectable levels of BDE-99, however, lipid normalized concentrations of BDE-99 in control birds were approximately 2.5× lower than the lowest dose group, and 42× lower than the highest dose group. The safflower control oil had no detectable PBDEs, so the BDE-99 in control birds was either due to possible cross-contamination between treatment groups within the nest, or background levels of BDE-99. Plasma BDE-99 was significantly and positively correlated with adipose tissue burdens. In future studies, this close relationship can be used to estimate body burdens from nonlethal plasma sample concentrations of BDE-99.

With respect to environmental relevance, the plasma BDE-99 concentrations in the lowest dose groups are in the same range as plasma samples collected from wild birds. There are reported plasma BDE-99 concentrations of up to 638 ng/g l.w. (4.15 ng/g w.w., 0.65% average lipid) in bald eagle (*Haliaeetus leucocephalus*) nestlings on the North American west coast (McKinney *et al.*, 2006) and of up to 566.23 ng/g l.w. (8.72 ng/g w.w., 1.54% average lipid) in Glaucous gulls (*Larus hyperboreus*) in the Norwegian arctic (Verreault *et al.*, 2005). In comparison, the 2.5 ng/g bw/day dose group in our zebra finch study averaged 843.33 ng/g plasma lipid. In peregrine falcon (*Falco peregrinus*) eggs, concentrations of up to 9200 ng BDE-99/g l.w. have been reported (Lindberg *et al.*, 2004).The nestling plasma concentrations in the highest dose group in the present zebra finch study were estimated at 14079.7 ng BDE-99/g l.w. In zebra finches, the lipid-normalized BDE-99 concentration is typically four times higher in day-old nestlings compared with eggs from the same mother (Margaret L. Eng, unpublished data); therefore, the

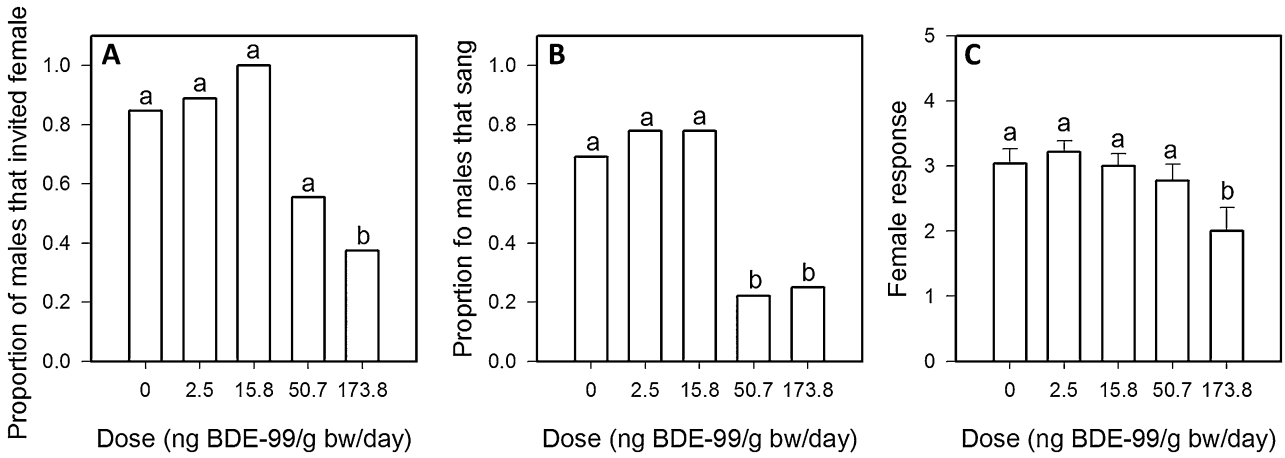

FIG. 3. Results from zebra finch mating trials between reproductively mature males that were orally exposed to 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) over the 21-day nesting period, paired with clean experienced females. (A) The proportion of males that engaged in courtship behavior was significantly lower than expected in the highest dose group ($p = 0.013$). (B) The proportion of males that sang during mating trials was significantly lower than expected in the two highest dose groups ($p = 0.023$). (C) The response of clean experienced females to BDE-99-exposed males was significantly reduced in the highest dose group ($p = 0.018$). Sample size (n) = 13, 9, 9, 9, and 8 for the control, 2.5, 15.8, 50.7, and 173.8 ng/g treatment groups, respectively. Significant difference between groups is indicated on the graph with different lower case letters ($p < 0.05$).

nestling concentrations in our highest dose group are environmentally relevant compared with reported concentrations of BDE-99 in wild bird eggs. We did not measure BDE-99 in brain tissue, however, BDE-99 is able to cross the blood-brain barrier and accumulate in the brain tissue of birds (Naert *et al.*, 2007). It is therefore possible that the effects that we observed may be the result of direct brain exposure to BDE-99, as well as indirect effects of endocrine disruption. Further studies examining the species-specific accumulation of BDE-99 in the brain would be informative for determining the underlying mechanisms of effects.

