Early life exposure to triphenyl phosphate: Effects on thyroid function, growth, and resting metabolic rate of Japanese quail (Coturnix japonica) chicks

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Abstract
Triphenyl phosphate (TPHP; CAS # 115-86-6), a commonly used plasticizer and flame retardant, has been reported in wild birds and identified as a potential high-risk chemical. We exposed Japanese quail (Coturnix japonica) by in ovo injection, and once hatched, orally each day for 5 days to safflower oil (controls) or TPHP dissolved in vehicle at low (5 ng TPHP/g), mid (50 ng TPHP/g), or high (100 ng TPHP/g) nominal TPHP doses. The low TPHP dose reflected concentrations in wild bird eggs, with mid and high doses 10x and 20x greater to reflect potential increases in environmental TPHP concentrations in the future. Despite no effects on mRNA expression in thyroid-related genes, TPHP exposure enhanced thyroid gland structure in high TPHP males, but in females, suppressed thyroid gland structure and activity (all TPHP females), and circulating free triiodothyronine (high TPHP females only). Consistent with thyroidal changes, and compared to controls, mid and high TPHP chicks experienced significantly reduced resting metabolic rate (13%) and growth (53%); mid TPHP males and high TPHP females were significantly smaller. The observed thyroidal effects and suppressed growth and metabolic rate of the quail chicks suggest that TPHP may adversely affect the health of wild birds.

1. Introduction
The global regulation of flame retardants (FRs) such as polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCDD) has led to the increased production and use of replacement FRs (e.g., organophosphate esters (OPEs)). In recent years, OPEs have been detected in biota including the tissues and eggs of aquatic-based birds (Eulaers et al., 2014; Greaves and Letcher, 2014; Su et al., 2015a; Fernie and Letcher, 2018; Verreault et al., 2018) and terrestrial birds (Fernie et al., 2017). Little is known about the toxicological properties of many replacement OPEs in birds, although some were shown to cause molecular effects in key metabolic pathways in vitro (Crump et al., 2012) and altered thyroid function in vivo (Fernie et al., 2015).

Triphenyl phosphate (TPHP) is an OPE most commonly used as a plasticizer and a flame retardant, and has been identified as a potentially high-risk chemical in Canada by the federal Chemicals Management Plan (Environment Canada, 2016). Production and use of TPHP in the United States amounted to 4,500 to 22,700 tonnes per year in the late 1990s; since 2005, its use has likely increased due to the phasing out of PBDEs (Van der Veen and de Boer, 2012). TPHP can enter the environment through household and industrial
activities (Carlsson et al., 2000; Van der Veen and de Boer, 2012). Because TPHP is an additive FR, it can easily be released into air, soil, and wastewater (Hosseini et al., 2011; Van der Veen and de Boer, 2012; Salamova et al., 2014). TPHP concentrations were measured in atmospheric particles in the Arctic (1.1–52 pg/m³) (Salamova et al., 2014), and may be at even higher concentrations near point sources (e.g., waste water treatment plants, landfills).

High detection rates of TPHP and/or its metabolite, diphenyl phosphate (DPPP) have been reported in wild birds, including glaucous gulls (Larus hyperboreus) (71% females, 59% males) (Verreault et al., 2018) and cinereous vultures (Aegypius monachus) (>50%) (Monclús et al., 2019), with concentrations measured in multiple species in North America, Europe, the Arctic and China, e.g., European starlings (Sturnus vulgaris), congeneric gull species (Greeves and Letcher, 2014, 2016a; Eulaers et al., 2014; Hallanger et al., 2015; Lu et al., 2017; Verreault et al., 2018), white-tailed eagles (Haliaeetus albicilla) (Eulaers et al., 2014), and cinereous vultures (Monclús et al., 2019). Concentrations of TPHP have been reported in eggs (≤MLQ, — 20 ng/g ww), maternal liver (26–165 ng/g lw) and other tissues (e.g., blood, fat: < 0.2–5.5 ng/g ww) (Eulaers et al., 2014 and references therein), and feathers (5.9–250 ng/g dw; Eulaers et al., 2014; 10.3 ± 2.3 ng/g dw, Monclús et al., 2019) for wild birds as well as domestic birds (0.06–21.0 ng/g dw) of domestic chickens (Gallus domesticus) and ducks (Anas platyrhynchos domesticus) (Eulaers et al., 2014 and references therein). These reported TPHP concentrations may underrepresent the actual TPHP concentrations that birds are exposed to in their environment given how rapidly birds appear to metabolize TPHP. In avian hepatocytes (chicken, herring gull (Larus argentatus)), TPHP is known to be dealkylated or hydrolyzed to DPHP but also forms hydroxylated metabolites (Su et al., 2014, 2015b; Greaves et al., 2016b).

