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Uptake, Deposition, and Metabolism of Triphenyl Phosphate in Embryonated Eggs and Chicks of Japanese Quail (*Coturnix japonica*)

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Abstract: The toxicokinetics of triphenyl phosphate (TPHP) in vivo including the uptake, deposition, and biotransformation into the metabolite diphenyl phosphate (DPHP) is presently reported in embryonated eggs and chicks of Japanese quail. Quail were dosed with TPHP at 3 concentrations by air cell egg injection on embryonic day 0, followed by daily oral dosing after chicks hatched (5 d). Vehicle-only exposed controls were also used. In dosed eggs, only 33% of the TPHP remained 2 d after injection (no hepatic development); after 10 d (post-hepatogenesis), only 2% remained. The estimated TPHP half-lives in the eggs ranged from 1.1 to 1.8 d for the 3 dosed groups. In all exposed eggs and chicks, DPHP significantly increased with dose ($0.001 < p < 0.044$). It appears that DPHP is an important metabolite in quail, making up 41 to 74% of all metabolites formed in embryonated eggs. In chicks, at medium and high doses, DPHP concentrations significantly exceeded those of TPHP ($p \leq 0.007$), making up 67 and 76% of the total burden, respectively. Our findings suggest that rapid TPHP metabolism occurred in chicks and embryonated quail eggs but that this may vary with the age of the embryonated egg and the stage of embryo development, which should be considered when evaluating concentrations of TPHP and DPHP measured in eggs of wild birds. *Environ Toxicol Chem* 2020;39:565–573. © 2019 Her Majesty the Queen in Right of Canada. *Environmental Toxicology and Chemistry* © 2019 SETAC

Keywords: Triphenyl phosphate; Toxicokinetics; Japanese quail; Birds; Embryonated eggs; Chicks

INTRODUCTION

Organophosphate esters (OPEs) are extensively used as flame retardants and plasticizers in a variety of products including building materials, textiles, and electronic equipment. In most cases, OPEs are incorporated by physical addition and are not chemically bound to polymers; thus, they can leach into the environment (van der Veen and de Boer 2012). Since the restriction on the use of older flame retardants such as penta- and octa-polybrominated diphenyl ethers (PBDEs), the production and consumption of OPEs is increasing. The concentrations of OPEs in indoor dust have overtaken those of PBDEs in some studies, which may provide evidence of their increased use (reviewed in van der Veen and de Boer 2012); and OPEs have been detected in a wide range of environmental compartments (reviewed in Greaves and Letcher 2017). Concentrations of OPEs in biota are generally much lower than those of PBDEs, suggesting that they may have comparatively lower bioaccumulative potential. However, considering the extensive occurrence of OPEs in the environment and biota (Greaves and Letcher 2017), there has been increasing attention on their toxicology and effects in exposed organisms, including humans.

Triphenyl phosphate (TPHP) is an important and current-use OPE. Production and use of TPHP ranged from 4500 to 22 700 metric tons per year in the United States in the 1990s, which has likely increased significantly since the phaseout of PBDEs (van der Veen and de Boer 2012). It enters the environment during industrial production as well as through household use and disposal of electronics (Carlsson et al. 2000; van der Veen and de Boer 2012) and breakdown from other compounds (organophosphite antioxidants; Liu and Mabury 2019), and it has been detected in air, soil, and water (Hosseini et al. 2011;

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van der Veen and de Boer 2012; Salamova et al. 2014). It has been detected in biota including tissues, feathers, and eggs of birds in Canada (Greaves and Letcher 2014; Greaves et al. 2016a; Lu et al. 2017) and Europe (Eulaers et al. 2014; Hallanger et al. 2015; Verreault et al. 2018). In 2010, mean concentrations of TPHP in herring gulls (*Larus argentatus*) from a Great Lakes colony were 0.89 ± 0.25 ng/g wet weight in egg yolk and 2.11 ± 1.11 ng/g wet weight in fat (Greaves and Letcher 2014).

The government of Canada has categorized TPHP as a potential high-risk chemical (Environment and Climate Change Canada 2016). It has been suggested that TPHP may have possible health effects, such as interference with reproductive function and endocrine axes (Greaves and Letcher 2017), the latter of which has been recently confirmed in Japanese quail chicks (*Coturnix coturnix japonica*; Guigueno et al. 2019). In vitro TPHP metabolism has been examined in animal and human cells (van den Eede et al. 2013, 2016; Su et al. 2014a, 2015) and one in vivo study in fish (Wang et al. 2016), and the results show that TPHP can be rapidly metabolized through phase I and phase II biotransformation to a number of metabolites, which are more hydrophilic and more readily eliminated than the parent compound. Diphenyl phosphate (DPHP) can be a major metabolite of TPHP, as shown in some studies across a wide variety of species, including zebrafish (*Danio rerio*; Wang et al. 2016) and polar bear (*Ursus maritimus*; Strobel et al. 2018), and has been detected in the addled eggs of Great Lakes bald eagles (*Haliaeetus leucocephalu*; geometric mean 1.0 ± 0.023 ng/g wet weight (Stubbings et al. 2018). Yet, to date, the metabolism of TPHP and formation of its major metabolite, DPHP, have not been investigated in vivo or in vitro in birds.

