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Developmental exposure to a brominated flame retardant: An assessment of effects on physiology, growth, and reproduction in a songbird, the zebra finch

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ABSTRACT

Mixtures of polybrominated diphenyl ethers (PBDEs) have been widely used as additive flame retardants, and BDE-99 is one of the most predominant congeners found in the environment. BDE-99 has been reported in avian samples worldwide, yet knowledge of its toxicity to birds is minimal. We assessed the short- and long-term effects of nestling exposure to environmentally relevant levels of BDE-99 in a model passerine, the zebra finch. Early exposure to BDE-99 did not affect hematocrit, oxidative stress, or thyroid hormones in either the juvenile or adult stages, and there were no effects on chick growth or survival. BDE-99 exposure caused a dose-dependent delay in timing of reproduction, but there were no other effects on reproductive success. In zebra finches, endpoints related to reproductive behavior appear to be the most sensitive to BDE-99. However, passerines overall appear to be less sensitive than birds of prey or mammals to PBDE exposure.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of hydrophobic and bioaccumulative chemicals that find commercial use as flame retardants, and have become ubiquitous in environmental, human, and wildlife samples (Hites, 2004). 2,2′,4,4′,5-Pentabromodiphenyl ether (BDE-99) is one of the most pervasive congeners (Hites, 2004). In mammals, dosing with BDE-99 has been shown to cause a wide range of adverse effects, including disruption of thyroid hormone homeostasis (Hakk et al., 2002; Kuriyama et al., 2007), oxidative stress (Albina et al., 2010; Belles et al., 2010), and interference in reproductive development and behavior (Talsness et al., 2005; Lilienthal et al., 2006).

BDE-99 is consistently found in avian tissue and egg samples throughout the world (Chen and Hale, 2010; Morrissey et al., 2010), yet its effects in birds are not well known due to a lack of pertinent toxicological literature. There have been some avian exposure studies using penta-BDE mixtures, but these show considerable variation in the sensitivity of bird species to PBDEs. American kestrels (*Falco sparverius*) exposed during development to a penta-BDE mixture containing 27.2% BDE-99 were reported to exhibit some mild effects on growth (Fernie et al., 2006), thyroid hormones and oxidative stress (Fernie et al., 2005), and kestrels exposed in ovo through maternal transfer showed effects on reproductive success (Marteinson et al., 2010). In ovo exposure to a penta-BDE mixture decreased pipping and hatching success in American kestrels, but had no effect on embryo survival endpoints in chickens (*Gallus gallus*) or mallard ducks (*Anas platyrhynchos*) (McKernan et al., 2009). Adult female European starlings (*Sturnus vulgaris*) exposed to a penta-BDE mixture through subcutaneous implants showed limited reproductive and endocrine disruption effects, and no biochemical or hematological effects (Van den Steen et al., 2009; Van den Steen et al., 2010). To our knowledge only one study has exposed birds to BDE-99: in captive mallards, in ovo exposure to BDE-99 altered the vitamin status of chicks (Murvoll et al., 2005). The use of penta-BDE mixtures makes it difficult to identify the specific components responsible for any putative toxic effects. Specifically the contribution of BDE-99, a dominant congener found in the environment and wildlife, to any observed effects of penta-BDE mixtures is not clear so further studies are required to assess the specific effects of BDE-99 in birds.

There is considerable evidence in oviparous organisms that early developmental life stages are more sensitive than adult stages to contaminant exposure (e.g. Peterson et al., 1993; Hutchinson et al., 1998). However, the toxicological effects of developmental

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exposure may not be evident until the individual reaches reproductive maturity, necessitating long-term studies to assess fitness implications of early, developmental exposure to contaminants. Here, we investigate the long-term effects of early developmental exposure to environmentally relevant levels of BDE-99 in birds, using the zebra finch (*Taeniopygia guttata*) as a model passerine species. The zebra finch is a useful model to monitor effects of contaminants under controlled laboratory conditions, as it readily breeds in captivity, has a short generation time (4 months), and has been widely used in toxicological dosing studies (e.g. Gill et al., 2004; Albert et al., 2008; Hoogesteijn et al., 2008; Kitulagodage et al., 2011). We have previously shown that reproductively mature male zebra finches exposed to BDE-99 as nestlings exhibit altered mating behavior (Eng et al., 2012). The objectives of the present study were to investigate the effects of early exposure to BDE-99 in the same cohort of zebra finches on non-behavioral endpoints including growth, physiology, and reproduction.

