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In ovo exposure to brominated flame retardants Part II: Assessment of effects of TBBPA-BDBPE and BTBPE on hatching success, morphometric and physiological endpoints in American kestrels

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ABSTRACT

Tetrabromobisphenol A bis(2,3-dibromopropyl ether) (TBBPA-BDBPE) and 1,2-bis(2,4,6-tribromophenoxy) ethane (BTPBE) are both brominated flame retardants (BFRs) that have been detected in birds; however, their potential biological effects are largely unknown. We assessed the effects of embryonic exposure to TBBPA-BDBPE and BTBPE in a model avian predator, the American kestrel (*Falco sparverius*). Fertile eggs from a captive population of kestrels were injected on embryonic day 5 (ED5) with a vehicle control or one of three doses within the range of concentrations that have been detected in biota (nominal concentrations of 0, 10, 50 or 100 ng/g egg; measured concentrations 0, 3.0, 13.7 or 33.5 ng TBBPA-BDBPE/g egg and 0, 5.3, 26.8 or 58.1 ng BTBPE/g egg). Eggs were artificially incubated until hatching (ED28), at which point blood and tissues were collected to measure morphological and physiological endpoints, including organ somatic indices, circulating and glandular thyroid hormone concentrations, thyroid gland histology, hepatic deiodinase activity, and markers of oxidative stress. Neither compound had any effects on embryo survival through 90% of the incubation period or on hatching success, body mass, organ size, or oxidative stress of hatchlings. There was evidence of sex-specific effects in the thyroid system responses to the BTBPE exposures, with type 2 deiodinase (D2) activity decreasing at higher doses in female, but not in male hatchlings, suggesting that females may be more sensitive to BTBPE. However, there were no effects of TBBPA-BDBPE on the thyroid system in kestrels. For the BTPBE study, a subset of high-dose eggs was collected throughout the incubation period to measure changes in BTBPE concentrations. There was no decrease in BTBPE over the incubation period, suggesting that BTBPE is slowly metabolized by kestrel embryos throughout their ~28-d development. These two compounds, therefore, do not appear to be particularly toxic to embryos of the American kestrel.

1. Introduction

In response to changes in regulations on the manufacture and use of several flame retardants (e.g. polybrominated diphenyl ethers, PBDEs), there has been an increase in the use of “novel” or “replacement” brominated flame retardants (BFRs) that are proposed alternatives for the regulated compounds (Covaci et al., 2011). Two important examples of these emerging BFRs are tetrabromobisphenol A bis(2,3-dibromopropyl) ether (TBBPA-BDBPE) and 1,2-bis(2,4,6-tribromophenoxy)ethane (BTPBE), which are used in a wide range of

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plastic products and resins. They are extremely hydrophobic (TBBPA-BDBPE log K_{ow} ~11.52, BTBPE log K_{ow} ~9.15) and are likely to associate with particles and persist in the environment (Harju et al., 2009; Howard and Muir, 2010). As well, both have demonstrated potential for bioaccumulation (de Jourdan et al., 2014; Giraudo et al., 2017; Law et al., 2006).

There are several reports of TBBPA-BDBPE and BTBPE detection in abiotic environmental samples worldwide, including air (Liu et al., 2016b; Sahlström et al., 2015), dust (Guo et al., 2018), soil (Liu et al., 2017; McGrath et al., 2017), and sediment (Chokwe et al., 2017). Both compounds have also been detected in biota such as birds (e.g. de Wit et al., 2010; Fernie and Letcher, 2018; Karlsson et al., 2006; Letcher and Chu, 2010; Sun et al., 2014; Verreault et al., 2007). Despite the increasing use and detection of these BFRs, very little is known of their potential effects, particularly for free-ranging birds. Previous avian assessments are limited to *in vitro* assays using embryonic chicken (*Gallus gallus domesticus*) hepatocytes, and an *in ovo* BTBPE study in chickens (Egloff et al., 2011; Ma et al., 2015). Those screening studies found that TBBPA-BDBPE and BTBPE both upregulated CYP1A4 expression, and BTBPE also downregulated genes involved in thyroid hormone metabolism. There were no other effects on pipping success (BTBPE only), mRNA expression, or cell viability (Egloff et al., 2011; Ma et al., 2015).

The objectives of the current study were to assess the effects of early developmental exposure to TBBPA-BDBPE and BTBPE in avian predators, using American kestrels (*Falco sparverius*) as a model species. Avian predators may be particularly vulnerable to the effects of environmental contaminants and can serve as sentinels for environmental pollution, as they feed at a higher trophic level and have the potential to accumulate high concentrations of contaminants (Espín et al., 2016; Golden and Rattner, 2003). Sensitivity to environmental contaminants can vary considerably among avian species (Farmahin et al., 2013; Heinz et al., 2009), and raptors have demonstrated a relatively high sensitivity to several brominated and organophosphate ester flame retardants (Guigueno and Fernie, 2017). For example, kestrel eggs injected with a PBDE mixture had significantly reduced pipping and hatching success compared to controls, but the same concentrations had no effects when injected into chicken or mallard (*Anas platyrhynchos*) eggs (McKernan et al., 2009). In the present study, kestrel embryos were exposed to TBBPA-BDBPE or BTBPE via egg injections, and then monitored for survival to hatching. Physiological measures of hatchlings, including markers of oxidative stress (lipid peroxidation, glutathione oxidation, thiol compounds, and oxidant-induced DNA damage) and thyroid hormone homeostasis (circulating and glandular thyroid hormone concentrations, thyroid gland histology, and deiodinase enzyme activity) were assessed. To characterize variation in species sensitivity, the results of this study will be compared to results from a concurrent study in zebra finches (*Taeniopygia guttata*), a model altricial songbird species (Eng et al., submitted).

2. Methods

2.1. American kestrel egg injections and tissue collection

American kestrels were selected as a model semi-altricial predator species, as they are a well-established avian model in toxicology and biology research (Bardo and Bird, 2009). All animal housing and procedures were approved by the U. S. Geological Survey (USGS) Patuxent Wildlife Research Center (PWRC) Animal Care and Use Committee. Freshly laid American kestrel eggs were obtained from a captive colony maintained at the USGS PWRC. Eggs were collected daily and stored in an egg chiller (13 °C, 55–65% relative humidity (RH), 60° rotation per hr) for up to 7 days to allow for simultaneous dosing of multiple eggs. The two compounds were tested over consecutive breeding seasons, with 111 fertile eggs collected to test for effects of TBBPA-BDBPE in 2014, and 83 fertile eggs collected to test for effects of BTBPE in 2015. Eggs were equilibrated to room temperature for 2 h, cleaned and dipped in a 0.1% povidone-iodine solution, warmed to 40 °C, rinsed in 40 °C ozone-treated carbon-filtered water, and air dried. Each egg was then weighed and set horizontally in sliding wooden trays within cabinet incubators (Kuhl Corporation, Flemington, NJ, USA) at 37.5 °C with 180° rotation every hour, and the humidity was adjusted to maintain mean egg weight loss of 14–16% over 26 days of incubation. On Embryonic Day 4 or 5 (ED4 or ED5) fertility was confirmed by candling, and only fertile eggs were included in the injection study.

