Assessment of the effects of early life exposure to triphenyl phosphate on fear, boldness, aggression, and activity in Japanese quail (*Coturnix japonica*) chicks[☆]

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ABSTRACT

Triphenyl phosphate (TPHP) is an organophosphate ester (OPE) used as a flame retardant (FR) and plasticizer. TPHP has previously been shown to disrupt behaviour in fish and mammals, but to our knowledge, this is the first study on the behavioural effects of TPHP in birds. Early life stage Japanese quail (*Coturnix japonica*) were exposed to nominal doses of 0 ng/g (vehicle-control), 5 ng/g (low dose), 50 ng/g (mid dose), and 100 ng/g (high dose) TPHP, both as embryos (via air cell injection prior to incubation) and as chicks (via daily gavage until 5 days post-hatch). The low dose reflects TPHP levels recorded in wild avian eggs, but actual environmental exposure levels may be higher given that TPHP is known to be rapidly metabolized in birds. We previously reported that the chicks exposed to TPHP in this study experienced reduced growth and resting metabolic rate, and sex-specific changes in thyroid function. The current study focuses on behavioural endpoints. We found that high-TPHP chicks exhibited less neophobia than vehicle-controls, and low-TPHP chicks exhibited more aggression towards conspecifics. No differences were observed in the responses of Japanese quail chicks to activity or tonic immobility (fear response) tests. These data add weight of evidence to previous findings suggesting that TPHP, among other OPEs, can disrupt ecologically-relevant behaviours in exposed vertebrates.

1. Introduction

Flame retardants (FRs) are chemical compounds that are applied to materials to reduce their flammability or combustion potential. Polybrominated diphenyl ethers (PBDEs) are brominated flame retardants (BFRs) that were once produced in large amounts, but have largely been phased out due to environmental persistence and association with adverse health effects (Gibson et al., 2019; van der Veen and de Boer, 2012). As suitable alternatives to PBDEs and other BFRs such as hexabromocyclododecane (HBCD), organophosphate esters (OPEs) are currently some of the more commonly used FRs internationally (Stapleton et al., 2012; Kajiwara et al., 2011). Temporal increases in concentrations of OPEs in human urine indicate that biotic exposure to these chemicals is becoming more widespread (Hoffman et al., 2017).

Triphenyl phosphate (TPHP) is a lipophilic OPE (log K_{ow} = 4.59) that is classified as a priority chemical under the Government of Canada's Chemical Management Plan. Like other OPEs, TPHP is not chemically bonded to the polymers it is added to and therefore has increased potential to be released into the environment (Wei et al., 2015). In addition to being used as a FR additive, TPHP can be used as a plasticizer (e.g., in PVC materials and beauty products), lubricant, and in paints, glues, and hydraulic fuel (Wei et al., 2015). TPHP has been detected in house and car dust (Brandsma et al., 2014;

Christia et al., 2018; Meeker and Stapleton, 2010), human breast milk (Sundkvist et al., 2010), human urine samples (Hoffman et al., 2017) and wildlife tissues (Brandsma et al., 2015; Sundkvist et al., 2010). Although TPHP is rapidly metabolized to diphenyl phosphate (DPHP) and other metabolites in avian cells (Su et al., 2014), it has been measured within the tissues and eggs of herring gulls (Greaves & Letcher, 2014; Greaves et al., 2016) and other birds (reviewed in Guigueno et al., 2019). This suggests that TPHP is continuously present in the birds' environment and that it is passed from mother to offspring.

Despite the widespread occurrence of OPEs such as TPHP in the environment, relatively little is known about their effects on biota. Many OPEs, including TPHP, are suspected endocrine disruptors, with several studies reporting disruption to androgen and estrogen signalling (Liu et al., 2012; Kojima et al., 2013). For example, exposure to TPHP was associated with intersex in males and abnormalities in reproductive behaviour in Japanese medaka (*Oryzias latipes*). These effects were linked to anti-androgenic activity (Li et al., 2018). Some OPEs also appear to interact with the thyroid system; they have been linked with alterations in T3 and T4 levels in fish (Wang et al., 2013; Kim et al., 2015), mammals (Springer et al., 2012), and birds (Farhat et al., 2013; Fernie et al., 2015; Guigueno et al., 2019). Brain organization and function are highly dependent on thyroid hormones, especially during early development (Bernal, 2007). OPEs are also suspected neurotoxicants and teratogens (Greaves and Letcher, 2017). Additionally, high throughput screening using an early life stage zebrafish (*Danio rerio*) photomotor assay suggested that many OPEs interfere with neurodevelopment (Noyes et al., 2015). These findings contribute to growing evidence that OPEs such as TPHP have the potential to cause behavioural abnormalities in early life stage organisms.

