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In ovo exposure to brominated flame retardants Part I: Assessment of effects of TBBPA-BDBPE on survival, morphometric and physiological endpoints in zebra finches

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ABSTRACT

Tetrabromobisphenol A bis(2,3-dibromopropyl) ether (TBBPA-BDBPE) is an additive flame retardant used in polyolefins and polymers. It has been detected in biota, including in avian eggs, yet little is known of its effects. We assessed the pattern of TBBPA-BDBPE concentrations in songbird eggs over the incubation period, and the effects of embryonic exposure to TBBPA-BDBPE in a model songbird species, the zebra finch (*Taeniopygia guttata*). To assess concentrations during embryo development, eggs were injected on the day they were laid with the vehicle control (safflower oil) or 100 ng TBBPA-BDBPE/g egg, and whole egg contents were collected throughout embryonic development on day 0 (unincubated), 5, 10 and 13. To evaluate effects of embryonic exposure to TBBPA-BDBPE, eggs were injected at Hamburger-Hamilton stage 18 (~80 h after initiation of incubation) with safflower oil only, 10, 50 or 100 ng TBBPA-BDBPE/g egg (albumin injection volume 1 µl/g). Eggs were monitored for hatching success, and nestlings were monitored for growth and survival. At 15 days post-hatch, tissues were collected to assess physiological effects. TBBPA-BDBPE was incorporated into the egg as the embryo developed, and concentrations started declining in late incubation, suggesting biotransformation by the embryo. There were no effects on hatching success, nestling survival, growth, organ somatic indices, or thyroid hormone homeostasis; however, there was evidence that body condition declined in a dose-dependent manner towards the end of the rapid nestling growth phase. This decreased body condition could be a delayed effect of early developmental exposure, or it may be the result of increased exposure to biotransformation products of TBBPA-BDBPE produced over the nestling period, which are predicted to be more bioaccumulative and toxic than the parent compound.

1. Introduction

Increasing restrictions on use of polybrominated diphenyl ether (PBDE) flame retardants have caused a shift towards use of alternative brominated flame retardants (BFRs), such as tetrabromobisphenol-A (TBBPA) and its derivatives (Covaci and Malarvannan, 2015). Tetrabromobisphenol A bis(2,3-dibromopropyl) ether (TBBPA-BDBPE) is a TBBPA derivative that is used as an additive flame retardant in polyolefin and polymer plastics (Covaci et al., 2011). It is very hydrophobic (est. log K_{ow} ~10 to 12) and has a high molecular weight (943.6). Based on its chemical properties, it is predicted to be persistent and associate with particles, leading to bioaccumulation (Harju et al., 2009; Howard and Muir, 2010). A mesocosm study found it to be persistent in sediments (de Jourdan et al., 2013). As such, TBBPA-BDBPE could have similar environmental fate and transport properties as decaBDE (Harju et al., 2009). It has been detected in several abiotic compartments worldwide, including air around the North American Great Lakes (Liu et al., 2016b), soil near chemical manufacturing plants in China (Liu et al., 2017), river sediments in Africa (Chokwe et al., 2017), and water and dust in Europe (Ali et al., 2011; Nyholm et al., 2013; Sawal et al.,

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2008).

TBBPA-BDBPE has also been detected in biota, including herring gull (*Larus argentatus*) eggs in Canada (Letcher and Chu, 2010) and in marine biota in China (Liu et al., 2016a). Probable degradation products of TBBPA-BDBPE potentially formed by debromination and ether bond breakage have also been detected in biota, and are predicted to be more bioaccumulative and toxic than TBBPA-BDBPE (Liu et al., 2016a, 2018; Qu et al., 2013). Aquatic mesocosm studies have demonstrated the bioaccumulative properties of TBBPA-BDBPE in fish (de Jourdan et al., 2014), and there is evidence of maternal transfer of TBBPA-BDBPE in both fish and birds (Letcher and Chu, 2010; Nyholm et al., 2008).

Despite evidence of increasing usage and environmental detections, very little is known of the metabolic processes or toxicological effects of TBBPA-BDBPE, particularly for birds. The objectives of the present study were to 1) determine the concentration of TBBPA-BDBPE and its fate in whole egg contents throughout the incubation period, and 2) assess the effects of exposure to TBBPA-BDBPE during embryonic development on hatching success, growth, condition, and thyroid hormone homeostasis in a model altricial songbird species, the zebra finch (*Taeniopygia guttata*). While songbirds are typically small mid-trophic level consumers, they have relatively high metabolic rates and food consumption requirements (Nagy, 1987), and songbirds at contaminated sites (e.g. landfills, former industrial sites, wastewater treatment plants) have been shown to accumulate concentrations of persistent organic pollutants (POPs) that are comparable to those seen in certain top predators (e.g. Chen et al., 2013; Cristol et al., 2008; Custer et al., 2003; Fernie and Letcher, 2018). Terrestrial songbirds could be exposed to contaminants that partition to particles, such as TBBPA-BDBPE, through ground foraging, and consumption of soil invertebrates or predatory invertebrates (Eens et al., 2013; Eng et al., 2014). Among avian species, there can be considerable variation in sensitivity to environmental contaminants (Farmahin et al., 2013; Heinz et al., 2009), and to characterize variation in species sensitivity, the results of this study will be compared to results from a concurrent study with American kestrels (*Falco sparverius*), a model semi-altricial predator species (Eng et al.,).

2. Methods

2.1. Animals and husbandry

The zebra finch is an altricial songbird species well established as a model for assessing the effects of contaminants in songbirds, including during the developmental period (e.g. Eng et al., 2012; Yu et al., 2016). Zebra finches readily breed in captivity, have a 14-day incubation period, and reach sexual maturity within 90 days. In the present study, zebra finch experiments were conducted at a captive colony maintained at the Simon Fraser University Animal Care Facility in Burnaby, British Columbia, Canada. Zebra finches were housed in a controlled environment (temperature 19–23 °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 Council permit (1070B-08) following guidelines from the Canadian Council 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 external nest boxes (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).