See Figure S1 for a summary flowchart of experimental procedures. Eggs were injected on ED5, which corresponds to Hamburger-Hamilton stage 18 for domestic chickens (Pisenti et al., 2001). Eggs were randomly assigned to treatments (0, 10, 50, or 100 ng/g ww), with eggs from the same parental pair being distributed evenly as possible across treatment groups. The injection site was wiped with alcohol and a 0.08 cm hole was removed from the shell using a Dremel drill, and a sterile 22 gauge needle was used to pierce the inner membrane. Dosing solutions had nominal concentrations of 10, 50 and 100 ng/μL, and a constant volume of 1 μl dosing solution per g egg (based on mean egg weight per batch) was injected into the air cell using a repeater pipette to achieve nominal target concentrations on average. The hole was sealed using waterproof clear bandage adhesive, and the egg was placed vertically, injection side up, in a portable incubator at 37 °C for 30 min to allow for the distribution of the injected solution over the inner cell membrane, then returned to the cabinet incubator in the horizontal position. In 2014, injected eggs from each dose group (n = 12 total) were collected on ED8 for TBBPA-BDBPE residue analysis. In 2015, a subset of kestrel eggs from the control and high BTBPE dose groups were sampled on ED12 (n = 4 control, 3 high), ED18 (n = 2 control, 5 high), ED21 (n = 3 control, 3 high), and ED25 (n = 3 control, 4 high) to measure BTBPE concentrations over the incubation period. Samples were stored individually at −20 °C until analysis.

On ED24, viable eggs were transferred into individual plastic mesh hatching cells and incubated without rotation at 37 °C and 70% RH until hatching. From ED27 to ED29, eggs were monitored for pipping and hatching. Pipped eggs were left to hatch up to 24 h, at which point they were considered failed to hatch. Once dry, all hatchlings were weighed and examined for physical deformities. Blood samples were taken from the jugular vein, then hatchlings were euthanized, and organs were removed and weighed. The right thyroid gland was frozen at −20 °C for thyroxine (T4) and triiodothyronine (T3) analyses, and the left thyroid gland was fixed in buffered formalin for histopathology. Two portions of liver (~100–200 mg) from the distal end of the largest lobe were flash frozen in liquid nitrogen then stored at −80 °C for hepatic deiodinase activity (BTBPE only) and oxidative stress measures. In 2014, subsets of hatchling livers from each dose group were pooled (3–5 livers per pool, n = 3 pools per dose group, 6 control group pools) for TBBPA-BDBPE analysis. Genetic sex was determined by real-time PCR following previously described methods (Brubaker et al., 2011), using primers sex 1 and sex 2 (0.75 μM each) of Wang and Zhang (2009).

2.2. Physiological measures

Details of physiological assays can be found in the Supporting Information. Briefly, plasma and glandular total (T) T3 concentrations were analyzed using the AccuBind® ELISA T3 Kit (Monobind Inc., Lake Forest, CA, USA), and TT4 concentrations were analyzed using the NT4 AccuBind ELISA kit (Monobind Inc.). Histological changes were assessed in the thyroid glands using previously described methods (Fernie et al., 2015, 2016, 2019; Park et al., 2011). Measurement of hepatic deiodinase activity was added in the second year of the study and was therefore measured for BTBPE samples only. The activity of two of the main deiodinase isoforms (D1, D2) was determined for a subset of samples following previously described methods (Fernie et al., 2015). A subset of 12 samples liver per treatment group were assayed for markers of oxidative stress, including thiobarbituric acid reactive

substances (TBARS), reduced glutathione (GSH), total glutathione (total GSH), oxidized glutathione (glutathione disulfide, GSSG; calculated as [(total GSH – GSH)/2]), the ratio between oxidized and reduced glutathione (GSSG:GSH), total thiols and protein bound thiols (calculated as [total thiols – GSH]), and 8-hydroxy-deoxy-guanosine (8-OH-dG) (BTBPE only). Assay methods were as previously described (Beyer et al., 2018), with minor modification (see Supporting Information). Intra-assay variability and inter-assay variability are presented in Tables S1 and S2.

2.3. Dosing solutions and analytical chemistry

Solid, pure TBBPA-BDBPE (CAS# 21850-44-2, Sigma-Aldrich) or BTBPE (CAS# 37853-59-1, Sigma-Aldrich) was dissolved in acetone and hexane, and the solution was combined with safflower oil (President's Choice® Organics). Solvents were allowed to volatilize and outgas, resulting in a stock solution of only TBBPA-BDBPE or BTBPE and safflower oil. The safflower oil vehicle was prepared in the same way without the TBBPA-BDBPE or BTBPE. Dosing solutions with nominal concentrations of 10, 50 and 100 ng/μL were made up from the stock solution and 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) (Letcher and Chu, 2010), and optimized for quantitative analysis and determination by UPLC-APPI(-)-tandem quadrupole MS (UPLC-APPI(-)-MS/MS). Target compound concentrations were inherently recovery-corrected by internal standards (BDE-206 and BDE-207). Percent recoveries were 94 ± 13% BDE-206 for the TBBPA-BDBPE dosing solutions, 71 ± 13% BDE-206 and 80 ± 7% BDE-207 for the hatchling liver, and 79 ± 16% BDE-206 for the eggs. The method limits of detection (MLODs) and method limits of quantification (MLOQs) were based on replicate analyses (n = 8) of matrix samples (chicken egg and pork liver) spiked with analytes at a concentration 3–5 times the estimated method detection limit. The MLOD (signal-to-noise (S/N) ratio of 3) was 0.014 ng/g ww in chicken egg and 0.026 ng/g ww in pork liver. The MLOQ (standard deviation of replicate matrix samples multiplied by the Student's t-value for a 99% confidence interval) was 0.03 ng/g ww in chicken egg, and 0.06 ng/g ww in pork liver.