Many contaminants are understood to influence the behaviour of organisms, causing alterations in, for example, mating behaviour, predator avoidance, activity levels, and social interactions (Peterson et al., 2017). Behaviour appears to be a sensitive endpoint for FRs in birds (Guigueno and Fernie, 2017) as well as in fish and mammals (Hendriks and Westerink, 2015). Less is known about the toxicological effects of OPEs specifically, but the available data suggest that they too can impact behaviour, and TPHP in particular has been identified as a compound with medium to high neurotoxicity (Hendriks and Westerink, 2015). In a screen for chemicals causing developmental neurotoxicity in zebrafish, TPHP was recommended as one of two OPEs that should be prioritized for further testing (Jarema et al., 2015; Behl et al. 2015). To our knowledge, a link between exposure to TPHP and changes in behaviour in birds has not been previously investigated. This is an important data gap because contaminant-induced alterations in behaviour can have a direct impact on ecologically-relevant outcomes such as growth, reproduction and survival.

The current study adds to our recent work exploring effects of TPHP on early life stage Japanese quail (*Coturnix japonica*). We previously reported that exposure of Japanese quail embryos and chicks to TPHP was associated with reduced growth and resting metabolic rate, as well as sex-specific alterations in thyroid gland histology and circulating T3 (Guigueno et al., 2019). In addition, TPHP was quickly metabolized to DPHP and other unknown metabolites (Marteinson et al., 2019). Here, we investigate behavioural effects of developmental exposure to TPHP in these same Japanese quail chicks, using tests that examine their fear response, activity level, boldness, and aggression.

2. Methods

2.1. Chemicals

A standard solution of TPHP (CAS # 115-86-6) was purchased

from Sigma-Aldrich (Oakville, ON, Canada) at a purity greater than 99%. Two sets of dosing solutions were prepared in organic safflower oil with different concentrations of TPHP; one set for egg injections and the other for daily oral dosing of the chicks after they hatched. The nominal and actual concentrations of TPHP for each treatment group can be found in Table 1. Actual concentrations of TPHP in the dosing solutions were analytically determined using ultra high-performance liquid chromatography—mass spectrometry, as described in Guigueno et al. (2019) and Marteinson et al. (2019).

2.2. Dosing of animals

Protocols describing the use of Japanese quail in this study were approved by McGill University's Animal Care and Use Committee (under the guidelines of the Canadian Council on Animal Care) (protocol #2016-7817). Detailed dosing procedures are described in Guigueno et al., (2019). Briefly, fertilized, unincubated Japanese quail eggs were purchased from Ferme Patrick Brodeur (Saint-François-du-Lac, Québec, Canada), and stored in a cool (15 - 18 °C), dry room for 1-4 days after being received. Eggs were randomly assigned to one of the following four treatment groups (nominal concentrations): vehicle control (organic safflower oil; 0 ng/g), low-TPHP (5 ng/g), mid-TPHP (50 ng/g), and high-TPHP (100 ng/g). The low-TPHP group was intended to reflect concentrations in the eggs and tissues of wild birds (e.g., Greaves and Letcher, 2014; Greaves et al., 2016; Lu et al., 2017). However, our more recent results indicate that this may be an underestimate, given how quickly TPHP was metabolized by the birds in this study (Marteinson et al., 2019).

To mimic an exposure that would occur naturally in the environment where TPHP is continuously present, each quail was exposed to organic safflower oil control or TPHP through two methods; 1) a single air cell injection of the test solution into the egg prior to incubation, and 2) daily oral dosing of chicks for 5 days starting at 24 h post-hatch. Egg injection involved pipetting a 10 μL volume of the appropriate dosing solution into each egg through a small hole in the shell above the air cell. This method was chosen (as opposed to injection into the yolk) to align with our recent proposal of a standardized method for early life stage toxicity testing in birds (Farhat et al., 2019). Post-hatch oral dosing was performed by inserting a pipette into the chick's mouth just past the glottis, and dispensing the appropriate dosing solution. Oral dosing took place at the same time each day (7:30—9:00 a.m.) and the volume of dosing solutions was adjusted to the chick's daily weight. Dosing procedures are described in additional detail in Guigueno et al., (2019).

