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# Investigating Endocrine and Physiological Parameters of Captive American Kestrels Exposed by Diet to Selected Organophosphate Flame Retardants

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Supporting Information

**ABSTRACT:** Organophosphate triesters are high production volume additive flame retardants (OPFRs) and plasticizers. Shown to accumulate in abiotic and biotic environmental compartments, little is known about the risks they pose. Captive adult male American kestrels (*Falco sparverius*) were fed the same dose (22 ng OPFR/g kestrel/d) daily (21 d) of tris(2-butoxyethyl) phosphate (TBOEP), tris(2-chloroethyl) phosphate (TCEP), tris(2-chloroisopropyl) phosphate (TCIPP), or tris(1,2-dichloro-2-propyl) phosphate (TDCIPP). Concentrations were undetected in tissues (renal, hepatic), suggesting rapid metabolism. There were no changes in glutathione status, indicators of hepatic oxidative status, or the cholinergic system (i.e., cerebrum, plasma cholinesterases; cerebrum muscarinic, nicotinic receptors). Modest changes occurred in hepatocyte integrity and function (clinical chemistry). Significant effects on plasma free triiodothyronine ($FT_3$) concentrations occurred with exposure to TBOEP, TCEP, TCIPP, and TDCIPP; TBOEP and TCEP had additional overall effects on free thyroxine ($FT_4$), whereas TDCIPP also influenced total thyroxine ($TT_4$). Relative increases (32%−96%) in circulating $FT_3$, $TT_3$, $FT_4$, and/or $TT_4$ were variable with each OPFR at 7 d exposure, but limited thereafter, which was likely maintained through decreased thyroid gland activity and increased hepatic deiodinase activity. The observed physiological and endocrine effects occurred at environmentally relevant concentrations and suggest parent OPFRs or metabolites may have been present despite rapid degradation.

## INTRODUCTION

Flame retardant (FR) compounds are used to hinder the ignition of fire. Some FRs (e.g., polybrominated diphenyl ether (PBDE) congeners in the PentaBDE and OctaBDE technical formulations) have been regulated and phased-out, resulting in the emergence and increasing usage of replacement FRs (e.g., organophosphate (OP) triester FRs).[1] OP triesters were introduced in the 1970s and are now widely used as additive flame retardants (OPFRs) (e.g., polyurethane foam, textiles) and plasticizers (e.g., floor polish, thermoplastics, lacquers). They are high production volume FRs that constituted 14% of the 1.5 million tonnes of FRs used globally in 2004,[1,2] and 20% of European FR usage in 2006.[1] More recently (2012) in the United States, the annual production volume of tris(2-chloroisopropyl) phosphate (TCIPP) (CAS#: 13674-84-5) was reported as ∼25 million kg and ∼4.5−22.5 million kg for tris(1,2-dicholor-2-propyl) phosphate (TDCIPP) (CAS#: 13674-87-8).[3] Total usage quantities of TCIPP in Denmark, Finland, Norway, and Sweden were reported to range approximately from 1.1 to 2 million kg from 2007 to 2011.[4] Some Nordic countries have also reportedly used TDCIPP[4] and tris(2-chloroethyl) phosphate (TCEP) (CAS#: 51805-45-9) (annual usage volume in 2008: 0.2−261 tonnes[1]).

OPFRs, including TCIPP, TDCIPP, TCEP, and tris(2-butoxyethyl) phosphate (TBOEP) (CAS#: 78-51-3), have been detected in abiotic environmental compartments.[5,6] In the Great Lakes, atmospheric concentrations of summed (∑)

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OPFRs exceeded those of brominated flame retardants (BFRs) by 2−3 orders of magnitude.[7] OPFRs have also been detected in airborne particles over the Southern, Arctic, Pacific, and Indian Oceans[8] and the North Sea,[9] suggesting long-range transport.

Various OPFRs, including TBOEP, TCEP, TCIPP, and/or TDCIPP, have also been detected in aquatic birds in the northern hemisphere[10−12] over the past 25 years.[12] Concentrations of 16 OPFRs varied in tissues of herring gulls (*Larus argentatus*) with TDCIPP and TCIPP dominant in egg yolk, maternal muscle, and red blood cells, TBOEP prevailing in albumen and maternal fat, and low concentrations of TCEP limited to eggs.[2] In the same gulls, plasma concentrations of triphenyl phosphate (TPHP) were also detected as were OPFR diester metabolites, indicating that OPFR triesters can be metabolized by birds.[13] Feather concentrations of six OPFRs in nestling white-tailed eagles (*Haliaeetus albicilla*) were much greater than PBDEs, non-PBDE FRs, polychlorinated biphenyl 153, and dichlorodiphenyldichloroethylene (*p,p′*-DDE).[14]

Little is known about the potential toxic effects of OPFR triesters in humans and biota, including birds. Exposure to TDCIPP and TCIPP altered mRNA abundance of genes associated with phase I and II metabolism, thyroid hormone and lipid regulation, and growth in chicken embryonic hepatocytes (CEH).[15] In chicken embryos exposed by egg injection, very high concentrations of TCIPP (9,240 and 51,600 ng/g) significantly delayed pipping and reduced growth (51,600 ng/g), and TDCIPP reduced embryo mass, gallbladder size (45,000 ng/g), and free thyroxine ($FT_4$) concentrations (7,640 ng/g).[16] Exposure of chicken embryos to TBOEP induced hepatic CYP3A37 mRNA, and the effects of triethyl phosphate (TEP) were similar to TCIPP and/or TDCIPP: decreased pipping success, altered growth and plasma bile acids, modulated genes associated with xenobiotic and lipid metabolism and the thyroid hormone pathway, and reduced plasma $FT_4$ levels.[17]

Recently, several OPFRs, including TBOEP, TCEP, TCIPP, and TDCIPP, were listed as priority chemicals for risk assessment by Environment Canada. The risk of some chlorinated OP esters is also being evaluated by the U.S. Environmental Protection Agency. Investigations of the biological effects of specific OPFRs on avian embryos following in vitro and egg-injection exposures were recently reported.[15−18] However, data from ecologically relevant models and other life stages are lacking. Here, the American kestrel (*Falco sparverius*) is used as a raptorial model species because it has been used extensively in avian ecotoxicological studies.[19−25] Kestrels were found to be more sensitive to the effects of PBDEs than galliform species (e.g., chickens[24] or common terns (*Sterna hirundo*)).[25] The in vivo uptake and effects of chronic, dietary exposure to TBOEP, TCEP, TCIPP, and TDCIPP were investigated with captive kestrels, focusing on physiological systems (e.g., hepatic oxidative status, thyroid, and hepatic function) previously shown to be sensitive to OPFR exposure.[15−18]

## MATERIALS AND METHODS

**Kestrel Dosing and Sampling.** This study was conducted in 2011 and 2012 under guidelines approved by the Animal Care and Use Committee at the USGS Patuxent Wildlife Research Center (PWRC; Laurel, MD, USA). Captive American kestrels were housed individually in large outdoor flight pens with a shade roof, perches, food tray, and water bowl, and shelter was available from the elements.[26] The birds were fed day-old dead cockerel ad libitum daily, receiving the dosed cockerel each morning and either nondosed cockerel or dead laboratory mice on alternate afternoons. We used the same dietary feeding-exposure methods of previous flame retardant studies in which captive American kestrels accumulated concentrations of PBDEs, hexabromocyclododecane (HBCDD), decabromodiphenyl ether (BDE-209), but not *β*-1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane.[19−23] Dosing solutions were injected into the cerebral hemispheres of the cockerel, a preferred dietary item of captive kestrels. Food consumption was monitored daily, and all dosed cockerel were consumed daily with minor exceptions.

A pilot study was conducted in 2011 using 7 adult two-year old male kestrels exposed daily by diet for 28 days to TDCIPP and 7 adult male kestrels of the same age exposed to only the safflower oil vehicle. The details and results of this pilot study are presented in the Supporting Information (SI). In 2012, a study was conducted to understand the potential uptake and effects of all four priority OPFRs. On the basis of the findings of this pilot study, a higher concentration of TDCIPP was used to characterize possible effects of elevated concentrations likely experienced by wild birds.[12,27] In this study, a separate set of 38 one-year old adult male kestrels (7 per exposure group with 10 controls) were exposed daily by diet to TDCIPP, TBOEP, TCIPP, or TCEP for 21 days because potential effects of the higher dose were anticipated to occur within this time frame. Control male birds of the same age were exposed to the safflower oil vehicle only. Each kestrel ate one cockerel per day that had been injected with 50 μL of a safflower oil solution containing one of each of the four OPFRs. On the basis of the mean weight (114 g) of the adult male kestrels at the initiation of the study, each kestrel received a daily dose of 2500 ng OPFR/50 μL safflower oil/day, corresponding to 21.93 ng OPFR/g kestrel/day.