Although few studies have examined the toxic effects of TPHP on biota, the available literature suggests that TPHP exposure can lead to metabolic and endocrine disruption in fish (Liu et al., 2013a, 2013b; Kim et al., 2015) and humans (Meeker and Stapleton, 2010). Zebrafish (Danio rerio) exposed to an environmentally relevant concentration of TPHP for 7 days experienced disruption in hepatic carbohydrate and lipid metabolism (Du et al., 2016). In addition to disrupting other hormonal axes, TPHP altered gene expression in zebrafish related to the hypothalamic–pituitary–thyroid axis, an important axis for growth and development (Liu et al., 2013a, 2013b; Kim et al., 2015). Increased concentrations of TPHP in house dust was associated with an increase in prolactin in adult men (Meeker and Stapleton, 2010). As such, TPHP is a potential endocrine and metabolic disruptor in fish and mammals, but to our knowledge, no information is available on its potential effects on birds.

Thyroid function, metabolism, and growth are important to the health and survival of birds, and have been adversely impacted by various FRs (Guigueno and Fernie, 2017). Thyroid function regulates cellular development, thermoregulation, metabolism, growth, and reproduction. Metabolic rate can affect survival through energy acquisition and expenditure (Drent and Daan, 1980; Hammond and Diamond, 1997). Growth rate is a strong predictor of post-cellular development, thermoregulation, metabolism, growth, and survival in birds (Maness and Anderson, 2013). In the current study, we examined the effects of TPHP on hatching parameters, the incidence of deformities, thyroid function, growth and metabolic rate within the same individual Japanese quail (Coturnix japonica) that were exposed in ovo and post-hatch to environmentally relevant concentrations of TPHP. To the best of our knowledge, this is the first study to assess the in vivo effects of TPHP on birds, and we expand on earlier research by assessing changes in multiple thyroid-related endpoints including changes in gene expression, thyroid function, and growth and metabolic rate of the same individuals.

## 2. Methods

### 2.1. Chemicals and preparation of dosing solutions

For chemical analysis, standard solutions of TPHP and DPHP were purchased from Sigma-Aldrich (Oakville, ON, Canada). Internal standard solutions of d15-TPHP and d15-DPHP were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada) and from Dr. Belov at the Max Planck Institute for Biophysical Chemistry (Germany). Dichloromethane (DCM) (HPLC Plus) and methanol (MeOH) (HPLC Plus) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Sodium sulfate was purchased from Sigma-Aldrich and treated at 600 °C overnight in a box furnace prior to use. All glassware, except volumetric flasks, were treated at 450 °C for 8 h (or overnight) in a box furnace prior to use to destroy any possible organic contamination. All other solvents and reagents used in the process were HPLC grade or better.

Using a standard solution of TPHP (CAS # 115-86-6, purity > 99%, Sigma-Aldrich; Oakville, ON, Canada), two sets of dosing solutions were prepared for the egg injections and chicks (oral dosing) because different volumes were required (eggs: 10 μL; chicks: 1.6–10 μL based on weight). For the egg dosing solutions, the following amounts of TPHP were dissolved into 2.2 mL of safflower oil: 16 μg TPHP (low TPHP), 163 μg TPHP (mid TPHP), or 327 μg TPHP (high TPHP). For the chick dosing solutions, TPHP was mixed into 6.9 mL of safflower oil as follows: 147 μg TPHP (low TPHP), 1470 μg TPHP (mid TPHP), or 2940 μg TPHP (high TPHP).

### 2.2. Study design

The experimental groups included a vehicle control (organic safflower oil only; measured: 0 ng TPHP/g) and three different concentrations of TPHP (low, nominal = 5 ng TPHP/g; mid, nominal = 50 ng TPHP/g; and high, nominal = 100 ng TPHP/g). Actual measured TPHP concentrations are reported subsequently. In the dosing solutions, the low TPHP dose reflected concentrations that were reported in bird eggs and tissues collected between 2008 and 2014 (Greeves and Letcher, 2014; Greeves et al., 2016a; Lu et al., 2017). Individuals were exposed to TPHP in ovo via a single injection into the air cell of the egg on embryonic day (ED) 0, and as chicks by oral dosing daily, from 1 day post-hatch (dph) through 5 dph inclusive. Each treatment group included a total of 42 eggs, which were incubated in four separate incubation batches (1–4). Each incubation batch contained a control group plus the low, mid and high TPHP treatment groups. For logistical purposes, the start of incubation of each incubation batch was off-set by 4, 14, and 18 days after batch one.

### 2.3. Egg source, injection, and incubation

All animal handling procedures and protocols were approved by McGill University’s Animal Care Committee (Protocol # 2016–7817) under the guidelines of the Canadian Council on Animal Care. Freshly laid, fertile, but unincubated Japanese quail eggs were purchased from Ferme Patrick Brodeur (Saint-François-du-Lac, PQ, Canada), and artificially incubated at McGill University (Sainte-Anne-de-Beaureve, PQ, Canada). To ensure that the embryos did not develop prior to injection, eggs were stored in a cool (~15–18 °C) dry room until injected within four days of receipt.