Understanding the toxicokinetics and metabolism of TPHP in vivo in birds is a critical first step in furthering our understanding of the exposure and fate of this emerging environmental contaminant and subsequently will be useful in the assessment of risks from exposure to this OPE for wildlife. The main objective of the present research was thus to determine the uptake and metabolism of TPHP by Japanese quail embryos and chicks as well as the formation of a major metabolite, DPHP. Other TPHP metabolites (e.g., OH-TPHPs) are known to form in other nonavian species but are beyond the scope of the present study. This is the first study to examine the toxicokinetics of known TPHP exposure concentrations in vivo in any avian model.

MATERIALS AND METHODS

Dosing solution and protocol

Dichloromethane (DCM), TPHP (>99% purity; Chemical Abstracts Service no. 115-86-6), methanol (MeOH), and sodium sulfate (Na_2SO_4) were purchased from Sigma-Aldrich. Both d_{15}-TPHP and d_{10}-DPHP were used as internal standards and purchased from Dr. Belov (Max Planck Institute for Biophysical Chemistry) and Wellington Laboratories. Sodium sulfate was treated in a box furnace at 600 °C and glassware at 450 °C for ≥8 h prior to their use to eliminate any organic contamination (except volumetric flasks). Otherwise, other solvents and reagents were HPLC grade. Working solutions of TPHP at 2 concentrations were prepared and used for either egg injection or oral dosing of chicks. For embryonated eggs, injection concentrations were 12.5, 67.0, and 145.9 μg TPHP/mL safflower oil and, for chicks, 19.4, 155.9, and 345.5 μg TPHP/mL safflower oil for low, medium, and high doses, respectively, in each case (Supplemental Data, Table S1; Guigueno et al. 2019). The low-dose category was designed to reflect environmentally relevant concentrations of TPHP that have been determined in field samples of eggs of wild birds (Greaves and Letcher 2014), whereas medium- and high-dose solutions represented a 10- and 20-fold increase above that, respectively (Guigueno et al. 2019). The control safflower oil solution contained background concentrations measured at 0.01 μg/mL.

The following egg injection and incubation methods have been well described elsewhere (Guigueno et al. 2019). Briefly, fertilized, unincubated Japanese quail eggs were purchased regionally (Ferme Patrick Brodeur) and housed at McGill University. To minimize embryonic development prior to chemical injection, eggs were stored at 15 to 18 °C (maximum 4 d). For egg injections, the air cell was located and delineated with a pencil and a small hole drilled in the middle using a rotary tool. The 10-μL dose was injected into the air cell using a repeater pipette, the hole was covered (AirPore Tape Sheets; Qiagen), and the egg was left upright for 45 min for absorption. Eggs were thus dosed on embryonic day 0, and subsequently artificial incubation was initiated. Eggs were placed on their sides in a commercial poultry incubator (Ova-Easy Advanced Series II cabinet incubators type 190; Brinsea Products) and incubated using an appropriate regime, as described (Guigueno et al. 2019). In total, 76 eggs were incubated in 4 batches with an equal number of eggs from the control and 3 doses in each batch.

For chemical analysis of eggs (see next section *Determination of TPHP and DPHP*), 12 eggs were removed and assessed 2 d later (4/group) prior to the commencement of liver development of the embryo (Ainsworth et al. 2010) and again 10 d later, following organogenesis. These sampling time points were selected to include differences in TPHP metabolism before the liver, the main metabolic organ, was present and after it began to develop. Hatching success was not affected by exposure to TPHP in the present study, and deformity rates were not elevated compared to controls (Guigueno et al. 2019). Following the complete 16 to 18 d of total incubation, the remaining 52 eggs were hatched and used for chemical analysis of chick carcasses with 12 to 14 individuals per group. These individuals were dosed in ovo and by diet after hatching to mimic the exposure regime that would be experienced by wild chicks based on concentrations detected in eggs of wild birds (Greaves and Letcher 2014). These are also the same chicks that experienced TPHP-related changes in thyroid function, resting metabolic rate, and growth (Guigueno et al. 2019). The eggs designated for hatching were monitored for pipping and placed in individual nest cells. When the chicks hatched, they were weighed, banded, and, when dry, placed in a commercial brooder separated by dose (model 0540; GQF Manufacturing). The temperature of the brooder was set to 34 °C, and the chicks

were fed quail starter feed ad libitum. Oral dosing of the birds occurred daily for 5 d immediately after the chicks were weighed, to mimic an exposure regime similar to that experienced by wild birds and to ensure sufficient exposure to elicit effects in relation to other objectives within the overall project (Guigueno et al. 2019). Chicks were dosed with a volume scaled to their body mass (1.5–10 µL) to obtain consistent concentrations of 4.5, 36.4, or 80.6 ng TPHP/g chick for low-, medium-, and high-dose chicks, respectively. Dosing solution was pipetted into the mouth, and chicks were held until the dose was swallowed. At age day 6, chicks were weighed and their tibiotarsus measured (digital calipers to the nearest mm); then, they were euthanized, and liver and brain masses were determined. Euthanasia was thus done at 26 to 35 h after the final oral dose, the timing of which was evenly distributed among doses. All methods were approved by McGill University's Animal Care Committee (protocol 2016-7817) and followed the guidelines set by the Canadian Council on Animal Care.