For the purposes of this study, TBOEP (>94% purity) and TCEP (>97% purity) were purchased from Sigma-Aldrich (St. Louis, MI, USA); TDCIPP (>95% purity) was purchased from TCI America (Portland, OR, USA), and TCIPP (>95% purity) was purchased from AK Scientific (Union City, CA, USA). The nominal (calculated) concentrations of OPFRs in the safflower oil dosing solutions were TBOEP at 49 μg/μL, TCIPP at 52 μg/μL, TCEP at 52 μg/μL, and TDCIPP at 51 μg/μL. The nominal concentrations were very accurate as the actual measured concentrations of each OPFR in the dosing solutions were as follows: TBOEP, 50 μg/μL; TCIPP, 59 μg/μL; TCEP, 41 μg/μL; and TDCIPP, 53 μg/μL.

The kestrels were weighed, and their general health assessed at the beginning of the exposure period and again at 7, 14, and 21 d of the exposure trial. Immediately after weighing, between 0.6 and 1.1 mL of whole blood was withdrawn from each kestrel by jugular venipuncture using a heparinized 1.0 mL syringe with a 27-gauge needle. Following their exposure, the birds were humanely euthanized by carbon dioxide. Further blood was immediately collected by cardiac venipuncture with a subsample analyzed for hematocrit and the remainder stored on ice until processing by centrifugation. Plasma was aliquoted into cryovials and stored at −80 °C. For each kestrel, the thyroid gland, liver, kidney, pectoral muscle (∼1 g), and brain were immediately collected, weighed, subsectioned, and stored in 10% buffered formalin for histological assessment or frozen at −80 °C. Somatic indices (liver, kidney, brain) were


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calculated as organ mass divided by body mass with the result then multiplied by 100.

**Clinical Chemistry Analysis.** All plasma samples were stored at −70 °C and shipped on dry ice for clinical chemistry analysis by Dr. Carolyn Cray (University of Miami). Only plasma samples free of lipemia and hemolysis were analyzed for alkaline phosphatase activity (ALP), alanine aminotransferase activity (ALT), gamma-glutamyl transpeptidase (GGT) enzymatic activity, total protein (TP), prealbumin and albumin (ALB), $\alpha_1$, $\alpha_2$, $\beta$, and $\gamma$ globulins (G), aspartate aminotransferase (ASAT), glutamate dehydrogenase activities (GLDH), lactate dehydrogenase isozymes (LDH), hemolysis, and lipemia indices. The A:G ratio was calculated as the sum of the prealbumin and albumin divided by the sum of all measured globulins. Heparinized plasma samples were used for biochemical determinations. Routine biochemical analyses (ALT, LDH, GGT, ALP, and TP) were conducted using an Ortho Vitros 250XR chemistry analyzer (Ortho, Rochester, NY, USA).[28] Further analytical details are provided in the SI.

**Neurochemical Assays.** Neurochemical assays were completed at the University of Michigan based on previous analytical methods.[29,30] Homogenate and membrane preparations from the cerebrum tissues were prepared as described previously.[29−31] Brains were homogenized in 50 mM Tris buffer (50 mM Tris HCl, 50 mM Tris Base, pH 7.4), and an aliquot of homogenate was saved for enzyme analyses. Membranes were isolated by centrifuging at 48000*g* for 15 min. The pellet was washed three times and resuspended in Tris buffer. Protein content of homogenate and membranes was measured using the Bradford assay, and samples were stored at −80 °C. The cholinergic assays (receptor binding to muscarinic and nicotinic receptors; enzyme activity of acetylcholinesterase and butrylcholinesterase) were performed as previously described.[30] A detailed description of these analytical methods is provided in the SI.

**Oxidative Status.** Livers collected in 2012 were analyzed for indicators of glutathione status and oxidative status. Reduced glutathione (GSH), oxidized glutathione (GSSG), and the GSSG/GSH ratio were analyzed using the DetectX Glutathione Fluorescent Detection Kit (Arbor Assays, Ann Arbor, MI, USA), total sulfhydryl (TSH) using the Measure-iT Thiol Assay Kit (Life Technologies - Molecular Probes, Inc., Eugene, OR, USA), protein bound sulfhydryl (PBSH) by calculation of the difference between TSH and GSH, and lipid peroxidation (thiobarbituric acid reactive substances, TBARS) using the QuantiChrom TBARS Assay Kit (Bioassay Systems, Hayward, CA, USA). All assays were validated prior to sample analysis to determine the detection limit and assess accuracy, precision, and dilutional parallelism and to identify any matrix effects (none). Each sample was analyzed in duplicate with a mean coefficient of variation (CV; mean ± standard deviation) of 2.01 ± 1.47%, 4.26 ± 2.09%, and 1.25 ± 0.68%/2.44 ± 1.53% for TBARS, TSH, and reduced/total GSH, respectively. Duplicate samples for which CVs exceeded 10% were reanalyzed. A detailed description of the analytical methods used for the neurochemical assays and oxidative stress assays are provided in the SI.

**Analysis of Thyroid Function.** A three-pronged assessment of thyroid function was performed by determining circulating triiodothyronine ($T_3$) and $T_4$ concentrations, hepatic $T_4$-outer ring deiodinase activity ($T_4$-ORD), and histological assessments of the structure of the thyroid gland. Circulating concentrations of free and total $T_3$ ($FT_3$, $TT_3$) and $T_4$ ($FT_4$, $TT_4$) were determined according to the manufacturer's instructions using commercially available radioimmunoassays (MP Biomedical, Santa Ana, CA, USA) and based on linear response versus concentration calibration curves ($R^2$: $T_3$ = 0.997, $T_4$ = 0.953) across the physiological range of hormone concentrations ($T_3$ = 0.5−8 ng/mL, $T_4$ = 20−200 ng/mL). Hepatic deiodinase activity was determined as previously described with American kestrels.[32] The thyroid gland of each kestrel was assessed for structural changes in epithelial cell height (ECH) and colloid parameters as previously described.[33] Full details for assessments of hepatic deiodinase activity and thyroid gland histology are provided in the SI.

**Chemical Analysis.** In the Organic Contaminants Research Laboratory (OCRL)/Letcher Laboratories at Environment Canada, concentrations of TBOEP, TCEP, TCIPP, and TDCIPP were determined in liver and kidney tissue samples in isolated fractions.[2,13,34] Liquid chromatography-electrospray-tandem quadrupole-mass spectrometry (LC-ESI(+)-MS/MS) was used to determine the OPFRs in the isolated fractions using the following MRM transitions: TCEP ($m/z$ 284 > 63), TCIPP ($m/z$ 329.1 > 99), TDCIPP ($m/z$ 430.9 > 99), TBOEP ($m/z$ 294.3 > 102). Mean recovery (±1 SD) of the internal standard $d_{27}$-TBP was 85 ± 15% for all liver and kidney analyses. An internal standard method was used for quantification and concentrations of target compounds were recovery-corrected. In 2012, the OCRL/Letcher Laboratories participated in the first international NORMAN PFR (OPFR) ILS and our OPFR results for this QA/QC exercise complied within 10% of the NORMAN PFR certified data.

A pork liver homogenate (Ottawa market), previously determined to be absent of any OPFRs, was fortified with each target OPFR and analyzed with the batch of samples ($n$ = 12, liver or kidney). On the basis of fortified pork liver, the estimated method limit of quantification (MLOQ) and detection (MLOD) of the OPFRs was defined as the minimum amount of analyte that produced a peak with a calculated signal-to-noise ratio of 10 and 3, respectively. As a result, the MLOQ and MLOD (ng/g ww) for each OPFR are TBOEP (0.27, 0.09), TCEP (0.1, 0.03), TCIPP (0.31, 0.1), and TDICPP (0.14, 0.04), respectively.