2.2. Zebra finch egg injections

Methods in this study were chosen to align with methods in a concurrent study following established protocols for American kestrels, which used a safflower oil vehicle and air cell injections (Eng et al., submitted). Albumen injections were used for zebra finches as air cell injections are less feasible due to the small size of the egg (~1 g) and air cell membrane. There is evidence that air cell and albumen injections can produce comparable results for certain contaminants. For example, effects on hatching success of 1.6 µg/g methylmercury in a corn oil vehicle injected into Mallard (*Anas platyrhynchos*) eggs at a volume of 1 µl/g was not statistically different for air cell injected (6.67%) or albumen injected (20%) eggs (Heinz et al., 2006).

Prior to injection, the injection location was cleaned with an ethanol wipe and allowed to dry. Insertion holes were made through the side of the egg using sterile 26 gauge needles, and then enlarged with sterile 22 gauge needles (BD PrecisionGlide™). The dosing solutions were injected into the albumen using a 2 µl pipette. Holes were sealed with cyanoacrylate glue (Loctite® Gel Control Super Glue). The injection volume was 1 µl/g egg, and eggs were injected on the day that the egg was laid for the time course study, and at Hamburger-Hamilton stage 18 (~80 h after initiation of incubation) (Murray et al., 2013) for the effects study.

For the time-course study (objective 1), eggs were injected with vehicle control (safflower oil) or 100 ng TBBPA-BDBPE/g egg. Whole egg contents were collected on day 0 (unincubated), 5, 10 and 13 of incubation (n = 5 eggs per dose per time point) by opening eggs and emptying contents into chemically cleaned vials. Unincubated eggs remained at room temperature for 1 h post-injection before contents were collected. Collected samples were homogenized and stored at −20 °C until chemical analysis.

For the effects study (objective 2), eggs were injected with safflower oil vehicle only, or 10, 50 or 100 ng TBBPA-BDBPE/g egg (nominal concentrations). Within each nest, treatments were assigned to eggs in a random order to account for the influence of heritable, nest, and laying/hatching order factors. Following injection procedures, all eggs were returned to the nest and incubated by the parents. Toward the end of the 12- to 14-day incubation period, nest boxes were checked two to three times per day to determine hatching success and hatching date, and to identify which hatchling came from which egg. Every 5 days until day 15, nestlings were weighed and the length of the right tarsus measured using digital calipers (to the nearest 0.01 mm). At day 15, birds were euthanized via exsanguinations with anesthesia (1:1 rompun:ketamine) and tissues were weighed and collected. Thyroid glands, gonads, spleen and bursa were removed and weighed, and thyroid glands were frozen at −80 °C for hormone analysis. Sex was identified by gonads. Blood samples were centrifuged at 3000 × *g* for 10 min to separate plasma from the red blood cells. Plasma was then stored frozen (−20 °C) for hormone analysis.

2.3. Thyroid hormone analysis

Total thyroxine (T4) and total triiodothyronine (T3) concentrations in plasma samples and thyroid glands were determined using modified enzyme-linked immunosorbent assay (ELISA) kits (Monobind 225–300, 125–300, Lake Forest CA). Thyroid glands were digested according to previously published methods (McNabb et al., 2004). Glandular extracts (74% EtOH) were diluted 1:10 with charcoal stripped hen plasma, and standards used in glandular assays were also prepared to contain 7.4% ethanol. Hen plasma from a plasma pool was included in each plate to assess reproducibility and intra-assay precision, as well as quality control standards (Monobind ML-300) that were verified to be in the expected range. All samples were run in duplicate. Duplicate

precision (% CV) averaged 3.2%, 2.9%, 4.1% and 16.5% for glandular total T3, glandular total T4, plasma total T3, and plasma total T4, respectively. Plasma total T3 and glandular samples were all above the limits of detection for the assay. For plasma total T4, 8 samples were below the limit of detection (4 ng/ml, based on 2 SD of the zero calibrator).

2.4. Dosing solutions and analytical chemistry

Solid, pure TBBPA-BDBPE (CAS# 21850-44-2, Sigma-Aldrich) was dissolved in 100 µl acetone and 100 µl hexane, and the solution was combined with safflower oil (President's Choice Organics). Solvents were allowed to volatilize and outgas for 3 h, resulting in a stock solution of only TBBPA-BDBPE and safflower oil. The safflower oil vehicle was prepared in the same manner but without the inclusion of TBBPA-BDBPE. Dosing solutions with nominal concentrations of 10, 50 and 100 ng/µl were prepared by dilution of the stock solution with safflower oil.

TBBPA-BDBPE was measured in dosing solutions and tissues based on elution and mass spectrometry parameters that were generated by high-performance liquid chromatography - quadrupole-time-of-flight-mass spectrometry with atmospheric pressure photoionization (APPI) in the negative mode (LC-APPI(-)-Q-ToF-MS) according to previously published methods (Letcher and Chu, 2010), and optimized for quantitative analysis and determination by UPLC-APPI(-)-tandem quadrupole MS (UPLC-APPI(-)-MS/MS). The method limit of detection (MLOD) for TBBPA-BDBPE was 0.02 ng/g ww based on a signal-to-noise ratio (S/N) of 3, and the method limit of quantification (MLOQ) was 0.08 ng/g ww based on a S/N of 10. Target compound concentrations were inherently recovery-corrected using an internal standard (based on BDE-206). Average percent recoveries were 81 ± 11% for day 0 and 5 eggs, and 75 ± 9% for day 10 and 13 eggs. Percent recovery of TBBPA-BDBPE in the dosing solutions averaged 93.9 ± 13%. The quantitative accuracy of the measured TBBPA-BDBPE in spiked reference material (pork) was 105.1%. The control oil had no detectable TBBPA-BDBPE. Measured dosing solutions concentrations (3.0, 13.7 or 33.5 ng/µL) were below nominal concentrations (10, 50 or 100 ng/µL), which indicates 33.5 ng/µL is the maximum solubility of TBBPA-BDBPE in safflower oil.

