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Sex-Specific Responses in Neuroanatomy of Hatchling American Kestrels in Response to Embryonic Exposure to the Flame Retardants Bis(2-Ethylhexyl)-2,3,4,5-Tetrabromophthalate and 2-Ethylhexyl-2,3,4,5-Tetrabromobenzoate

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Abstract: Bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (BEH-TEBP) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB), flame retardant components of FireMaster 550® and 600® have been detected in tissues of wild birds. To address the paucity of information regarding potential impacts of flame retardants on the brain, brain volume regions of hatchling American kestrels (*Falco sparverius*) were evaluated following in ovo injection at embryonic day 5 with safflower oil or to 1 of 3 doses of either BEH-TEBP (13, 64, or 116 μg/g egg) or EH-TBB (12, 60, or 149 μg/g egg). The doses for both chemicals reflected concentrations reported in wild birds. The volumes of the hippocampus and telencephalon and volumetric differences between left and right hemispheres were measured in hatchlings (embryonic day 28). A sex-specific effect of BEH-TEBP on relative hippocampus volume was evident: the hippocampus was significantly enlarged in high-dose females compared to control females but smaller in low-dose females than the other females. There was no significant effect of EH-TBB on hippocampus volume in female kestrel hatchlings or of either chemical in male hatchlings and no effects of these concentrations of EH-TBB or BEH-TEBP on telencephalon volume or the level of symmetry between the hemispheres of the brain. In sum, embryonic exposure of female kestrels to these BEH-TEBP concentrations altered hippocampus volume, having the potential to affect spatial memory relating to ecologically relevant behavior such as prey capture, predator avoidance, and migration. *Environ Toxicol Chem* 2018;37:3032–3040. © 2018 SETAC

Keywords: American kestrel; FireMaster 550; Hippocampus; Avian toxicity; Flame retardants; Neurotoxicity

INTRODUCTION

Thousands of chemicals are present in the environment, yet the environmental effects of many of these chemicals are not well known (US Environmental Protection Agency 2014; Environment Canada 2016). The United States and Canadian federal risk assessment and regulatory agencies have identified several flame retardants as priority chemicals for which there are limited toxicity data for wildlife (US Environmental Protection Agency 2014; Environment Canada 2016). To that end, the present study investigated 2 such priority and currently used brominated flame retardants: bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (BEH-TEBP; Chemical Abstracts Service [CAS] no. 26040-51-8) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB; CAS no. 183658-27-7), components of several flame retardant technical mixtures (e.g., Firemaster® 550, Firemaster BZ-54; Stapleton et al. 2008; Bearr et al. 2010), to determine if BEH-TEBP and/or EH-TBB may pose a hazard to exposed wildlife. Research has indicated that atmospheric concentrations of BEH-TEBP and

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EH-TBB have been rapidly increasing (Ma et al. 2011) and that these 2 flame retardants have been detected globally in biota (Covaci et al. 2011), including birds of prey (Fernie et al. 2017).

Avian predators are widely used as sentinels for chemical pollution because they feed at high trophic levels, integrate signals across space (through long-distance movements) and time, and can be abundant in the environment (Golden and Rattner 2003; Sergio et al. 2006; Elliott and Elliott 2013). Predatory avian species occupying high trophic positions in food webs can be exposed to and accumulate some of the highest concentrations of chemical pollutants, including flame retardants (Guerra et al. 2012; Elliott et al. 2015). To date, many avian toxicology studies involving flame retardants have identified changes in reproduction and endocrine function (e.g., Fernie et al. 2009, 2005; Marteinson et al. 2017), but extremely few toxicology studies in general have addressed possible changes in neuroanatomy (Guigueno and Fernie 2017). Given that the brain is particularly sensitive to some environmental contaminants (Iwaniuk et al. 2006; Hoogesteijn et al. 2008) and that changes in the brain can have direct ramifications on ecologically relevant endpoints such as behavior, the development of brain-related biomarkers would improve our understanding of the biological significance of the effects of chemical pollutants.

Exposure to chemicals, including flame retardants, may affect the brain and more specifically neuroanatomy. In the avian brain, only the song control nuclei were previously examined in the context of the potential toxicity of brominated flame retardants (Eng et al. 2012, 2014). Exposure to 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) increased the volume of the song control nuclei in female songbirds (Eng et al. 2012). Various regions of the brain may also be sensitive to flame retardants as reported with other chemicals. For instance, the telencephalon of zebra finches (*Taeniopygia guttata*) increased in size when the birds were exposed to methylmercury (MeHg; Yu et al. 2017). The telencephalon is the most highly developed part of the forebrain, consisting primarily of the cerebral hemispheres and containing the hippocampus. The hippocampus of some laboratory mammals can also be sensitive to chemicals, notably polychlorinated biphenyls (PCBs; Gilbert and Liang 1998). Female mice exposed to 2,3,7,8-TCDD had a significantly smaller intra- and infrapyramidal mossy fiber field, a region of the hippocampus in mammals closely associated with spatial memory (Powers et al. 2005). As with mammals, the hippocampus plays an important role in the spatial memory of birds (Sherry et al. 1992); and dysfunction in the hippocampus could affect a bird's ability to avoid predators, locate food, and capture prey.