BTBPE dosing solutions, kestrel eggs, and yolk sacs were analyzed using gas chromatography-single quadrupole mass spectrometry operating in the electron-capture negative ionization mode (GC-MS [ECNI]), following methods developed for analysis of polybrominated and other halogenated flame retardants, as previously described for avian eggs (Chen et al., 2012; Fernie and Letcher, 2018). For quality assurance, with each batch of samples one method blank sample was analyzed, and a reference material sample consisting of egg homogenate of double-crested cormorants (*Phalacrocorax auritus*) was extracted and analyzed to ensure consistency of data acquisition. Target compound concentrations were inherently recovery-corrected using an internal standard approach. That is, the response factor of the analyte relative to that of the appropriate internal standard (the relative response factor) was used for quantification. The mean recovery of the internal standard for BTBPE (BDE-156) was 86 ± 21%. The MLOD based on a S/N ratio of 3 was 0.5 ng/g, and the MLOQ based on a S/N of 10 was 1.5 ng/g. The control safflower oil had no detectable TBBPA-BDBPE or BTBPE. Measured dosing solution concentrations (3.0, 13.7 or 33.5 ng TBBPE-BDBPE/μL and 5.3, 26.8, 58.1 ng BTBPE/μL) were below nominal concentrations (10, 50 or 100 ng/μL), which indicates the maximum solubility in safflower oil is 33.5 ng/μL for TBBPA-BDBPE, and 58.1 ng/μL for BTBPE.

2.4. Statistical analysis

Statistical analysis was conducted using SAS® 9.4 (SAS Institute, Cary, NC, USA). Assumptions of normality and homogeneity of variance were tested for using Shapiro-Wilk and Levene's tests, and by examining Q-Q plots, and, where necessary, data were log transformed to meet assumptions. Effects of treatment on binary variables (e.g. hatching success) were assessed using generalized linear mixed models (proc GLIMMIX), and the parental pair was included as a random factor. Treatment effects on continuous variables were assessed using general linear mixed models (proc MIXED) with treatment as a fixed effect, and parental pair as a random factor for all analyses. To compare body mass among treatments, egg mass was included as a covariate. For thyroid gland histological analysis, individual nested within treatment was included as a random effect to account for the multiple measurements recorded for each bird, and summary statistics were calculated from mean values for each individual bird. Organ somatic indexes were calculated from the mass of the organ divided by the body mass. To correct for the effect of structural size, body condition index was estimated using the residuals from a linear regression of body mass on crown-rump length (CRUMP) (Schulte-Hostedde et al., 2005), as CRUMP was the structural size measure most strongly correlated with mass. Thyroid gland mass was the summed value of the left and right thyroid. 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. For statistical purposes, censored data (i.e. below reporting limits) were replaced by a random number between the reporting limit and zero. If more than 50% of the samples had concentrations below the reporting limit, then we did not run statistical tests and only report the ranges of concentrations. A significance level of $\alpha = 0.05$ was used for all tests.

3. Results

3.1. Survival and morphology

There was no effect of TBBPA-BDBPE treatment on hatching success (Table 1; $F_{3,68} = 0.06$, $p = 0.982$) or pipping success ($F_{3,68} = 1.20$, $p = 0.317$). There was also no treatment effect on body mass, controlling for the effect of egg mass ($F_{3,28} = 1.34$, $p = 0.280$), and there was also no sex effect ($F_{1,27} = 0.03$, $p = 0.874$) or sex*treatment interaction ($F_{3,24} = 2.30$, $p = 0.103$). There was also no effect of TBBPA-BDBPE on body condition ($F_{3,27} = 0.77$, $p = 0.521$), and there was also no sex effect ($F_{1,26} = 0.09$, $p = 0.763$) or sex*treatment interaction ($F_{3,23} = 2.47$, $p = 0.088$). The difference between treatments for the bursa somatic index was marginally non-significant ($F_{3,28} = 2.72$, $p = 0.063$), with the medium dose birds having the smallest average bursa somatic index and the controls having the highest. None of the other organ somatic indices were significantly different between TBBPA-BDBPE treatments (liver somatic index $F_{3,28} = 0.26$, $p = 0.855$; spleen somatic index $F_{3,29} = 1.13$, $p = 0.354$; thyroid somatic index $F_{3,27} = 1.00$, $p = 0.408$), and there were no differences between sex ($p \geq 0.101$) or sex*treatment interactions ($p \geq 0.319$) for any of the indices.

BTBPE had no effects on hatching success ($F_{3,43} = 0.80$, $p = 0.498$) or pipping success ($F_{3,43} = 0.28$, $p = 0.837$). Body mass was also not affected by BTBPE ($F_{3,27} = 1.18$, $p = 0.334$; controlling for egg mass). Females were heavier than males ($F_{1,27} = 4.96$, $p = 0.035$), but there was no sex*treatment interactions ($F_{3,24} = 0.92$ $p = 0.444$) for mass. There was also no effect of BTBPE on body condition ($F_{3,28} = 1.26$, $p = 0.307$), and there was also no sex effect ($F_{1,27} = 0.62$, $p = 0.438$) or sex*treatment interaction ($F_{3,24} = 1.18$, $p = 0.340$). There were no effects of BTBPE on any of the somatic indices (liver somatic index $F_{3,29} = 0.71$, $p = 0.554$; bursa somatic index $F_{3,28} = 0.42$, $p = 0.740$; spleen somatic index $F_{3,29} = 0.52$, $p = 0.370$; thyroid somatic index

Table 1
Percentages for survival and mean (SE) values for morphological and physiological measures in American kestrel hatchlings that were exposed to either TBBPA-BDBPE or BTBPE on embryonic day 5 via egg injection. The range only is reported for plasma TT4 where > 50% of samples were below the reporting limit. TT4 = total thyroxine, TT3 = total triiodothyronine, ECH = epithelial cell height, rT3 = reverse 3,3′,5′-triiodothyronine, T2 = 3,3′-diiodothyronine, TBARS = thiobarbituric acid reactive substances, GSH = glutathione, GSSG = glutathione disulfide, 8-OH-dG = 8-hydroxy-deoxy-guanosine. Deiodinase activity and 8-OH-dG were added in the second year of the study.