2.5. Statistical analysis

Statistical analysis was completed using SAS® 9.4. Day 15 somatic indices for liver (LSI), bursa (BSI), spleen (SSI) and thyroid (TSI) were calculated by dividing organ mass by total body mass, and a body condition index (BCI) was estimated using the residuals from a linear regression of body mass on tarsus length (Schulte-Hostedde et al., 2005). The SSI, BSI, glandular T3 and t4, glandular T3:T4 ratio, and plasma T3:T4 ratio were log-transformed to meet assumptions of normality. To assess the effects of TBBPA-BDBPE on binary variables (hatching success, survival), generalized linear mixed models were used with a binomial distribution and a logit link (proc GLIMMIX). The effect of TBBPA-BDBPE dose on growth from 0 to 15 days was assessed using linear mixed models (proc MIXED) and the REPEATED statement, with bird ID as a repeated subject effect. The effect of dose on continuous variables was analyzed using linear mixed models (proc MIXED) with dose as a fixed effect. Nest was included as a random factor for all analyses. Sex and the sex*treatment interaction were included as fixed factors, but non-significant terms were removed from final models. *Post-hoc* tests for differences between means were adjusted for multiple comparisons following the Tukey–Kramer method. Sample sizes of injected eggs in the effects study were 28, 24, 29 and 23, and sample sizes at day 15 were 21, 17, 17 and 15 in the vehicle control, 0, 10, 50 and 100 ng TBBPA-BDBPE/g dose groups, respectively. Target sample size was a minimum of 20 eggs injected per treatment. Final sample sizes were not identical between treatments due to factors unrelated to treatment that are characteristic of using parental incubation in captive zebra finches (e.g. parents remove eggs from nest or do not incubate eggs). For statistical purposes, censored data were replaced by a random number between the limit of detection (LOD) and zero. A significance level of $\alpha = 0.05$ was used for all tests.

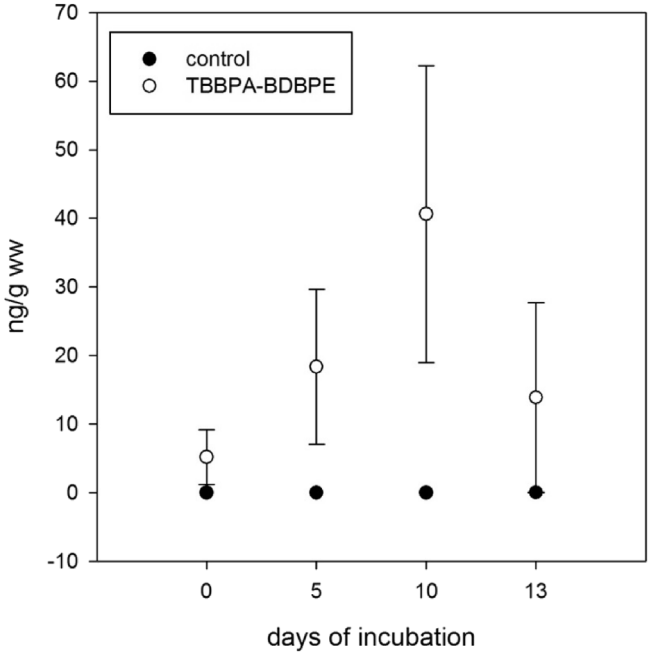

Fig. 1. Effect of incubation time on TBBPA-BDBPE concentrations (mean ± SE) in zebra finch eggs injected on day 0 with either the vehicle control (safflower oil) or 100 ng/g egg TBBPA-BDBPE.

3. Results

3.1. Embryonic metabolism

For the time-course study, TBBPA-BDBPE was not detected in 19/20 vehicle injected eggs, and was at the MLOQ in one vehicle egg. Eggs injected with 100 ng/g nominal (33.5 ng/g measured) had the lowest measured concentrations when they were unincubated, and the maximum concentrations were detected at day 10, which was followed by a decline in average TBBPA-BDBPE in late incubation (Fig. 1). The average concentration measured in the eggs just prior to hatch (day 13) was 34% of the average concentration measured at day 10.

3.2. Potential effects of TBBPA-BDBPE

In the effects study, overall hatching success was 71.1%, and there was no effect of TBBPA-BDBPE dose on hatching success ($F_{3,99} = 0.69$, $p = 0.559$; Table 1) or survival to day 15 ($F_{3,43} = 0.01$, $p = 0.998$). There was no effect of treatment on incubation time, controlling for laying order ($F_{3,42} = 0.81$, $p = 0.495$). There was no effect of TBBPA-BDBPE on LSI ($F_{3,38} = 1.35$, $p = 0.274$), SSI ($F_{3,35} = 0.34$, $p = 0.794$), BSI ($F_{3,37} = 0.14$, $p = 0.936$), or TSI ($F_{3,38} = 1.18$, $p = 0.331$) in day 15 nestlings. Females had a higher LSI than males ($F_{1,38} = 11.01$, $p = 0.002$), and there were no other effects of sex or interactions between sex and dose for the various organ somatic indices.

Glandular T4 was not different across treatments ($F_{3,38} = 0.62$, $p = 0.606$; Table 1) or between sexes ($F_{1,37} = 1.02$, $p = 0.318$), and there was no dose*sex interaction ($F_{3,34} = 0.58$, $p = 0.631$). Similarly, the glandular T3 did not differ across treatments ($F_{3,38} = 0.86$, $p = 0.472$) or sexes ($F_{1,37} = 0.11$, $p = 0.748$), and there was no dose*sex interaction ($F_{3,34} = 0.31$, $p = 0.816$). Glandular T3 and T4 were positively correlated with each other ($r = 0.872$, $p < 0.0001$). The

Table 1
Hatching success and mean (SE) organ somatic indices (% of body mass), and thyroid hormone concentrations (T4 = thyroxine, T3 = triiodothyronine) in zebra finches exposed to TBBPA-BDBPE *in ovo* via egg injection. Dose concentrations in the table are nominal, measured concentrations were (0, 3.0, 13.7 or 33.5 ng/μL).