Because of their known effects on thyroid function, flame retardants have the potential to affect brain development, notably that of the hippocampus (Kuriyama et al. 2007). Changes in thyroid function of birds, including American kestrels (*Falco sparverius*), were observed when they were exposed to various flame retardants (e.g., Rattner et al. 2013; Fernie et al. 2015; Fernie and Marteinson 2016; Guigueno and Fernie 2017); and the variation in circulating thyroxine (T4) of nestling peregrine falcons (*Falco peregrinus*) was explained by circulating concentrations of specific historical and novel flame retardants, including EH-TBB (Fernie et al. 2017). Thyroid function partially influences hippocampus development and neurogenesis (Kapoor et al. 2015) and consists of the tight regulation of circulating T4 and triiodothyronine (T3) through feedback mechanisms influencing receptors and thyroid-related hormones in the hypothalamus and pituitary gland, T4 production and release from the thyroid gland, and deiodinase enzymes in the brain and elsewhere in the body (e.g., McNabb 2007). Hypothyroidism during critical embryonic developmental periods can affect expression of thyroid-related genes in the avian brain that regulate brain development. Type 2 deiodinase (D2), responsible for most of the local production of T3 in the brain, is strictly regulated by T3 and T4 concentrations within the brain (Bianco et al. 2002).

Little is known about which, if any, regions of the brain are affected by flame retardants, despite the sensitivity of the brain to TCDD, PCBs, MeHg, and BDE-99. The present study begins to address this knowledge gap by examining changes in the volumes of the hippocampus and telencephalon and in symmetry between hemispheres of hatchling American kestrels following their embryonic exposure to BEH-TEBP or EH-TBB. We hypothesized that BEH-TEBP and/or EH-TBB would affect the neuroanatomy of American kestrels, an excellent model for avian predators because their sensitivity to other brominated flame retardants has been shown to be greater than that of chickens, ducks, or other avian species (McKernan et al. 2009; Rattner et al. 2013; Guigueno and Fernie 2017). Given the sex-specific findings of Eng and colleagues (2012) involving zebra finches exposed to BDE-99, we predicted that EH-TBB and BEH-TEBP may also differentially affect the sexes in hatchling neuroanatomy in the present study. Our approach should help to develop a tool that could be used in future environmental research and applied in risk assessment of other priority chemicals.

METHODS

Egg injections, experimental groups, and tissue collection

All of the animal handling procedures and protocols used in the present study were approved by the Animal Care and Use Committee at the Patuxent Wildlife Research Center of the US Geological Survey (USGS). In the spring of 2015, 60 eggs were obtained from 45 breeding pairs of American kestrels from a captive colony at the USGS Patuxent Wildlife Research Center in Laurel, Maryland. Freshly laid eggs were collected daily and stored chilled at 5 °C for up to 7 d to ensure synchronous hatching. Fertility was confirmed on embryonic day 5 (Pisenti et al. 2001), at which time only eggs of known fertility were injected into the air cell with either organic safflower oil only (Irresistible Brand®; BioOrganic; controls) or 1 of 3 fixed doses (nominally 10, 50, or 100 ng/g egg) of either BEH-TEBP (>99% purity) or EH-TBB (>99% purity; both from Wellington Laboratories) dissolved in organic safflower oil. The low-dose concentration of 10 ng/g egg for both chemicals approximated concentrations commonly reported in wild bird eggs and other tissues (Sagerup et al. 2010; Gentes et al. 2012; Guerra et al.

2012; Lazarus et al. 2016), whereas the mid- and high-dose concentrations of 50 and 100 ng/g egg are more rarely encountered but are similar to levels reported for several raptor species by Jin et al. (2016).

After being injected, the injection hole was taped, and eggs were placed upright for 1 h. Eggs were then set on their side to incubate and rotated 180° hourly. Incubation occurred at 37.5 °C with a 10:14-h light: dark photoperiod, with humidity set between 35 and 40% and readjusted accordingly to maintain a mean weight loss of 16% over incubation (Miller et al. 2002). At embryonic day 12, a subset of 9 eggs was collected from the control group ($n = 3$) and from each group of eggs exposed to the high dose of BEH-TEBP ($n = 3$) or EH-TBBB ($n = 3$) to determine in ovo concentrations of, and hence embryonic exposure to, BEH-TEBP and EH-TBB. On embryonic day 24, incubation temperature was set to 37 °C and relative humidity at 70% until hatching (embryonic day 28). Embryos hatched independently, without any assistance, at approximately embryonic day 28. Hatchlings were euthanized by decapitation and their tissues collected immediately.