Eggs were injected as previously described (Francis et al., 2018) with minor modifications. We injected 10 μL of organic safflower oil (pure or with dissolved TPHP) into the air cell using a repeater pipette. An equal volume of dosing solution was injected into each egg regardless of egg mass (mean egg mass ± SE: 13.32 ± 0.07 g (n = 250)) for final measured concentrations of 8.3 (low), 46 (mid),
Consequently, the incubator temperature was correctly set at 37.5°C/C/14 with 55% humidity and the eggs were rotated hourly. For the final 3 d of incubation, the eggs ceased, the temperature was set to 37.2°C/C/14 and humidity at 70%. Eggs were checked daily for pipping, and then placed individually in nesting cells for culling. After hatching, chicks were unable to escape from their individual nesting cells.

2.4. Hatching, brooder conditions, and oral dosing

Hatchlings were weighed and individually identified with numbered leg bands. Deformities were assessed in hatchlings and those that failed to hatch. When dry, they were transferred to a 5-tier brooder (GQF Manufacturing Co. Stacked Brooder, Model #0540, Savannah, GA, USA) at 34°C/C/14 and fed ad libitum using chick starter feed. To avoid cross-contamination among treatment groups, each tier held the same treatment group of chicks housed in plastic bins by incubation batch, descending from controls (top group) each tier held the same treatment group of chicks housed in plastic bins by incubation batch, descending from controls (top tier), to low, mid, and high (bottom tier) TPHP birds. Quail embryos from batches 1 and 2 hatched nearly 24 h earlier than expected. A secondary thermometer within the incubator suggested that the temperature was ≤1°C/C/14 above the intended set point of 37.5°C/C/14 that had been consistently registered by the incubator throughout incubation. Incubation temperature can impact the length of incubation and development of chicken embryos (Shim and Pesti, 2011). Consequently, the incubator temperature was correctly set at 37.5°C/C/14 for batches 3 and 4 and monitored thereafter using multiple independent thermometers within the incubator. Each batch contained eggs from all treatments, and thus all experienced the same temperature conditions throughout the study. We controlled for influence of incubation temperature by including ‘incubation batch’ in our statistical models (see below).

Oral dosing of the birds occurred daily from 1 to 5 dph (730–900) immediately following recording of growth measurements (discussed below). The oral dose was administered by pipetting the respective dosing solution into the mouth and holding the bird until it was observed swallowing the solution. The volume of dosing solution administered varied daily by individual body mass (1.6–10μL) to maintain consistent final TPHP concentrations of 4.5 (low), 36.4 (mid), or 80.6 (high) ng TPHP/g chick, based on the concentrations measured in the dosing solutions. The measured TPHP concentrations of the dosing solutions were very close to the nominal concentrations of 5, 50 and 100 ng TPHP/g chick.

2.5. Growth measurements

Growth and thyroid function were measured in the same individuals (i.e., all hatchlings), with a subset of those same individuals used to assess resting metabolic rate due to logistical constraints. Sample sizes of each treatment group (regardless of batch) for the growth and thyroid function assessments were as follows: controls (6F, 8M), low TPHP (4F, 7M), mid TPHP (8F, 5M), and high TPHP (6F, 7M). Following Fernie et al. (2006), chicks were weighed daily (1–6 dph), and the length of the tibiometatarsus bone was recorded to the nearest 1 mm using digital calipers on 1, 3 and 6 dph by the same individual. Growth rates in body mass and bone length divided by the total number of days that measurements were made.

2.6. Resting metabolic rate

Following previously described protocols with minor modifications (Elliott et al., 2013), resting metabolic rate of individual chicks was assessed at 5 dph, using a subset of the same individuals for the growth and thyroid assessments; sample sizes for each treatment group (regardless of batch) were as follows: controls (6F, 8M), low TPHP (4F, 7M), mid TPHP (8F, 5M), and high TPHP (6F, 7M). Chicks were tested in a pseudorandom order by treatment with individuals from the same treatment tested evenly between 9:30 to 00:30. Because of their rapid growth and high metabolic rate, the minimum resting metabolic rate of each chick was measured within 30 min when oxygen consumption stabilized. Chicks were held in a quiet, dark environment during this assessment, with each chick placed in a cylindrical respirometry chamber (Qubit Systems Inc; Kingston, ON, Canada) within a Styrofoam® box inside of a cardboard box. The respirometry chamber was connected to a Field Metabolic System (Sable Systems International; North Las Vegas, NV, USA; hereafter FMS) with a pull setup (i.e., air was pulled through the chamber into the FMS). We added scrubbers in two locations. First, we added a column containing only Drierite desiccant (W.A. Hammond Drierite Co. Ltd.; Xenia, OH, USA) after the water vapour meter, but before the CO2 meter. Second, a soda lime/Drierite column was added after the CO2 meter, but before the O2 meter. We added Drierite after the air passed through soda lime because soda lime itself can produce water vapour. Filters were included at either end of each column to ensure dust from the scrubbing agents did not enter the FMS System. We set the pump drive to 14.5% and the flow rate to 330 mL/min. Finally, the FMS was connected to a computer with Expedata Data Analysis Software (Sable Systems International), allowing for data acquisition and baselining for data analysis.