We observed no effects of early exposure to BDE-99 on neuroanatomy of the song-control system or song quality in reproductively mature male zebra finches, which was contrary to our hypotheses. BDE-99 exposure was limited to the nestling period, and there was no *in ovo* exposure in our study. In studies that have observed effects of halogenated organic contaminants on the song-control nuclei, *in ovo* exposure was present (Hoogesteijn *et al.*, 2008; Iwaniuk *et al.*, 2006). Under natural conditions, birds are exposed to environmental contaminants *in ovo* via maternal transfer, and it is possible we did not see effects in male neuroanatomy and song quality due to a lack of *in ovo* exposure. However, significant development of the song-control system occurs posthatch (Bottjer *et al.*, 1985; Mooney and Rao, 1994); therefore, there was a window for potential effects of nestling exposure to BDE-99. Future studies examining the effect of *in ovo* exposure to BDE-99 on the song-control system would help identify whether the lack of significant effect in males in our study was the result of timing of exposure. In addition, future studies should examine cellular properties of the song-control regions of exposed birds. Although we found no effect on the size of song-control nuclei in this study, we cannot rule out potential effects on cellular phenotypes.

Although there was no effect of BDE-99 exposure on measured male neuroanatomy, females in the lowest exposure and second highest exposure groups had significantly smaller HVC volumes compared with the control group. As these effects were not dose dependent, it is not clear whether smaller HVC volumes were the result of BDE-99 exposure. Further studies in females examining *in ovo* exposure and cellular properties of the song-control system could help clarify whether there are BDE-99 effects on female neuroanatomy. Sex differences in effects of BDE-99 on the song-control system would not be surprising as it has been well established that there are profound sex differences in the connectivity, volume, and cellular properties of this system in zebra finches (Ball and MacDougall-Shackleton, 2001; Nottebohm and Arnold, 1976). There is also some evidence of sex differences in the sensitivity of T4 and sex steroid hormone homeostasis to BDE-99 exposure (Kuriyama *et al.*, 2007; Lilienthal *et al.*, 2006), which could potentially result in sex differences in the song-control system. All existing studies comparing male and female PBDE-induced changes are in mammals, and sex differences in effects of BDE-99 exposure should be further explored in avian systems.

Early exposure to BDE-99 appeared to decrease motivation of zebra finch males to mate, as the proportion of males that participated in courtship behavior was significantly lower in the highest dose group and the proportion of males that sang was significantly lower in the two highest dose groups. This reduction in mating behavior might be connected with a reduction in sex steroid hormones, as male sexual behavior is mediated by androgens and estrogens (Ball and Balthazart, 2004), and BDE-99 has been shown to reduce levels of these hormones in mammals (Alonso *et al.*, 2010; Lilienthal *et al.*, 2006). There are reports in birds that congeners in penta-BDE

technical mixtures may reduce reproductive behavior and circulating testosterone levels (Fernie *et al*., 2008; Marteinson *et al*., 2011). In the present zebra finch study, male mating behavior was affected by BDE-99 exposure, but the male song-control system was not; this suggests that the neural circuits that underlie the expression of male sexual behavior may be more sensitive to changes in sex steroids than the song-control nuclei. Future work should examine the effects of early BDE-99 exposure on brain regions controlling sexual motivation and the motivation to sing, such as the medial preoptic area of the hypothalamus (Riters and Ball, 1999).

In mating trials, just the males had been exposed to BDE-99, but there were still effects of treatment on female behavior. Clean experienced female zebra finches paired with BDE-99-exposed males responded the least to the highest dose group, which is likely a consequence of the reduced singing and courtship behavior exhibited by males in the higher dose groups. The behavioral changes caused by BDE-99 could potentially lead reduced reproductive success. Observations in captive American kestrels (*Falco sparverius*) studies have shown that unexposed females lowered their investment in the number and size of eggs laid when paired with penta-BDE–exposed males that exhibited reduced reproductive behavior, such as fewer copulations and fewer mating calls (Marteinson *et al*., 2010).

In conclusion, this study shows that early exposure to BDE-99 has adverse long-term effects on the behavior of zebra finches. Although previous studies in birds have looked at the effects of PBDEs on growth, physiology, and reproduction (e.g., Fernie *et al*., 2005, 2006; Marteinson *et al*., 2011), ours was the first to look at possible effects of a PBDE on neuroanatomical measures and song quality and to assess mating behavior using repeatable variables from observer-blind mating trails. Male mating behavior was significantly reduced by BDE-99 exposure, and female response to exposed males was lowest in the highest dose group. Male behavior was more sensitive to BDE-99 exposure than male neuroanatomy, which highlights the importance of considering behavior when assessing biological effects of contaminant exposure. Our study has shown that current environmental levels of BDE-99 are high enough to have significant effects on mating behavior in birds and may therefore be impacting the reproductive rates of free-living birds. These results warrant further investigation in birds into the mechanisms of action of BDE-99 and the consequent effects on behavior and reproductive success, in order to quantify the importance of BDE-99 exposure in free-living bird populations.

FUNDING

This work was primarily supported by the Chemicals Management Plan of Environment Canada and was also funded by a Natural Sciences and Engineering Research Council (NSERC) Postgraduate Scholarship to M.L.E.

ACKNOWLEDGMENTS

The authors thank Sarah Parker and Martha Fronstin (Simon Fraser University), Luke Periard (Organic Contaminants Research Laboratory [OCRL], National Wildlife Research Centre), and Yong Seok An and Zach Hall (University of Western Ontario) for lab assistance.

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