Brains were removed from 51 hatchlings ($n = 6$–8 per group, for a total of 51 brains; Supplemental Data, Table S1) and placed in 10% formalin for 2 wk. They were then immersed in a 30% sucrose solution for approximately 30 h to ensure cryoprotection (until the brains sank to the bottom of the vial) and then frozen at –80 °C. The brains were shipped frozen on dry ice from USGS Patuxent Wildlife Research Center to McGill University, where they were stored at –80 °C until they were processed for neuroanatomical measurements as described below (*Histological processing of brains*) following Guigueno et al. (2018, 2016). The sex of the hatchlings was determined genetically following the method of Brubaker et al. (2011).

Histological processing of brains

Each brain was sectioned into 40-μm coronal sections using a cryostat. Every fifth section was collected and placed in a well on a 24-well plate filled with 0.1 M phosphate-buffered saline (pH 7.5; BioShop®). Sections were then mounted on gelatin-coated slides using a paintbrush with synthetic bristles. The mounted sections were then Nissl-stained with thionin (Spectrum Chemical Manufacturing), serially dehydrated in ethanol, and cleared in solvent (NeoClear; EMD Chemicals). Finally, slides were coated with Permount (Fisher Scientific) and protected with coverslips.

Image analysis

Images of the brain sections were captured with a high-resolution (4800 dpi) flatbed scanner (Epson Perfection 4490 Photo) to delineate the telencephalon. Zoomed-in images of the hippocampus were captured with a ×2.5 objective on a Zeiss Imager M2 light microscope equipped with an AxioCam HRc digital camera (Carl Zeiss) connected to a computer with Zen 2 (Blue Edition [2012]; Carl Zeiss) image analysis software. The volumes of the telencephalon and the hippocampus were measured in the left and right hemispheres of each brain to assess symmetry. The perimeters of the telencephalon from every second collected section (every 10th total section) and of the hippocampus from every collected section (every fifth total section) were traced in ImageJ (Schneider et al. 2012) to measure cross-sectional surface areas. The boundary between hippocampus and the lateral boundary with the hyperpallium apicale was determined by identifying changes in cell size, density, and alignment, a boundary that resembles that of songbirds (Figure 1; Guigueno et al. 2016). The same observer (M.F. Guigueno), who was blind to the experimental groups and sex, completed all tracings. The volumes of the telencephalon and hippocampus were estimated using the formula for a frustum (i.e., portion of a cone lying between 2 parallel planes). The frusta volumes were then summed between each tissue section (400 μm for telencephalon and 200 μm for hippocampus) to estimate the total volume of each region of interest. If the selected tissue section was poorly stained or damaged, the next intact and well-stained section was used, and the sampling interval was adjusted accordingly.

Preparation of the chemical dosing solutions and chemical analysis

All chemical preparations and analyses of tissues for the present study were conducted by the Letcher Organics Research Contaminants Laboratory at the National Wildlife Research Center, Environment and Climate Change Canada in Ottawa, Ontario, Canada. The entire contents of each of the 9 eggs collected at embryonic day 12 were individually homogenized in preparation for chemical analysis. The analyses of EH-TBB and BEH-TEBP concentrations in the embryonic day 12 egg homogenates and in the 4 dosing solutions were conducted according to the methods fully described elsewhere (Chen et al. 2013; Su et al. 2017). Briefly, samples were thawed for a period of 30 to 45 min at ambient room temperature (22 °C) prior to extraction. Samples were weighed, and after combining with diatomaceous earth, the mixture was quantitatively transferred

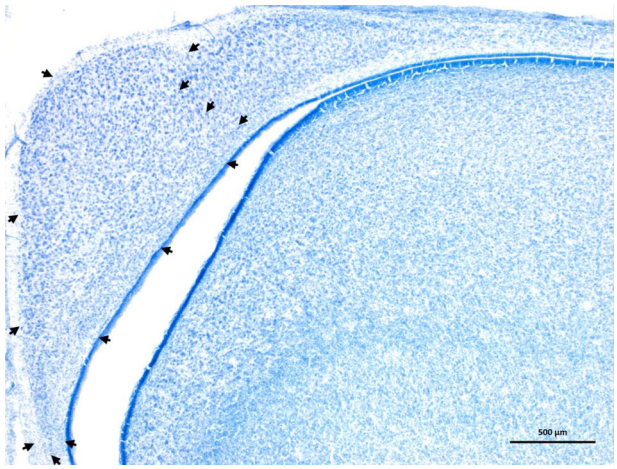

FIGURE 1: An example of a coronal section of a hatchling American kestrel Nissl-stained hippocampus taken with a ×2.5 objective on a microscope. Boundaries of the hippocampus are indicated by arrows.