	TBBPA-BDPBE Dose (ng/g egg)					BTBPE Dose (ng/g egg)				
	0	10	50	100	*p*	0	10	50	100	*p*
Injected eggs (N)	26	27	31	27		20	22	22	19	
Hatching success %	61.5	59.3	58.1	63	0.982	85	86.4	77.3	68.4	0.498
Pipping success %	92.3	81.5	96.8	85.2	0.317	90	95.5	86.4	89.5	0.837
Body mass (g)	10.2 (0.2)	10.5 (0.2)	10.9 (0.3)	10.4 (0.2)	0.171	10.4 (0.2)	10.6 (0.1)	10.5 (0.2)	9.9 (0.4)	0.327
Liver somatic index (10x)	0.24 (0.01)	0.23 (0.01)	0.24 (0.01)	0.24 (0.01)	0.855	0.72 (0.07)	0.81 (0.08)	0.69 (0.07)	0.82 (0.08)	0.554
Spleen somatic index (1000x)	0.60 (0.06)	0.64 (0.05)	0.58 (0.05)	0.57 (0.05)	0.354	0.59 (0.04)	0.58 (0.04)	0.52 (0.04)	0.59 (0.06)	0.370
Bursa somatic index (1000x)	1.05 (0.09)	1.00 (0.07)	0.75 (0.07)	0.89 (0.08)	0.063	0.92 (0.06)	0.98 (0.10)	0.98 (0.06)	0.83 (0.09)	0.740
Thyroid somatic index (1000x)	0.145 (0.008)	0.143 (0.009)	0.145 (0.012)	0.162 (0.007)	0.408	0.154 (0.008)	0.156 (0.009)	0.150 (0.008)	0.154 (0.007)	0.970
Thyroid measures (N)	15	16	15	17		12	12	12	11	
Glandular TT4 (ng/mg)	170.25 (15.55)	170.13 (14.08)	172.84 (12.38)	154.95 (14.47)	0.840	184.4 (14.5)	198.1 (17.7)	165.6 (14.7)	197 (17.9)	0.895
Glandular TT3 (ng/mg)	2.18 (0.19)	2.34 (0.21)	2.28 (0.17)	2.25 (0.20)	0.971	1.07 (0.15)	1.04 (0.11)	1.17 (0.1)	1.21 (0.16)	0.540
Glandular TT3:TT4 ratio (10x)	0.165 (0.017)	0.175 (0.016)	0.167 (0.017)	0.185 (0.016)	0.844	0.069 (0.007)	0.07 (0.011)	0.091 (0.012)	0.078 (0.013)	0.363
Plasma TT4 (ng/ml)	ND to 1.12	ND to 0.87	ND to 1.05	ND to 1.83	n/a	ND to 1.56	ND to 2.04	ND to 2.08	ND to 2.08	n/a
Plasma TT3 (ng/ml)	0.73 (0.14)	0.56 (0.10)	0.60 (0.09)	0.61 (0.10)	0.535	0.64 (0.07)	0.79 (0.09)	0.74 (0.08)	0.67 (0.11)	0.651
Thyroid gland histology (N)	16	16	18	17		11	10	12	9	
Mean ECH (μm)	6.50 (0.22)	7.10 (0.29)	6.83 (0.18)	6.81 (0.22)	0.365	6.99 (0.23)	7.09 (0.26)	7.58 (0.31)	6.94 (0.13)	0.175
Mean colloid area (μm^2)	726.3 (78.8)	610.4 (59.8)	646.2 (54.4)	661.9 (61.3)	0.555	603.7 (46.6)	776.2 (198.9)	511 (42.9)	596 (72.4)	0.433
Mean colloid diameter:ECH	4.99 (0.34)	4.27 (0.31)	4.51 (0.23)	4.51 (0.25)	0.262	4.19 (0.20)	4.36 (0.34)	3.59 (0.24)	4.12 (0.30)	0.185
Deiodinase activity (N)						12	12	12	11	
D1 activity (pg rT3 & T2/mg protein/min)	n/a	n/a	n/a	n/a		11.8 (1.3)	10.9 (2.1)	10.3 (1.7)	11.5 (2.9)	0.898
D2 activity (pg T3/mg protein/min)	n/a	n/a	n/a	n/a		36.3 (3.3)	41.2 (4.9)	32.8 (4.2)	38.1 (7.7)	0.425
Oxidative stress (N)	12	12	12	12		12	12	12	10	
TBARS (nmol/g protein)	57.9 (5.8)	51.5 (3.6)	52.7 (3.8)	54.3 (3.2)	0.765	33.4 (2.3)	32.8 (1.5)	34.9 (1.8)	33.3 (1.3)	0.875
Reduced GSH (umol/g tissue)	5.0 (0.3)	5.2 (0.4)	5.0 (0.3)	5.4 (0.3)	0.771	5.3 (0.3)	5.5 (0.3)	5.6 (0.3)	5.4 (0.4)	0.933
Total GSH (umol/g tissue)	7.6 (0.4)	8.2 (0.6)	7.7 (0.4)	8.2 (0.5)	0.759	7.7 (0.4)	8.1 (0.4)	8.3 (0.5)	8.0 (0.6)	0.852
GSSG (umol/g tissue)	1.3 (0.1)	1.5 (0.1)	1.4 (0.1)	1.4 (0.1)	0.645	1.2 (0.1)	1.3 (0.1)	1.4 (0.1)	1.3 (0.1)	0.768
Ratio GSSG/GSH	0.27 (0.01)	0.29 (0.01)	0.27 (0.01)	0.26 (0.01)	0.606	0.24 (0.01)	0.24 (0.02)	0.24 (0.01)	0.24 (0.01)	0.949
Thiol (umol/g tissue)	35.7 (1.5)	37.4 (2.4)	36.4 (1.7)	39.3 (2.0)	0.578	34.3 (2.1)	34.2 (1.7)	36.7 (2.3)	36.1 (2.6)	0.768
Protein Bound Thiol (umol/g tissue)	30.7 (1.2)	32.1 (2.0)	31.4 (1.4)	33.9 (1.6)	0.582	29.0 (1.7)	28.8 (1.5)	31.1 (2.1)	30.7 (2.3)	0.756
8-OH-dG (pg/ug DNA)	n/a	n/a	n/a	n/a		37 (3.8)	34.9 (3.5)	46.9 (4.5)	45.8 (4.3)	0.092

$F_{3,29} = 0.08$, $p = 0.970$). Males had a significantly higher bursa somatic index than females ($F_{1,28} = 7.65$, $p = 0.010$; males 0.00103 ± 0.00006, females 0.00082 ± 0.00005), but there were otherwise no differences in somatic indices between sexes ($p \geq 0.261$), and no sex*treatment interactions ($p \geq 0.329$).