The method blank consisted of internal standard $d_{27}$-TBP spiked diatomaceous earth (DE) that was spiked with 200 $\mu$L chicken egg albumin (also previously determined to be absent of any OPFRs and used to maintain desirable IS recovery through volatility) and was used throughout the entire extraction, concentration, and analysis procedure. This was conducted in triplicate with the batch of 12 samples to demonstrate freedom from cross-contamination and contaminants that would interfere with analysis and to determine the levels of contamination associated with the processing and analysis of samples. The method blank concentration ranges for each of the four OPFRs were as follows: 1.7−4.1 ng/g ww for TCEP, 2.6−6.5 ng/g ww for TCIPP, 0.9−1.5 ng/g ww for TDCIPP, and 1.4−5.5 ng/g ww for TBOEP. OPFR blank subtraction was performed as follows: the highest level of contamination determined in the method blank triplicate was selected for sample correction, as well as the subtraction of one standard deviation of the three blanks.

**Statistical Methods.** End points were compared for each treatment to vehicle-treated controls by parametric analysis of variance (ANOVA) (e.g., hepatic $T_4$-ORD), repeated measures (RM) ANOVA (i.e., thyroid hormones), or nonparametric ANOVA and RM ANOVA on ranked data (i.e., clinical


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chemistry) using SAS 9.3. Residuals were tested for homogeneity of variance and normality. Only the concentrations of circulating thyroid hormones were successfully log transformed. Because each histological parameter of the thyroid gland required multiple measurements per bird, nested ANOVAs on ranked data were performed with treatment as the factor and the individual bird as the nested factor; Scheffe's *posthoc* tests followed. Hepatic $T_4$-ORD was examined with an ANOVA followed by least-squares means *posthoc t*-tests of all pairwise comparisons without adjustments. RM ANOVAs initially identified overall significant treatment effects on thyroid hormones or clinical chemistry for each OPFR compared to controls, followed by 1-way ANOVA to identify significant treatment differences at individual time periods when overall significance was identified. Spearman's correlation analysis was used to determine statistical associations between hepatic lipid and TBOEP concentrations and plasma concentrations of albumin and thyroid hormones. Means ± SEM are presented and statistical significance occurred when $p \leq 0.05$. Only significant comparisons relative to the controls are reported unless noted.

## RESULTS AND DISCUSSION

**Kestrel Exposure and Uptake of OPFRs.** After method blank-correction, hepatic concentrations of TCEP, TCIPP, and TDCIPP and all renal concentrations of the four OPFRs were less than MLOD (≤0.1 ng/g ww). Hepatic concentrations of TBOEP were quantifiable in TBOEP-exposed (5.0 ± 0.6; 3.2−7.9 ng/g ww) and control birds (4.0 ± 0.9; 0.3−9.4 ng/g ww), suggesting potential cross-contamination of the latter, but were not associated with lipid concentrations ($p = 0.10$). Although these results suggest the potential for the OPFRs failing to be absorbed by the kestrels during digestion, the same dietary exposure methodology used in the current study had previously resulted in kestrels accumulating PBDEs,[19,23] HBCDD,[20] and BDE-209[21] in their tissues or eggs. Furthermore, hepatic concentrations of these and other OPFRs were not detected in maternal herring gulls,[2] suggesting that OPFRs do not accumulate in hepatic tissues of birds. In a previous study, concentrations of TBOEP and TEP were negligible in pipped chickens exposed by egg injection,[17] but TCIPP and TDCIPP increasingly accumulated in various tissues (e.g., liver) in a dose-dependent manner in pipped chickens exposed to much higher dosing concentrations[16] than used in the current study.

The lack of detectable OPFR concentrations in the current kestrels is consistent, although not directly comparable, with the undetectable concentrations of these OPFRs in the tissues of some wild birds, including the eggs of common terns,[35] and the liver and brain of adult herring gulls.[2] However, low ppb concentrations of several OPFRs were reported in the plasma[13] and eggs of herring gulls from the Great Lakes.[2,12,34] Concurrently, nestling peregrine falcons (*Falco peregrinus*) had low plasma concentrations of TBOEP (1.0−7.5 ng/g ww), TCEP (0.2−2.0 ng/g ww), TCIPP (0.9−5.5 ng/g ww), and/or TDCIPP (0.3−1.0 ng/g ww) (Fernie et al., Environment Canada, unpublished data). Similar low ppb concentrations of these and other OPFRs were found in the feathers of white-tailed eagles[14] and livers of kittiwakes (*Rissa tridactyla*) and common eiders (*Somateria mollissima*).[11] Together, the results of these studies suggest that accumulation of chlorinated and nonhalogenated OPFRs occurs in (wild) birds, presumably exposed continuously for longer periods or to higher concentrations than experienced by the captive kestrels in the current study.

The undetectable residues of TCEP, TCIPP, TDCIPP, and to a lesser extent TBOEP, in the captive kestrels likely reflects rapid metabolism through enzyme-mediated processes. OP diesters, including BDCIPP and BCIPP derived from TDCIPP and TCIPP, respectively, were recently reported in humans,[36] suggesting the potential for in vivo metabolism of the four OPFRs used in the current study. In an in vitro rat liver microsomal assay, more than 95% of TDCIPP was rapidly metabolized (<5 min) to BDCIPP.[37] Similarly, TPHP was metabolized in vitro by CEH and mainly to the glucuronide conjugate of *para*-OH-TPHP.[27] Genes associated with xenobiotic phase I and II metabolism and lipid regulation, specifically CYP3A37, CYP2H1, and UGT1A9, were up-regulated in CEH and chicken neuronal cells exposed to TDCIPP or TCIPP.[15] CYP3A37 and CYP2H1 are phase I cytochrome P450 metabolizing enzymes involved in oxidation of lipophilic compounds, including xenobiotics, and UGT1A9 is a phase II conjugation enzyme that joins small lipophilic molecules with larger polar glucaronic acids into excretable metabolites.[15,18] These in vitro results were substantiated in vivo with pipped chicks that exhibited induction of hepatic CYP3A37 mRNA and type I deiodinase by TCIPP, CYP3A37, and CYP2H1 by TDCIPP[16] and hepatic CYP3A37 mRNA by TBOEP.[17]

**Biological Effects.** The exposure to TBOEP, TCEP, TCIPP, or TDCIPP had no significant effect on the body mass or temporal weight gain of the American kestrels (p-values ≥ 0.22) and reflect similar results when adult male kestrels were exposed to DE-71[19,38] but not a technical mixture of HBCDD (HBCDD-TM).[32] However, the current OPFR-kestrel findings contrast with the reduced body mass of pipped chickens following in vivo developmental exposure to a much higher dose of TBOEP (45,400 ng/g).[17]

Traditional clinical chemistry measures of avian organ function are sensitive but nonspecific indicators of the effects of various persistent organic pollutants.[39,40] Increases in circulating A:G ratios were reportedly associated with increasing plasma concentrations of ∑PBDE and ∑OH-PBDE metabolites in wild peregrine falcon nestlings.[40] The kestrels exposed to TCIPP or TDCIPP demonstrated significant overall changes in plasma A:G ratios (ranked RM ANOVA: $p \leq 0.01$) with significantly suppressed ratios at 21 d exposure to TDCIPP (ANOVA: $F_{1,16} = 5.71$, $p = 0.03$). Exposure to TBOEP had a significant overall effect on plasma albumin (RM ANOVA: $F_{3,13} = 6.95$, $p = 0.005$) with increased concentrations evident at 7 and 21 d exposure (ranked ANOVAs: $p \leq 0.05$). Changes in circulating ALP and ALT in some raptor species,[39,40] but not all,[41] have been associated with mirex, chlordanes, or PBDE concentrations in their tissues and were significantly affected in the current kestrels exposed to TCEP, TBOEP, or TDCIPP (ALT only) (ranked RM ANOVA: $p \leq 0.02$). Although increased plasma bile acids,[17] liver,[18] and gall bladder[16] size were reported in pipped chickens following exposure to TEP and TCP, there were no significant effects on bile acids or any somatic organ indices of the adult kestrels exposed to TBOEP, TCEP, TCIPP, or TDCIPP. Although significant, the changes in A:G, albumin, ALP, and ALT in the kestrels were generally within the reported normal range of other raptors,[40−43] albeit not kestrels, and thus may represent marginal changes in hepatocyte function and integrity from the OPFR exposure conditions in the current study.