Determination of TPHP and DPHP

When eggs were collected, they were placed in chemically cleaned jars. Following euthanasia of the 6-d-old chicks and organ extraction to examine the physiological effects of TPHP (i.e., brain, liver, thyroid glands; Guigueno et al. 2019), the remaining chick carcasses were wrapped in chemically cleaned aluminum foil for chemical analysis to determine if TPHP and/or DPHP were accumulated during development. Both eggs and chick carcasses were stored frozen until chemical analysis.

Embryonated eggs and chick carcasses were assessed for concentrations of TPHP and its known metabolite, DPHP, following the methods detailed elsewhere (Guigueno et al. 2019). All egg contents or carcass samples were homogenized, and approximately 1 g was weighed and spiked with 20 µL of the internal standard mixture (50 ng/mL each d_{15}-TPHP and d_{10}-DPHP in MeOH), followed by 2.5 g of Na_2SO_4 and 4 mL of DCM 1 h later. This mixture was vortexed for 1 min and then placed in an ultrasonication bath for 10 min of extraction at room temperature. Following extraction, the mixture was centrifuged at 3315 *g* (4000 rpm) for 10 min. This extraction procedure was repeated 2 more times. The 3 extracts were combined and concentrated, and the solvent was exchanged with 1 mL of MeOH. High-dose samples weighed approximately 0.2 g and so were spiked with 40 µL of the internal standard solution for a final solution volume of 2 mL MeOH. A freeze-lipid separation method was used for bulk lipid removal from sample extracts. The sample tube containing the MeOH solution was sealed and frozen (–20 °C) overnight, after which it underwent 5 min of centrifugation (466 *g* [1500 rpm], –5 °C); 100 µL of the supernatant was placed into a total recovery HPLC vial for analysis.

For analysis of the safflower oil dosing solutions, they were diluted with ethyl acetate (1 v:100 v), followed by MeOH (1 v:100 v). Then, 100 µL of this mixture was combined with 100 µL of the internal standard mixture and directly analyzed. Analysis of both TPHP and DPHP was carried out using a Xevos TQ-S ultra-high performance (Acquity) liquid chromatograph–mass spectrometer system (UHPLC-MS/MS; Waters). The UHPLC system operated with a Kinetex® EVO C18 analytical column (Phenomenex; 50 mm L × 2.1 mm i.d. × 1.7 µm particle size) at a temperature of 50 °C for the column and 25 °C for the 10-µL sample. The UHPLC mobile phase and gradient were made up of (A) 2 mM ammonium acetate in methanol and (B) 2 mM ammonium acetate in water.

The target compounds were determined using electrospray ionization in the positive (TPHP) or negative (DPHP) ion mode. The quantitative multiple reaction monitoring (MRM) (+) ion transitions were *m/z* 342.2 > 160.2 and *m/z* 327.0 > 152.0 for d_{15}-TPHP and TPHP, respectively. The confirmation MRM (+) ion transitions were *m/z* 342.2 > 82.2 and *m/z* 327.0 > 77.0 for d_{15}-TPHP and TPHP, respectively. The quantitative MRM (–) ion transitions were *m/z* 259.1 > 98.2 and *m/z* 249.0 > 93.2 for d_{10}-DPHP and DPHP, respectively. The confirmation MRM (–) ion transitions were *m/z* 259.1 > 159.1 and *m/z* 249.0 > 155.1 for d_{10}-DPHP and DPHP, respectively.

Quality control and assurance of TPHP and DPHP determination

For quality control during TPHP and DPHP analysis, a series of 6 standards (dissolved in MeOH) with graded concentrations of the target compounds (0–10 ng/mL) and one constant concentration standard (1 ng/mL) were prepared and analyzed by UHPLC-MS/MS. From these readings, calibration curves were generated which were linear and had a correlation coefficient >0.99. Mean percentage of recovery efficiencies were very high at 98 ± 6 and 94 ± 8% for d_{15}-TPHP and d_{10}-DPHP, respectively, and for spiked egg and carcass homogenates.

For each batch of 10 samples, one blank was prepared and analyzed. In these blanks, the average TPHP concentration was 0.05 ± 0.03 ng/g and the average DPHP concentration was 0.06 ± 0.04 ng/g; thus, all samples were blank-corrected by batch. The method limit of quantification (MLOQ) and method limit of detection (MLOD) were 0.005 and 0.003 ng/g wet weight for TPHP and 0.012 and 0.007 ng/g wet weight for DPHP, respectively. The MLODs and MLOQs were based on a minimum signal-to-noise ratio of 3 and 10, respectively.

Data treatment and statistical analyses

For eggs, the mass of the initial TPHP in the injections was determined and the mass of TPHP or DPHP measured in the eggs calculated (egg sample mass × egg concentration) and used to determine the measures outlined herein for each dose. First, the half-life was calculated for TPHP as follows: $N(t) = N_0(1/2)^{t/t_{1/2}}$, where $N(t)$ is the mass of TPHP remaining in the egg, N_0 is the initial injected TPHP, t is time, and $t_{1/2}$ is the half-life. Because only 2 measurements were possible, the half-lives are considered to be estimates. Second, the rate of depletion of TPHP per day was determined in the first 2 d and subsequent 8 d (estimated using means from embryonated eggs calculated at 2 and 10 s after injection). Third, we determined the proportion of the initial TPHP dose that 1) remained as TPHP, 2) was converted to DPHP, or 3) was unaccounted for by the mass of either of these compounds

(i.e., proportion formed into other metabolites which were not measured) 2 and 10 d after injection. Finally, we calculated the proportion of metabolized TPHP that was converted to DPHP.