2.7. Analysis of thyroid function

Thyroid function was assessed at 6 dph in the same chicks used to assess changes in growth, including the subset of chicks used to assess resting metabolic rate. Chicks were euthanized by decapitation at 6 dph. Immediately following decapitation, blood was collected directly into a heparinized vial for each individual bird. The liver and brain were immediately collected, weighed, and stored in liquid nitrogen (LN2) and then at −80°C for biochemical assays. Thyroid glands were weighed and placed in formalin. Blood samples were centrifuged and plasma aliquots stored in LN2, then at −80°C for hormone analysis. The remaining carcass was wrapped in chemically-cleaned aluminum foil and frozen for chemical analysis.

Thyroid function was assessed by examining thyroid-related hepatic gene expression, plasma hormone levels and thyroid gland histology. We examined the expression of iodothyronine deiodinase 2 (DIO2), thyroid hormone responsive spot 14 (THRSP), and thyroid hormone receptor alpha (THRA) mRNA in liver. For each target gene, samples (n = 55) were run in duplicate on a single plate along with no-reverse transcriptase (NRT) controls and a no template control (NTC). Elongation factor-1 alpha 1 (EEF1A1) was selected as the reference gene based on GeNorm analysis (Vandesompele et al., 2002). No amplification was observed in the no template or no RT controls and reference gene expression was invariant across the treatment groups. A complete description of the related molecular methods is provided in the Supplementary Information (SI).

Plasma concentrations of free (F) and total (T) triiodothyronine
(T3) and thyroxine (T4), and histological changes in the structure and functioning of the thyroid glands, were determined using previously described methods (Fernie et al., 2015, 2016; 2019; Park et al., 2011). Detailed methods are presented in the Supplementary Information (SI) regarding assessments of epithelial cell height (ECH), colloid area (μm²) (CA), colloid diameter (CD), and mean ECH:CD ratio (thyroid gland activation and potential thyroid hormone production) (Bocian-Sobkowska et al., 2007).

2.8. Sample analysis and determination of TPHP and DPHP concentrations

We measured the concentrations of TPHP and its major metabolite DPHP in the dosing solutions, and homogenized egg samples and chick carcasses, according to published methods with minor modifications (Su et al., 2015a; Greaves et al., 2016b; Lu et al., 2017). Analytical details and quality control and assurance details are provided in the SI.

2.9. Statistical analyses

All statistical analyses were performed using SAS (version 9.3, SAS Institute Inc., Cary, NC). Model type was selected based on the type of dependent variable (dichotomous or continuous) and whether measures were repeated. We incorporated treatment and incubation batch in all models, and sex into the models when possible. To analyze hatchling success and the incidence of deformities, we used logistic regression (PROC LOGISTIC) with treatment and incubation batch as factors. We used a general linear model (PROC GLM) to analyze time to hatching and incorporated treatment and batch as factors. We examined potential changes in the thyroid-related genes (DIO2, THRSP, and THRA) normalized to a reference gene (EEFI1)A1 and in circulating concentrations of FT3, FT4, TT3, and TT4, using GLMs with treatment, sex and incubation batch as main effects and all two-way interactions (7 GLMs in total). Log-transformation (natural log) was required to produce normally distributed data for THRSP but not for DIO2 or THRA. Thyroid hormone data were combined for batches 1 and 2 because of the lack of data (empty cells) for some treatment groups in these batches. However, there were no effects related to incubation batches on circulating thyroid hormones. To assess structural and functional changes in the thyroid glands, a linear mixed model (PROC MIXED) was used with an unstructured covariance matrix for each of the three dependent variables (i.e., ECH, CD, ECH:CD) while controlling for the 20 repeated measurements (RM) of these parameters within each thyroid gland. Each model included treatment, sex, batch, and RM as main effects, and all two-way interactions between treatment, sex, and incubation batch. A linear mixed model (PROC MIXED) with an unstructured covariance matrix and age in the repeated statement was used to analyze growth parameters. We incorporated age, treatment, incubation batch, all two-way interactions, and treatment-by-age-by-sex and incubation batch-by-age-by-sex as three-way interactions. A general linear model (PROC GLM) was also used to identify changes in metabolic rate with sex, treatment, and incubation batch, and all interactions of these three variables as explanatory variables, along with time of day and body weight as covariates. When residuals were not normally distributed, data were log-transformed. The statistical models allowed us to identify the potential effects of TPHP treatment and sex while accounting for any differences among the four incubation batches. Because we had strong a priori predictions for thyroid measurements, growth and metabolism, we further analyzed significant effects with one-tailed Fisher least significance difference (LSD) post hoc tests for comparisons between control and TPHP. Except for time to hatching, data are presented as marginal means (least squares means in SAS) ± SE generated from the model results, to account for the effects of the covariates. Results were considered significant if p ≤ 0.05.