to a stainless-steel cell for accelerated solvent extraction. The sample was then spiked with a volume of 50 μL of BDE-30, BDE-156, $^{13}C_{12}$-BDE-209 (Wellington Laboratories), and $^{13}C_6$-*anti*-Dechlorane Plus (DDC-CO) and $^{13}C_6$-*syn*-DDC-CO (Cambridge Isotope Laboratories) internal standards, each with concentrations of 500 pg/μL, and then subjected to accelerated solvent extraction. After accelerated solvent extraction, the extracts were solvent (dichloromethane [DCM])–evaporated to approximately 2 mL and filtered through a 5-mL column of sodium sulfate to remove residual moisture. The sodium sulfate and sample were washed with DCM, and the eluent was collected in a 15-mL tube and brought to exactly 10 mL. A 1-mL aliquot (10% of the original sample extract) was taken for gravimetric lipid determination. The remaining sample volume was evaporated to 1 mL; 0.5 mL of this was added to a centrifuge filter tube (0.45 μM, polyvinylidene difluoride) and then centrifuged at 10 000 rpm for 2 min. The filtrate was then subjected to gel permeation chromatography (GPC) and DCM, and the remaining 0.5 mL of sample was added to the centrifuge filter tube, centrifuged, and added to the GPC tube. The centrifuge filter tube was then rinsed with an additional 0.5 mL of DCM and centrifuged, and the rinsings were added to the sample GPC tube. Using a gentle flow of nitrogen gas, the sample volume was brought down to 2.5 mL and further cleaned up by GPC at a flow rate of 5 mL/min of DCM. The dump time was 12 min, and thus, the first 60 mL was discarded. The "collect" time was set to 16 min with a 3-min wash time in between samples. The collected fraction of eluent was concentrated with nitrogen to a volume of 2 mL and transferred to another tube with 3 rinses of DCM, concentrated, and then solvent-exchanged into 5% DCM/hexane to a volume of 1 mL.

The sample was cleaned using an LC-Si solid phase extraction (SPE) cartridge (500 mg, 6 g; J.T. Baker; Su et al. 2017). The SPE cartridge was conditioned, and then the sample was loaded and eluted with 8 mL of 5% DCM/hexane into a test tube. The eluent was concentrated under a gentle stream of ultra-high-purity nitrogen and then solvent-exchanged with iso-octane to a final volume of approximately 250 μL. The exact mass of each sample was recorded and the final volume determined by dividing by the density of 2,2,4-trimethylpentane. After the exact mass of the fraction volume was recoded and quantitatively transferred to a preweighed GC vial with insert and cap, the final sample fraction was then ready for GC-MS (electron-capture negative ion) analysis.

The method limit of detection (MLOD) was 0.3 ng/g wet weight, and the method limit of quantification (MLOQ) was 1.0 ng/g for both BEH-TEBP and EH-TBB. We substituted half of the MLOQ concentration for samples that were below the MLOQ but above the MLOD and half of the MLOD value for samples that were below the MLOD. If >50% of the samples had concentrations below the MLOD, then we report only the ranges of concentrations.

The measured concentrations of EH-TBB in the safflower oil dosing solutions used for the EH-TBB exposures were as follows: low dose (12 μg/g or 11 ng/μL), medium dose (60 μg/g or 55 ng/μL), and high dose (149 μg/g or 137 ng/μL). For the eggs exposed to BEH-TEBP, the measured concentrations of this flame retardant in the related dosing solutions were as follows: low dose (13 μg/g or 12 ng/μL), medium dose (64 μg/g or 60 ng/μL), and high dose (116 μg/g or 107 ng/μL). In the control safflower oil, EH-TBB and BEH-TEBP were below chemical detection (<0.3 ng/g).

Statistical analyses

Statistical analyses were conducted using SAS statistical software, Ver 9.4 (SAS Institute). Because neuroanatomical patterns can be sex-specific, we analyzed the sexes separately for each chemical. We previously reported that female kestrels had a larger hippocampus (~13% larger) than males (Guigueno et al. 2018). In the present study, we used general linear models with the experimental treatment groups (all 3 doses of either EH-TBB or BEH-TEBP plus the control) as independent variables to analyze the potential effects of the chemicals on the different volumetric components of the brain (relative symmetry between hemispheres for hippocampus, relative symmetry between hemispheres for telencephalon, mean relative hippocampus volume, and mean relative telencephalon volume). The statistical analyses involving hippocampus as the dependent variable (symmetry and mean between hemispheres) incorporated telencephalon volume minus hippocampus volume as a covariate to correct for brain size, whereas the analyses with telencephalon as the dependent variable (symmetry and mean between hemispheres) incorporated crown-to-rump length as a covariate to correct for body size. Crown-to-rump length is an accepted measure of body size in birds, including those exposed to stressors (Miller et al. 2002; Finch et al. 2011). To measure crown-to-rump length, each hatchling was placed on a centimeter ruler, aligning the body from the head to the feet along the same plane of the ruler, by the same individual. The ruler was screwed into a wooden block, which created a fixed point against which the head of the animal was placed.