2. Materials and methods

2.1. Animals and husbandry

The present study was conducted on a captive colony of zebra finches maintained at the Simon Fraser University Animal Care Facility in Burnaby, British Columbia. Zebra finches were housed in a controlled environment (temperature 19–23 °C; humidity 35%–55%; photoperiod 14 h light–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 (864B-08) following 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, which is a standard diet for breeding zebra finches (Williams and Martyniuk, 2000). 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). Nest boxes were 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 using digital calipers (to the nearest 0.01 mm) every 5 days until day 30, and again at day 90.

2.2. Experimental protocol

Prior to this experiment, a tissue residue study was carried out which validated oral dosing methods and confirmed environmental relevance of doses (see Eng et al., 2012 for details). All dosing was done with technical grade BDE-99 (>98% purity, Cambridge Isotope Labs, Andover, MA). 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. Dosing solutions were analyzed via gas chromatography (GC)-mass spectrometry (MS) (electron capture negative ionization mode [ECNI]), and a full description of the procedures used for the extraction and determination of PBDEs in the dosing solutions have been described elsewhere (Eng et al., 2012). The only bromide ion that was quantifiable was BDE-99. There were four dose levels (2.5, 15.8, 50.7, and 173.8 ng BDE-99/g body weight [bw]/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. 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 started to form. Blood samples were collected at 30 and 90 days of age. All birds were blood-sampled from the brachial vein following puncture with a 26G needle and blood was collected into heparinized hematocrit tubes. Blood samples were centrifuged at 3000 *g* for 10 min to separate plasma from the red blood cells, and hematocrit was measured by packed cell volume. Plasma was then stored frozen (−80 °C) until analysis.

Once birds reached sexual maturity (day 90), female breeding trials were conducted. Each exposed female was paired with an experienced, clean male and bred following the standard protocol as described above (see "Animals and Husbandry"). Following completion of breeding trials, birds were anesthetized and then exsanguinated, and blood was centrifuged and plasma was stored frozen until analysis.

2.3. Plasma analysis

Total antioxidant capacity (TAC) was measured using a colorimetric method adapted from Erel (2004), where the colored 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) is decolorized by antioxidants in the plasma according to their concentrations and antioxidant capacities. This reaction can be monitored spectrophotometrically, and the final absorbance is inversely related to the TAC of the sample. The reaction is calibrated using Trolox for the standard curve, and results are expressed as mmol Trolox equivalent/L. The total oxidant status (TOS) was measured using a colorimetric method adapted from Erel (2005), where oxidants in the plasma oxidize the ferrous ion–*o*-dianisidine complex to the ferric ion, which then reacts with xylenol orange to make a colored complex. The color intensity can be measured spectrophotometrically, and is proportional to the total amount of oxidant molecules in the plasma sample. The assay is calibrated using hydrogen peroxide (H_2O_2) for the standard curve, and the results are expressed in mmol H_2O_2 equivalent/L. The oxidative status index (OSI) was calculated as the ratio of TOS:TAC for each individual, with high ratios reflecting high oxidative stress. A hen plasma pool was used as an avian standard to compare inter-assay variation. The TAC assay had an average intra-assay coefficient of variation (CV) of 3.8% ($n = 2$ replicates) and the inter-assay CV was 3.3% ($n = 2$ assay plates). For TOS the average intra-assay CV was 5.1% ($n = 2$ replicates) and the inter-assay CV was 3.5% ($n = 2$ assay plates).

Total and free thyroxine (T4 and FT4) and total and free triiodothyronine (T3 and FT3) levels in plasma samples were determined using enzyme-linked immunosorbent assay (ELISA) kits (Monobind 225-300, 1225-300, 125-300, and 1325-300, Lake Forest CA). Kits were validated for parallelism (see Plikaytis et al., 1994 for methods) and recovery using plasma from non-experimental zebra finches. Hen plasma from a plasma pool was included in each plate to assess reproducibility and intra-assay precision. Quality control standards (Monobind ML-300) were included in each plate and were verified to be in the expected range. The T4 assay had an average intra-assay coefficient of variation (CV) of 12.0% ($n = 2$ replicates) and the inter-assay CV was 7.7% ($n = 6$ assay plates). For FT4 the average intra-assay CV was 12.8% ($n = 2$ replicates) and the inter-assay CV was 11.4% ($n = 3$ plates). The average intra-assay CV for T3 was 5.4% ($n = 2$ replicates) and the inter-assay CV was 5.9% ($n = 4$ plates). For FT3 the average intra-assay CV was 8.5% ($n = 2$ replicates) and the inter-assay CV was 8.3% ($n = 3$ plates).