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OPFRs were characterized as having similar or more potent in vitro neurotoxicity as known or suspected neurotoxins.[44] Exposure of PC12 cells to TDCIPP promoted differentiation into dopaminergic and cholinergic neuronal phenotypes, and TCEP and TCIPP promoted the cholinergic phenotype.[44] In another study, TCIPP and TDCIPP were sequestered in the cerebral hemisphere of chicks, but there was no assessment of potential neurotoxic effects.[16] A range of cholinergic markers were studied in both brain (cerebrum) and plasma of the kestrels (Table S1, SI). In the pilot study, the exposure to TDCIPP resulted in a statistically significant but small (5%) suppression of cerebrum cholinesterase activity (see SI) that was likely to be of little biological relevance. Yet the kestrels' exposure to the higher concentration of TDCIPP, or to TBOEP, TCEP, or TCIPP at the concentrations used in the current study, did not elicit significant changes in AChE or BChE enzyme activity in plasma or brain, or nicotinic or muscarinic cholinergic receptor levels in the brain (ANOVAs: all $p \geq 0.13$) (Table S1, SI).

Multiple indicators of oxidative status have been altered in response to in vitro and in vivo exposures to various flame retardants. The in vitro exposure of PC12 cells to TDCIPP increased lipid peroxidation, but exposure to TCIPP or TCEP had no effect on this indicator.[44] Exposure to PBDE congeners has elicited changes in oxidative status in raptors[45] but not passerines.[46,47] Hepatic glutathione ratio and lipid peroxidation were induced in juvenile American kestrels exposed embryonically and as nestlings to a mixture of BDE-47, -99, -100, and -154.[45] Although a similar antioxidant-rich diet involving cockerel was fed to the adult male kestrels in the current study, the exposure to TBOEP, TCEP, TCIPP, or TDCIPP did not alter glutathione status or indicators of hepatic oxidative status (all $p \geq 0.11$).

**Thyroid Function.** Exposure to some OPFRs has elicited endocrine changes in fish,[48] and notably altered thyroid hormones in fish[49] and pipped chickens,[16,17] with modeling demonstrating an association between TDCIPP exposure and $FT_4$ in humans.[50] Generally, circulating thyroid hormone concentrations are closely regulated in vertebrates through feedback mechanisms involving the hypothalamus, pituitary and thyroid glands, and peripheral tissues. Circulating $TT_3$ and $TT_4$ are bound to transthyretin (TTR) and albumin with varying importance depending on the class of vertebrate being considered. However, biological effects are generally elicited by circulating unbound $FT_3$ (predominantly) and $FT_4$, and these unbound THs are also thought to exert the greatest negative feedback influence on the thyroid gland.

The exposure of the adult kestrels to each of the four OPFRs had significant overall effects on circulating $FT_3$ and to TBOEP on plasma $TT_3$ concentrations (all RM ANOVA $p \leq 0.05$). Circulating $FT_4$ concentrations (TBOEP, TCEP) and $TT_4$ (TDCIPP) in the adult kestrels were also significantly altered (all RM ANOVA $p \leq 0.05$), as were temporal changes in $FT_4$ and $FT_3$ plasma concentrations (see SI). Consistent significant increases in the circulating thyroid hormones were observed specifically at 7 d exposure to each OPFR (Figure 1a and b and Figure S1a and b in the SI). At 7 d, the exposure to TBOEP significantly increased the $FT_3$ concentrations by 32% ($F_{1,15}$ = 5.41, $p$ = 0.03) and $TT_3$ concentrations by 96% ($F_{1,15}$ = 20.23, $p$ = 0.0005) (Figure 1a and Figure S1a in the SI), whereas the TCEP treatment significantly increased the concentrations of $FT_3$ (57%) ($F_{1,16}$ = 19.27, $p$ = 0.0005) (Figure 1a) and $FT_4$ (96%) ($F_{1,14}$ = 7.42, $p$ = 0.02) (Figure 1b). At 7 d exposure to

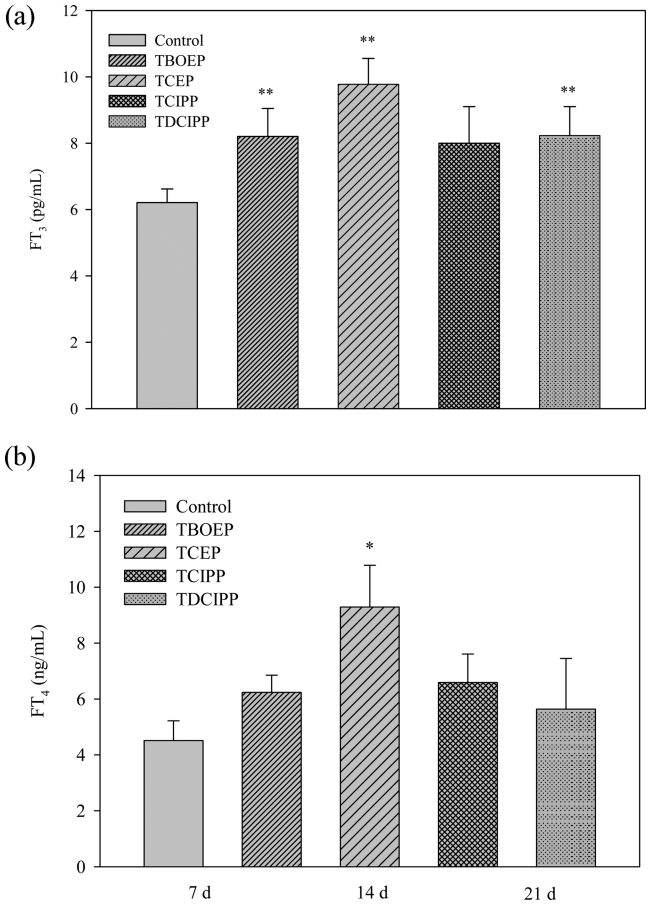


**Figure 1.** Circulating concentrations of (a) $FT_3$ and (b) $FT_4$ thyroid hormones at 7 d in adult male American kestrels exposed daily to TBOEP, TCEP, TCIPP, or TDCIPP (22 ng OPFR/g kestrel/d) for 21 d. All comparisons are made to the adult male control kestrels. Concentrations of $TT_3$ and $TT_4$ are reported in Figure S1 a and b in the SI. Means ± standard errors about the means (SEMs) are presented. Sample sizes for each thyroid hormone measured consisted of 10 control kestrels and 7 kestrels per OPFR treatment group, with the exception of the TBOEP ($n$ = 6) and TDCIPP ($n$ = 6) treatment groups, when assessing $FT_4$ concentrations. Statistically significant p-values are indicated as follows: $p \leq 0.0001$ (***), $p \leq 0.01$ (**), and $p \leq 0.05$ (*).

TDCIPP, $FT_3$ (32%) (Figure 1a) and $TT_4$ (31%) (Figure S1b, SI) concentrations were significantly increased; in comparison, the limited suppression of $TT_4$ (13%) at 21 d may be of little biological importance (all $p \leq 0.05$).

The changes reported here in the circulating thyroid hormones of the adult kestrels contribute to our understanding of the potential and varying effects of OPFRs[16−18] and other FRs[32,45,46,51] on thyroid function in birds. In pipped chickens, developmental exposure to TDCIPP or TEP, but not TBOEP or TCIPP, modulated genes associated with the thyroid hormone pathway and decreased plasma $FT_4$ but not $FT_3$ levels or glandular $TT_4$ concentrations.[16,17] However, $FT_4$ and glandular $TT_4$ were unaltered following exposure of chicken embryos to tris(methylphenyl) phosphate (TMPP; formerly TCP).[18] The reported changes in circulating thyroid hormones in several bird species exposed to various OPFRs builds on existing knowledge of thyroid sensitivity to multiple FRs, including HBCD,[32] and PBDEs in some[45,46] but not all[38,51] avian studies.


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Changes in circulating thyroid hormones may reflect changes in hormone transport to target tissues by transthyretin (TTR) and/or albumin. Although TTR was downregulated following in vitro exposure of CEH to TCIPP but not TDCIPP,[15] it was unaltered in pipped chickens exposed in vivo to either OPFR.[16] In the current kestrels, circulating levels of albumin were significantly and positively correlated with $FT_3$, $FT_4$, and $TT_4$ at 7 d and with $TT_4$ and $TT_3$ at 14 and 21 d ($n$ = 38, Spearman's $R$-values $\geq$ 0.42, $p \leq 0.01$). Significant changes, mostly increases, in the concentrations of $FT_3$, $FT_4$, and $TT_4$, but generally not in albumin, were evident at these time points, suggesting complex changes and other mechanisms were involved in eliciting effects on circulating thyroid hormones in the kestrels.