We ensured that residuals from all analyses were normally distributed. To achieve normally distributed residuals for the models with hippocampus, we log-transformed the covariate (telencephalon minus hippocampus). We first tested for the potential effects of any of the 3 doses of BEH-TBP or EH-TBB on volumetric differences between the hemispheres for hippocampus and telencephalon. Because there was no significant effect of the 2 chemicals (experimental treatment group) on the absolute relative volumetric difference between hemispheres for hippocampus and telencephalon in females and in males (Supplemental Data, Table S1), we averaged the left and right hemispheres to include mean hippocampus and mean telencephalon volumes in subsequent statistical analyses. To identify significant differences among the different dosing concentrations of each chemical (i.e., individual chemical treatment groups), post hoc *t* tests were employed. We did not correct for running multiple post hoc tests because they reduce statistical power and their use has been questioned by statisticians in the biological sciences (Nakagawa 2004). Values are presented as marginal means ± standard error of the mean to correct for covariates. The statistical significance level was set to $p < 0.05$.

RESULTS

Exposure concentrations

Concentrations of EH-TBB and BEH-TEBP in the subset of 9 eggs collected at embryonic day 12 are presented in Table 1. Concentrations of EH-TBB were only detected in the 3 EH-TBB-injected eggs collected from the high-dose EH-TBB group and demonstrate that the embryos had been exposed to EH-TBB during the previous 7 d of development (3.0–59.7 ng/g wet wt). Much lower concentrations of BEH-TEBP were detected in the 3 eggs collected at embryonic day 12 from the BEH-TEBP high-dose group (1.4–2.5 ng/g wet wt), as well as in one egg from the EH-TBB group (1.0 ng/g wet wt) and one control egg (1.9 ng/g wet wt; Table 1).

Hemispherical differences: Hippocampus or telencephalon volume (symmetry)

There was no significant effect of either BEH-TEBP or EH-TBB on the absolute relative volumetric difference between hemispheres for hippocampus or telencephalon in the female or male hatchlings (Supplemental Data, Table S1).

Mean hippocampus volume

We identified a sex-specific effect of BEH-TEBP on mean hippocampus volume (corrected for telencephalon size). For female kestrel hatchlings, there was a significant effect of exposure to BEH-TEBP ($F_{3,9} = 6.87$, $p = 0.01$; Figure 2) and of telencephalon minus hippocampus volume as a covariate ($F_{1,9} = 24.69$, $p = 0.0008$). Specifically for the female hatchlings, there was a significant difference in mean hippocampus volume between the control and high-dose BEH-TEBP groups ($t_6 = 2.36$, $p = 0.04$), between the low- and high-dose groups ($t_6 = 4.48$, $p = 0.001$), and between the low- and medium-dose groups ($t_4 = 2.67$, $p = 0.03$): hippocampus volume of female hatchling kestrels was enlarged following embryonic exposure to the medium and high doses of BEH-TEBP compared to the low-dose female hatchlings and/or the control female hatchling kestrels (Figure 2). The hippocampus volume was marginally different between the control and low-dose female hatchlings (2-tailed test: $t_6 = 1.93$, $p = 0.09$) and became significantly different with a one-tailed test ($t_6 = 1.93$, $p = 0.04$), suggesting that female hatchling kestrels exposed as embryos to an environmentally relevant concentration of BEH-TEBP had a significantly smaller hippocampus relative to controls (Figure 2). For male hatchlings, there was no significant effect of exposure to BEH-TEBP ($F_{3,11} = 1.07$, $p = 0.40$; Figure 2). Our covariate, telencephalon minus hippocampus volume, was a significant influence (factor)