2.4. Statistical analysis

All statistical analyses were carried out using SAS 9.1.3 (SAS Institute, 2003). Data were tested for normality and homogeneity of variance following Shapiro-wilk and Levene's tests, and by inspecting q–q plots. Sample sizes of birds that survived to 90 days consisted of 21 (8 female, 13 male), 20 (8 female, 12 male), 19 (5 female, 14 male), 20 (6 female, 14 male), and 20 (12 female, 8 male) nestlings exposed to control oil, 2.5, 15.8, 50.7, and 173.8 ng BDE-99/g bw/day, respectively. To assess the effect of dose on each endpoint, initially sex and dose were both included for each analysis and if there was no effect or interaction with sex, sex was removed from the model. Contingency tables and fisher's exact test (FET) for small sample sizes were used to assess effects of BDE-99 dose on survival. An index of body condition was estimated as the residuals from a linear regression of body mass on tarsus length (Schulte-Hostedde et al., 2005). The effect of BDE-99 dose on growth from 0 to 90 days was assessed using the REPEATED statement in the MIXED procedure. The effect of BDE-99 dose on body condition and physiological variables was assessed using generalized linear models (GLM procedure), and *post-hoc* tests for differences between means were adjusted for multiple comparisons following the Tukey–Kramer method. A linear regression was used to test whether laying interval increased with dose. Variables that were not parametric were log transformed before analysis. Treatment effects on binary variables (hatching and fledging success) were tested with generalized linear models (GENMOD procedure) in a two level structure with individual eggs nested within broods, using a binomial distribution and the logit link function.

3. Results

BDE-99 tissue residue values following oral exposure for the 21-day nestling period have been previously reported in Eng et al. (2012). Based on the tissue residue study, the estimated 30-day plasma concentrations were 332.7 ± 141.0, 843.4 ± 454.0, 1597.3 ± 314.5 and 4450.2 ± 1396.2 ng/g lipid weight (lw) for the 0, 2.5, 15.8, and 50.7 ng BDE-99/g bw/day dose groups, respectively. The predicted concentration for the 173.8 dose group based on the

Table 1
Physiological variables in juvenile (30-day-old) and adult zebra finches that were orally exposed to BDE-99 for the 21 day nesting period. Mean (SE). There was no significant effect of dose on any variable ($p \geq 0.095$). TAC = total antioxidant capacity, TOS = total oxidant status, OSI = oxidative status index, T4 = total thyroxine, T3 = total triiodothyronine, FT4 = free thyroxine, FT3 = free triiodothyronine.

	Dose (ng/g BDE-99/g bw/day)				
	0	2.5	15.8	50.7	173.8
Day 30					
Hematocrit	0.53 (0.01)	0.52 (0.01)	0.51 (0.02)	0.52 (0.01)	0.51 (0.01)
TAC (mM Trolox)	1.33 (0.1)	1.29 (0.07)	1.16 (0.07)	1.31 (0.07)	1.29 (0.15)
TOS (mM H_2O_2)	0.47 (0.06)	0.5 (0.07)	0.42 (0.05)	0.36 (0.04)	0.43 (0.06)
OSI	0.39 (0.07)	0.41 (0.08)	0.37 (0.04)	0.29 (0.04)	0.37 (0.07)
T4 (nmol/L)	11.03 (1.05)	10.72 (1.25)	14.36 (2.35)	12.74 (2.98)	11.4 (1.3)
Adult					
Hematocrit	0.54 (0.01)	0.53 (0.01)	0.53 (0.01)	0.53 (0.01)	0.55 (0.01)
TAC (mM Trolox)	1.53 (0.09)	1.3 (0.09)	1.54 (0.08)	1.48 (0.12)	1.24 (0.12)
TOS (mM H_2O_2)	0.68 (0.08)	0.55 (0.06)	0.48 (0.06)	0.57 (0.11)	0.69 (0.13)
OSI	0.48 (0.07)	0.49 (0.09)	0.32 (0.04)	0.51 (0.13)	0.95 (0.39)
T4 (nmol/L)	5.07 (0.99)	5.83 (1.29)	5.22 (0.87)	7.18 (1.33)	5.75 (1.15)
T3 (nmol/L)	1.3 (0.07)	1.29 (0.12)	1.33 (0.09)	1.21 (0.08)	1.47 (0.11)
FT4 (pmol/L)	2.81 (0.5)	2.69 (0.4)	2.89 (0.46)	2.67 (0.41)	2.09 (0.43)
FT3 (pmol/L)	2.58 (0.13)	2.94 (0.19)	2.47 (0.19)	2.49 (0.26)	3.21 (0.27)

tissue residue data was 14079.69 ng/g lw. 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.