3. Results

3.1. TPHP concentrations in dosing solutions and 6-day old chicks

Tissue concentrations measured in the homogenized 6 dph chick carcasses are provided in Table 1. Although TPHP was quantifiable in the control chicks, concentrations were close to the quantification limit. There were significant differences in TPHP concentrations measured in the egg homogenates and chick carcasses amongst the four treatment groups overall (p-values < 0.004), with post-hoc pairwise comparisons identifying significantly higher TPHP carcass concentrations in the high TPHP group compared to controls only (p = 0.005). The uptake, metabolism and depletion of TPHP and DPHP is discussed in detail separately (Letcher, Fernie, et al., manuscript in preparation).

3.2. Hatching success, deformities

Overall hatching success (n = 139; 43%) showed no effects of the in ovo exposure to the different TPHP concentrations (χ² = 2.70, p = 0.44) or across the four incubation batches (χ² = 3.70, p = 0.30). Similarly, the overall incidence of deformities (n = 109; 25%) did not vary across the TPHP treatments (χ² = 2.98, p = 0.40) or the incubation batches (χ² = 4.47, p = 0.21). Time to hatching showed no influence of the TPHP concentrations (F₃,5₂ = 0.57, p = 0.64), but varied among the incubation batches (F₃,5₂ = 9.68, p < 0.0001); chicks in incubation batches 1 and 2 hatched 24 h earlier than in the other two batches. Incubation batch was accounted for in all statistical models.

3.3. Thyroid function

There were no significant effects of the TPHP concentrations, sex of the chicks, or TPHP treatment × sex interaction (difference between males and females in relation to the TPHP concentrations) on the expression of the measured thyroid-related genes (Table S2). There were significant differences in the expression of THRSP among the four incubation batches, and for both THRSP and THRA, there were significant differences between males and females within the incubation batches (batch × sex interactions) (Table S2).

Circulating concentrations of FT3 showed a significant effect of the TPHP × sex interaction (F₃,1₇ = 4.51 p = 0.008); the high TPHP females had significantly lower circulating FT3 than control females (p = 0.0074) and mid TPHP females (p = 0.0044). There were no significant differences in circulating FT3 for the male chicks among the TPHP groups, and no significant effects of TPHP, sex, incubation

Table 1

| Concentrations of TPHP and its metabolite, DPHP, measured in Japanese quail chicks. Homogenized carcasses (minus brain, liver, blood) were collected at 6 dph. There were significant differences overall in TPHP carcass concentrations (F₃,₄₆ = 2.9, p = 0.04), with concentrations significantly higher in high TPHP carcasses than controls (p = 0.05). While the effects of TPHP on the quail are discussed here, the uptake and metabolism of TPHP and DPHP in these carcasses is discussed separately (Letcher, Fernie, et al., manuscript in preparation). Mean ± SE are reported. **p = 0.005. |
|-----------------|-----------------|-----------------|
|                | TPHP (ng/g ww)  | DPHP (ng/g ww)  |
| Control        | 0.25 ± 0.04     | 0.17 ± 0.06     |
| Low-dose TPHP  | 0.39 ± 0.10     | 0.24 ± 0.05     |
| Mid-dose TPHP  | 0.40 ± 0.05     | 0.80 ± 0.13     |
| High-dose TPHP | 0.54 ± 0.06***  | 1.75 ± 0.30     |
batch, or interactions on circulating FT4, TT4 or TT3 (Table S3). Circulating concentrations of these thyroid hormones are reported in Table S4.

TPHP significantly affected the structure of the thyroid gland (follicular ECH: $F_{3,34} = 3.85\ p = 0.02$). Glandular structure (ECH, CD) significantly differed between the sexes ($p$-values $\leq 0.02$) with significant interactions of sex X TPHP ($p$-values $\leq 0.02$) and sex X incubation batch ($p$-values $\leq 0.05$) (Table 2). ECH significantly differed among the female chicks only, and was significantly higher in controls than any TPHP females (low TPHP: $p = 0.0029$; mid TPHP: $p = 0.024$; high TPHP: $p = 0.0003$) (Fig. 1), and in mid vs. high TPHP females ($p = 0.028$) (Fig. 1). In contrast, follicular colloid reserves (CD) significantly differed among TPHP treatments for the males only (sex: $p = 0.02$) (Fig. 1) with a significant interaction of sex X TPHP ($p = 0.02$) (Table 2): follicular colloid reserves were significantly greater for high TPHP males than control males ($p = 0.0242$) and low TPHP males ($p = 0.0043$) (Fig. 1). CD also differed among the incubation batches ($p = 0.0004$).

Controlling for significant differences among the incubation batches ($F_{3,34} = 8.73\ p = 0.0002$; incubation batch X TPHP: $F_{3,34} = 4.59\ p = 0.0005$; incubation batch X sex: $F_{3,34} = 10.07\ p = 0.0001$) (Table 2), thyroid gland activity (ECH:CD) was significantly affected by TPHP ($F_{3,34} = 5.05\ p = 0.005$), with significant differences evident between sexes in response to TPHP (TPHP x sex interaction: $F_{3,34} = 3.50\ p = 0.03$). All TPHP females had significantly less active thyroid glands than the control females (low: $p = 0.0059$; mid: $p = 0.0125$; high $p = 0.0026$) (Fig. 2), while the high TPHP males had significantly more active thyroid glands than the low ($p = 0.017$) or mid TPHP ($p = 0.0391$) males (Fig. 2).