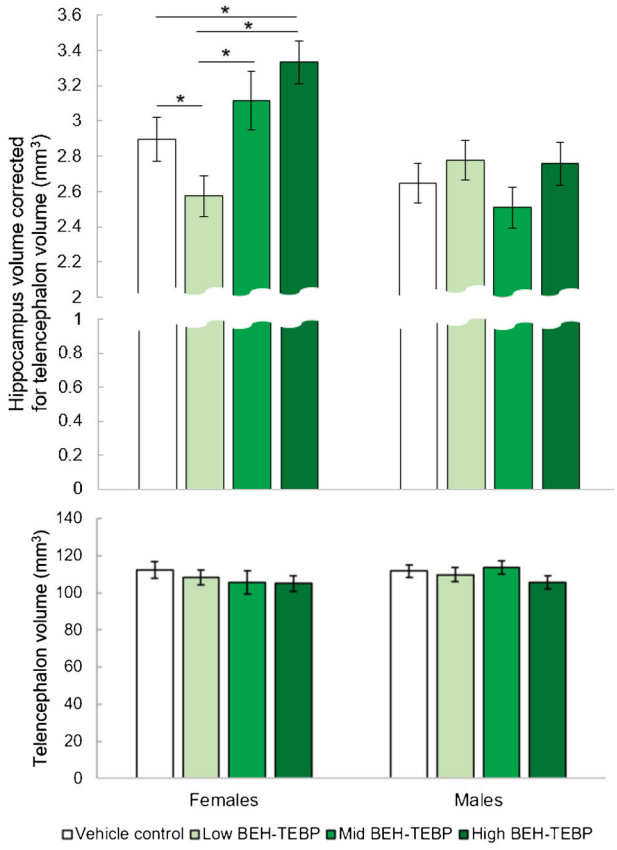

FIGURE 2: Hippocampus and telencephalon volumes (means from left and right hemispheres) in hatchling American kestrels exposed to bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (BEH-TEBP). Hippocampus volume relative to the telencephalon of females, but not males, was altered by embryonic exposure to the environmentally relevant dose of BEH-TEBP (13 μg/g egg) and the high dose of BEH-TEBP (116 μg/g egg). Telencephalon volume did not differ between groups for females and males. $^*p < 0.05$ using 2-tailed tests, except for the difference between the low and mid groups exposed to BEH-TEBP for hippocampus volume in females, which is significant using a one-tailed test. Error bars indicate standard error of the mean. Sample sizes were as follows according to group: control = 8; low BEH-TEBP = 8; mid BEH-TEBP = 6; and high BEH-TEBP = 8.

TABLE 1: Range of bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (BEH-TEBP) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) concentrations in exposure eggs on embryonic day 12 of American kestrel embryos exposed to either organic safflower oil only, BEH-TEBP (116 ng/g egg), or EH-TBB (149 ng/g egg) on embryonic day 5[a]

		Chemical exposure	
Experimental group	*n*	BEH-TEBP (ng/g wet wt)	EH-TBB (ng/g wet wt)
Control	3	<MLOD–1.9	<MLOD
BEH-TEBP	3	1.4–2.5	<MLOD
EH-TBB	3	<MLOD–1.0	3.0–59.7

[a] The method limit of detection was 0.3 ng/g.
MLOD = method limit of detection.

on the mean hippocampus volume of the male hatchlings too ($F_{1,10} = 6.42$, $p = 0.03$), indicating that the hippocampus size of both sexes in the BEH-TEBP treatment groups increased with increasing size of the telencephalon in which it is located.

We observed no significant effect of EH-TBB on the mean hippocampus volume for either the female ($F_{3,9} = 0.89$, $p = 0.48$) or male ($F_{3,10} = 0.83$, $p = 0.51$) hatchling kestrels at these 3 specific dosing concentrations. As with the BEH-TEBP-exposed hatchlings, those exposed to EH-TBB demonstrated significant effects of the covariate, telencephalon minus hippocampus volume: hippocampus volume increased with telencephalon volume of females ($F_{1,9} = 12.96$, $p = 0.006$) and males ($F_{1,10} = 6.00$, $p = 0.03$).

Mean telencephalon volume

There was no effect of embryonic exposure to the 3 dosing concentrations of BEH-TEBP on the telencephalon volume of the female hatchling kestrels ($F_{3,9} = 0.44$, $p = 0.73$) or of the male hatchling kestrels ($F_{3,11} = 0.93$, $p = 0.46$; Figure 2), nor was the covariate, crown-to-rump length, significant in female ($F_{1,9} = 0.23$, $p = 0.64$) or male ($F_{1,11} = 0.06$, $p = 0.82$) hatchling kestrels, indicating that telencephalon volume was not related to body size of these hatchlings exposed to BEH-TEBP. Similarly, there was no significant effect of embryonic exposure to the 3 dosing concentrations of EH-TBB on telencephalon volume in female ($F_{3,10} = 1.16$, $p = 0.38$) or male ($F_{3,10} = 1.07$, $p = 0.40$) hatchlings (Figure 3), nor was the covariate, crown-to-rump length, significant in either sex (females: $F_{1,9} = 1.49$, $p = 0.25$; males: $F_{1,10} = 1.27$ $p = 0.29$) in the hatchlings exposed to EH-TBB.