Concentrations of TPHP or DPHP were used for statistical analysis and compared between the 4 treatment groups. For egg concentrations from each measurement time point, Kruskal-Wallis tests were conducted separately; further pairwise comparisons were not conducted because of the small sample size of successfully analyzed eggs ($n = 3$–4/group). Differences in the concentrations of TPHP or DPHP in carcass samples between the 4 groups ($n = 12$–14/group) were compared using general linear models (GLMs) with least significant difference (LSD) post hoc tests and included the number of hours since the last dose as a covariate only when significant (results for TPHP only have been previously but more briefly reported [Guigueno et al. 2019]). Data were log-transformed to achieve normality. The relationship between DPHP and TPHP concentrations was tested with generalized linear mixed models (GLMMs) with data categorized by treatment group. To determine if the sex, body condition index (body mass/tarsus length), and liver or brain mass of the chicks were related to carcass concentrations of TPHP or DPHP at 6 d after hatching, a GLMM for each chemical was conducted, again with data categorized by treatment group. Body mass alone was not examined because daily oral doses of chicks were scaled daily to this measure. All statistics were conducted using IBM SPSS© 23 with a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

Uptake, metabolism, and depletion of TPHP in embryonated quail eggs

Egg-injection studies are particularly useful for characterizing the metabolism of contaminants in vivo because known quantities of contaminants are injected into the contained egg system. In all of the injected quail egg samples TPHP was measurable (Supplemental Data, Table S2) with concentrations that increased with dose at 2 d ($\chi^2 = 14.12$, $p = 0.003$) and 10 d ($\chi^2 = 12.04$, $p = 0.007$) after injection, as we have briefly reported elsewhere (Guigueno et al. 2019). Also, DPHP was measurable in all eggs (Supplemental Data, Table S2) and at concentrations that increased with the TPHP dose level (2 d, $\chi^2 = 14.14$, $p = 0.003$; 10 d, $\chi^2 = 13.52$, $p = 0.004$; Figure 1), confirming that TPHP is biotransformed and into the DPHP metabolite in the embryonated quail eggs. Both TPHP and DPHP were determined at low concentrations in control eggs (Supplemental Data, Table S2); the mass of TPHP in control eggs, representing background levels for commercial birds, exceeded the amount injected with the safflower oil vehicle (Table 1).

The results of the present study point to rapid metabolic depletion of TPHP in embryonated Japanese quail eggs at similar rates for the 3 dose levels. At 2 d after egg injection, with an average depletion rate of 29 to 35% per day (Supplemental Data, Table S3), only 33% of the initial dose remained in the eggs (Supplemental Data, Figure S1; Figure 2 and Table 1), suggesting an estimated TPHP half-life of <2 d. After another 8 d, TPHP was almost fully depleted from eggs (at a rate of 12% per day between 2 and 10 d) and was measured at concentrations (Supplemental Data, Figure S1 and Table S3; Figure 2 and Table 1) that accounted for only 2% of the initial injection mass of TPHP (Figure 1; Supplemental Data, Table S2). These findings from the quail embryos are consistent with the findings from other TPHP depletion studies that employed in vitro assays based on the liver microsomes of herring gulls, which demonstrated rapid depletion of TPHP at a rate of 22 ± 2 pmol/min/mg protein (Greaves et al. 2016b). The calculated estimates for TPHP half-lives in the present quail eggs ranged from 1.1 to 1.8 d with comparable values determined over the first 2 or 10 d after injection (Supplemental Data, Table S3), which are consistent with previous reports in vivo in fish (<1 d [Wang et al. 2016] and 1.25 d [Muir et al. 1983]).

A significant proportion of the TPHP injected into the embryonated quail eggs was metabolized by dealkylation to