	Dose (ng/g egg)				
	0	10	50	100	*p*
Injected eggs (N)	28	24	29	23	
Hatching success %	78.6	75	62.1	69.6	0.559
Liver somatic index	3.4 (0.1)	3.7 (0.1)	3.6 (0.1)	3.4 (0.1)	0.274
spleen somatic index	0.095 (0.011)	0.088 (0.011)	0.088 (0.01)	0.076 (0.008)	0.794
bursa somatic index	0.17 (0.01)	0.18 (0.02)	0.17 (0.02)	0.16 (0.01)	0.936
thyroid somatic index	0.0048 (0.0005)	0.005 (0.0006)	0.0058 (0.0006)	0.0056 (0.0006)	0.331
glandular T4 (ng/mg)	1.34 (0.19)	1.44 (0.27)	1.54 (0.38)	0.99 (0.11)	0.606
glandular T3 (ng/mg)	0.012 (0.003)	0.012 (0.003)	0.012 (0.004)	0.008 (0.001)	0.472
glandular T3:T4 ratio	0.0093 (0.0007)	0.0093 (0.0008)	0.0090 (0.0011)	0.0098 (0.0008)	0.612
plasma T4 (ng/ml)	7.49 (0.39)	6.6 (0.63)	7.35 (0.60)	7.22 (0.67)	0.682
plasma T3 (ng/ml)	1.65 (0.09)	1.5 (0.06)	1.45 (0.08)	1.56 (0.10)	0.399
plasma T3:T4 ratio	0.28 (0.02)	0.33 (0.05)	0.27 (0.03)	0.31 (0.05)	0.898

glandular T3:T4 ratio was not affected by TBBPA-BDBPE treatment ($F_{3,38} = 0.61$, $p = 0.612$) or sex ($F_{1,37} = 0.29$, $p = 0.595$), and there was no dose*sex interaction ($F_{3,35} = 1.28$, $p = 0.296$). Plasma thyroid hormones were not affected by treatment (T4: $F_{3,38} = 0.50$, $p = 0.682$; T3: $F_{3,38} = 1.01$, $p = 0.399$) or sex (T4: $F_{1,37} = 3.03$, $p = 0.090$, T3: $F_{1,37} = 2.74$, $p = 0.107$), and there was no dose*sex interaction (T4: $F_{3,34} = 0.33$, $p = 0.803$; T3: $F_{3,34} = 0.49$, $p = 0.693$). Plasma T4 was not correlated with plasma T3 ($r = -0.12$, $p = 0.304$), and there was no treatment effect on the plasma T3:T4 ratio ($F_{3,37} = 0.20$, $p = 0.898$). Females had a higher plasma T3:T4 ratio than males ($F_{1,37} = 5.17$, $p = 0.029$), but there was no dose*sex interaction ($F_{3,34} = 0.05$, $p = 0.986$).

Body mass significantly increased with age ($F_{3,262} = 30007.98$, $p < 0.0001$) and males were heavier than females ($F_{1,262} = 4.41$, $p = 0.037$); however there was no effect of TBBPA-BDBPE treatment on growth (dose*day interaction $F_{9,262} = 1.09$, $p = 0.374$), or on overall body mass across all time points ($F_{3,262} = 0.72$, $p = 0.544$), and there was no interaction between dose and sex ($F_{3,259} = 0.77$, $p = 0.510$). In a repeated measures analysis of body condition, there was a significant interaction between TBBPA-BDBPE dose and age ($F_{9,262} = 1.98$, $p = 0.042$), with body condition increasing with age in the control and to a lesser extent in the low dose group, but decreasing with age in the medium and high dose group (Fig. 2). There was no effect of sex ($F1_{,262} = 1.46$, $p = 0.228$), overall age across all doses ($F3_{,262} = 0.07$, $p = 0.978$), or overall dose across all ages ($F_{3,262} = 0.26$, $p = 0.852$) on the BCI. At day 15, there was a significant difference in BCI ($F_{3,38} = 4.34$, $p = 0.010$), with BCI decreasing as dose increased. For all other ages there were no effects of treatment on BCI ($p \geq 0.147$). There were no other effects of sex or age on the BCI ($p \geq 0.228$). The BCI pattern at day 15 is the result of structural size (tarsus) being similar across all treatments ($p = 0.791$), while body mass decreased in a dose-dependent manner ($p = 0.018$).

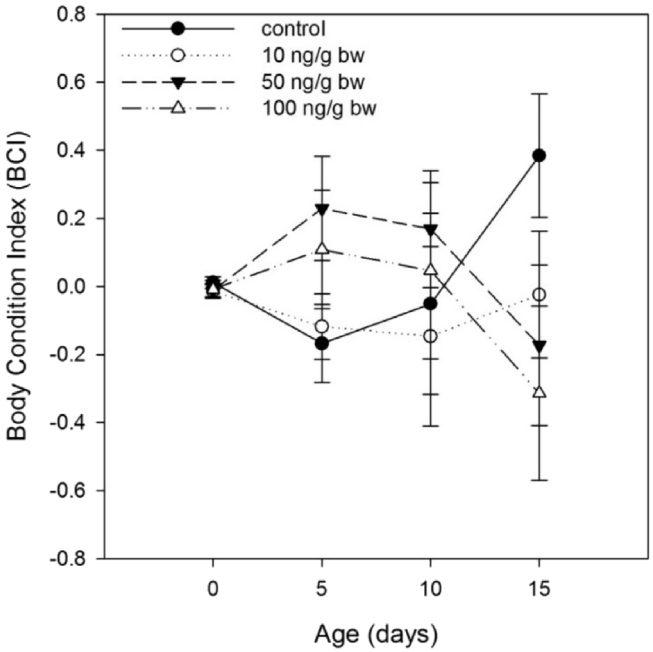

Fig. 2. Mean body condition index (BCI) from day 0 (hatch) to day 15 in zebra finches that were exposed to TBBPA-BDBPE *in ovo* via egg injection. Error bars represent standard errors about the means. Body condition was estimated using the residuals from a linear regression of body mass on tarsus length for each age. There was a significant interaction between dose and time for body condition ($p = 0.043$). From hatch to day 10 there were no treatment differences in BCI, but at the end of the rapid growth phase there was a dose dependent change in body condition, with the control group BCI increasing and the high dose BCI decreasing.