2.3. Chick incubation and care

Quail eggs were incubated on their side, in an Ova-Easy Advanced Series II Cabinet Incubator, type 190 (Brinsea) at 37.5 °C, 55% humidity, with hourly rotation for the first 15 days of incubation, and at 37.2 °C, 70% humidity, with no rotation thereafter. To avoid too many chicks hatching simultaneously, eggs were injected and incubated in 4 different batches. Each incubation batch included all treatment groups, and was staggered so that incubation for batches 2, 3 and 4 began 4, 14 and 18 days after batch 1 respectively. Batches 1 and 2 included 9 eggs per treatment, and batches 3 and 4 included 12 eggs per treatment, however, not all individuals were used in the behavioural testing or final data analysis (see section 2.5.1. 'consideration of batch effects', and Table 2 for final sample sizes).

After hatching, chicks were assessed for deformities, then weighed and given a white plastic leg band (2.8—3.0 mm) (Red Bird

Table 1
Nominal and actual concentrations of TPHP administered to each experimental group of Japanese quail chicks as embryos (via air cell injection into the egg) and as chicks (via daily gavage until 5 days post-hatch). Actual doses were calculated from the measured TPHP concentration of the dosing solutions.

	Experimental groups			
	Vehicle	Low–TPHP	Mid–TPHP	High–TPHP
Nominal concentration (ng/g)	0	5	50	100
Actual concentration (eggs; ng/g)	0.01	8.4	46	97.2
Actual concentration (chicks; ng/g)	0.01	4.5	36.4	80

Products, Inc., Mount Aukum, CA, USA) with a unique number for identification. Once their down was dry, they were transferred to a 5-tier brooder (GQF Manufacturing Co. Stacked Brooder, Model #0540, Savannah, GA, USA). Each tier measured $81.3 \times 96.5 \times 30.5$ cm and housed individuals from a single treatment group. The tier was further divided into subgroups with two plastic bins, to prevent interactions between chicks from overlapping batches. In the brooder, chicks were kept at 34 °C and a 12-hr light/12-hr dark cycle. They were given a nutritionally balanced food mixture (produced by Ferme Patrick Brodeur) and water *ad libitum*, which was refreshed twice daily when their plastic bin enclosures were cleaned.

2.4. Behavioural tests

Behavioural tests were conducted at 2 days post hatch (dph; tonic immobility) and 4 dph (neophobia, aggression, and activity level). In an effort to keep the chick's surroundings as consistent as possible, a surplus level of the brooder was used as the testing arena. The testing arena was placed on the floor away from the main brooder stack. A digital camera was mounted on a tripod next to the testing arena and used to record all behavioural tests from above. The tonic immobility trials were run directly within the testing arena, whereas a plastic bin, identical to the one in which chicks were housed, was placed inside the testing arena for the neophobia, aggression, and activity level trials (Fig. 1).

2.5. Tonic immobility (2 days post hatch)

The tonic immobility test examines the propensity of an animal to "freeze" (or "play dead"), often in response to restraint (Abe et al., 2013). A section of cardboard was folded into an M shape, with a centre angle of approximately 100°, and the cardboard was taped to the floor of the testing arena (Fig. 1). Once secured, a sheet of lab paper was placed over the cardboard structure to prevent contamination between treatment groups. Test subject selection was done by cycling through the treatment groups and randomly selecting a chick from each group until all chicks were tested. The chick was placed on its back within the main angle of the M-shaped cardboard, and two fingers were gently held against the crop for 5 s (Abe et al., 2013). After 5 s, the pressure against the bird's crop was lifted, and the bird's behaviour was observed. If the chick displayed 'freeze behaviour' (i.e., remained in an immobile state on its back despite the removal of pressure on its crop), a timer was started to measure the duration of the freeze response. The frozen state was considered to have ended when the chick rolled into a standing position. If the chick did not enter an immobile state immediately after the removal of pressure from its crop, the test was repeated up to four additional times (for a maximum total of five attempts) and the number of times to freezing was recorded.