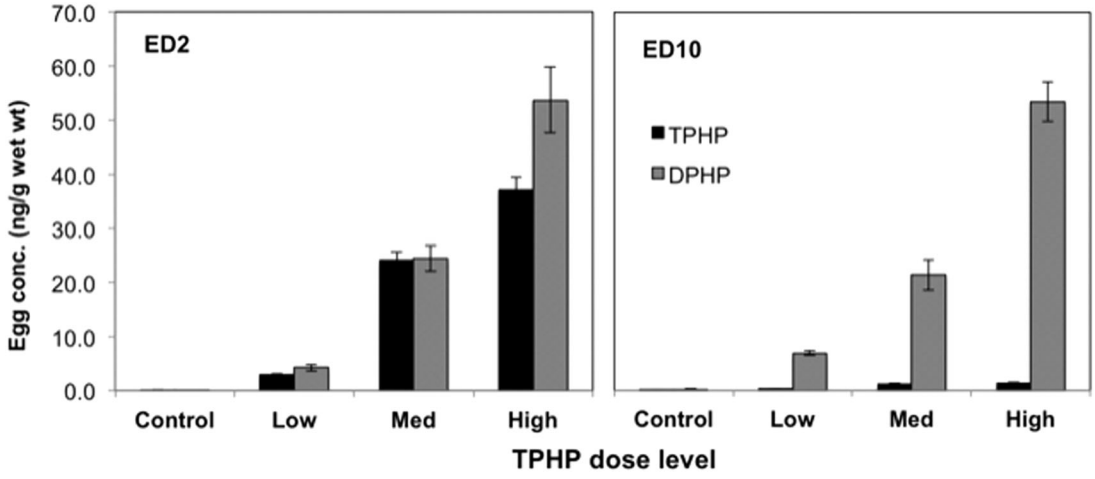

FIGURE 1: Triphenyl phosphate and diphenyl phosphate metabolite concentrations in Japanese quail eggs on embryonic day (ED) 2 and ED10. Dosed groups were compared with controls that were similarly dosed with the vehicle only. Dosed groups significantly differed from controls for both contaminants at ED2 and ED10 (Kruskal-Wallis: low, $\chi^2 = 12.04$, $p = 0.003$; high, $\chi^2 = 14.14$, $p = 0.007$). DPHP = diphenyl phosphate; TPHP = triphenyl phosphate.

TABLE 1: Mass of parent triphenyl phosphate (TPHP) and diphenyl phosphate (DPHP) metabolite in eggs of Japanese quail (*Cortunix cortunix japonica*) following a single egg injection at embryonic day 0[a]

ED		Mass TPHP injected (ng)	TPHP	DPHP	Total TPHP + DPHP	Other metabolites
2	Vehicle	0.1 mass%	0.4 ± 0.2	0.1 ± 0.2	0.5 ± 0.2	na
			na	na	na	na
	Low	125 mass%	35.1 ± 7.1	52.4 ± 15.2	87.5 ± 22.0	37.5 ± 22.0
			28 ± 5	42 ± 12	70 ± 18	30 ± 18
	Medium	689.5 mass%	289.0 ± 44.4	295.9 ± 77.2	584.9 ± 120.9	104.6 ± 120.9
			42 ± 6	43 ± 11	85 ± 18	15 ± 18
	High	1458.6 mass%	421.1 ± 51.7	604.9 ± 103.5	1026.1 ± 132.4	432.58 ± 132.44
			29 ± 4	42 ± 7	70 ± 9	30 ± 9
10	Vehicle	0.1 mass%	0.5 ± 0.3	1.6 ± 3.3	2.2 ± 3.6	na
			na	na	na	na
	Low	125 mass%	2.8 ± 2.0	71.0 ± 10.6	82.8 ± 10.7	42.2 ± 10.7
			2 ± 2	64 ± 9	66 ± 9	34 ± 9
	Medium	689.5 mass%	13.3 ± 6.2	260.9 ± 55.7	274.2 ± 51.0	415.3 ± 51.0
			2 ± 1	38 ± 8	40 ± 8	60 ± 7
	High	1458.6 mass%	14.8 ± 5.5	652.9 ± 49.8	667.7 ± 53.0	790.9 ± 53.0
			1 ± 0.4	45 ± 3	46 ± 4	54 ± 4

[a]Percentages of the initial dose of TPHP or DPHP remaining in the egg were calculated as well as the percentage of the initial dose that was metabolized into other compounds.
ED = embryonic day; na = not applicable.

DPHP. At 2 d after injection, concentrations of DPHP in the eggs had reached those of the remaining TPHP in low- and medium-dosed eggs and had increased above TPHP concentrations in high-dose eggs (Figure 1; Supplemental Data, Table S2). Another 8 d later, egg profiles were highly dominated by DPHP relative to the largely depleted TPHP (Figure 1; Supplemental Data, Table S2). On average, 42% of the TPHP injected into the embryonated eggs was metabolized to DPHP after 2 d and 50% by 10 d, which made up most of the remaining burden of TPHP and DPHP. This finding is in agreement with a previous report in TPHP-exposed zebrafish, which demonstrated 3 to 3.5 times higher concentrations of DPHP relative to TPHP in liver and intestines (Wang et al. 2016). That DPHP concentrations remained elevated in the quail eggs suggested that DPHP itself was depleted more slowly than TPHP. Nevertheless, relative to more persistent contaminants, DPHP still appeared to be cleared quickly, having been largely depleted within 3 d of depuration in TPHP-exposed zebrafish (Wang et al. 2016).

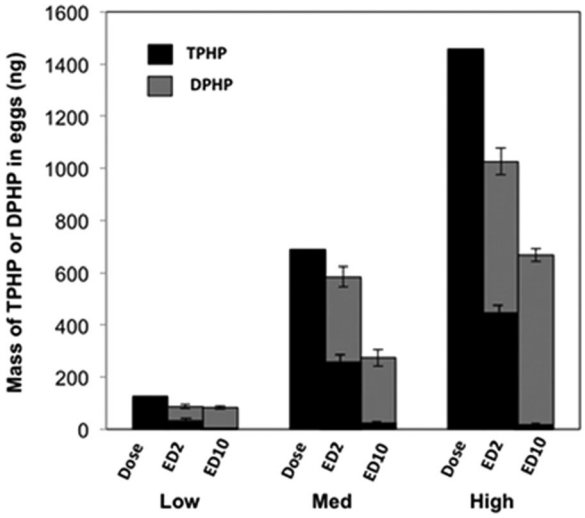

FIGURE 2: Mass of triphenyl phosphate (TPHP) injected into embryonated eggs compared to TPHP and the metabolite diphenyl phosphate in Japanese quail eggs at embryonic day (ED) 2 and ED10. DPHP = diphenyl phosphate.