### 3.4. Growth: body weight

Controlling for significant differences among the incubation batches (batch X treatment: $F_{9,36} = 3.29,\ p = 0.005$; batch X age: $F_{18,36} = 45.45,\ p < 0.0001$) (Table S5), body weight increased

### Table 2
Statistical outcomes for histological assessments of the thyroid glands of Japanese quail (6 dph) following exposure to TPHP. Num/Den DF – numerator or denominator degrees of freedom.

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<td>19</td>
<td>34</td>
<td>4.94</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Incubation Batch x Sex</td>
<td>3</td>
<td>34</td>
<td>3.5</td>
<td>0.0257</td>
</tr>
<tr>
<td>Incubation Batch x Sex</td>
<td>9</td>
<td>34</td>
<td>4.99</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

Fig. 1. Histological changes in epithelial cell height (ECH) and follicular colloid diameter (CD) of the thyroid glands of female and male Japanese quail chicks (6 dph) exposed to TPHP. Marginal means ($\pm$SE) were generated from a linear mixed model that incorporated 20 measurements per chick. Different letters indicate statistically significant differences between groups ($p \leq 0.05$).
3.5. Growth: tarsus length

There was also a significant TPHP treatment X age X sex interaction ($F_{6,36} = 3.27, p = 0.01$; Fig. 5B and C) on bone growth (Table S5); as with body weight, females and males responded differently in bone growth to the mid and high TPHP doses. At 6 dph, high TPHP females had a significantly shorter tarsus (11% lower) than control females (Fisher LSD post hoc test: $p = 0.02$), but tarsus length was similar between control females and low or mid TPHP females ($p \leq 0.48$) (Fig. 4B). Bone growth rate was 52% lower for the high TPHP females (0.3 mm/d) than the control females (0.7 mm/d). The mid TPHP males had a significantly shorter tarsus at 3 dph (9% shorter; $p = 0.004$) and at 6 dph (10% shorter; $p = 0.02$) compared to control males, with no differences in bone length between controls and low TPHP or high TPHP males ($p \geq 0.06$; Fig. 4C). The bone growth rate of the mid TPHP males (0.4 mm/d) was 42% lower than the control males (0.8 mm/d).

3.6. Metabolic rate

There were no differences in metabolic rate between the sexes (main effect of sex: $F_{1,26} = 0.47, p = 0.50$), between the sexes in response to TPHP (sex X TPHP treatment: $F_{3,26} = 0.14, p = 0.93$), or relating to the time of day when tested ($F_{1,26} = 0.07, p = 0.80$). Consequently, the statistical analysis was repeated with sex and time of day removed from the model. Controlling for the significant difference among incubation batches (batch X TPHP: $F_{3,26} = 2.53, p = 0.009$) (Fig. S1), there were significant effects of TPHP ($F_{3,34} = 10.02, p < 0.0001$) and body weight (covariate: $F_{3,34} = 48.51, p < 0.0001$) on oxygen consumption (Table S6). Oxygen consumption, controlled for body weight, decreased across the treatment groups, with greater reductions observed as TPHP dose increased (Fig. 5). Oxygen consumption was significantly reduced in the high TPHP chicks (12.8%; Fisher LSD post hoc test: $p = 0.02$) and the mid TPHP chicks (8.7%; $p = 0.05$) compared to control chicks (Fig. 5). Mean oxygen consumption of the low TPHP chicks was intermediate between that of the control and mid-TPHP chicks, but was not significantly different from the control chicks ($p = 0.17$; Fig. 5).

4. Discussion

Our findings demonstrate that the quail were exposed to and accumulated TPHP concentrations throughout development, and likely metabolised parent TPHP to DPHP, as shown in the chick carcasses at 6 dph (Letcher, Fernie et al. manuscript in preparation). These findings are consistent with the in vitro metabolism of TPHP by avian hepatocytes (Su et al., 2014, 2015b; Greaves et al., 2016b), and with the TPHP and DPHP concentrations reported in tissues of wild birds (e.g., Eujaers et al., 2014; Hallanger et al., 2015; Verreault et al., 2018, Monclus et al., 2019). We hypothesize that the measured concentrations of TPHP and DPHP in the control carcasses likely reflect background exposure, e.g., from commercial quail feed used in study. The changes observed in the quails’ thyroid system, growth and metabolic rate may result from their exposure to the parent TPHP and/or the metabolites of TPHP including DPHP itself; these potential relationships, the uptake of TPHP and its metabolism to DPHP, in the present quail are discussed in more detail separately (Letcher, Fernie et al. manuscript in preparation).