There was no effect of BDE-99 dose on day 30 ($F_{4,95} = 0.63$, $p = 0.645$) or adult ($F_{4,95} = 1.06$, $p = 0.383$) hematocrit levels (Table 1), and there was no effect of sex on day 30 ($F_{1,90} = 0.96$, $p = 0.330$) or adult ($F_{1,90} = 0.19$, $p = 0.663$) hematocrit levels.

On day 30 there was no effect of BDE-99 dose on TAC ($F_{4,51} = 0.61$, $p = 0.655$), TOS ($F_{4,51} = 0.93$, $p = 0.456$) or OSI ($F_{4,46} = 1.04$, $p = 0.399$; Table 1). Similarly in adults, there was no effect of dose on TAC ($F_{4,70} = 1.21$, $p = 0.312$), TOS ($F_{4,83} = 0.88$, $p = 0.478$) or OSI ($F_{4,78} = 0.81$, $p = 0.521$). Sex had no effect on 30-day TAC or TOS, or on adult TOS ($p > 0.082$). The 30-day OSI was significantly higher in females than in males ($F_{1,54} = 4.19$, $p = 0.046$), but there was no interaction between dose and sex for OSI ($F_{4,46} = 0.81$, $p = 0.524$). In adults, males had significantly higher TAC than females ($F_{1,79} = 8.12$, $p = 0.006$), and no sex*dose interaction ($F_{4,79} = 1.26$, $p = 0.295$) for TAC. The adult OSI was significantly higher in females than in males ($F_{1,78} = 5.44$, $p = 0.022$), but there was no interaction between sex and dose ($F_{4,78} = 1.21$, $p = 0.312$).

There was no effect of BDE-99 dose on day 30 T4 ($F_{4,90} = 0.57$, $p = 0.683$), or on adult T4 ($F_{4,88} = 0.46$, $p = 0.765$), T3 ($F_{4,93} = 0.93$, $p = 0.449$), FT4 ($F_{4,70} = 0.38$, $p = 0.487$) or FT3 ($F_{4,51} = 2.09$, $p = 0.095$; Table 1). There was no effect of sex on thyroid hormone concentrations for day 30 T4, or for adult T3 or FT3 ($p > 0.226$). Adult males had significantly higher T4 and FT4 than adult females ($F_{1,88} = 6.89$, $p = 0.010$ and $F_{1,70} = 53.18$, $p < 0.0001$, respectively), but there was no sex*dose interaction for T4 or FT4 ($F_{4,88} = 0.43$, $p = 0.785$ and $F_{4,70} = 0.87$, $p = 0.487$, respectively).

Chick mass over time was not affected by BDE-99 dose ($F_{4,95} = 0.31$, $p = 0.868$) and chick growth was not affected by dose as there was no interaction between dose and age ($F_{28,665} = 0.72$, $p = 0.855$; Fig. 1). There was also no effect of dose on body condition on day 30 ($F_{4,95} = 0.19$, $p = 0.945$) or day 90 ($F_{4,95} = 1.47$, $p = 0.218$). Sex had no effect on body condition or growth ($p > 0.149$). The overall survival of BDE-99 exposed nestlings to reproductive maturity was 93.5%, and there was no effect of dose on survival ($p = 0.755$, FET).

Among experimental females dosed with BDE-99 as nestlings, 89.7% successfully laid eggs when paired with clean experienced males at 90 days of age, and there was no effect of dose on whether a female laid eggs or not ($p = 0.385$, FET). Of the females that laid eggs, there was also no significant difference between dose groups in the clutch size ($F_{4,30} = 0.69$, $p = 0.604$), average egg mass ($F_{4,30} = 0.28$, $p = 0.889$), hatching success ($p > 0.140$), fledging success ($p > 0.123$), or the laying interval (number of days between pairing and laying of the first egg) ($F_{4,30} = 1.57$, $p = 0.207$; Table 2). However, the average laying interval did increase in a dose dependent manner (Table 2), and there was a significant positive relationship between BDE-99 dose and laying interval ($F_{1,33} = 6.09$, $p = 0.019$, adj. $R^2 = 0.130$; Fig. 2). Laying interval increases as dose increases according to the equation: laying interval $= 10^{(0.8693 + 0.0011*\text{dose})}$. There was an average delay of