Thyroid hormonal changes can be transient either temporally or in a dose-dependent manner under the influence of contaminant stress. Changes in thyroid gland structure and circulating hormone concentrations, as well as deiodinase enzymes, are sensitive indicators of overall changes in thyroid function and gland activation. In pipped chickens, glandular $T_4$ was unaltered following TBOEP or TEP exposure.[17] Histological assessments of the thyroid glands of the current adult kestrels revealed that exposure to the four OPFRs significantly altered ECH:colloid (Figure 2a) (nested nonparametric ANOVA: $F_{30,699}$ = 9.50, $p < 0.0001$) as well as ECH and colloid size (Figure S2a and b, SI) compared to controls. The ECH:colloid ratio, an integrated measure of thyroid gland activity,[52] was significantly increased in the kestrels exposed to TCEP, TCIPP, or TDCIPP for 21 d (*posthoc* $p \leq 0.008$) (Figure 2a), suggesting reduced activity of their thyroid glands.[52] Compared to the controls, TDCIPP exposure significantly reduced ECH and increased colloid area (*posthoc* $p \leq 0.004$). TCEP exposure significantly reduced ECH (*posthoc* $p < 0.0001$) (Figure S2a and b, SI).

Thyroid hormone activation (i.e., conversion from $T_4$ to $T_3$) and degradation (conversion to reverse $T_3$ or $T_2$ and further deiodinated products) are mediated by various deiodinase enzymes primarily in the liver, but also in the kidney, small intestine, and brain. In vitro changes were evident in deiodinase genes from chickens following embryonic exposure to TCIPP but not TDCIPP, whereby Type I deiodinase enzymes were induced in hepatocytes.[16] In the current in vivo study with adult kestrels, exposure to the four OPFRs significantly affected hepatic $T_4$-ORD activity (ANOVA: $F_{4,34}$ = 3.24, $p$ = 0.025) (Figure 2b) with exposure to TBOEP, TCIPP, and TDCIPP significantly increasing hepatic $T_4$-ORD activity involved in the conversion of $T_4$ to $T_3$ (*posthoc* $p \leq 0.04$). Although activity of other deiodinase enzymes was not assessed, the pattern of the results suggests that the OPFR exposure altered thyroid function of the kestrels in vivo. We hypothesize that the increased hepatic $T_4$-ORD activity in combination with the decreased gland activity was potentially sufficient to maintain circulating thyroid hormone concentrations at 21 d of exposure. Additional research is required to test this hypothesis.

In the current study, the exposure of captive kestrels to low concentrations of TBOEP, TCEP, TCIPP, or TDCIPP is likely similar to or lower than that encountered by wild birds[2,13,34] because no measurable concentrations were detected in the kestrels. Some clinical chemistry measures suggested moderate alterations in hepatocyte integrity and function. Exposure to each of the four OPFRs elicited overall changes in circulating thyroid hormones, particularly in $FT_3$, with relative increases of 32−96% at 7 d of exposure; thereafter, the only change in

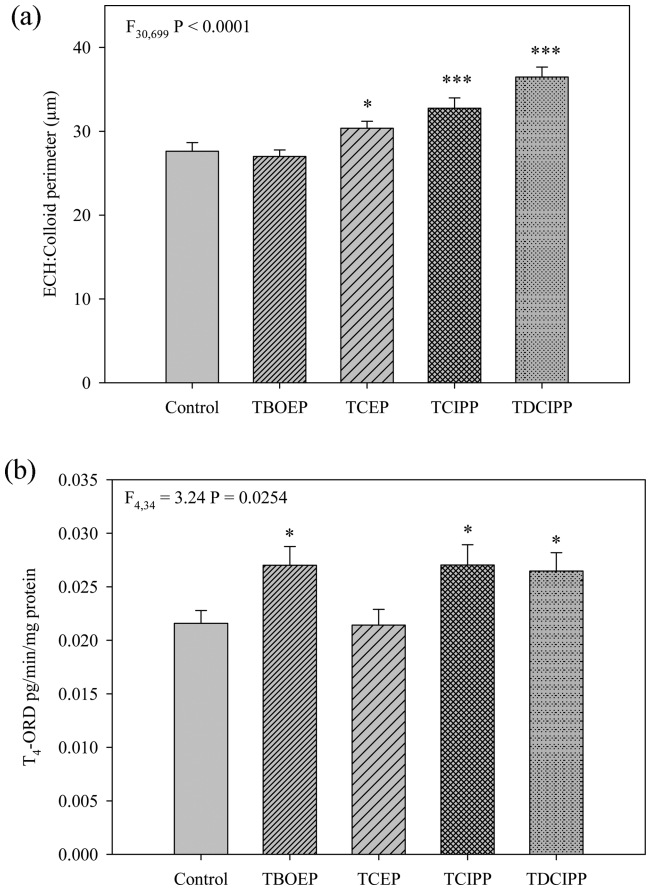


**Figure 2.** Significant changes in (a) thyroid gland histology (ECH:colloid perimeter) and (b) hepatic $T_4$-outer ring deiodinase ($T_4$-ORD) were observed in adult male kestrels at 21 d exposure to TBOEP, TCEP, TCIPP, or TDCIPP when compared to control kestrels. The ECH:colloid ratio represents an integrated measure of thyroid gland activity (Bocian-Sobkowwska et al. 1997). Significant changes in epithelial cell height and colloid area are reported in Figure S2a and b in the SI. Means ± SEMs are presented. Sample sizes consist of 7 birds per treatment group, including controls. Statistically significant p-values are indicated as follows: $p \leq 0.0001$ (***), $p \leq 0.01$ (**), and $p \leq 0.05$ (*).

circulating thyroid hormone concentrations was minor, and the thyroid hormones were likely maintained during this time through reduced activity of the thyroid gland and/or the increased activity of hepatic $T_4$-ORD enzymes. The results of the current in vivo study with adult kestrels and the previous studies with pipped chickens[16,17] demonstrate that exposure to these particular OPFRs has the potential to elicit effects in both young and adult birds. Further research is required, however, to determine potential endocrine and physiological effects of OPFRs on wild birds given their chronic, longer-term exposure to OPFRs compared to that experienced by the American kestrels in the current study.

## ■ ASSOCIATED CONTENT

### Supporting Information

This section describes the pilot study conducted with TDCIPP, and the detailed methodology and results, appropriate figures, and table of neurochemistry and clinical chemistry values for American kestrels from the subsequent study involving the exposure of the captive birds to TBOEP, TCEP, TCIPP, or TDCIPP. The Supporting Information is available free of



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### Notes

The authors declare no competing financial interest.

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**SUPPLEMENTARY INFORMATION: COVER SHEET**

# Investigating Endocrine and Physiological Parameters of Captive American kestrels Exposed by Diet to Selected Organophosphate Flame Retardants

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**SUPPLEMENTARY INFORMATION**

# Investigating Endocrine and Physiological Parameters of Captive American kestrels Exposed by Diet to Selected Organophosphate Flame Retardants

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## Introduction

In the Supplemental Information section, the methodology and results of a pilot study conducted with TDCIPP in 2011 are presented. In addition, the detailed methodology and results, including appropriate figures and table, from the main study conducted in 2012, are presented for the neurochemistry, oxidative status, thyroid function, and clinical chemistry assessments of the captive adult male American kestrels exposed to TBOEP, TCEP, TCIPP or TDCIPP.

## Materials and Methods

*Pilot Study*

A pilot study was conducted in 2011 using 7 adult two-year old male American kestrels exposed daily by diet for 28 days to TDCIPP and 7 adult male kestrels of the same age exposed to only the safflower oil vehicle. The initial exposure dose in the pilot study was 226 ng TDCIPP /d; each kestrel ate one cockerel/d that had been injected with 50 µL of a safflower oil solution containing TDCIPP at 4.52 ng/µL. Based on the mean weight (114 g) of the adult male kestrels at the initiation of the study, each kestrel received a daily dose of 1.98 ng TDCIPP/g kestrel/d. This dosing concentration was 10x less than the TDCIPP exposure concentration used in the 2012 main study. The kestrels were weighed and their general health assessed at the beginning of the exposure period, and again at 7, 14, 21, and 28 d of the pilot exposure trial. All housing, handling, blood sampling and dissecting procedures were the same as those used in the main study conducted in 2012, and are described in Fernie et al. (submitted with this SI section).