In the present study, there were no effects of TPHP on the hatching success or deformities of the quail, although their overall hatching success was low (43%) compared to Japanese quail eggs that were not exposed to chemicals or injected (64% or higher) (Luna et al., 2012). The cause(s) for the low hatching success across the treatments are unknown. The variation in incubation temperatures in our study is experienced by other incubating birds (Coe et al., 2015). All four treatment groups in our study were represented in each incubation batch and thus experienced the same background exposure, e.g., from commercial quail feed used in study. The changes observed in the quails’ thyroid system, growth and metabolic rate may result from their exposure to the parent TPHP and/or the metabolites of TPHP including DPHP itself; these potential relationships, the uptake of TPHP and its metabolism to DPHP, in the present quail are discussed in more detail separately (Letcher, Fernie et al. manuscript in preparation).

Controlling for the significant differences among incubation batches (main effect of incubation batch: $F_{3,36} = 15.40, p < 0.0001$; batch X treatment interaction: $F_{3,36} = 2.53, p = 0.02$; batch X age interaction: $F_{6,36} = 18.92, p < 0.0001$) (Table S5), tarsus length increased significantly with age ($F_{2,36} = 146.08, p < 0.0001$) and was affected by TPHP (treatment X age interaction: $F_{6,36} = 3.13, p = 0.01$) (Fig. 4A, Table S5). Specifically, the rate of bone growth was significantly slower in the high and mid TPHP chicks than control chicks, although it was similar in the mid TPHP chicks and controls by 6 dph (Fig. 4A).
conditions, and there are no consistent batch-related patterns in the observed TPHP-related changes (Fig. S1). Additionally, we controlled for potential effects of temperature variation in our statistical models.

Many FRs, including some OPEs, are well-known disruptors of the avian thyroid system (Fernie et al., 2005, 2015; Fernie and Marteinson, 2016; Guigueno and Fernie, 2017; Marteinson et al., 2017a, b), potentially leading to impacts on metabolic rate and growth that are partially thyroid-mediated (McNabb, 2007). Compared to earlier studies, the current study examined potential chemical effects in vivo on avian thyroid function in the same individuals, specifically at the level of gene expression (DIO2, THRSP, THRA), thyroid gland histology (structure: ECH, CD; function: ECH:CD), and circulating thyroid hormones (FT3, FT4, TT3, TT4). In the present quail, the TPHP exposure concentrations had no effects on the expression of these thyroid-related genes but altered circulating T4 and thyroid gland structure and activity in a sex-specific manner. The high TPHP female chicks had suppressed circulating FT3, while in all TPHP females, thyroid gland activity (ECH:CD) and structure (shorter ECH) was suppressed, although circulating T4 was unchanged. Similarly, other OPEs suppressed circulating FT3, reduced thyroid gland activity but increased hepatic deiodinase activity in adult American kestrels (Fernie et al., 2015). Since T4 is converted to the biologically active, T3, outside of the thyroid gland, the reductions in circulating FT3 in the female quail suggest possible alterations in T4 deiodination to T3, e.g., hepatic T4-outer ring deiodinase (T4-ORD) activity, as observed with other OPEs in kestrels (Fernie et al., 2015). The activity of the DIO2 enzyme is regulated by the interplay of transcriptional, posttranscriptional, and posttranslational mechanisms (Wagner et al., 2007) and although no changes were observed in DIO2 gene expression, enzyme activity may be altered nonetheless. Alternatively, activity of hepatic deiodinase 1 (DIO1) may play a greater role in regulation of thyroid hormone levels in TPHP exposed birds and its role in the effects of this FR on thyroid hormone levels could be explored in future studies. If thyroid function of the TPHP female quail was sufficiently compromised, reductions in their growth and metabolism would be anticipated. Thyroid gland structure and activity was enhanced for the high TPHP male chicks contrasting with the suppression observed for female chicks. These glandular alterations may have increased thyroid hormone production and/or release to maintain circulating concentrations since there were no differences in circulating T3 or T4 among the males. The high TPHP male chicks had more glandular colloid than the control or low TPHP males, and more active thyroid glands (ECH:CD) than the low or mid TPHP males. The high TPHP concentrations stimulated thyroid gland activity and the increased colloid suggests that more T4 was created and/or stored in the thyroid glands. Increased thyroid function may allow for compensatory changes in growth and/or metabolism, despite being physiologically costly. Compared to control males, growth of the high TPHP males was similar, whereas the mid TPHP males were

Fig. 3. Weight gain of Japanese quail chicks from hatch through to 6 dph following embryonic and post-hatch exposure to varying concentrations of TPHP. Marginal adjusted means ± SE are presented. There was a significant treatment × age interaction (A), whereby mid and high TPHP chicks had a reduced rate of growth relative to control chicks. There was also a significant treatment × age × sex interaction (B: females, C: males): high TPHP females (B) were significantly lighter and had a lower growth rate (53%) than control females, while mid-TPHP males (C) were significantly lighter each day, and gained weight more slowly than control males. Asterisks indicate significant differences between control and at least one TPHP treatment at p < 0.05 and the days that these significant differences occurred.
much smaller (22%) and grew more slowly, suggesting the lack of thyroidal compensation of the mid TPHP males (i.e., similar to control males) may have prevented adaptable changes in growth. In the Athabasca Oil Sands Region, tree swallow nestlings with more active glands were heavier and had better survival than those nestlings lacking thyroidal changes that were smaller and had poorer survival (Fernie et al., 2019).