4. Discussion

We injected zebra finch eggs with TBBPA-BDBPE to assess the changes in concentrations over the embryonic period, and the effects of early exposure on songbird survival and development. We found that injected TBBPA-BDBPE was not well incorporated into the egg until embryos started to develop. There were no effects on survival or physiological measures; however, there was some evidence that TBBPA-BDBPE exposure affected nestling body condition, which was not evident until the latter end of the rapid growth phase.

The low detection of injected TBBPA-BDBPE in unincubated eggs, followed by an increase in egg concentration through mid-incubation, suggests that in early incubation the TBBPA-BDBPE was not well incorporated into the egg, and it was better incorporated as the embryo developed. In an egg injection study, it would be expected that the highest concentration is detected at injection, followed by a decrease in concentration as the embryo becomes metabolically competent (e.g. Eng et al., 2017; Farhat et al., 2013). However, the pattern of uptake and distribution of the contaminant within the egg can be affected by the method of injection and the physico-chemical characteristics of the contaminant. For example, eggs injected into the air cell with hydrophobic contaminant mixtures (pentaBDE and octaBDE) in an oil vehicle, only had 1.4–29.6% incorporated into the egg at pipping, and there was an inverse relationship between log K_{ow} and the amount of compound that crossed the air cell membrane (McKernan et al., 2010). In contrast, substances with relatively low log K_{ow} (≤ 3.75) in a vehicle that dissolves both polar and non-polar compounds (dimethyl sulfoxide) were detected at close to 100% of injection concentration in

unincubated avian eggs, regardless of whether the injection site was the air cell or the albumen (e.g. Eng et al., 2017; Farhat et al., 2013). An alternative route of exposure to egg injections that likely results in a more natural distribution of contaminants is to use maternal transfer by dosing egg-laying females. However, egg injections have an advantage of providing more control over the concentrations in individual eggs. Additionally, maternal transfer requires a much larger sample size, as all eggs from the same mother are pseudoreplicates. Egg injections also remove potential contaminant effects on maternal behaviour, and randomizing treatments within a nest can control for other maternal effects. In the present study, a large hydrophobic molecule dissolved in an oil vehicle was injected into the albumen. A possible explanation for the low detection of TBBPA-BDBPE in eggs sampled at day 0 is that instead of distributing through the egg contents it sorbed to the shell membrane and was lost during sample preparation. Increasing concentrations were detected in eggs at mid incubation, and it is possible that as embryos developed and consumed the albumen, the TBBPA-BDBPE was better incorporated into the tissues of the embryos and the samples. Additional measures of both TBBPA-BDBPE and its metabolites (e.g. debrominated compounds) in separate egg fractions (e.g. shell membranes, yolk, albumen, embryo) throughout the incubation period would be valuable for elucidating the pattern of uptake and metabolism by avian embryos.

The decrease in TBBPA-BDBPE concentration starting between day 10 and 13 (Fig. 1) and at the end of egg incubation was possibly due to embryonic metabolism, although metabolites were not measured in this study. There is evidence from exposure studies in fish that TBBPA-BDBPE taken up from the diet is rapidly eliminated (Nyholm et al., 2009). In rats, there is a low uptake efficiency of orally administered TBBPA-BDBPE (< 5%), but once it is taken up, metabolism is slow (Knudsen et al., 2007). The metabolites of TBBPA-BDBPE and their mechanism of formation have not been well characterized, but possible transformation mechanisms include debromination, ether-bond breakage, hydroxylation and Phase II conjugate metabolite formation (Liu et al., 2017, 2018; Qu et al., 2013). For example, female rats (Charles River) orally exposed to TBBPA (0–1000 mg/kg/day for 28 days) resulted in the *in vivo* formation of both TBBPA-glucuronide and TBBPA-sulfate metabolites, presumably via Phase II UDP-glucuronosyl transferases and sulfotransferases, respectively (Borghoff et al., 2016).

Based on the pattern of concentrations measured in TBBPA-BDBPE injected eggs, it is likely that embryos were not exposed to TBBPA-BDBPE until mid-to late-incubation; however, that may be similar to the timing of natural exposure, which would most likely be through embryonic utilization of the yolk. Egg partitioning has not been specifically assessed for TBBPA-BDBPE, but hydrophobic contaminants typically partition to the lipid-rich yolk. Rapid utilization of the yolk starts in mid-incubation, then the remaining yolk sac is drawn into the body at the end of incubation and the last of the yolk reserves are assimilated in the first few days post-hatch (Nangsuay et al., 2011; Sotherland and Rahn, 1987). Altricial eggs typically have proportionally less yolk than precocial eggs, and the yolk sac reserves are retained for a longer period in precocial young (Sotherland and Rahn, 1987), which could have implications for species differences in the timing of exposure to egg contaminants.

TBBPA-BDBPE concentrations injected into zebra finch eggs were higher than concentrations that have been reported for free-ranging birds (up to 0.36 ng/g ww in herring gull eggs sampled in 2008–2009) (Letcher and Chu, 2010); however, there is very little recent data on TBBPA-BDBPE concentrations in avian eggs. Environmental concentrations of TBBPA-BDBPE may have increased since these earlier studies given the ongoing or potentially increased use of this FR (Covaci and Malarvannan, 2015). Consequently, the higher doses used in the present study would be useful to evaluate the potential health risks of this FR. Our doses were within the range of concentrations that have been detected in marine biota (up to 2782.8 ng/g lw at 16% lipid dry mass in Japanese blue crabs, *Portunus trituberculatus*) (Liu et al., 2016a). At the concentrations presently used, there were no effects on hatching success or nestling survival or on any organ somatic indices of the zebra finches. Similar to the zebra finches, American kestrel embryos exposed to similar concentrations of TBBPA-BDBPE via egg injection also showed no effects on pipping or hatching success, oxidative stress, or somatic indices for liver, bursa or spleen (Eng et al., submitted). The few other studies that have assessed the effects of TBBPA-BDBPE have also observed minimal or no effects. A mesocosm exposure study in fathead minnows found no adverse effects on physical, biochemical, or reproductive parameters following exposure to environmentally-relevant concentrations of TBBPA-BDBPE (de Jourdan et al., 2011). In chicken embryonic hepatocyte assays, TBBPA-BDBPE upregulated CYP1A4 expression, but had no effects on mRNA expression of 26 other gene targets or on cell viability (Ma et al., 2015). *In vitro* assays found no evidence of TBBPA-BDBPE interference with androgenic, progestagenic or AhR-mediated pathways (Hamers et al., 2006), or steroidogenic enzyme activity (Cantón et al., 2006). TBBPA-BDBPE also did not interfere with estrogen receptor regulation, although there was some evidence it inhibited sulfation of estradiol, which could result in increased availability of endogenous estrogens (Hamers et al., 2006). However, these *in vitro* effects were based on the technical mixture that may contain TBBPA, which is a potent inhibitor of estradiol sulfation.