2.6. Neophobia, aggression, and activity (4 days post hatch)

For the neophobia, aggression, and activity tests, chicks were randomly sorted into social groups comprised of four chicks; one from each of the treatments. Chicks remained in the same social group for all three tests, and each chick only participated in a single group. The head of each chick was marked with a different colour tape for identification and tracking purposes. The colour of tape was assigned randomly, and was independent of the individual's

Table 2
Effects of exposure to triphenyl phosphate (TPHP) on behaviour in Japanese quail chicks. Behavioural endpoints are expressed as means ($\pm$SE). Significant differences between treatment groups are indicated with superscript letters ($p < 0.05$). Tonic immobility was measured individually in 2 day old chicks, with one low-TPHP chick and one mid-TPHP chick not being included in this analysis because they did not freeze within the pre-determined maximum number of freezing attempts (i.e., five). Sample sizes are indicated for all tests, and these varied depending on the requirements of the test itself. For tonic immobility, all available chicks from batches 3 and 4 were used (n = 6–12; however, the final sample size for the Low-TPHP group was 5 because of the one bird that did not freeze). The remaining tests were performed on social groups consisting of one individual from each treatment group. For these tests, the sample size was limited to the number of individuals in the least populated group in the study (n = 6).

|	Experimental groups			
	Vehicle	Low-TPHP	Mid-TPHP	High-TPHP
Sample Size	*12*	*5*	*6*	*9*
Tonic Immobility				
Number of attempts to freeze	2.4 ± 0.3	3.4 ± 0.7	3.2 ± 0.7	2.4 ± 0.4
Freeze duration (s)	21.0 ± 3.3	35.8 ± 14.4	38.6 ± 13.1	21.9 ± 6.7
Sample Size	*6*	*6*	*6*	*6*
Neophobia				
Time spent near novel object (s)	43.0 ± 15.2	59.7 ± 20.1	74.4 ± 30.0	89.7 ± 24.4
# of pecks to novel object	9.5 ± 2.8[a]	10.3 ± 1.8[a]	14.2 ± 3.0[a,b]	18.0 ± 5.7[b]
Aggression				
# of pecks to conspecifics	9.0 ± 3.0[a]	20.5 ± 6.6[b]	6.2 ± 1.6[a]	10 ± 3.0[a]
# of hip check to conspecifics	8.2 ± 3.8	8.8 ± 4.3	6.2 ± 2.0	9.7 ± 1.7
Time in food bowl (s)	202.7 ± 75.2	293.2 ± 75.5	197.5 ± 67.6	287.0 ± 49.0
Activity				
Total distance (cm)	249.8 ± 30.4	273.1 ± 40.2	297.5 ± 24.9	294.9 ± 50.5
Time spent immobile (s)	49.2 ± 4.5	45.0 ± 4.5	43.6 ± 4.44	46.3 ± 5.5
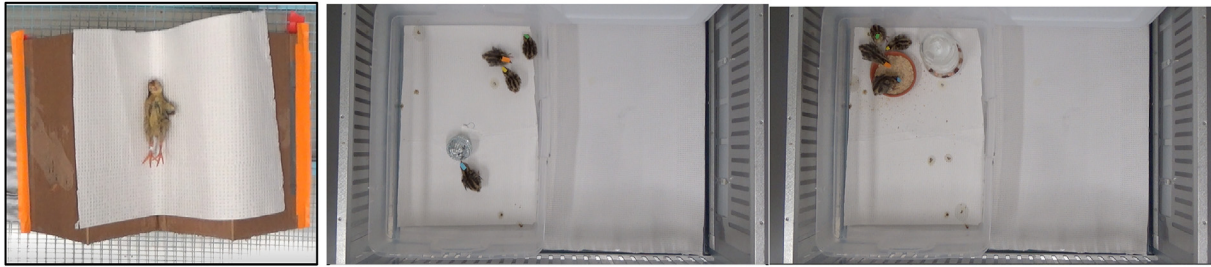
Fig. 1. Tonic immobility (left), neophobia (middle), and aggression (right) tests. Dimensions of the arena were 81.3 × 96.5 × 30.5 cm. For the neophobia, aggression, and activity trials, chicks were tested in a plastic bin within the arena that was identical to the one they were housed in. The activity test was performed following the aggression test, after food and water dishes were removed from the testing bin.