*Neurochemical Assays*

Neurochemical assays were completed for the 2011 pilot study at the National Wildlife Research Center (Environment Canada, Ottawa, ON, Canada). Brains were dissected from the

cranium at the time of necropsy, fixed in formalin and stored in a scintillation vial until processing. Neurochemical analysis was conducted at the National Wildlife Research Center (Environment Canada, Ottawa, ON) in 2011 and at the University of Michigan (Basu Laboratory) in 2012. In 2011, the whole brain was homogenized in buffer/Triton-X-100 at a ratio of 1 part brain and 3 part of 1% Triton solution to a final concentration of 250 mg/mL. The homogenate was centrifuged to eliminate interferences during spectrophotometric analysis and 10 µL of the supernatant homogenate was analyzed in duplicate. Cholinesterase (ChE) analysis was completed as described in MET-BMK-CHE-01J, as modified by Hill and Fleming (1982). The cuvette contained 3 mL of DTNB solution (5,5'-dithiobis-nitrobenzoic acid in $2.5x10^{-4}$ M in phosphate buffer 0.05M, pH 7.9), 10 µL of brain homogenate and 100 µL of acetylthiocholine iodide 0.0312 M (for a final concentration of 1.0 mM). ChE activity was determined with a spectrophotometer (Hewlett Packard Diode Array HP8453, S/N 02502465) at 25°C. The change in absorbance at 405 nm over one minute was recorded, with readings every 15 seconds, and a wait time of 60 seconds to ensure readings were made during the linear segment of the reaction. ChE analysis was conducted in duplicate for each bird.

In 2012, neurochemical assays were completed at the University of Michigan (NB) based on previous analytical methods (Basu et al. 2009; Rutkiewicz et al. 2010, 2013). Cellular membranes were prepared from brain tissues using protocols described elsewhere (Basu et al., 2009). Binding to the muscarinic (mACh) and nicotinic nACh) receptors, was performed in Na/K buffer (50 mM $NaH_2PO_4$, 5 mM KCl, 120 mM NaCl, pH 7.4). Thirty µg of membrane preparation was re-suspended in buffer and added to microplate wells containing a 1.0 µM GF/B glass filter (Millipore, Boston, MA, USA). For muscarinic and nicotinic receptor binding, samples were incubated with 1 nM [$^3$H]-QNB (42 Ci/mmol; NEN/Perkin Elmer, Boston, MA,

USA) and 1 nM [$^3$H]-Cytisine (26.8 C/mmol; NEN/Perkin Elmer), respectively, for 60 min. All assays were carried out with gentle shaking at room temperature and binding reactions were terminated by vacuum filtration. The filters were rinsed three times with buffer and then allowed to soak for 96 hrs in 25 µL of OptiPhase Supermix Cocktail (Perkin Elmer). Radioactivity retained by the filter was quantified by liquid scintillation counting in a microplate detector (Wallac Microbeta, Perkin Elmer) having a counting efficiency of approximately 50%. Specific binding to both receptors was defined as the difference in radioligand bound in the presence and absence of 100 µM unlabeled atropine. Binding was reported as fmol of radioisotope bound per mg of membrane protein (fmol/mg). All samples were assayed in quadruplicate for total and non-specific binding.

The enzymatic activities of cholinesterase (acetylcholinesterase in plasma and brain tissue, and butyrylcholinesterase in plasma) were completed according to published procedures with slight modifications. Tissues were sonicated for 30 s in cold Na/K buffer that included 0.5 % (v/v) Triton X-100. Following a 10 min centrifugation (15,000 x *g*, 4 ºC) the supernatant was removed. For cholinesterase activity, 0.5 µg of supernatant protein was mixed with 100 µM 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red, Molecular Probes), 200 mU horseradish peroxidase, 20 mU choline oxidase, and 100 µM acetylcholine. For acetylcholinesterase, iso-OMA was used to inhibit butyrylcholinesterase activity. For butyrylcholinesterase, galanthamine was used to inhibit acetylcholinesterase activity. Following a 30 min incubation period for both assays, the reaction end-product (resorufin: $\lambda_{ex}$ = 540, $\lambda_{em}$ = 590) was monitored between 30 and 60 min (CytoFluor 2350, Millipore). Specific activities were expressed as nmol of resorufin formed per min per unit (µg or mg) protein. Each sample was assayed in triplicate.

*Oxidative Status*

In 2012, a portion of each liver was analyzed for seven indicators of oxidative stress: reduced glutathione (GSH), oxidized glutathione (GSSG), GSSG:GSH, total glutathione (tGSH), total sulfhydryl (TSH), protein bound sulfhydryl (PBSH), and thiobarbituric acid reactive substances (TBARS). Frozen liver samples were thawed and homogenized on ice in 2X PBS (pH 7.4; Fisher BioReagents, Waltham, MA) at 200 µg/µl. The homogenate was centrifuged at 14,000 rpm for 10 minutes at 4°C and 60 µl aliquots of the supernatant were transferred to 0.2 ml tubes and frozen at -80°C until analysis. For GSH, GSSG, GSSG/GSH, and tGSH analysis, the liver supernatant was thawed and diluted to 12.5 µg/µl in 1X PBS and analyzed using the DetectX® Glutathione Fluorescent Detection Kit (Arbor Assays, Ann Arbor, MI) following the manufacturer's protocol. For determination of TBARS, the sample supernatant was thawed on ice and diluted to 100 µg/µl in 1X PBS. TBARS levels were determined using the QuantiChrom™ TBARS Assay Kit (Bioassay Systems, Hayward, CA) following the manufacturer's instructions. TSH was determined in thawed supernatant that had been diluted to 6.25 µg/µl in 1X PBS using the Measure-iT™ Thiol Assay Kit (Life Technologies - Molecular Probes, Inc., Eugene, OR) following the manufacturer's instructions. Concentrations of PBSH were calculated as the difference between TSH and GSH concentrations.

*Thyroid Function: Deiodinase Activity and Histology of the Thyroid Gland*

In 2012, a three-pronged assessment of thyroid function was performed (LP, VP) by determining circulating triiodothyronine ($T_3$) and $T_4$ concentrations, hepatic $T_4$-outer ring deiodinase activity ($T_4$-ORD), and histological assessments of the structure of the thyroid gland. For determination of deiodinase activity, a 100-200 mg sample of each liver was homogenized in ice cold 0.1 M sodium phosphate buffer (pH=7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM dithiothrietol (DTT), centrifuged at 9,000 X g and 4 $^{\circ}$C for 15 minutes,

and the resulting S9 supernatant recovered. The S9 homogenate was frozen and stored at -80°C until analysis. Protein concentration was determined in a separate aliquot of S9 homogenate using the Bradford (1976) method. To determine deiodinase activity, 10 µl of S9 homogenates were incubated at 37°C with 900 µl of sodium phosphate buffer. At timed intervals, 5 µl of dimethylsulfoxide containing 3000 ng of $T_4$ were added to each sample to begin the reactions. Reactions were terminated 90 minutes later by adding 1 ml of ice cold methanol. Two types of blanks were included with each analytical run: one with all assay constituents except S9 and another containing all constituents except $T_4$. To determine $T_3$ produced by the reactions, 100 ul of the final reaction volume was used in commercially available radioimmunoassays (MP Biomedical, Santa Ana CA, USA). Deiodinase activity was expressed as pmol of $T_3$ liberated per minute per mg of protein, with blank correction included as previously described for American kestrels (Marteinson et al. 2011).