We measured thyroid hormones at day 15, which is near the estimated peak of thyroid hormone concentrations in altricial birds, while thyroid hormones in precocial birds peak just prior to hatch (McNabb, 2006). In zebra finches, there were no effects of *in ovo* TBBPA-BDBPE on glandular or circulating thyroid hormones at day 15. Similarly, in kestrel hatchlings exposed *in ovo*, TBBPA-BDBPE did not affect any measure of thyroid hormone homeostasis, including circulating and glandular thyroid hormone concentrations, thyroid gland histology, or deiodinase enzyme activity (Eng et al., submitted). There is evidence from *in vitro* competitive binding assays that the TBBPA-BDBPE technical mixture is a moderate inhibitor of the human thyroid transport protein transthyretin (TTR), although it is significantly less competitive than T4 (Hamers et al., 2006). TBBPA, which may be a component of the TBBPA-BDBPE technical mixture and is also a potential transformation product of TBPPA-BDBPE, was a more competitive ligand than T4 for TTR (Hamers et al., 2006). However, it should be noted that human and avian TTR can have different relative binding affinities for thyroid hormones and contaminants (Ucan-Marin et al., 2009). While we did not observe changes in stored or circulating thyroid hormone concentrations in zebra finches, we did see a decline in zebra finch body condition (mass corrected for structural size) in the higher dose groups towards the end of the rapid growth phase, while the body condition of the control and low dose group birds increased. The mechanism for this effect on body condition is not clear. While there were no differences in thyroid concentrations at day 15, we did not measure hormones earlier in the nestling period during the rapid growth phase, which may be a more critical window of development when conditions could have latent effects. Thyroid hormones interact in a permissive way with growth hormones and insulin-like growth factors, and they are important for normal growth and development in nestling birds (McNabb, 2007). It is also possible that the metabolites of TBBPA-BDBPE have more of an effect than the parent compound (Liu et al., 2018), which could explain delayed effects of exposure. The timing of assessment in the present study was selected to facilitate comparison with the concurrent American kestrel study (Eng et al., submitted); however, longer-term studies and further investigation into how TBBPA derivatives affect factors involved in growth and development are warranted.

TBBPA and its derivatives are currently not restricted in any country (Covaci and Malarvannan, 2015), although they share many characteristics with the BFRs they are potentially replacing (hydrophobic, aromatic moieties, halogenated). There are increasing reports of detections in environmental samples including biota, although studies monitoring concentrations of TBBPA-BDBPE and other TBBPA derivatives are still limited. Even less is known of the effects of TBBPA-BDBPE

in wildlife. Here we present evidence that *in ovo* exposure decreases body condition later in the nesting period in altricial songbirds, but does not affect survival, thyroid hormone homeostasis, or body condition at hatch. A concurrent study of *in ovo* exposure to TBBPA-BDBPE in a semi-altricial top predator, the American kestrel, found no effects of TBBPA-BDBPE, although exposed birds were only assessed at hatching, and effects on post-hatching hormone effects, growth and development were not measured (Eng et al., submitted). Further studies measuring factors that could link body condition and exposure, and studies of longer-term effects, would be valuable for understanding the mechanisms of action and potential fitness consequences of TBBPA-BDPBE exposure.

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References

Ali, N., Harrad, S., Goosey, E., Neels, H., Covaci, A., 2011. Novel" brominated flame retardants in Belgian and UK indoor dust: implications for human exposure. Chemosphere 83, 1360–1365.

Borghoff, S.J., Wikoff, D., Harvey, S., Haws, L., 2016. Dose-and time-dependent changes in tissue levels of tetrabromobisphenol A (TBBPA) and its sulfate and glucuronide conjugates following repeated administration to female Wistar Han Rats. Toxicology reports 3, 190–201.

Cantón, R.F., Sanderson, J.T., Nijmeijer, S., Bergman, Ã. k., Letcher, R.J., van den Berg, M., 2006. In vitro effects of brominated flame retardants and metabolites on CYP17 catalytic activity: a novel mechanism of action? Toxicol. Appl. Pharmacol. 216, 274–281.

Chen, D., Martin, P., Burgess, N.M., Champoux, L., Elliott, J.E., Forsyth, D.J., Idrissi, A., Letcher, R.J., 2013. European starlings (*Sturnus vulgaris*) suggest that landfills are an important source of bioaccumulative flame retardants to Canadian terrestrial ecosystems. Environ. Sci. Technol. 47, 12238–12247.

Chokwe, T.B., Okonkwo, J.O., Nwamadi, M.S., 2017. Occurrence and distribution of tetrabromobisphenol A and its derivative in river sediments from Vaal River Catchment, South Africa. Emerging Contaminants 3, 121–126.

Covaci, A., Harrad, S., Abdallah, M.A.E., Ali, N., Law, R.J., Herzke, D., de Wit, C.A., 2011. Novel brominated flame retardants: a review of their analysis, environmental fate and behaviour. Environ. Int. 37, 532–556.

