Exposure to organophosphate flame retardants alters thyroid function and hepatic status in American kestrels.

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Introduction

Tris(2-butoxyethyl) phosphate (TBOEP), tris(2-chloroethyl) phosphate (TCEP), tris(2-chloroisopropyl) phosphate (TCIPP), and tris(1,2-dicholor-2-propyl) phosphate (TDCIPP), are priority organophosphate flame retardants (OPFRs) for risk assessment by the Environment Canada (Chemicals Management Plan) and the U.S. Environmental Protection Agency. TBOEP, TCIPP and TDCIPP have been detected in indoor air (Markland et al. 2005), sediment (Meyer and Bester 2004), water (Fries and Püttmann 2003), fish (LeBel and Williams 1986, Sundkvist et al. 2010), herring gull tissues and eggs from their maternal clutches (Chen et al. 2012; Greaves and Letcher, submitted), and in human breast milk (Sundkvist et al. 2010) and adipose tissue (LeBel and Williams 1986). Sources of OPFRs include municipal waste water effluent, precipitation (Fries and Püttmann 2003), and air (Regenery and Puttman 2009). Little is known about the potential toxic effects of these OPFRs. Sager and Little (1989) concluded that TBOEP may affect the biology and pharmacology of the β-adrenergic signaling system involved in brain, organ and immune function. In chicken embryos exposed to very high concentrations of OPFRs by egg injection, TCIPP significantly delayed pipping (9,240 and 51,600 ng/g) and reduced tibiotarsal bone length (51,600 ng/g), and TDCIPP reduced embryo mass, gallbladder size (45,000 ng/g), and free thyroxine concentration in plasma (FT4) (7,640 ng/g) (Farhat et al. 2013). Our objectives were to determine the dietary uptake and potential effects of these four OPFRs on American kestrels (*Falco sparverius*), a raptorial model species (Bardo and Bird 2009), found to be more sensitive to the effects of polybrominated diphenyl ethers than galliform species (McKernan et al. 2009).

Materials and Methods

This study was conducted in 2011 and 2012 under approved animal care and use guidelines. Captive American kestrels were housed individually in large outdoor flight pens, with shelter available from the elements. They were fed *ad libitum* day-old dead cockerel daily, with the dosed cockerel fed each morning, and either non-dosed cockerel or dead laboratory mice provided on alternative afternoons. Following exposure methods used in previous flame retardant studies with captive kestrels (e.g., Fernie et al. 2011, Marteinson et al. 2012, Sullivan et al. 2013), 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.

In 2011, 7 captive adult two-year old male American kestrels were exposed daily by diet for 28 days to TDCIPP and compared to 7 adult male kestrels of the same age similarly exposed to the safflower oil vehicle only. In 2012, a separate set of 38 one-year old adult male kestrels, 7 per exposure group with 10 controls, were exposed daily by diet for 21 days to a higher concentration of TDCIPP, or to one of three other OPFRs, specifically TBOEP, TCIPP, or TCEP. Once again, control male birds of the same age were exposed to the safflower oil vehicle only. Each kestrel received 250 ng TDCIPP/50 ul safflower oil/day in 2011, or the same concentration for each of the four OPFRs, 2500 ng OPFR/50 uL safflower oil/day in 2012.

In 2011 and 2012, the kestrels were weighed and their general health assessed at the beginning of the exposure period, and again at 7 d, 14 d, 21 d and 28 d (2011 only) of the exposure trial. Concurrently, between 0.6 and 1.1 ml of whole blood was withdrawn by jugular venipuncture using a heparinized 1.0 ml syringe with 27-gauge

needle from each kestrel at weekly intervals. Following their exposure for 21 d (2012) or 28 d (2011), the birds were humanely euthanized. Further blood was immediately collected by cardiac venipuncture, with a sub-sample analyzed for hematocrit, and the remainder stored on ice until processing by centrifugation. Plasma was aliquoted into cryovials and stored at -80^{o}C. For each kestrel, the thyroid gland, liver, kidney, pectoral muscle (1 gram), and brain were also immediately collected, weighed, sub-sectioned, stored in 10% buffered formalin for histological assessment, or placed on ice until frozen at -80^{o}C within 4 hours of collection.