Pipping success was similar between years. However, we observed a higher mortality rate across all treatments in 2014 due to a lower than expected mean weight loss and greater moisture retention in the eggs. Relative humidity was adjusted in 2015 to allow for a more optimal 14–16% weight loss over the 26-day incubation (McKernan et al., 2009), resulting in a higher % hatch rate in 2015.

3.2. Thyroid effects (hormones, glands, enzymes)

Overall in the TBBPA-BDBPE treatment groups, males had greater plasma TT3 concentrations than females ($F_{1,23} = 5.20$, $p = 0.032$; male 0.72 ± 0.07 ng/ml, female 0.51 ± 0.07 ng/ml), but there was no interaction between sex and treatment ($F_{3,20} = 2.29$ $p = 0.109$), and no effect of treatment for plasma TT3 ($F_{3,23} = 0.75$, $p = 0.535$; Table 1). For plasma TT4, 29 out of 56 samples were below the reporting limit for TBBPA-BDBPE (0.45 ng/ml), which precluded statistical comparison of means. However, the proportion of samples below the reporting limit was not different across TBBPA-BDBPE treatment groups ($\chi^2_3 = 3.14$, $p = 0.370$).

For the glandular TT4 concentrations, there was a significant interaction between TBBPA-BDBPE treatment and sex ($F_{3,19} = 5.00$ $p = 0.010$), with control females and high dose females having significantly lower thyroid gland TT4 than control males, but all other treatment*sex groups were not significantly different from each other. On average across all treatments, males had higher glandular TT4 than females ($F_{1,22} = 8.65$, $p = 0.008$; male 186.8 ± 9.1 ng/mg, female 145.6 ± 9.6 ng/mg) although there was no effect of treatment overall ($F_{3,22} = 0.28$, $p = 0.840$). Total glandular T3 was also higher in males than females ($F_{1,22} = 4.54$, $p = 0.044$; male 2.47 ± 0.14 ng/mg, female 2.05 ± 0.12 ng/mg) but there was no effect of TBBPA-BDBPE treatment ($F_{3,22} = 0.08$ $p = 0.971$), and no interaction between sex and treatment ($F_{3,19} = 0.96$ $p = 0.433$). Glandular TT3 and TT4 were positively correlated (r = 0.35, $p = 0.006$), but the ratio of glandular TT3:TT4 was not affected by treatment ($F_{3,23} = 0.27$ $p = 0.844$) or sex ($F_{1,22} = 0.66$, $p = 0.424$), and there was no sex*treatment interaction ($F_{3,19} = 1.38$ $p = 0.281$).

The cellular structure of the thyroid glands was also not affected by TBBPA-BDBPE treatment, although there were some sex differences for certain measures. Females had smaller average colloid area ($F_{1,40} = 11.36$, $p = 0.002$; female 533.4 ± 38.8 μm^2, male 734.7 ± 38.0 μm^2), and less active thyroid glands (a smaller average

colloid diameter:ECH ratio) than males ($F_{1,40} = 6.44$, $p = 0.015$; female 4.13 ± 0.22, male 4.81 ± 0.17), although mean ECH was not different between sexes ($F_{1,40} = 0.02$, $p = 0.878$). Neither the structure of the thyroid glands (ECH, colloid area), or the activity of the thyroid gland (colloid diameter:ECH) were affected by TBBPA-BDBPE treatment ($p \geq 0.262$), and there were no interactions between treatment*sex for any of the thyroid gland histology measures ($p \geq 0.672$).

In the BTBPE experiment, there were no differences across treatments for plasma total T3 ($F_{3,14} = 0.56$, $p = 0.651$), and there were also no effects of sex (T3 $F_{1,13} = 0.75$, $p = 0.403$) or interactions between sex and treatment (T3 $F_{3,10} = 0.53$ $p = 0.672$) for plasma thyroid hormone concentrations. For plasma TT4, 28 out of 47 samples were below the reporting limit for BTBPE (0.78 ng/ml), and we therefore did not compare means. The proportion of samples below the reporting limit was not different across BTBPE treatment groups ($\chi^2_3 = 1.26$, $p = 0.738$).

For glandular thyroid hormones, there were no BTBPE treatment effects ($p \geq 0.479$) or interactions between treatment and sex ($p \geq 0.875$), although males had higher TT3 than females (sex $F_{1,13} = 7.32$, $p = 0.018$; male 1.28 ± 0.10 ng/mg, female 0.96 ± 0.07 ng/mg). The relationship between glandular TT3 and TT4 was positive, but not significantly correlated (r = 0.27, $p = 0.066$). There was no effect of BTBPE treatment on the TT3:TT4 ratio ($F_{3,14} = 1.15$, $p = 0.363$) and no sex ($F_{1,13} = 0.52$, $p = 0.483$) or sex*treatment interaction ($F_{3,10} = 0.06$ $p = 0.980$) effects.

Thyroid gland histology showed that there were no significant effects of BTBPE treatment on glandular structure or function ($p \geq 0.175$), but there were some sex differences. Females had larger mean ECH than males ($F_{1,37} = 5.73$, $p = 0.022$; females 7.44 ± 0.22 µm, males 6.88 ± 0.10 µm), but there were no sex differences for the colloid area ($F_{1,37} = 0.06$, $p = 0.803$) or the mean colloid diameter:ECH ratio ($F_{1,37} = 0.52$, $p = 0.477$). There were also no interactions between BTBPE treatment and sex for any of the thyroid histology measures ($p \geq 0.186$).

Hepatic deiodinase activity was only measured for the second year of the study. BTBPE treatment had no effect on D1 activity ($F_{3,14} = 0.20$, $p = 0.898$), and no sex*treatment interaction for D1 ($F_{3,10} = 1.88$ $p = 0.197$). There was a non-significant trend for males to have higher D1 activity than females ($F_{1,13} = 4.28$, $p = 0.059$; male 13.2 ± 1.7 pg rT3 & T2/mg protein/min, female 9.1 ± 1.0 pg rT3 & T2/mg protein/min). While there was no overall effect of treatment on D2 activity when sexes were combined ($F_{3,10} = 1.02$, $p = 0.425$), there was a significant interaction between treatment and sex ($F_{3,10} = 8.73$ $p = 0.004$), and a significant effect of sex on D2 activity (sex $F_{1,10} = 25.12$, $p = 0.0005$). Overall, males had higher D2 activity than females (male 44.8 ± 3.7, female 29.7 ± 2.8 pg T3/mg protein/min). Male D2 activity did not significantly change across dose groups, but there was a non-significant tendency for seemingly greater activity at higher dosages. In contrast, female D2 activity declined in a dose dependent manner, and females in the 100 ng/g dose group (D2 range: 14.8–30.3 pg T3/mg protein/min) had significantly lower D2 activity than control females (D2 range: 32.0–61.3 pg T3/mg protein/min), and significantly lower activity than males in all three dose groups (Fig. 1; male D2 range 22.8–100.0 pg T3/mg protein/min).