Covaci, A., Malarvannan, G., 2015. Brominated flame retardants. In: Alaee, M. (Ed.), Dioxin and Related Compounds: Special Volume in Honor of Otto Hutzinger. Springer International Publishing, Cham, pp. 379–410.

Cristol, D.A., Brasso, R.L., Condon, A.M., Fovargue, R.E., Friedman, S.L., Hallinger, K.K., Monroe, A.P., White, A.E., 2008. The movement of aquatic mercury through terrestrial food webs. Science 320 335-335.

Custer, C.M., Custer, T.W., Dummer, P.M., Munney, K.L., 2003. Exposure and effects of chemical contaminants on tree swallows nesting along the Housatonic River, Berkshire County, Massachusetts, USA, 1998–2000. Environ. Toxicol. Chem. 22, 1605–1621.

de Jourdan, B.P., Hanson, M.L., Muir, D.C.G., Solomon, K.R., 2013. Environmental fate of three novel brominated flame retardants in aquatic mesocosms. Environ. Toxicol. Chem. 32, 1060–1068.

de Jourdan, B.P., Hanson, M.L., Muir, D.C.G., Solomon, K.R., 2014. Fathead minnow (*Pimephales promelas Rafinesque*) exposure to three novel brominated flame retardants in outdoor mesocosms: bioaccumulation and biotransformation. Environ. Toxicol. Chem. 33, 1148–1155.

de Jourdan, B.P., Oakes, K.D., Hanson, M.L., Sibley, P.K., Servos, M.R., Muir, D.C., Solomon, K.R., 2011. Physiological Effects of 3 Non-PBDE Brominated Flame Retardants on Pimephales promelas (Fathead Minnow) Exposed in Outdoor Mesocosms. Interdisciplinary Studies on Environmental Chemistry - Environmental Pollution and Ecotoxicology. Center for Marine Environmental Studies, Ehime University, Matsuyama, Japan, pp. 55–66.

Eens, M., Jaspers, V.L.B., Van den Steen, E., Bateson, M., Carere, C., Clergeau, P., Costantini, D., Dolenec, Z., Elliott, J.E., Flux, J., Gwinner, H., Halbrook, R.S., Heeb, P., Mazgajski, T.D., Moksnes, A., Polo, V., Soler, J.J., Sinclair, R., Veiga, J., Williams, T.D., Covaci, A., Pinxten, R., 2013. Can starling eggs be useful as a biomonitoring tool to study organohalogenated contaminants on a worldwide scale? Environ. Int. 51, 141–149.

Eng, M.L., Elliott, J.E., MacDougall-Shackleton, S.A., Letcher, R.J., Williams, T.D., 2012. Early exposure to 2,2 ',4,4 ',5-Pentabromodiphenyl ether (BDE-99) affects mating behavior of zebra finches. Toxicol. Sci. 127, 269–276.

Eng, M. L., Karouna-Renier, N. K., Henry, P. F. P., Letcher, R. J., Peters, L. E., Palace, V. P., Williams, T. D., Elliott, J. E., Fernie, K. J.. In Ovo exposure to brominated flame retardants Part II: assessment of effects of TBBPA-BDBPE and BTBPE on hatching success, morphometrics and physiology in American kestrels. Ecotoxicol. Environ. Saf..

Eng, M.L., Letcher, R.J., Williams, T.D., Elliott, J.E., 2017. In ovo tris(2-butoxyethyl) phosphate concentrations significantly decrease in late incubation after a single exposure via injection, with no evidence of effects on hatching success or latent effects on growth or reproduction in zebra finches. Environ. Toxicol. Chem. 36, 83–88.

Eng, M.L., Williams, T.D., Letcher, R.J., Elliott, J.E., 2014. Assessment of concentrations and effects of organohalogen contaminants in a terrestrial passerine, the European starling. Sci. Total Environ. 473–474, 589–596.

Farhat, A., Crump, D., Chiu, S., Williams, K.L., Letcher, R.J., Gauthier, L.T., Kennedy, S.W., 2013. *In ovo* effects of two organophosphate flame retardants - TCPP and TDCPP - on pipping success, development, mRNA expression, and thyroid hormone levels in chicken embryos. Toxicol. Sci. 134, 92–102.

Farmahin, R., Manning, G.E., Crump, D., Wu, D.M., Mundy, L.J., Jones, S.P., Hahn, M.E., Karchner, S.I., Giesy, J.P., Bursian, S.J., Zwiernik, M.J., Fredricks, T.B., Kennedy, S.W., 2013. Amino acid sequence of the ligand-binding domain of the aryl hydrocarbon receptor 1 predicts sensitivity of wild birds to effects of dioxin-like compounds. Toxicol. Sci. 131, 139–152.

Fernie, K.J., Letcher, R.J., 2018. Waste-water treatment plants are implicated as an important source of flame retardants in insectivorous tree swallows (*Tachicyneta bicolor*). Chemosphere 195, 29–39.

Hamers, T., Kamstra, J.H., Sonneveld, E., Murk, A.J., Kester, M.H.A., Andersson, P.L., Legler, J., Brouwer, A., 2006. In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol. Sci. 92, 157–173.

Harju, M., Heimstad, E.S., Herzke, D., Sandanger, T., Posner, S., Wania, F., 2009. Emerging "new" Brominated FLame Retardants in FLame Retarded Products and the Environment. Report 2462 Oslo, Norway. Norwegian Pollution Control Authority, pp. 113.

Heinz, G.H., Hoffman, D.J., Klimstra, J.D., Stebbins, K.R., Kondrad, S.L., Erwin, C.A., 2009. Species differences in the sensitivity of avian embryos to methylmercury. Arch. Environ. Contam. Toxicol. 56, 129–138.

Heinz, G.H., Hoffman, D.J., Kondrad, S.L., Erwin, C.A., 2006. Factors affecting the roxicity of methylmercury injected into eggs. Arch. Environ. Contam. Toxicol. 50, 264–279.

Howard, P.H., Muir, D.C.G., 2010. Identifying new persistent and bioaccumulative organics among chemicals in commerce. Environ. Sci. Technol. 44, 2277–2285.