Plasma was used to assess multiple biomarkers, including circulating free and total triiodothyronine (FT3, TT3) and thyroxine (FT4, TT4), and clinical chemistry measures related to hepatic integrity and function. Plasma was also used for assessing acetyl- and butyrylcholinesterase activity, and the brain for these two cholinergic enzymes as well as the muscarinic and nicotinic receptors. Red blood cells and pectoral muscle were retained for amino acid analysis in a separate study. Kidney and liver were used to determine concentrations of the four OPFRs. Other hepatic aliquots were used to assess T4- outer ring deiodinase (T4-ORD) and indicators of oxidative stress (glutathione levels and lipid peroxidation). Thyroid glands were placed in phosphate-buffered formalin for histological assessments. All analyses were conducted following standard analytical procedures. The MLOQ and MLOD (ng/g ww) for each OPFR are listed respectively: TBOEP (0.27, 0.09), TCEP (0.1, 0.03), TCIPP (0.31, 0.1), and TDICPP (0.14, 0.04).

The four OPFRs were determined in liver and kidney tissue samples in fraction isolated according to the method described elsewhere (Chen et al., 2012; Greaves and Letcher, submitted). 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, where the concentrations of target compounds were inherently recovery-corrected by internal standard. In 2012, the Letcher Laboratory at the National Wildlife Research Center participated in the first international NORMAN PFR (OPFR) ILS. For this QA/QC exercise, the OPFR results from this lab complied within 10% of the NORMAN PFR certified data. In all cases where an OPFR was quantifiable in a liver or kidney sample fraction, the concentration was background corrected.

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). Based on the 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 listed respectively: TBOEP (0.27, 0.09), TCEP (0.1, 0.03), TCIPP (0.31, 0.1), and TDICPP (0.14, 0.04).

The method blank consisted of internal standard d_{27}-TBP spiked diatomaceous earth (DE) (spiked with 200 µL chicken egg albumin, also previously determined to be absent of any OPFRs and used to maintain desirable IS recovery through volatility) was carried through the entire extraction, concentration and analysis procedure. This was done in triplicate with the batch of the 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. In the case of OPFR analysis, 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 analysis, conducted with SAS 9.3®, consisted of non-parametric tests, or when appropriate, parametric ANOVAs or repeated measures (RM) ANOVAs when multiple measures were recorded over time on the same individuals. Post-hoc statistical means were calculated for comparisons among groups, but particularly to controls. Statistical significance is considered at the $p \leq 0.05$ level.

Results and Discussion

Exposure. Based on the determination of TBOEP, TDCIPP, TCIPP and TCEP dose solutions, the original solutions for the exposed cohort were prepared accurately as shown by the nominal versus measured concentrations being

different by <13.5% RSD (except TCEP which was <20 %RSD). After method blank-correction, TCEP, TCIPP or TDCIPP were <MLOD in all liver samples from dosed and control birds. However, TBOEP was quantifiable in the liver samples of both the TBOEP-dosed and control birds, with method blank-correct concentrations ranging between 0.3 and 10 ng/g ww. After method blank-correction, TBOEP, TCEP, TCIPP or TDCIPP were <MLOD in all kidney samples from the control and the OPFR-dosed kestrels. However, TBOEP was quantifiable in the liver samples of both the TBOEP-dosed and control kestrels; TCEP, TCIPP and TDCIPP were also <MLOD in the hepatic tissues, with the exception of low ppb levels in one sample in each of the OPFR dosing groups.

It is likely that the four OPFRs in the present study were rapidly metabolized by the kestrels through enzyme-mediated processes over the exposure periods, and thus there were generally no detectable residues in either the liver or kidney tissue samples. Our findings are consistent with the rapid metabolism reported in fish and rats exposed to TCIPP and TDCIPP (European Union, 2008a,b). Using a rat liver microsomal assay, Chu and colleagues (2011) demonstrated that TDCIPP was completely metabolized to BDCIPP within 5 min. of *in vitro* incubation. Further, the present results with the kestrels are consistent with those of Greaves and Letcher (submitted), in which all four OPFRs were quantified in egg yolk and albumen, and maternal fat and muscle of herring gulls, but none was detectable in the liver, brain and plasma samples of the same female gulls from the Laurentian Great Lakes of North America.

Overt Effects. Overall, the exposure of the kestrels to the four OPFRs at these concentrations had no dramatic effects on the endpoints measured in the study; all reported comparisons were made with the control birds. The body mass of the kestrels was unaffected by the exposure to the OPFRs, and there were no apparent overt effects on their health. Exposure to the four OPFRs at these exposure concentrations had no effect on hepatic oxidative stress, cholinesterase enzymatic activity in plasma or brain, or nicotinic or muscarinic receptor levels in the brain, of the kestrels.