3.3. Oxidative stress measures

There were no effects of TBBPA-BDBPE treatment on any of the oxidative stress measures (Table 1). There was also no interaction between treatment and sex ($p \geq 0.365$), and no difference between males and females ($p \geq 0.170$) for any measure. There was minimal evidence that BTBPE induced oxidative stress. None of the hepatic oxidative stress measures were affected by BTBPE treatment overall ($p \geq 0.756$; Table 1). There was a significant interaction between treatment and sex for both total thiols ($F_{3,10} = 5.30$ $p = 0.019$) and protein bound thiols ($F_{3,10} = 5.03$ $p = 0.022$); however, there were no significant differences between any BTBPE treatment by sex groups in post-hoc tests adjusted for multiple comparisons. None of the other measures had significant sex*treatment interactions ($p \geq 0.090$), and there was no difference between males and females for any measures ($p \geq 0.405$). 8-OHdG was marginally higher in the medium and high BTBPE dose groups than in the control and low dose group; however, this effect was not statistically significant ($F_{3,14} = 2.62$, $p = 0.092$), and there was no effect of sex ($F_{1,13} = 0.17$, $p = 0.684$) or sex*treatment interaction ($F_{3,10} = 1.06$ $p = 0.408$) for 8-OHdG.

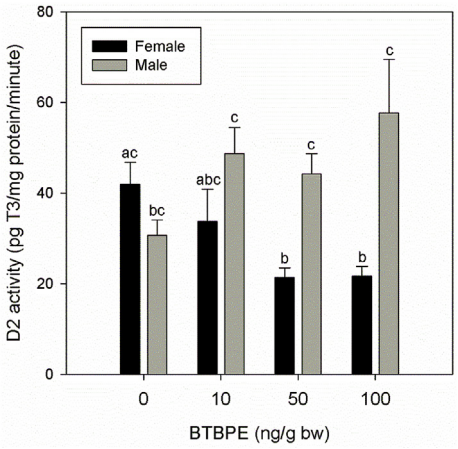

Fig. 1. Effect of *in ovo* BTBPE exposure on hepatic deiodinase activity for the D2 isoform in American kestrel hatchlings. There was a significant interaction between sex and treatment for D2 activity ($p = 0.004$). Female D2 activity decreased in a dose dependent manner, while there was a non-significant trend for male D2 activity to increase with BTBPE exposure. Error bars represent standard error of the mean. Significant differences between groups indicated by no shared lowercase letters. N = 5 in male 100 ng/g group, and 6 in all other groups.

3.4. TBBPA-BDBPE hatchling liver concentrations and egg

TBBPA-BDBPE concentrations in hatchling livers were very low or not detected (ND), regardless of treatment group. Five out of six control group liver pools had no detectable TBBPA-BDBPE (range ND to 0.6 ng/g ww). Low dose ranged from ND to 0.8 ng/g ww, medium dose ranged from ND to 0.03 ng/g ww, and high dose from 2.5 to 5.3 ng/g ww. Similarly, egg concentrations were low across treatment groups. TBBPA-BDBPE was below MLOQ in 2 of 4 control eggs (range ND to 0.1 ng/g ww) but was detectable in all dosed eggs. Mean (± SE) TBBPA-BDBPE was 1.43 ± 1.2 ng/g ww in the low dose group, 2.4 ± 0.5 ng/g ww in the medium dose group, and 2.3 ± 2.1 ng/g ww in the high dose group.

3.5. BTBPE egg and yolk sac concentrations

All vehicle (safflower oil) injected controls had BTBPE concentrations below detection limit (0.5 ng/g) throughout incubation. The concentration of BTBPE in eggs injected with 58.1 ng/g egg (100 ng/g nominal concentration) was not statistically different across the four time points (ED 12, 18, 21, 25) ($F_{3,10} = 0.19$, $p = 0.899$), although the egg with the highest measured concentration (30.0 ng/g) was collected on ED12 (Fig. 2a). On average, the percentage of BTBPE injected into the air cell on ED5 that was detected in egg contents was 22% on ED12, 19% on ED18, 14% on ED21, and 19% on ED25. In yolk sacs collected from hatchlings, there was a significant dose dependent increase in BTBPE ($F_{3,45} = 11.67$, $p < 0.0001$; Fig. 2b), and the high dose group had a higher BTBPE concentration compared to all other dose groups (compared to control and low $p < 0.001$, compared to medium $p = 0.026$).

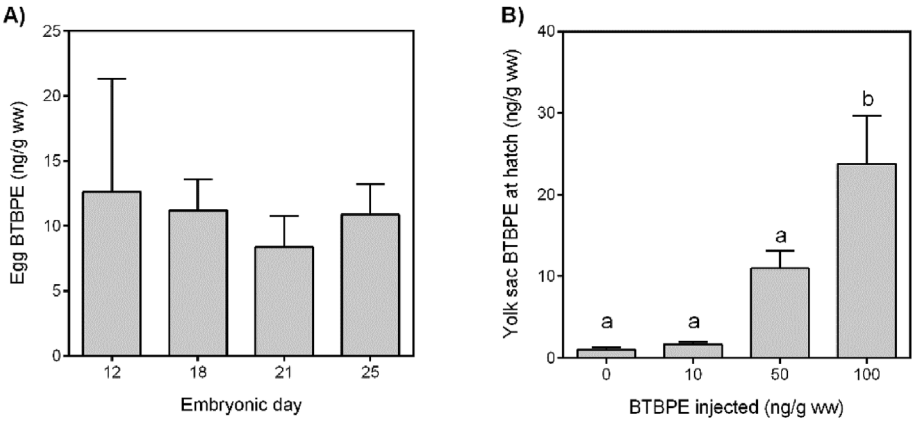

Fig. 2. A) Mean (SE) concentration of BTBPE in American kestrel eggs injected into the air cell with 58 ng/g egg (100 ng/g nominal concentration) on embryonic day 5 (ED5). BTBPE was below the detection limit (0.5 ng/g) in all vehicle (safflower oil) injected controls throughout incubation. There was no difference between egg concentrations at different ages ($F_{3,10} = 0.19$, $p = 0.899$). B) Mean (SE) concentration of BTBPE in yolk sacs of hatchlings (ED28) injected with BTBPE on ED5. BTBPE was significantly higher in yolk sacs from the highest dose group compared to the control and low ($p < 0.0001$) and the medium ($p = 0.026$) dose groups. Significant differences between groups are indicated different lowercase letters.