Formalin-fixed tissues were processed and embedded in paraffin using standard procedures (Luna 1968). Tissues were sectioned at approximately 7 µm, mounted on slides, and stained with hematoxylin and eosin (H & E). Each histological slide was examined using a Carl Zeiss EMS stereoscope with an APoLumar SI.2Y objective, and an AxioCam HRC digital camera at 26 milliseconds (ms) exposure to capture images of the thyroid tissues for measurement. Digital images of two separate fields of view for each slide were taken at 40x magnification. For each thyroid gland, ten complete follicles per image (for a total of 20 follicles) were selected and analyzed as described by Park et al. (2011). Briefly, epithelial cell heights (ECH) were measured (µm) at 4 locations per follicle, approximately 90 degrees from each other, and then averaged to calculate a mean ECH per follicle. Area ($\mu m^2$) of the colloid inside the same 20 thyroid follicles was also measured. The data were expressed as mean

thyroid ECH and mean colloid area per individual bird. The mean ratio of colloid perimeter:ECH was also calculated as an indicator of thyroid gland activation and the potential for thyroid hormone production (Bocian-Sobkowwska et al. 1997). All analyses were performed using Zen Lite 2012 (2012) software.

*Clinical Chemistry*

The Ortho Vitros 250XR chemistry analyzer (Ortho, Rochester, NY, USA) was maintained by the recommended manufacturer and internal quality assurance programs. Standard QA/QC measures were run to assure analyzer performance. The following methods were used: AST- aspartate + alpha ketoglutarate method; ALT – alanine + alpha ketoglutarte method; LDH – pyruvate + NADH method; GTT – L-gammaglutamyl p-nitroanilde method; ALK PHOS – p-nitrophenyl phosphate method; Total protein – biuret method. GLDH was analyzed on the Randox Daytona analyzer (Kearneysville, WV, USA) and the reagents were also purchased from and implemented according to Randox guidelines. QA/QC for the GLDH involved a 2-tier control, and employed the alpha oxoglutarate + NADH method. Hemolysis, lipemia and bile acids were analyzed according to Cray and Andreopoulos (2003), and analysis of bile acids utilized the MP Biomedical radioimmunoassay kit. Electrophoresis was used to determine prealbumin, albumin, and globulins, and run using the Helena system according to Cray et al. (2011).

*Statistical Methods*

As described in the main study (Fernie et al., submitted), endpoints were compared for each treatment to vehicle-treated controls only, by parametric analysis of variance (ANOVAs) (e.g., hepatic T4-ORD) or repeated measures (RM) ANOVAs (i.e., thyroid hormones), or non-parametric ANOVAs and RM ANOVAs on ranked data (i.e., clinical chemistry), using SAS

9.3®. Residuals were tested for homogeneity of variance and normality. Only the concentrations of circulating thyroid hormones were successfully log transformed. Since each histological parameter of the thyroid gland required multiple measurements per bird, nested ANOVAs on ranked data were performed with treatment as the factor and the individual bird as the nested factor; Scheffe's *post-hoc* tests were then applied. Hepatic T4-ORD was examined with an ANOVA followed by least square means *post-hoc t*-tests of all pairwise comparisons without adjustments. RM ANOVAs initially identified overall significant treatment effects on thyroid hormones or clinical chemistry for each OPFR compared to controls, followed by 1-way ANOVAs to identify significant treatment differences at individual time periods when overall significance was identified. Spearman's correlation analysis was used to determine statistical associations between hepatic lipid and TBOEP concentrations, and plasma concentrations of albumin and thyroid hormones. Means ± SEM are presented and statistical significance is considered when $p \leq 0.05$.

**Results**

*The Pilot Study, 2011*

In the pilot study, the exposure of the kestrels to the lower concentration of TDCIPP had no effect on circulating $TT_3$ and $TT_4$ levels (P-values $\geq 0.10$) or plasma cholinesterase activity (KW Chi-square P = 0.65), but resulted in lower brain cholinesterase activity (41.9 ± 0.8 μmol/min/g) compared to controls (44.4 ± 0.6 μmol/min/g) (KW Chi-square = 4.45 df = 1 P = 0.03). This represented a 5% reduction in the brain cholinesterase activity of the TDCIPP-exposed kestrels compared to the control kestrels. Based on numerous studies with cholinesterase-inhibiting pesticides in birds, this degree of suppression of cholinesterase activity

in brain does not seem to be associated with physiological or behavioral deficits (Grue et al. 1991, 1997).

*Cholinesterase activity, clinical chemistry, and plasma thyroid hormones: 2012*

For the adult male American kestrels involved in the main study conducted with the four OPFRs in 2012, cholinesterase activity and clinical chemistry values are presented in Table S1. To the best of our knowledge, this is the first report of such information captured in one publication.

In 2012, there were significant effects identified for each chemical in terms of changes in concentrations of one or both thyroid hormones in the male kestrels. All comparisons are made to control male kestrels only. For the kestrels exposed to TBOEP, there was a significant overall effect on plasma $FT_4$ (RM ANOVA: Trt: $F_{3,10} = 3.60$ P = 0.05) and $TT_3$ (RM ANOVA: Trt: $F_{3,12} = 5.87$ P = 0.01), as well as the temporal changes in $FT_4$ (RM ANOVA: Trt*Time: $F_{2,11} = 4.96$ P = 0.03). In addition, the exposure to TBOEP for 7 d also significantly increased $FT_3$ concentrations ($F_{1,15} = 5.41$ P = 0.03) and $TT_3$ concentrations ($F_{1,15} = 20.23$ P = 0.0005).

When the captive kestrels were exposed to TCEP, there was a significant overall effect on $FT_4$ (RM ANOVA: Trt: $F_{3,11} = 6.16$ P = 0.01) and $FT_3$ concentrations (RM ANOVA: Trt: $F_{3,13} = 6.83$ P = 0.005), and the temporal changes in $FT_4$ (RM ANOVA: Trt*Time: $F_{2,12} = 8.28$ P = 0.006) and $FT_3$ concentrations (RM ANOVA: Trt*Time: $F_{2,14} = 6.81$ P = 0.009). The exposure to TCEP for 7 d resulted in increased concentrations of both $FT_3$ ($F_{1,16} = 19.27$ P = 0.0005) and $FT_4$ ($F_{1,14} = 7.42$ P = 0.02).

The exposure of the male kestrels to TCIPP had a significant effect overall on their plasma $FT_3$ concentrations (RM ANOVA: Trt: $F_{3,13} = 4.04$ P = 0.03) and the temporal changes in $FT_3$ (RM ANOVA: Trt*Time: $F_{2,14} = 6.03$ P = 0.01), with a significant suppression of $FT_3$

concentrations at 14 d exposure ($F_{1,16}$ = 4.91 P = 0.04). There were no significant effects of the TCIPP exposure on plasma $FT_4$ or $TT_4$ concentrations in the kestrels.

The exposure to the higher TDCIPP concentration used in 2012, had a significant overall effect on the plasma $FT_3$ concentrations (RM ANOVA: Trt: $F_{3,13}$ = 3.48 P = 0.05) and the temporal changes in plasma $FT_3$ (RM ANOVA: Trt*Time: $F_{2,14}$ = 5.49 P = 0.02), with a significant elevation of $FT_3$ concentrations at 7 d exposure ($F_{1,16}$ = 5.28 P = 0.04). The exposure to TDCIPP also significantly increased plasma concentrations of $TT_4$ at 7 d ($F_{1,16}$ = 11.02 P = 0.005) and suppressed them at 21 d ($F_{1,16}$ = 4.37 P = 0.05), contributing to significant overall effects on plasma $TT_4$ levels (RM ANOVA: Trt: $F_{3,13}$ = 7.25 P = 0.004) and temporal changes in $TT_4$ concentrations (RM ANOVA: Trt*Time: $F_{2,14}$ = 11.31 P = 0.001). There was also a significant overall effect of TDCIPP on the temporal changes of $FT_4$ (RM ANOVA: Trt*Time: $F_{2,11}$ = 4.03 P = 0.05).