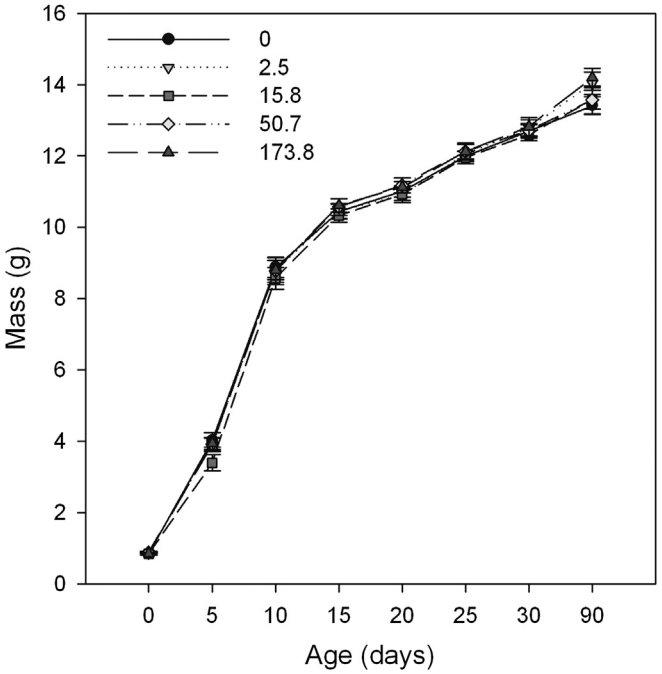

Fig. 1. Growth of zebra finches orally exposed to 0, 2.5, 15.8, 50.7, or 173.8 ng BDE-99/g bw/day for the 21-day nesting period. There was no interaction between dose and age ($F_{28,665} = 0.72$, $p = 0.855$).

Table 2
Reproductive variables for adult zebra finches that had been orally exposed to BDE-99 for the 21 day nesting period. Mean (SE). There was no significant effect of dose for any variable ($p > 0.123$).

Reproductive variable	Dose (ng/g BDE-99/g bw/day)				
	0	2.5	15.8	50.7	173.8
Laying interval	7.13 (0.74)	7.83 (0.98)	8.50 (0.65)	10.67 (3.31)	13.00 (2.46)
Clutch size	6.25 (0.53)	5.17 (0.70)	5.25 (0.25)	5.83 (0.31)	5.36 (0.53)
Average egg mass (grams)	1.06 (0.04)	1.01 (0.03)	1.05 (0.05)	1.02 (0.03)	1.02 (0.03)
Proportion of eggs to hatch per clutch	0.58 (0.14)	0.74 (0.15)	0.65 (0.22)	0.25 (0.16)	0.58 (0.12)
Proportion of hatched eggs to fledge per brood	0.89 (0.07)	0.9 (0.07)	0.81 (0.10)	0.38 (0.38)	0.82 (0.13)

approximately 3 days in the 50.7 ng/g bw/day dose group, and about 6 days in the 173.8 ng/g bw/day dose group compared to the control group.

4. Discussion

In this study we assessed the long-term effects of early developmental exposure to the polybrominated diphenyl ether congener BDE-99 on selected physiological variables, growth, and reproduction in zebra finches. As previously demonstrated through tissue residue analysis (Eng et al., 2012), our doses resulted in plasma and adipose burdens that fall within the range of concentrations reported in free-living birds (e.g. Lindberg et al., 2004; McKinney et al., 2006; Voorspoels et al., 2007). At these ecologically-relevant dose concentrations, there were no effects on a range of endpoints including hematocrit, oxidative stress, thyroid hormone homeostasis, chick growth and survival. There was also no effect on female reproductive success in adults, although there was evidence that females exposed to higher concentrations of BDE-99 during early development were more likely to delay onset of egg laying. Overall we observed very few effects in 30-day-old or adult birds following early developmental exposure to BDE-99, similar to the results of a study of PBDE exposure in European starlings, which concluded that passerine species may be relatively less sensitive to the effects of PBDEs (Van den Steen et al., 2010).