While there were effects of all three TPHP doses evident on thyroid function of the female quail chicks, the effects of TPHP on growth and metabolic rate were evident in the mid and high TPHP birds only and not in the low TPHP quail exposed to TPHP levels measured in wild bird eggs (Greaves and Letcher, 2014; Greaves et al., 2016a; Lu et al., 2017). The pronounced reductions in growth (9–13%) and metabolic rate (38–53%) of the mid and high TPHP birds were consistent with the reduced growth of weanling rats fed 1% w/w of TPHP (Hinton et al., 1987), and of female American kestrels exposed to technical DE-71 whose weight was correlated with lower circulating TT3 (Fernie and Marteinson, 2016 but see Fernie et al., 2006). We hypothesize that the sex-specific growth responses of the quail to TPHP likely reflects the sex-

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**Fig. 4.** The growth of the tibiometatarsus bone of Japanese quail chicks from 1 dph through to 6 dph following exposure to TPHP. Marginal means ± SE are presented. There was a significant treatment × age interaction (A): mid and high TPHP chicks had significantly shorter bones than controls at 3 dph, but at 6 dph, only high TPHP chicks had significantly shorter bones. There was also a significant treatment × age × sex interaction (B: females, C: males): for high TPHP females (B), the tibiometatarsus grew significantly more slowly (52% less) over the 6 d exposure period, resulting in a significantly shorter tibiometatarsus at 6 dph. Whereas for males (C), the mid TPHP males had significantly shorter tibiometatarsus (3, 6 dph) and slower growth rates (42%) than control males. Asterisks indicate significant differences between control and at least one TPHP treatment at \( p < 0.05 \), and the days when significant differences occurred.

**Fig. 5.** Resting metabolic rate (controlled for body weight) of Japanese quail chicks exposed to varying concentrations of TPHP. Marginal adjusted means ± SE are presented. Chicks from the mid and high TPHP groups had a significantly lower resting metabolic rate than control chicks. Different letters indicate statistically significant differences between groups (\( p < 0.05 \)).
specific changes in thyroid function (discussed earlier). The high TPHP females and mid TPHP males were smaller and grew more slowly. Endocrine responses to many chemicals, including FRs, are sex-dependent (see Fernie et al., 2007; Marteinson et al., 2011, 2017a). Sex differences in liver metabolic pathways may have implications for physiological processes, growth, regulation of nuclear receptors (e.g., thyroid hormones), and many cytochrome P450 enzymes; female livers may be more efficient at neutralizing substances (Rando and Wahlil, 2011). In birds, species differences occur in liver metabolic pathways, but sex differences in these pathways are currently unknown but appear likely based on multiple avian studies.

In many species, chicks that grow more slowly typically have lower survival (Maness and Anderson, 2013). Juvenile survival of birds increased with fledging body weight and/or wing length resulting from higher growth rates (Monros and Beldaroba, 2002; Morrison et al., 2009). In our study, slower growth rates (<53%) were observed in the TPHP-exposed chicks, and the high TPHP females and mid TPHP males were smaller (10–34%) than respective controls at 6 dph (Figs. 4 and 5). Conceivably, the smaller these chicks were at the end of our study (6 dph), the less likely they would have been to survive. Although TPHP did not affect the size of the chicks at hatching (Figs. 3 and 4) but influenced their growth after hatching, we hypothesize that the cumulative exposures (embryonic and post-hatch) may act in concert to affect body weight and tarsus length with chick age.

While there was no sex-specific effects of TPHP on metabolic rate, we observed a more rapid decline in metabolic rate with increasing TPHP exposure concentrations, paralleling the rate, we observed a more rapid decline in metabolic rate (embryonic and post-hatch) may act in concert to affect body after hatching, we hypothesize that the cumulative exposures (embryonic and post-hatch) may act in concert to affect body weight and tarsus length with chick age.

In summary, developmental exposure (in ovo and post-hatch) to the commonly used flame retardant, TPHP, altered thyroid function including circulating FT3 (females only), and suppressed growth and metabolism of Japanese quail chicks. These TPHP concentrations had no effect on hatching rates, deformity rates, or the thyroid-related genes, in the quail. As with other flame retardants, there were sex-specific effects of TPHP exposure, with thyroid suppression occurring predominantly in females, but increased thyroid function evident in high TPHP males. The effects on thyroid function, but not growth or metabolism, were observed at TPHP levels similar to those reported in wild bird eggs. Future research should investigate the uptake and metabolism of TPHP and the potential effects of TPHP on avian behaviour. It is possible that TPHP-related reductions in thyroid function, metabolic rate and growth may be ecologically important for wild birds.

Conflicts of interest

The authors declare no conflict of interest in conducting this study.

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The datasets generated and/or analyzed during the current study are available from the corresponding author.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.05.110.

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