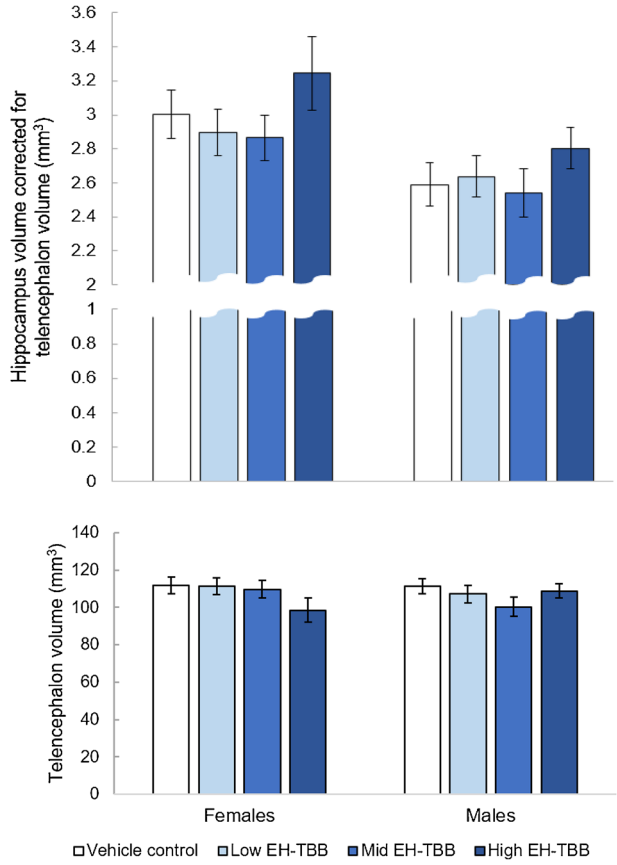

FIGURE 3: Hippocampus and telencephalon volumes (means from left and right hemispheres) in hatchling American kestrels were not altered by embryonic exposure to the 3 doses of 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) used in the present study (12, 60, or 149 μg/g egg). There was no significant difference in hippocampus and telencephalon volumes for either females or males. Standard error bars indicate the standard error of the mean. Sample sizes were as follows according to group: control = 8; low-dose EH-TBB = 8; mid-dose EH-TBB = 7; high-dose EH-TBB = 6.

DISCUSSION

The results of the present study suggest that exposure to BEH-TEBP during most of embryonic development (82% or 23 d) significantly affected neuroanatomy in hatchling American kestrels. The measurable concentrations of BEH-TEBP and EH-TBB in the subset of 9 eggs collected 7 d after egg injection with the high dosing concentrations demonstrate that the kestrel embryos in the present study were exposed to these chemicals during the earlier stages of development. The findings also suggest that much of the original dosing concentration of BEH-TEBP had been metabolized during these 7 d in the early half of the kestrel embryonic period, whereas much of the original high dose of EH-TBB injected into the fertile eggs remained. The source of the cross-contamination of the BEH-TEBP within these 2 eggs is unclear. Nevertheless, there was a sex-specific effect of BEH-TEBP on the mean hippocampus volume (corrected for telencephalon volume): only female hatchling kestrels, and not males, showed a significant effect on hippocampus size following their embryonic exposure to BEH-TEBP. Female hatchlings exposed as embryos to the high dose of BEH-TEBP had a larger relative hippocampus than control females (Figure 2). Perhaps more importantly, our results suggest that exposure through much of embryonic development to the environmentally relevant level of BEH-TEBP resulted in female hatchling kestrels likely having a smaller hippocampus than control females and a significantly smaller hippocampus than the females exposed to the medium or high dose of BEH-TEBP. Different mechanisms may be responsible for the opposing effects of BEH-TEBP at low versus high doses. Although different regions of the brain were sensitive in the zebra finches exposed to BDE-99 (Eng et al. 2012) and mice exposed to TCDD (Powers et al. 2005), the consistency of only females demonstrating volumetric changes in various regions of the brain in these 2 studies and in the present study is interesting to observe and potentially worthy of further investigation.

How did BEH-TEBP influence hippocampus volume? In other studies, BEH-TEBP has been described as an endocrine disruptor (Patisaul et al. 2013). One possible mechanism is through disruption of thyroid function, particularly in the brain, which develops from early embryogenesis onward in birds (McNabb 2007) and can be disrupted in a sex-specific way, potentially leading to sex-specific effects on hippocampus volume. For example, thyroid function was altered in a sex-specific manner by PBDEs in American kestrel nestlings (Fernie and Marteinson 2016), and similarly, plasma thyroid hormones were disrupted

when adult American kestrels were exposed to 1,2-dibromo-4-(1,2-dibromoethyl)-cyclohexane (Marteinson et al. 2017). Thyroid hormones are vital for establishing brain architecture during the development of the central nervous system in vertebrates, including chicken embryos (McNabb 2007). In particular, thyroid hormones play an important role in neural development and neurogenesis of the hippocampus (Kapoor et al. 2015), and the production of T3 within the brain is regulated by localized D2 activity. Development of the avian brain during embryogenesis can also be disrupted by hypothyroidism affecting expression of thyroid-related genes at critical developmental periods. Alterations in thyroid-related receptors of the hypothalamus and/or pituitary gland may also be impacted by chemical exposure. We hypothesize that the alterations in the relative hippocampus volume of the female hatchling kestrels (Figure 2) following their embryonic exposure to the high-dose and environmentally relevant dose of BEH-TEBP may reflect thyroid-related changes in the brain, including potentially localized T3 production and D2 activity and/or thyroid-related receptors or related gene expression governing brain development at critical developmental periods. Future studies examining thyroid-related disruption in the brain may determine the mechanism underlying the volumetric differences we observed in the present hatchling American kestrel females.