4. Discussion

We used egg injections in American kestrels to assess the effects of two BFRs, TBBPA-BDBPE and BTBPE, in avian predators. Exposure concentrations were environmentally realistic (nominal concentrations of 0, 10, 50 or 100 ng/g egg; measured concentrations 0, 3.0, 13.7 or 33.5 ng TBBPE-BDBPE/g egg and 0, 5.3, 26.8 or 58.1 ng BTBPE/g egg). While injected TBBPA-BDBPE concentrations were higher than has been reported for avian eggs to date (up to 0.36 ng/g ww in herring gull eggs) (Letcher and Chu, 2010), they were within the range of concentrations reported in biota. For example, Japanese blue crabs (*Portunus trituberculatus*) had concentrations of up to 2782.8 ng/g lw at 16% lipid dry mass (Liu et al., 2016a). BTBPE concentrations were similar to the concentrations found in tree swallow (*Tachicyneta bicolor*) eggs at a sewage lagoon (mean 15 ng/g ww, range 1.5–38 ng/g ww) (Fernie and Letcher, 2018), and within the range of concentrations measured in the plasma of a top avian predator (peregrine falcon, *Falco peregrinus*) in urban landscapes (mean 40.93 ng/g ww, range 0.02–569 ng/g ww) (Fernie et al., 2017). At these concentrations, we found evidence that the exposure to BTBPE disrupted one indicator of thyroid function in females. However, no other significant effects were detected for either compound despite measuring multiple endpoints, which suggests that these BFRs may not be very toxic at these concentrations or they may not have reached the developing kestrel embryos in toxic amounts from the air cell. The proportion of the injected dose that crosses the air cell membrane decreases with increased bromination and at higher log K_{ow} (McKernan et al., 2010). For example, in kestrel eggs that were injected with PBDEs, 18.8% of the injected dose was detected at pipping for penta-BDEs (log K_{ow} 6.8–7.8), and only 2.2% of the dose had crossed the air cell membrane for octa-BDEs (log $K_{ow} \geq 7.8$) (McKernan et al., 2010).

Concentrations of TBBPA-BDBPE in the eggs and hatchling livers were very low across all treatment groups. Concentrations were measured in egg contents collected 3 days after injection (at ED8), and it is possible that the low concentrations are due to only small amounts crossing the air cell membrane in the time between injection and sampling, with the rest remaining between the inner and outer shell membranes. In a parallel study, Eng et al. (submitted) found that TBBPA-BDBPE was not incorporated in zebra finch eggs immediately after injection into the albumen, but increased over time to a peak at 10 days of incubation. It is unlikely that metabolism contributed to the low concentrations in the eggs, as avian hepatic differentiation starts at approximately Hamburger-Hamilton stage 30 (Wong and Cavey, 1992), while on ED8 kestrels are at Hamburger-Hamilton stage 25 (Pisenti et al., 2001). In the hatchlings, the low concentrations detected in livers may reflect metabolism of the parent compound by the later-stage embryos, or be due to a low proportion of TBBPA-BDBPE crossing the air cell membrane. 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.

TBPPA-BDBPE and BTBPE did not affect the survival of kestrel embryos to pipping or hatching, and had no effect on body mass at hatch. There were also no differences in the hatchling organ somatic indices among any of the treatments. Similarly, *in ovo* BTBPE (48–3008 ng/g egg) did not affect pipping success in chickens (Egloff et al., 2011), and zebra finches exposed to TBBPA-BDBPE via egg injection showed no effects on hatching success, fledgling survival, or organ somatic indices (Eng et al., submitted). However, zebra finches in the highest TBBPA-BDBPE dose group had significantly reduced body condition, but that effect was not observed until 15 days post-hatch. Kestrels were only monitored until hatch, and it is possible effects of developmental exposure to TBBPA-BDBPE could manifest at later life stages. Additionally, kestrel eggs were injected into the air cell, whereas eggs in the zebra finch study were injected into the albumen, and potentially less of the TBBPA-BDBPE reached the developing embryos in the kestrel eggs.

A review of previous evidence suggests that elements of the avian thyroid system are moderately sensitive to BFRs (Guigueno and Fernie, 2017). We conducted a comprehensive examination of potential effects of *in ovo* TBBPA-BDBPE and BTBPE on the thyroid system in kestrels, including measures of circulating plasma hormones and stored glandular hormones, histology of the thyroid gland, and activity of the hepatic deiodinase enzymes. In the TBBPA-BDBPE study, males had more active thyroids than females based on hormone concentrations as well as histology; however, there were no effects of TBBPA-BDBPE treatment on any of the thyroid measures in the kestrel hatchlings. The same histological methods used in the current study were also utilized in studies that identified changes in thyroid gland structure and functioning in birds exposed to organophosphate esters (Fernie et al., 2015), polyaromatic hydrocarbons (Fernie et al., 2019) and air pollutants (Fernie et al., 2016). The lack of TBBPA-BDBPE thyroidal effects in the present kestrel hatchlings contrasts with the thyroidal effects found for nestling kestrels (17–20 days post hatch) exposed to PBDEs as embryos via maternal transfer from mothers exposed to 320 ng/g kestrel/d for 75 days (Fernie and Marteinson, 2016), and adult kestrels exposed to one of four organophosphate ester flame retardants at 22 ng/g kestrel/day for 21 days (Fernie et al., 2015) or a mixture of volatile organic pollutants via inhalation for 18 days (Fernie et al., 2016). This contrast in findings may reflect the differences in ages and exposure scenarios or duration of assessment across the studies - thyroidal effects of these FRs may not be evident in hatchling birds. Alternatively, there may truly be no effects of TBBPA-BDBPE on the avian thyroid system. Certainly, the absence of an effect on the thyroid system corresponds with a concurrent study involving TBBPA-BDBPE egg injection in zebra finches, which also did not observe effects on nestling plasma or glandular TT3 or TT4 concentrations (Eng et al., submitted). In female kestrels

exposed to BTBPE, we found a significant dose-dependent decrease in D2 enzyme activity, although no effects on D1 or any other thyroid measures. However, this effect should be interpreted with caution due to the large number of physiological variables tested and the possibility of type I errors. Vertebrates have three types of deiodinases (D1, D2, D3), and of these, D2 is the most important enzyme in the activation of thyroid hormones, catalyzing the conversion of T4 to the more biologically active T3 via outer ring deiodination (ORD). D1 is primarily involved in the ORD of rT3 to T2, and D3 catalyzes inner ring deiodination (IRD) from T4 to rT3, or T3 to T2 (Darras and Herck, 2012). In the female kestrels studied here, the reduced D2 activity may have been a compensatory response to maintain circulating thyroid hormones by reducing the conversion of T4 to T3.