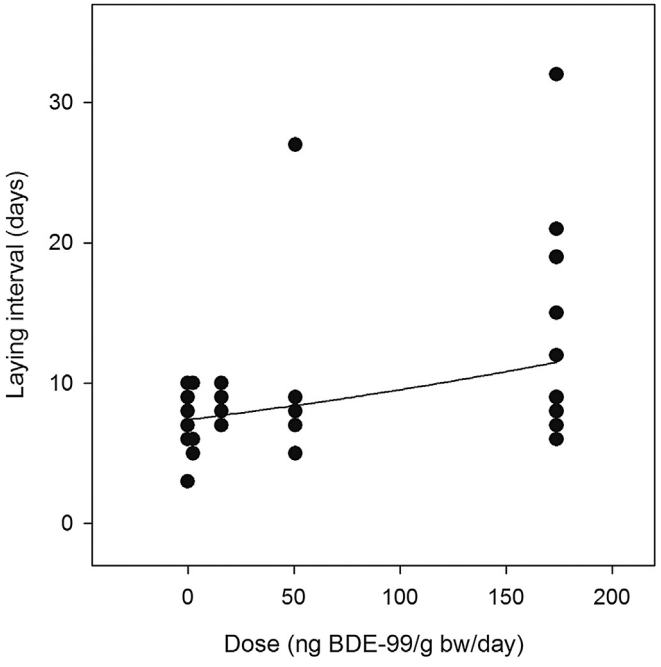

Fig. 2. Laying interval (number of days between pairing and laying of the first egg) of reproductively mature female zebra finches that were exposed to BDE-99 for the 21-day nestling period. Laying interval increases as dose increases according to the equation: laying interval $= 10^{(0.8693 + 0.0011*\text{dose})}$.

It has been suggested that hematocrit can be used as an indicator of whether a pollutant has caused changes in the red blood cell volume or number (Handy and Depledge, 1999). In the present study, 30-day and adult hematocrit levels were unaffected by nestling exposure to BDE-99. Other studies that have looked at the effect of BDE-99 or penta-BDE exposure in birds also found no effect on hematocrit (Murvoll et al., 2005; Van den Steen et al., 2010; Winter et al., 2013). In contrast, there is some evidence in mammals that PBDE exposure has hematological effects. In free living harbor seals (*Phoca vitulina*) the red blood cell count was inversely related to ΣPBDEs in the blood (Neale et al., 2005), and in ranch mink (*Mustela vison*) hematocrit decreased following exposure to a commercial PBDE mixture (Martin et al., 2007). However, at least in the mink study the dose groups that showed an effect had tissue PBDE concentrations approximately three orders of magnitude higher than in our study (18,505 μg/g lw and 27,909 μg/g lw versus up to 14079.69 ng/g lw in the present study). Alternatively, it is possible that the red blood cells in birds may be less sensitive to PBDE exposure than in mammals, as the hematological cells differ between birds and mammals. Notably, avian erythrocytes are nucleated whereas mammalian erythrocytes are not (Glomski and Pica, 2011).

Oxidative stress results from an imbalance between reactive oxygen species (ROS) and antioxidant defenses, and an increase in ROS can potentially damage cell components or activate specific signaling pathways (Finkel and Holbrook, 2000). Oxidative stress has been proposed as one of the potential mechanisms for the toxic effects of BDE-99 (Albina et al., 2010; Belles et al., 2010). Adult rats acutely exposed to BDE-99 (single dose of 0, 600 or 1200 ng/g bw) showed decreased antioxidant capacity in the brain, kidney and liver (Albina et al., 2010; Belles et al., 2010), and altered oxidative stress markers in the serum and erythrocytes (Alonso et al., 2010). Similarly, an in vitro study in a human cell line found that BDE-99 exposure increased markers of ROS formation and lipid peroxidation (Tagliaferri et al., 2010). In contrast, we found that BDE-99 exposed zebra finches did not show changes in plasma oxidative stress compared to control birds at either the juvenile (day 30) or adult stages. Other studies in birds have also found limited evidence supporting an effect of PBDEs on oxidative state. A study in American kestrels reported that in ovo and nestling exposure to a penta-BDE mixture (18.7 μg PBDE per egg, 15.6 ng/g bw for 29 days post-hatch) increased markers of oxidative stress in the liver (Fernie et al., 2005); however, the increase was not significant ($p = 0.09$). In a passerine species, the European starling, adults exposed to a penta-BDE mixture through subcutaneous implants (~1740 ng/g bw) showed no significant differences from control birds in the total plasma antioxidative capacity (Van den Steen et al., 2010), which is consistent with our results. Overall, there is little evidence that PBDE exposure in birds causes oxidative stress, which suggests that birds may produce fewer ROS following PBDE exposure than mammals.