TPHP dosing treatment in order to avoid the potential effect of colour on behaviour. In addition, we avoided using the colour red as there is evidence this colour is a general signal of intimidation in a number of animal species (Pryke, 2009). The order of testing was as follows: neophobia, aggression, activity. Before the neophobia test, chicks were given 5 min in the testing bin to adjust to the unfamiliar individuals in their group. After 5 min, the chicks were gently held in the corner of the testing bin while a novel object (a 5 cm mirror ball ornament) was securely taped in the centre. Chicks were then left to interact with the novel object for 7 min. After the neophobia test, chicks were given a 5 min reprieve in the testing arena, with fresh food and water. Chick social groups were moved from the testing arena into a separate bin where they were deprived of food for 1−1.25 h until the start of the aggression trials (fresh water was available during this period). Before the start of the aggression trial, a mason jar lid was filled with food, and placed in the middle of the testing arena, along with a water dispenser. Chicks were then transferred back into the testing arena, and video-recorded for a 12-min session. After the 12-min aggression trial, food and water were removed, and behaviour in the empty bin was video-recorded for the 5-min activity trial. Timing between tests was optimized during a pilot study in which we noted the time required for chicks to resume their normal behaviour (e.g. preening, foraging, walking around, eating).

2.7. Data analysis

2.7.1. Analysis of behavioural videos

Endpoints analyzed for each behavioural test are summarized in Table 2. Tonic immobility was scored live based on the number of attempts to freeze and freeze time recorded. Data for activity and neophobia trials were analyzed using Ethovision® XT 12 Tracking Software (Noldus Information Technology Inc., Leesburg, Virginia). Tracking was done from a video source, using the colour tape markers to register the individual movements of each quail. For the neophobia trial, time spent near novel object was defined as the total time that the chick's head (i.e., colour marker) was within the novel object zone (defined as within 5 cm of the novel object). Data for the number of pecks to the novel object, and all endpoints for aggression (pecks to conspecifics, hipchecks to conspecifics, and time spent inside the food bowl) were collected manually from the videos. Data were scored blind to the treatment group, with the tape colour being randomly assigned by a person who did not score the videos.

2.7.2. Consideration of batch effects

As documented in our previous publication (Guigueno et al., 2019), chicks from incubation batches 1 and 2 hatched nearly 24 h earlier than expected. We suspect that this was due to an incubator malfunction which resulted in the temperature being ≤1 °C higher than intended. The problem was fixed, and batches 3 and 4 hatched at the expected time. In addition to early hatching (Hepp et al., 2006), elevated heat during incubation has previously been shown to result in lower baseline corticosterone levels in hatched chicks (DuRant et al., 2009), which could, in turn, influence behavioural responses. To test for potential impacts on behaviour, we compared responses in vehicle-treated individuals from batches 1 and 2 (combined) to batches 3 and 4 (combined), and observed a significant effect on time frozen (1&2 > 3&4; $p = 0.0062$), pecks to novel object (1&2 > 3&4; $p < 0.0001$), and number of hip checks given (1&2 < 3&4; $p = 0.02$), with no difference in the other behavioural endpoints ($0.0682 < p < 0.90$). Given these results, we decided to include behavioural data from chicks in batches 3 and 4 only, in the analysis for this publication (see Table 2 for sample sizes). This resulted in a smaller sample size, however, similar results were obtained by including data from all batches and adding "batch" in our models, an approach taken in Guigueno et al. (2019). The full data set is available in the Supplementary Material (Table S1). The remainder of the main paper only includes data for batches 3 and 4.

2.7.3. Statistical analysis

All statistical analyses were run using SAS version 9.3 (SAS Institute Inc, Cary, NC). Hatching success for batches 3 and 4 was calculated as the percentage of fertilized eggs that hatched within 24 h of pipping. We ran a logistic regression (PROC LOGISTIC) to determine whether hatching success varied across treatments.