By using the egg, which would have contained all of the initial dose, we were also able to determine what proportion of this TPHP was transformed into uncharacterized metabolites other than DPHP. At 2 d after injection, 25% of the initial TPHP dose was metabolized into other by-products; and 10 d after injection, 48% of the initial dose had been converted to metabolites other than DPHP. These basic trends were similar across the dose groups but with some small variations in the proportions (Supplemental Data, Figure S1; Figure 2 and Table 1). This result is consistent with a number of recent studies that have identified several TPHP metabolites. In the only other known in vivo study, TPHP metabolites from phase I (mono- or dihydroxylated TPHP, monohydroxylated DPHP) and phase II biotransformation (TPHP- and DPHP-glucuronide conjugates) were identified in TPHP-dosed zebrafish (Wang et al. 2016). Also, in chicken embryonic hepatocytes after 36 h or incubation with one dose of TPHP, only 0.2% of the initial dose remained as TPHP, approximately 17% was metabolized in vitro into DPHP (Su et al. 2014a, 2015). A large proportion (60%) was metabolized to *para*-OH-TPHP and a minor proportion into *meta*-OH-TPHP, but both were entirely present as phase II glucuronide conjugates and measured only in the cell incubation media (Su et al. 2015). This demonstrated that in these cells the main metabolic pathway was phase I *para*-hydroxylation followed by phase conjugation.

Toxicokinetic studies have shown some variation in the TPHP metabolites that are metabolically formed. The high DPHP concentrations in the present dosed quail eggs alone confirm that DPHP is an important metabolite of TPHP. However, in addition, at 2 d after injection, DPHP mass in embryonated eggs made up 58, 74, and 41% of all metabolites

formed in low-, medium-, and high-dose eggs, respectively; and after 10 d, DPHP accounted for 58, 39, and 45%, respectively. This demonstrated that DPHP was the dominant metabolite in the quail, making up more than half of the burden under some conditions. These results are consistent with the findings in zebrafish dosing studies with TPHP (Wang et al. 2016) but are in contrast with the majority of in vitro studies where DPHP was not the major metabolite of TPHP including in human liver fractions (22% of all metabolites [van den Eede et al. 2013]), chicken hepatocytes (*Gallus domesticus*, 17% of initial dose [Su et al. 2015]), and liver microsomal assays for herring gull (Greaves et al. 2016b) and ringed seals (*Pusa hispida*; Strobel et al. 2018). Further research is warranted to determine if metabolism differs significantly between whole animals and isolated liver cells, but the fact that DPHP was the dominant metabolite in vitro in polar bear liver microsomal assays (Strobel et al. 2018) suggests that interspecies differences may also be a factor.

Mechanisms of TPHP metabolism in the embryonated quail egg

Several studies have demonstrated that TPHP can be metabolized into a number of metabolites by phase I (cytochrome P450 [CYP]) and phase II liver enzymes (sulfotransferases, glutathione transferases, and glucuronyl transferases) in whole fish (Wang et al. 2016) and bird and human hepatocyte assays (van den Eede et al. 2013; Su et al. 2014a, 2015). However, by isolating the period prior to liver development and the onset of a functional enzymatic capacity of the quail embryos (2 d), our results demonstrated that TPHP was metabolized to DPHP and other products in the absence of developed capacity for hepatic metabolism and that the majority of the TPHP was depleted (~70%) under these conditions. This suggested that other breakdown pathways occurred in the early embryonated quail eggs. The first possibility is that enzyme-mediated metabolism occurred in the extrahepatic embryonic tissue. After 23 to 26 h, quail embryos have a visible notochord and head fold and possibly one somite (structural precursor) between 23 and 26 h (Ainsworth et al. 2010). The central nervous system can contain CYP enzymes, as confirmed in mammals (Strobel et al. 2001); and TPHP can accumulate in the brain of fish (Wang et al. 2016), which suggests potential metabolism of TPHP by neural tissue (Gram et al. 1986) in the present early quail embryos. Adding some support for this hypothesis, and identifying a possible neurological effect of this OPE, was the significant and negative relationship between the brain mass of the present chicks and their carcass concentrations of TPHP ($t_{1,48} = -2.83$, $B = -0.06$, $p = 0.007$). The second possibility is that nonembryonic elements of the egg may have been responsible for enzymatic breakdown of TPHP in the first 2 d following injection. Both the yolk sac and chorioallantoic membrane (the latter analogous to the placenta in mammals) are known to have CYP-enzyme activity and can act as a first defense against contaminant exposure (Annas et al. 1999 and references therein). The chorioallantoic membrane only forms 4 to 5 d after incubation commences, suggesting that either its precursor mesodermal layers (Annas et al. 1999) or the yolk sac membrane may have contributed to TPHP metabolism in the early quail eggs (as well as thereafter). Su et al. (2016) showed that base catalyzed hydrolysis occurs to a minor degree in aqueous solutions at pH of 8 to 9, which is in the range of the present quail eggs (pH 7.9–9.2 [Romanoff and Romanoff 1929]). Further, the base catalyzed hydrolysis half-life of TPHP was shown to be very long at 112 d (Su et al. 2016), and this process has not been shown to contribute greatly to TPHP breakdown in vitro in liver microsomal systems (van den Eede et al. 2013; Su et al. 2014a). Therefore, base catalyzed hydrolysis is an unlikely mechanism of TPHP breakdown in the present study. Further research is warranted to characterize the metabolites and pathways of enzymatic breakdown and depletion of TPHP in avian eggs at various stages of embryonic development.