BDE-99 and its hydroxylated (OH) metabolites are structurally similar to thyroid hormones, and have the potential to disrupt thyroid hormone homeostasis through various mechanisms, such as competitive displacement of thyroid hormones from thyroid hormone transport proteins (Ucan-Marin et al., 2009). There is evidence in mammals that BDE-99 can alter T4 concentrations (Hakk et al., 2002; Kuriyama et al., 2007). However, studies of free living birds have found that in bald eagles (*Haliaeetus leucocephalus*) and glaucous gulls (*Larus hyperboreus*), there were no associations between PBDE concentrations and thyroid hormone concentrations (Verreault et al., 2007; Cesh et al., 2010), although circulating T3 concentrations in nestling bald eagles were positively correlated with OH-PBDEs (Cesh et al., 2010). In laboratory studies of birds, there is limited evidence that penta-BDE mixtures can affect thyroid hormones. Adult female European starlings exposed to a penta-BDE mixture through subcutaneous implants showed no differences in T3 and T4 concentrations between control and exposed groups in the six months after implantation. When egg-laying starlings were excluded from these results, there was a non-significant trend for lower T3 two weeks after implantation, which disappeared by two months after implantation (Van den Steen et al., 2010). American kestrels exposed to a penta-BDE mixture through egg injection and nestling gavage had lower circulating T4 than controls, although the difference was not significant, and their T3 concentrations and thyroid glandular structure were unaffected by exposure (Fernie et al., 2005). There were also no effects on T3 or T4 concentrations in American kestrels exposed to penta-BDEs through maternal transfer (low exposure 289 ng/g ww, high exposure 1131 ng/g ww; Marteinson et al., 2011). Thyroid hormones in zebra finches exposed in ovo to BDE-99 (10, 100 or 1000 ng/g ww) showed no significant differences between dosed and control birds (Winter et al., 2013). Similarly in our study, thyroid hormone homeostasis was unaffected by early exposure to BDE-99. Overall, the majority of field and laboratory studies in birds have not found significant effects of PBDE exposure on thyroid hormone homeostasis. The lack of significant effects may be because PBDE concentrations are too low to disrupt thyroid hormone binding to transport proteins or receptor sites. It has recently been demonstrated that OH-PBDE metabolites bind to gull thyroid hormone transport proteins (transthyretin and albumin) with a higher affinity than T3 or T4 do, but non-OH-PBDEs have a lower binding affinity than T3 and T4, and reported environmental concentrations of OH-PBDEs are likely too low to have a substantial effect on T3 or T4 binding (Ucan-Marin et al., 2009, 2010).

In our study, the chick growth and survival of zebra finches exposed as nestlings to BDE-99 was not affected. In immature birds, thyroid hormones are important for growth and development, and act on growth in a permissive way in conjunction with growth hormones and insulin-like growth factors (McNabb, 2007); therefore, in the present study the absence of effect of BDE-99 exposure on thyroid hormone homeostasis is consistent with the lack of effect on growth. In ovo exposure to BDE-99 in zebra finch had no effect on growth in the first-generation, BDE-treated chicks (Winter et al., 2013). However, the offspring from the high-dose group of these in ovo exposed birds had significantly lower body weight as juveniles, although this effect disappeared by sexual maturity (Winter et al., 2013). Therefore, we might have observed effects of BDE-99 exposure in nestlings if we had followed multiple generations. Alternatively, zebra finches may be more sensitive to in ovo exposure than nestling exposure. Additionally, in contrast to the decreased weight observed in the offspring of zebra finches exposed in ovo to BDE-99 by Winter et al. (2013), penta-BDE exposed female kestrels were reported to grow faster and were larger than control birds (Fernie et al., 2006). In the kestrels, the BDE-99 congener was not positively associated with weight gain. The absence of an effect of BDE-99 on survival in the present study is consistent with a study in zebra finches that assessed the long-term effects of in ovo exposure to BDE-99 (Winter et al., 2013), as well as with a penta-BDE exposure study of American kestrels exposed in ovo and as nestlings (Fernie et al., 2006), that also found no effect of exposure on survival.