Clinical Chemistry. Based on plasma clinical chemistry measures, there was evidence of modified hepatic integrity and function of the kestrels at 7 d exposure to TBOEP, TCIPP, and TDCIPP. At this stage, the birds exposed to TBOEP had significantly elevated albumin:globulin (A:G) ratios ($P = 0.02$) and alkaline phosphatase activity ($P = 0.02$), while those exposed to TCIPP or TDCIPP had elevated bile acids ($P \leq 0.01$) that aid in fat absorption and modulate cholesterol levels. Farhat et al. (2013) suggested that TDCIPP disturbed lipid metabolism and was a likely mechanism of toxicity in chicken embryos. The TDCIPP-exposed kestrels also had lower A:G ratios at 7 d ($P = 0.02$), and continued to demonstrate changes in hepatic integrity and function, with reduced alanine aminotransferase activity at 14 d of exposure ($P = 0.05$) and lower A:G ratios at 21 d ($P = 0.04$). Moreover, exposure to all four OPFRs suppressed gamma-glutamyl transpeptidase (GGT) enzymatic activity scores in the kestrels ($P \leq 0.01$). It should be noted that there were no significant differences in concentrations of total protein, pre-albumin and albumin, various globulins, aspartate aminotransferase or glutamate dehydrogenase activities, lactate dehydrogenase isozymes, hemolysis indices, or lipemia indices.

Thyroid Function. In 2011, exposure to the lower levels of TDCIPP for 28 d had no significant effect on TT3 or TT4 levels of the kestrels. However, in 2012, exposure to the higher concentrations of the four OPFRs as a category, had significant overall effects on circulating TT4 and TT3 concentrations (RM ANOVA: Treatment $P = 0.01$, $P = 0.05$ respectively) and the temporal changes in plasma TT4 and FT3 (RM ANOVA: Treatment*Time $P \leq 0.03$).

At the individual sampling periods, there was evidence of changes in circulating thyroid hormones in the kestrels. At 7 d exposure, TT3 and the TT3/TT4 ratio were elevated in the TBOEP exposed birds, while TCEP-exposed kestrels had much higher plasma FT3 and FT4 levels. At 14 d, the TCIPP-exposed kestrels had moderately suppressed FT3 levels and FT3/FT4 ratio. Finally, circulating TT4 levels in the TDCIPP-exposed kestrels was significantly elevated at 7 d exposure and suppressed at 21 d, with no significant changes in hormone levels at the other sampling times.

While hormonal changes can appear transiently under the influence of contaminant stress, thyroid gland changes are sensitive indicators of overall gland activation. Structure of the thyroid gland and hepatic deiodinase activity were assessed at 21 d of exposure. While exposure to these OPFRs may no longer have affected circulating thyroid hormones at 21 d (with the exception of TDCIPP suppressing plasma TT4), changes in the glandular structure and hepatic T4 deiodinase activity were evident. The decrease in epithelial cell height (ECH) and/or

increased colloid in the follicles of the thyroid gland, suggest that there was decreased activation of the glands of the kestrels exposed to TCEP, TCIPP or TDCIPP, but not TBOEP. Elevated hepatic T4-ORD was evident at 21 d exposure to TBOEP, TCIPP and TDCIPP ($P = 0.03$). Type I deiodinase was induced by TCIPP, and plasma FT4 levels reduced by TDCIPP (7,640 ng/g) in chicken embryos (Farhat et al. 2013).

Conclusions. The exposure of captive kestrels to these concentrations of TBOEP, TCEP, TCIPP, or TDCIPP, is likely below those encountered by wild herring gulls (e.g., *in ovo* concentrations of TBOEP ≤ 2.2 ng/g ww; TCEP ≤ 0.55 ng/g ww; TCIPP ≤ 4.1 ng/g ww; TDCIPP ≤ 0.17 ng/g ww; Chen et al. 2012), and/or of shorter duration than experienced by wild birds, since no measureable concentrations were detected in their hepatic and renal tissues. Overall, there were no overt effects of this dietary exposure on the survival of the kestrels during the period of study, although moderate effects were apparent, particularly at 7 d of exposure. Some clinical chemistry measures suggested alterations in hepatocyte integrity and function. Changes in circulating thyroid hormones were evident at 7 d of exposure and more 'ephemeral' thereafter, disappearing by 21 d of exposure with the exception of reduced plasma TT4 levels in TDCIPP-exposed birds. However, at that time, deactivation of the thyroid gland and/or increased hepatic T4-ORD deiodinase activity had occurred in the kestrels exposed to most of these OPFRs. Further research is required to determine possible effects on the health of wild birds given the higher concentrations of these OPFRs reported in their tissues.

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