Chick carcass concentrations of TPHP and DPHP

We detected TPHP in all chick carcasses from the dosed groups, which demonstrated that this OPE accumulated in chicks when exposed in ovo and posthatch (and excluding liver and brain concentrations; Guigueno et al. 2019). Because the majority of TPHP was metabolized even 10 d after injection in embryonated eggs (see section *Uptake, metabolism, and depletion of TPHP in embryonated quail eggs*), it is likely that TPHP concentrations measured in the chick carcasses reflected uptake from the oral doses rather than residual exposure from egg injections. Chick carcass concentrations of TPHP were generally 1 to 3 orders of magnitude lower than eggs (Supplemental Data, Table S2), increased with dose (Figure 3), and significantly differed between the dose levels (as we previously reported [Guigueno et al. 2019];

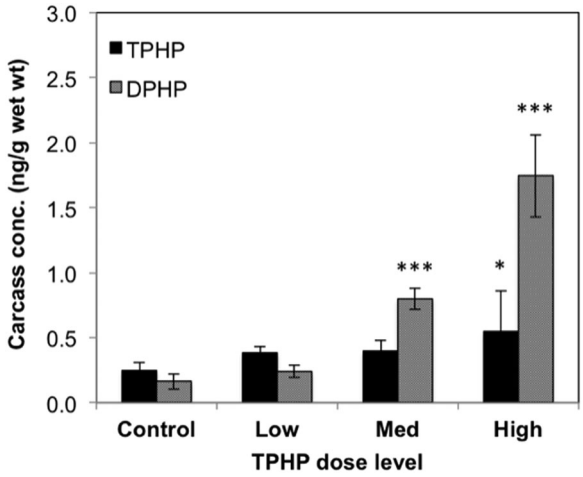

FIGURE 3: Concentrations of triphenyl phosphate (TPHP) and the metabolite diphenyl phosphate (DPHP) in carcass homogenates of chick Japanese quail exposed at low, medium, and high TPHP doses by egg injection, followed by oral dosing of chicks (days 1–5) with controls exposed to vehicle only ($n = 12$/group). Dosed groups were compared with controls (general linear models with least significant differences: main effects TPHP, treatment main effect, $F_{3,46} = 2.9$, $p = 0.044$; DPHP $F_{3,46} = 22.9$, $p < 0.001$).

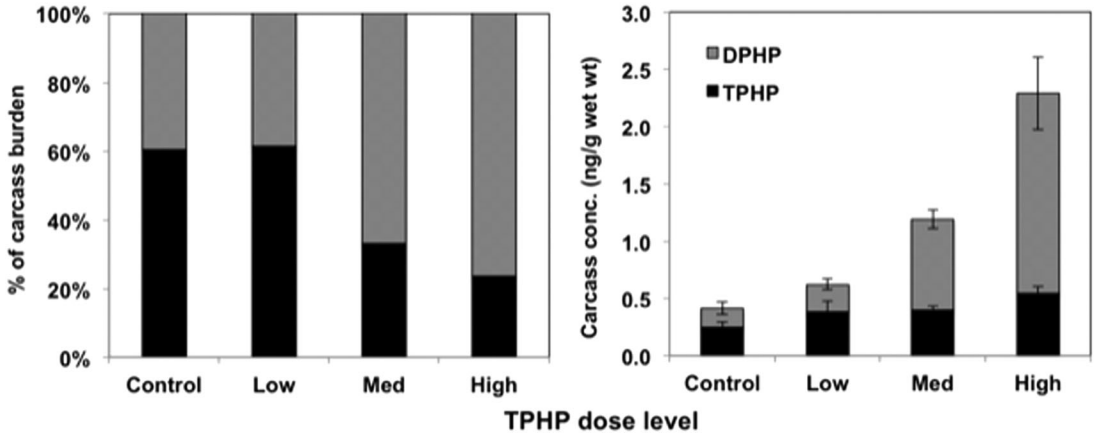

FIGURE 4: Percentage of measured burden (left) and concentrations (right) of triphenyl phosphate (TPHP) and its metabolite diphenyl phosphate (DPHP) in Japanese quail carcasses exposed at low, medium, and high TPHP doses via egg injection followed by oral dosing of chicks (days 1–5) with a control set exposed to vehicle only ($n = 12$/group).