We did find some effect of early developmental BDE-99 exposure on the timing component of reproduction in these same birds as adults: as BDE-99 dose increased, the laying interval increased. Mean laying interval was not statistically different among dose groups, likely due to the large variability in laying interval in the two highest dose groups. Our results agree with those from zebra finches exposed to BDE-99 in ovo, which also showed a trend of longer laying intervals in exposed birds compared to control birds (Winter et al., 2013). If BDE-99 were to similarly delay onset of egg laying in free-living birds this could have important consequences since timing of breeding is one of the main determinants of reproductive success and lifetime fitness (Williams, 2012). Evidence that PBDEs increase laying interval is variable from other experimental studies of captive birds. Following exposure to a penta-BDE mixture, American kestrels exhibited longer laying intervals (Fernie et al., 2009), while European starlings did not (Van den Steen et al., 2009). Nevertheless, our data suggest that mechanisms related to timing of breeding should be included as an important endpoint in future studies of effects of PBDEs.

Other than the effect on timing of breeding, we did not observe adverse effects of early exposure to BDE-99 on any other reproductive endpoints including breeding propensity, clutch size, egg mass, and hatching or fledging success. In contrast, zebra finches that were exposed to BDE-99 in ovo had significantly smaller clutch sizes than control birds (Winter et al., 2013), which again suggests that the impact of BDE-99 might depend on the timing of exposure relative to offspring development. In adult European starlings exposed to penta-BDEs, exposed birds laid larger eggs than controls, and there was a non-significant trend for exposed birds to lay less frequently (Van den Steen et al., 2009), but there were no effects on any other reproductive measures or on sex steroid hormones (Van den Steen et al., 2010). In American kestrels, both in ovo and adult exposure to PBDEs have been reported to cause negative effects on reproductive success and mating behavior (Fernie et al., 2008, 2009; Marteinson et al., 2010; Marteinson et al., 2011). In free living birds, a negative relationship between ΣPBDEs in eggs and average brood size was reported for peregrine falcons (*Falco peregrinus*; Johansson et al., 2009), and there is correlative evidence that high concentrations (>1000 ng/g ww in eggs) of ΣPBDEs may negatively affect reproductive productivity in ospreys (*Pandion haliaetus*; Henny et al., 2009), although there is evidence that PBDE residues have declined in ospreys from this region following the cessation of penta- and octa-BDE manufacturing in North America in 2004 (Henny et al., 2011). BDE-99 exposure also has significant effects on reproductive variables in mammals. Gestational and lactational exposure to BDE-99 (single dose to the mother of 60 or 300 ng/g bw) resulted in reproductive tract changes and lower fertility in female rats (Talsness et al., 2005). In male rats exposed to BDE-99 (single dose 600 or 1200 ng/g bw) as adults, circulating sex steroids were significantly decreased (Alonso et al., 2010). A separate study of developmental BDE-99 exposure also showed a decrease in circulating sex steroids and evidence of feminization in male rats, and a decreased number of ovarian follicles in female rats, although doses were relatively high (1000 or 10,000 ng/g bw injected gestational days 10–18; Lilienthal et al., 2006). Overall, studies in passerines have reported fewer reproductive effects relative to birds of prey and mammals, which again suggests that passerines may be less sensitive to PBDE exposure. Further studies of toxicity of developmental exposure to BDE-99 or

related chemicals that examine additional reproductive endpoints such as sex steroid hormone concentrations, histology of the reproductive tract, and female mating behavior could be informative for understanding effects on avian reproduction.

In conclusion, early developmental exposure to BDE-99 at concentrations relevant to free-living birds had very few long-term negative effects in zebra finches. Similarly, a study in European starlings concluded passerines were less sensitive than other bird species to PBDE exposure (Van den Steen et al., 2010). Another possibility for the lack of observed effects in our study may be that while our doses covered a range of concentrations comparable to those reported in free-living birds, higher concentrations or mixtures of PBDE congeners may be needed to cause more overt adverse effects. Alternatively, the embryonic life stage may be more sensitive than the nestling period, and *in ovo* exposure may be necessary to see effects on physiology and reproduction. Future studies looking at higher concentrations and timing of exposure would be needed to confirm this. We did observe negative effects on laying behavior in female zebra finches exposed to BDE-99 as nestlings, and have previously reported that BDE-99 exposure reduced the sexual behavior of male zebra finches (Eng et al., 2012). Endpoints related to reproduction, particularly behavior, might therefore be the most sensitive to BDE-99 exposure in zebra finches and should be included as endpoints in future studies. There is also now a body of evidence in mammals and other bird species that exposure to BDE-99 or PBDE mixtures can have negative consequences. All together, these results suggest that wild birds, particularly birds of prey, in environments with high PBDE exposure could suffer from negative effects on reproductive performance.

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