We did not observe a significant effect of either BEH-TEBP or EH-TBB on hemispherical differences for hippocampus and telencephalon volumes (Supplemental Data, Table S1). A previous study had shown that 2,3,7,8-TCDD induced a dose-related increase in the severity of brain asymmetry (Henshel et al. 1997). Despite structural differences among the 3 chemicals, the results of the study by Henshel and colleagues (1997) stimulated us to measure the same endpoint in the hatchling kestrels exposed to EH-TBB and BEH-TEBP. Although the embryonic exposure to the currently used concentrations of EH-TBB and BEH-TEBP did not affect brain symmetry in the present study, it is possible that exposure for a longer period to either chemical (e.g., embryonic exposure in combination with nestling exposure) and/or exposure to higher concentrations of these flame retardants could influence hemispherical symmetry, a question that future research could explore.

A change in hippocampus volume, either hippocampal enlargement or shrinkage as with the females from the high-dose and low-dose BEH-TEBP treatment groups, respectively, could directly impact ecologically relevant behavior. The hippocampus plays an important role in spatial memory and navigation in birds and mammals (Sherry et al. 1992). Lesioning or inactivating the avian hippocampus selectively disrupts spatial memory (Sherry and Vaccarino 1989; Hampton and Shettleworth 1996; Broadbent and Colombo 2000; Shiflett et al. 2003), which may affect abilities to avoid predators, locate food, and capture prey. Shrinkage of the hippocampus can lead to reduced spatial memory performance of animals. Female mice exposed to 2,3,7,8-TCDD made more errors on spatial memory tasks and thus performed poorly relative to controls (Powers et al. 2005). Although female hatchling kestrels exposed to the high dose of BEH-TEBP had a larger hippocampus than control females (Figure 2), this does not necessarily result in better spatial memory. This chemically induced increase in volume could be attributable to neuroinflammation, as has been suggested for the increased telencephalon volume observed in zebra finches when exposed to MeHg (Yu et al. 2017). Furthermore, an increase in hippocampus volume could be attributable to abnormal endocrine signaling, including thyroid-related changes at the cellular or molecular level in the brain. We recommend that future research involving embryonic exposure to BEH-TEBP or other chemicals examines the brain at the cellular and molecular levels (e.g., presence of proinflammatory cytokines and level and/or numbers of neurotransmitters and receptors). In addition, linkages between hippocampal changes and possible behavioral effects, such as on spatial memory, should be investigated in future research to help determine the biological significance of these hippocampal changes.

In the present study, we investigated changes in the size (volume) of the hippocampus and telencephalon and symmetry within the brain of the kestrels, but we did not investigate potential fine-scale changes in the brain, which may be a better measure of brain function in relation to the exposure to either flame retardant. In a review, Roth et al. (2010) concluded that changes in volume are likely a coarse proxy for the more relevant measures of neuron number and size in the hippocampus related to memory. In conjunction with assessments of volume, future studies should investigate these cellular characteristics using techniques such as immunohistochemistry in relation to embryonic exposure to EH-TBB, BEH-TEBP, and other chemical pollutants.

CONCLUSIONS

In summary, we found evidence for a sex-specific effect of BEH-TEBP on the hippocampus of female, but not male, hatchling American kestrels, previously reported to be among the more sensitive avian species to flame retardants (McKernan et al. 2009). We did not find changes in the telencephalon or hemisphere symmetry of the hatchlings exposed to BEH-TEBP regardless of their sex or changes in the neuroanatomical measures of hatchling kestrels exposed to EH-TBB. Changes in hippocampal volume, such as an inflammation response, could affect spatial memory, altering the ability of predators such as kestrels to navigate. The flame retardant BEH-TEBP is widely manufactured and used (Covaci et al. 2011) and is widely detectable including in wildlife tissues (Lam et al. 2009) and birds (Sagerup et al. 2010; Gentes et al. 2012; Guerra et al. 2012; Lazarus et al. 2016; Fernie et al. 2017), suggesting the likelihood of potentially similar effects in wild birds. In addition to determining whether BEH-TEBP crosses the blood–brain barrier (BEH-TEBP levels in the brain were not measured in the present study), future research should examine the potential impacts of BEH-TEBP on bird behavior, specifically on movement in the wild and on spatial memory, while concurrently measuring neuroanatomy in the same individuals. Examination of fine-scale changes in the avian brain, such as neuron number and size, as well as thyroid-related function in the brain (e.g., receptors, gene expression, D2 activity), in relation to exposure to BEH-TEBP and/or EH-TBB may reveal effects which are unrelated to volume

but nonetheless good indicators of altered brain function. In sum, we encourage further research to concurrently investigate and determine potential associations between chemical exposures, the brain (volumetric and fine-scale changes), and behavior, to better understand the risks of flame retardants and other chemicals on ecologically relevant endpoints.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4238.

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Data Accessibility—Data, associated metadata, and calculation tools are available from the corresponding author (kim.fernie@canada.ca) and through FigShare.

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