Table S1. Cholinesterase activity and plasma clinical chemistry concentrations measured in captive adult male American kestrels.

| Variable | Unit of Measure | N | Minimum | Maximum |
|---|---|---|---|---|
| *Cholinesterase Activity* | | | | |
| Brain Acetylcholinesterase Activity | OD/min/mg | 35 | 5 | 8 |
| Brain Butrylcholinesterase Activity | OD/min/mg | 35 | 0 | 0 |
| Brain Muscarinic Cholinergic Receptor | fmol/mg | 35 | 269 | 843 |
| Brain Nicotinic Cholinergic Receptor | fmol/mg | 35 | 14 | 40 |
| Plasma Acetylcholinesterase Activity | OD/min/mg | 36 | 1 | 5 |
| Plasma Butrylcholinesterase Activity | OD/min/mg | 34 | 0.0 | 0.2 |
| | | | | |
| *Clinical Chemistry* | | | | |
| Albumin:Globulin Ratio | | 38 | 0.5 | 2 |
| Alanine aminotransferase activity (ALT) | U/L | 38 | 34 | 297 |
| Gamma-glutamyl transpeptidase (GGT) | U/L | 38 | 4 | 7 |
| Albumin (%) | % | 38 | 21 | 55 |
| Albumin | g/dL | 38 | 1 | 3 |
| Bile acids | umol/L | 33 | 1 | 161 |
| Total Proteins (TP) | g/dL | 38 | 3 | 6 |
| Aspartate aminotransferase (ASAT) | U/L | 38 | 37 | 366 |
| Lactate dehydrogenase isozymes (LDH) | U/L | 38 | 1708 | 10632 |
| Alkaline Phosphotase (ALP) | U/L | 38 | 51 | 842 |
| Glutamate dehydrogenase activities (GLDH) | U/L | 38 | 0.1 | 17 |

Fig. S1. Circulating concentrations of $TT_3$ and $TT_4$ at 7 d exposure in adult male American kestrels that were exposed daily to TBOEP, TCEP, TCIPP or TDCIPP (22 ng OPFR/g kestrel/d) for a total of 21 d. Means ± standard errors about the means (SEMs) are presented. Statistically significant p-values are indicated as follows: $p \leq 0.0001$ (***); $p \leq 0.01$ (**); $p \leq 0.05$ (*).

S1a. Circulating $TT_3$ concentrations.

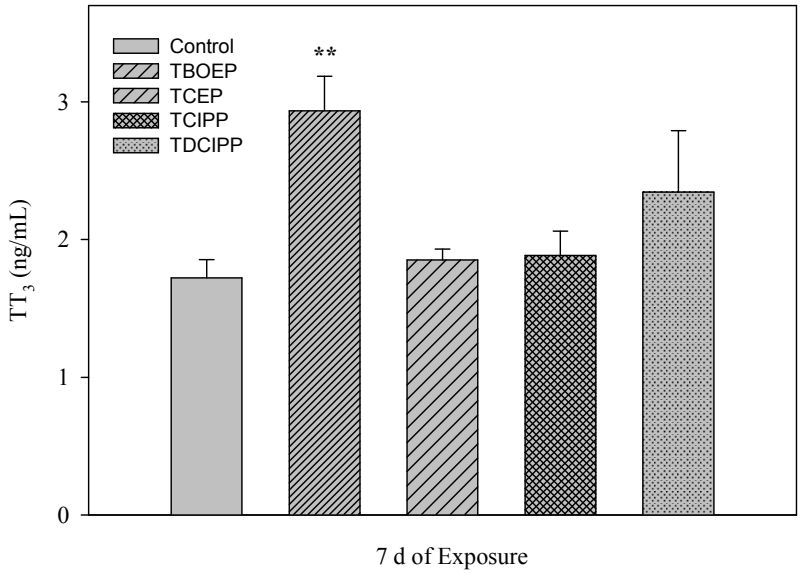
S1b. Circulating $TT_4$ concentrations.

Fig. S2. Significant changes in thyroid gland histology measures, notably epithelial cell height and volume of colloid, were observed in adult male kestrels at 21 d exposure to TBOEP, TCEP, TCIPP or TDCIPP. Means ± SEMs are presented. Statistically significant p-values are indicated as follows: $p \leq 0.0001$ (***); $p \leq 0.01$ (**); $p \leq 0.05$ (*).

S2a. Thyroid gland: epithelial cell height (ECH).

S2b. Thyroid gland: colloid area.

S1a. Circulating $TT_3$ concentrations.




S1b. Circulating $TT_4$ concentrations.

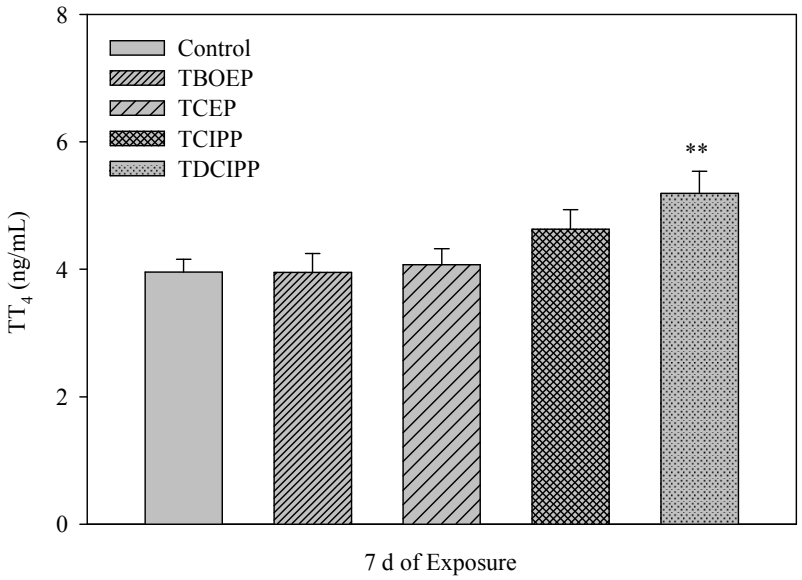
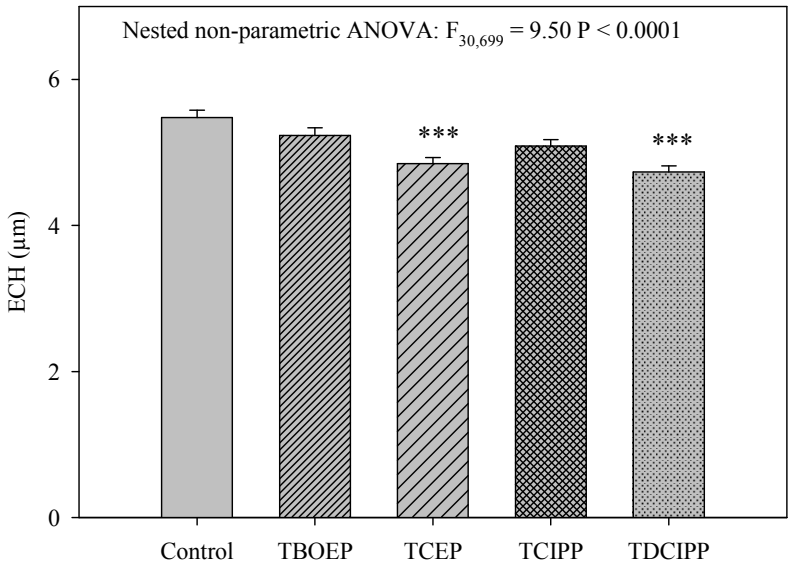
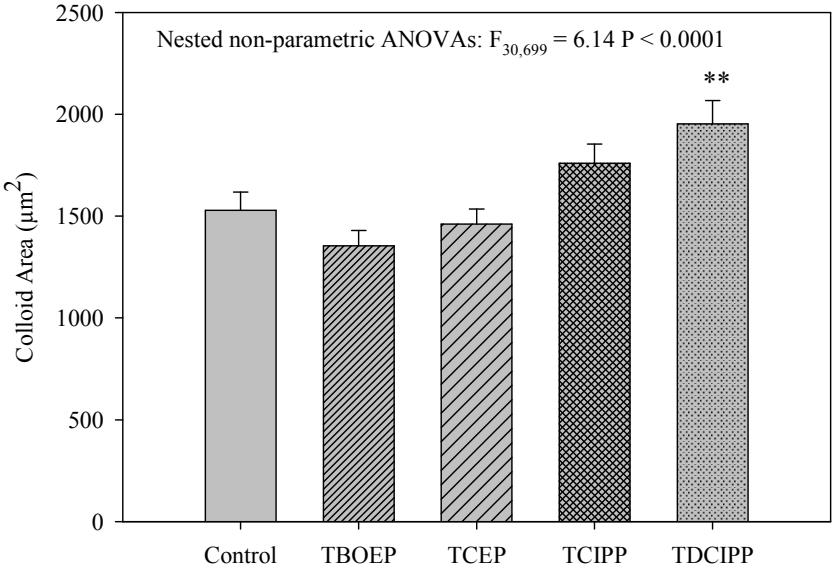


Fig. S2a. Thyroid gland: epithelial cell height (ECH).




S2b. Thyroid gland: colloid area (data for perimeter of colloid not shown).



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