In the current study, females appear to be more sensitive to BTBPE exposure, as we did not see any effects of BTBPE on the male thyroid system. Previous studies of American kestrels exposed as embryos to BFRs found a similar pattern of female sensitivity. Females, but not males, exposed to a PBDE mixture through maternal transfer had altered thyroid gland function and reduced plasma total T3 concentrations, which was associated with smaller body mass (Fernie and Marteinson, 2016). Egg injections of 116 μg/g egg of the BFR, bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (BEH-TEBP), altered the size of the hippocampus in the brain of female kestrels, but not males (Guigueno et al., 2018). The authors proposed a possible link between those neuroanatomy effects and thyroid-related changes in the brain, such as changes in D2 activity and subsequent T3 production. Further evidence for BTBPE thyroid disruption comes from *in vitro* assays with chicken embryonic hepatocytes, which found BTBPE at 0.03 and 0.1 μ*M* down-regulated genes coding for D3, although there were no effects on D2 mRNA expression (Egloff et al., 2011). Hepatic D3 was also down-regulated *in vivo* at ED22 in chicken embryos that had been injected with 3008 ng BTBPE/g egg prior to incubation (Egloff et al., 2011). However, in contrast, BTBPE at concentrations up to 0.364 μ*M* did not inhibit any deiodinase activity in human liver microsomal and cytosolic *in vitro* assays (Smythe et al., 2017). Thyroid hormones are involved in a multitude of processes in birds (McNabb, 2007). Changes in the deiodinase activity related to BTBPE exposure could have implications for development and reproduction, and further assessment of thyroid related effects of BTBPE in birds is warranted.

Changes in oxidative stress biomarkers are commonly used to evaluate physiological responses of biota to pollutants. Previous studies in kestrels have suggested that some flame retardants (Fernie et al., 2005) but not others (Fernie et al., 2015) might alter oxidative status. In rainbow trout (*Oncorhynchus mykiss*) exposed via their diet, BTBPE altered the hepatic transcription of genes related to oxidative stress (Giraudo et al., 2017). In contrast, a study of fathead minnows (*Pimephales promelas Rafinesque*) found no adverse effects on oxidative stress markers, or on any other physical or biochemical parameters following exposure to environmentally-relevant concentrations of TBBPA-BDBPE or BTBPE (de Jourdan et al., 2011). In kestrel hatchlings, *in ovo* TBBPA-BDBPE or BTBPE exposure did not have effects on any measures of oxidative stress, including TBARS, GSH, total GSH, GSSG/GSH, total or protein bound thiols, and 8-OH-dG, suggesting that these compounds do not likely lead to the generation of reactive oxygen species at current environmental levels.

In the second year of the study, we analyzed the change in BTBPE concentrations in hatchling yolk sacs, and in eggs injected with the high dose over the incubation period (ED12 to ED25). The concentrations in the yolk sac confirm that exposure was in a dose-dependent manner. There was no statistical difference in the egg concentration at any of the time points measured, with eggs retaining an average of 14–22% injected BTBPE regardless of the time point. The amount of BTBPE to cross the air cell membrane starting from injection day (ED5) was not specifically measured, but due to its high log K_{ow}, it is likely that a portion of the dose remained between the inner and outer shell membranes (McKernan et al., 2010). Although BTBPE metabolites were not measured, the lack of change in BTBPE concentrations over the incubation period suggests there was minimal metabolism of BTBPE by kestrel embryos. In contrast, rapidly metabolized compounds show steep declines over the incubation period. For example, organophosphate ester flame retardants (log $K_{ow} \leq 3.75$) injected into chicken eggs were detected at close to 100% of the target concentration immediately following injection, then dropped to < 1% of the injected concentration by ED19 (Farhat et al., 2013). There is evidence from fish that both TBBPA-BDBPE and BTBPE are slowly metabolized and have a tendency to accumulate in tissues. In fathead minnows in mesocosms, TBBPA-BDBPE and BDBPE both significantly accumulated in tissues over a 42 day exposure, and did not decrease over a 28 day depuration period (de Jourdan et al., 2014). BTBPE was resistant to metabolism by fish microsomes from three different species (Zheng et al., 2018), and exhibited significant trophic magnification in lake food webs in Canada (TMF = 1.86) and China (TMF = 2.83) (Law et al., 2006; Zheng et al., 2018). Over a 28 day BTBPE dietary exposure, rainbow trout accumulated approximately 76% of the daily dose in their tissues (Giraudo et al., 2017). Rats orally exposed to TBBPA-BDBPE absorbed < 5% of the dose, but that which was absorbed was retained by the liver and slowly metabolized (Knudsen et al., 2007).

Many current-use flame retardants have similar physical-chemical properties to the regulated historical flame retardants (Zhang et al., 2016), but their toxicology is poorly characterized. Here we evaluated the effects of early developmental exposure to TBBPA-BDBPE and BTBPE in a model avian predator. There was some evidence of sex-specific sensitivity of the thyroid system to BTBPE at concentrations within the range of what has been reported in avian wildlife. In recent years, BTBPE has been detected in a higher proportion of samples and at higher concentrations than previously reported (Jin et al., 2016), and with ongoing use, it is likely the trajectory will continue. Effects on the thyroid system could have cascading effects on other physiological, behavioral, and reproductive endpoints. There were no significant effects of *in ovo* TBBPA-BDBPE exposure in kestrels at hatching. However, the breakdown products of TBBPA-BDBPE could be more harmful than the parent compounds (Liu et al., 2016c, 2018), and longer-term studies may be needed to fully assess the potential impact of environmental contamination by these flame retardants on avian wildlife.

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Data generated during this study are available as a USGS data release (Karouna-Renier, 2019).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.04.047.

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