GLM, treatment main effect, $F_{3,46} = 2.9$, $p = 0.044$; although in pairwise comparisons only high-TPHP chicks differed from controls, LSD, $p = 0.005$). The covariate of number of hours between the last dose and euthanasia was not significant, suggesting that the majority of the last TPHP oral dose was metabolized in the 24 h thereafter.

The concentrations of DPHP measured in the carcasses of quail chicks supported the more detailed conclusions made from the analyses of embryonated eggs of the present study. First, DPHP was detected in all of the quail carcasses from dosed groups (at concentrations 1–3 orders of magnitude lower than egg concentrations), which strongly suggested that TPHP was also metabolized to DPHP in the whole chicks. Although we cannot separate newly metabolically formed DPHP from the chick and from what remained from the egg injection, this conclusion is supported by the fact that carcass concentrations of TPHP and DPHP were highly significant and positively associated (GLMM, $F_{1,49} = 19.39$, $p < 0.001$) and suggested transformation of the oral dose of TPHP to DPHP. In addition, liver mass was negatively related to both TPHP ($F_{1,48} = 4.95$, $p = 0.031$) and DPHP ($F_{1,48} = 6.71$, $p = 0.013$) concentrations in the carcasses, which was further suggestive of a link with liver metabolism. Carcass DPHP concentrations differed between the 4 treatment groups (GLM, treatment main effect $F_{3,46} = 22.9$, $p < 0.001$; Supplemental Data, Table S2; Figure 3), where medium and high, but not low, TPHP-dosed chicks had significantly higher carcass concentrations compared to controls (LSD, $p = 0.001$ and $p < 0.001$, respectively). Carcass concentrations of DPHP increased incrementally and significantly with each dose (LSD, $p \leq 0.003$; Figure 3) and were unrelated to the number of hours since the last oral dose, which suggested that DPHP levels remained constant while repeated TPHP exposure was ongoing.

Similar to the embryonated quail eggs, carcass concentrations confirmed that metabolism of TPHP to DPHP also occurred in the chicks. At the low TPHP dose level, carcass TPHP and DPHP concentrations were similar; but at the medium-dose and high-dose exposure levels, carcass concentrations of DPHP exceeded those of TPHP (medium, $U_{14,28} = 40.0$, $p = 0.007$; high, $U_{12,24} = 4.0$, $p < 0.001$, respectively; Figures 3 and 4). In these medium- and high-dose eggs, DPHP constituted on average 67 and 76% of the total burden, respectively, after only 6 d of oral exposure and one in ovo injection on embryonic day 0 and illustrated the rapid formation of DPHP in the quail chicks as well as in the eggs. However, whereas transformation rates of TPHP to DPHP were consistent for all dose categories in the eggs, these results suggest that the rates of TPHP metabolism may have increased with dose in the chicks.

Environmental implications for birds and other wildlife

In the present study, rapid metabolism of TPHP occurred in Japanese quail eggs, and concentrations of TPHP and its metabolites may vary greatly with the age of the embryonated egg and the stage of embryo development. Hence, for wild birds, maternal exposure and deposition of TPHP may be difficult to elucidate from collected eggs, where measured concentrations may mainly indicate presence in the environment. In addition, the rapid depletion of TPHP and DPHP metabolite formation in the quail is consistent with the results of other avian field studies demonstrating that TPHP concentrations are generally low or undetectable in egg and tissue samples from wild birds in the Great Lakes (Greaves and Letcher 2014; Greaves et al. 2016a; Lu et al. 2017; Stubbings et al. 2018) and in Arctic mammals (Strobel et al. 2018). The present results suggest that DPHP may be a useful indicator of TPHP exposure in the short term, but if DPHP is rapidly depleted, it may not be indicative of TPHP exposure over longer periods. This is supported by the findings that DPHP was determined at much lower concentrations than TPHP in addled eggs of Great Lakes bald eagles (*Haliaeetus leucocephalus*; with unknown embryonic staging [Stubbings et al. 2018]) and was undetectable in Great Lakes herring gull tissue and egg samples (Su et al. 2014b). In addition, DPHP is

not a metabolite specific to TPHP alone and can form from 2-ethylhexyl diphenyl phosphate and resorcinol bis(diphenylphosphate) (Dodson et al. 2014). It is unlikely that OH-TPHP metabolites would be measurable and useful as bioindicators of TPHP exposure. In vitro studies with chicken embryonic hepatocytes showed that >60% of the TPHP dose resulted in *meta*- and *para*-mono-OH-TPHP metabolites and that essentially all were phase II conjugated to glucuronides (Su et al. 2014a, 2015). Recently, using a Wistar-Han rat–based, hepatic microsomal assay, Chu and Letcher (2019) examined TPHP in vitro metabolism in the presence of glutathione (GSH). Metabolism was rapid, and phase I epoxidation of TPHP led to mono- and di-OH-TPHP metabolites, which were then rapidly phase II–conjugated with GSH, forming 7 different GSH S-conjugate metabolites. More research is also warranted on the toxicity of TPHP and other OPEs and their metabolites in exposed birds because we presently report that TPHP and DPHP accumulate in Japanese quail following oral exposure and that at even the earliest stages of development, embryos are exposed to a mixture of TPHP and DPHP and potentially other uncharacterized metabolites.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4637.

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Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (robert.letcher@canada.ca, kim.fernie@canada.ca).

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