Knudsen, G.A., Jacobs, L.M., Kuester, R.K., Sipes, I.G., 2007. Absorption, distribution, metabolism and excretion of intravenously and orally administered tetrabromobisphenol A [2,3-dibromopropyl ether] in male Fischer-344 rats. Toxicology 237, 158–167.

Letcher, R.J., Chu, S., 2010. High-sensitivity method for determination of tetrabromobisphenol-S and tetrabromobisphenol-A derivative flame retardants in great lakes herring gull eggs by liquid chromatography-atmospheric pressure photoionization-tandem mass spectrometry. Environ. Sci. Technol. 44, 8615–8621.

Liu, A.-f., Qu, G.-b., Yu, M., Liu, Y.-w., Shi, J.-b., Jiang, G.-b., 2016a. Tetrabromobisphenol-A/S and nine novel analogs in biological samples from the Chinese Bohai Sea: implications for trophic transfer. Environ. Sci. Technol. 50, 4203–4211.

Liu, A., Shi, J., Qu, G., Hu, L., Ma, Q., Song, M., Jing, C., Jiang, G., 2017. Identification of emerging brominated chemicals as the transformation products of tetrabromobisphenol A (TBBPA) derivatives in soil. Environ. Sci. Technol. 51, 5434–5444.

Liu, A., Zhao, Z., Qu, G., Shen, Z., Shi, J., Jiang, G., 2018. Transformation/degradation of tetrabromobisphenol A and its derivatives: a review of the metabolism and metabolites. Environ. Pollut. 243, 1141–1153.

Liu, L.-Y., Venier, M., Salamova, A., Hites, R.A., 2016b. A novel flame retardant in the Great Lakes atmosphere: 3, 3', 5, 5'-tetrabromobisphenol A bis (2, 3-dibromopropyl) ether. Environ. Sci. Technol. Lett. 3, 194–199.

Ma, M., Crump, D., Farmahin, R., Kennedy, S.W., 2015. Comparing the effects of tetrabromobisphenol-A, bisphenol A, and their potential replacement alternatives, TBBPA-bis(2,3-dibromopropyl ether) and bisphenol S, on cell viability and messenger ribonucleic acid expression in chicken embryonic hepatocytes. Environ. Toxicol. Chem. 34, 391–401.

McKernan, M.A., Rattner, B.A., Hatfield, J.S., Hale, R.C., Ottinger, M.A., 2010. Absorption and biotransformation of polybrominated diphenyl ethers DE-71 and DE-79 in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), American kestrel (*Falco sparverius*) and black-crowned night-heron (*Nycticorax nycticorax*) eggs. Chemosphere 79, 100–109.

McNabb, F.M.A., 2006. Avian thyroid development and adaptive plasticity. Gen. Comp. Endocrinol. 147, 93–101.

McNabb, F.M.A., 2007. The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. Crit. Rev. Toxicol. 37, 163–193.

McNabb, F.M.A., Jang, D.A., Larsen, C.T., 2004. Does thyroid function in developing birds adapt to sustained ammonium perchlorate exposure? Toxicol. Sci. 82, 106–113.

Murray, J.R., Varian-Ramos, C.W., Welch, Z.S., Saha, M.S., 2013. Embryological staging of the zebra finch, *Taeniopygia guttata*. J. Morphol. 274, 1090–1110.

Nagy, K.A., 1987. Field metabolic rate and food requirement scaling in mammals and birds. Ecol. Monogr. 57, 111–128.

Nangsuay, A., Ruangpanit, Y., Meijerhof, R., Attamangkune, S., 2011. Yolk absorption and embryo development of small and large eggs originating from young and old breeder hens. Poultry Sci. 90, 2648–2655.

Nyholm, J.R., Grabic, R., Arp, H.P.H., Moskeland, T., Andersson, P.L., 2013. Environmental occurrence of emerging and legacy brominated flame retardants near suspected sources in Norway. Sci. Total Environ. 443, 307–314.
Nyholm, J.R., Norman, A., Norrgren, L., Haglund, P., Andersson, P.L., 2008. Maternal transfer of brominated flame retardants in zebrafish (Danio rerio). Chemosphere 73, 203–208.
Nyholm, J.R., Norman, A., Norrgren, L., Haglund, P., Andersson, P.L., 2009. Uptake and biotransformation of structurally diverse brominated flame retardants in zebrafish (Danio rerio) after dietary exposure. Environ. Toxicol. Chem. 28, 1035–1042.
Qu, G.B., Liu, A.F., Wang, T., Zhang, C.L., Fu, J.J., Yu, M., Sun, J.T., Zhu, N.L., Li, Z.N., Wei, G.H., Du, Y.G., Shi, J.B., Liu, S.J., Jiang, G.B., 2013. Identification of tetrabromobisphenol A allyl ether and tetrabromobisphenol A 2,3-dibromopropyl ether in the ambient environment near a manufacturing site and in mollusks at a coastal region. Environ. Sci. Technol. 47, 4760–4767.
Sawal, G., Feibicke, M., Meinecke, S., Warmbrunn-Suckrow, E., Lepom, P., 2008. Identification of a novel brominated flame retardant in dust. Organohalogen Compd. 70, 2029–2032.
Schulte-Hostedde, A.I., Zinner, B., Millar, J.S., Hickling, G.J., 2005. Restitution of mass-size residuals: validating body condition indices. Ecology 86, 155–163.
Sotherland, P.R., Rahn, H., 1987. On the composition of bird eggs. Condor 89, 48–65.
Ucan-Marin, F., Arukwe, A., Mortensen, A., Gabrielsen, G.W., Fox, G.A., Letcher, R.J., 2009. Recombinant transthyretin purification and competitive binding with organohalogen compounds in two gull species (*Larus argentatus* and *Larus hyperboreus*). Toxicol. Sci. 107, 440–450.
Yu, M.S., Eng, M.L., Williams, T.D., Basu, N., Elliott, J.E., 2016. Acute embryotoxic effects but no long-term reproductive effects of in ovo methylmercury exposure in zebra finches (*Taeniopygia guttata*). Environ. Toxicol. Chem. 35, 1534–1540.