For all behavioural analyses, treatment was included as a fixed factor. For the data on aggression, body weight was included as a covariate because it was predicted that larger chicks would be better able to stay inside the food bowl and would differ in pecking and hip checking conspecifics. For tonic immobility, a Poisson regression model was used for the count data (i.e., number of attempts until freeze; Proc GENMOD) and a general linear model (Proc GLM) for the time spent frozen, with the data log-transformed to produced normally distributed residuals. For activity level, general linear models were used for total distance (log transformed) and time spent immobile. For neophobia, a Poisson regression model was used for the number of pecks to the novel object, and general linear models for time near novel object using log-transformed data. Finally, for aggression-related analyses, a Poisson regression model was used with treatment as a fixed factor and body weight as a covariate for the number of pecks and number of hipchecks given to conspecifics, and a general linear model for the time spent inside the food bowl; residuals were normally distributed. Significant effects of the TPHP treatment were further analyzed using Tukey-Kramer post-hoc tests. Means and standard errors were calculated for all endpoints. Results were considered statistically significant at a p-value of <0.05.

3. Results

3.1. Hatching

Overall hatching success for all four batches of Japanese quail chicks was previously reported in Guigueno et al., (2019), and was unaffected by TPHP treatment. Hatching success of fertile eggs from batches 3 and 4 alone was 43% ($n = 84$). The value was highest for the vehicle-treated embryos ($n = 22$, 55%), but not significantly different from the low-TPHP ($n = 22$, 32%), mid-TPHP ($n = 17$, 42%), and high-TPHP ($n = 23$, 43%) treatment groups ($X^2_3 = 2.3$, $p = 0.51$).

3.2. Tonic immobility

Of the 5-12 chicks individually tested from each treatment group, one low-TPHP chick and one mid-TPHP chick did not freeze at all. Excluding these two chicks from the analysis, chicks from different groups did not differ in the number of attempts to first freeze ($X^2_3 = 1.89$, $p = 0.60$), nor in the time spent freezing ($F_{3,28} = 0.95$, $p = 0.43$) (Table 2). Among chicks that froze, it took between 1 and 5 attempts to initiate freezing. Freezing times ranged from 1.7 to 99.4 s among individuals. The full data set for all behavioural endpoints is available in the Supplementary Material (Table S1).

3.3. Neophobia

The time spent near the novel object varied from 10.3 to 168.4 s across treatment groups and the number of pecks delivered to the object varied from 2 to 44 pecks. Chicks did not differ significantly in their time spent near the object ($F_{3,20} = 0.57$, $p = 0.64$), (Fig. 2A). However, they differed significantly in the number of pecks to the novel object ($X^2_3 = 20.68$, $p = 0.0001$), with the number of pecks increasing with TPHP dose (Fig. 2B). There was a significant difference between vehicle-control and high-TPHP chicks (adjusted $p = 0.0005$) and between low-TPHP and high-TPHP chicks (adjusted $p = 0.0028$), with no significant difference between other groups ($0.09 < p < 0.968$) (Fig. 2B).

3.4. Aggression

All chicks gathered around the food bowl and began eating immediately upon release into the testing bin for the aggression trail. Individuals controlled access to the food bowl by standing inside it. The length of time that each chick stayed inside the food bowl varied from 2.4 to 540.5 s. Given the small size of the food dish, the chicks competed for optimal position. Chicks pecked at each other's head, body, and legs, and delivered hip checks by hitting other chicks in the hips with their own hips, usually displacing the other chick by a couple of cm. Chicks often pushed out the chick inside the food bowl by pecking or hip checking. Chicks differed significantly between treatments in the number of pecks directed towards other individuals in their social group ($X^2_3 = 49.8$, $p < 0.0001$), with the chicks' body weight having a significant influence on the number of pecks given to conspecifics (covariate: $X^2_1 = 8.02$, $p = 0.0046$). Chicks from the low-TPHP treatment pecked others significantly more than any other group (adjusted $p = < 0.0001$ for all comparisons with the low-TPHP chicks), with no significant differences among the other groups ($0.25 < p < 0.999$) (Fig. 3). There was no significant difference among treatment groups in the number of hip checks given to other individuals ($X^2_3 = 3.01$, $p = 0.39$), nor in the amount of time spent inside the food bowl ($F_{3,19} = 0.18$, $p = 0.92$). Body weight was also a significant covariate for the number of hip checks ($X^2_1 = 5.73$, $p = 0.02$) and the time spent inside the food bowl ($F_{1,19} = 5.09$,
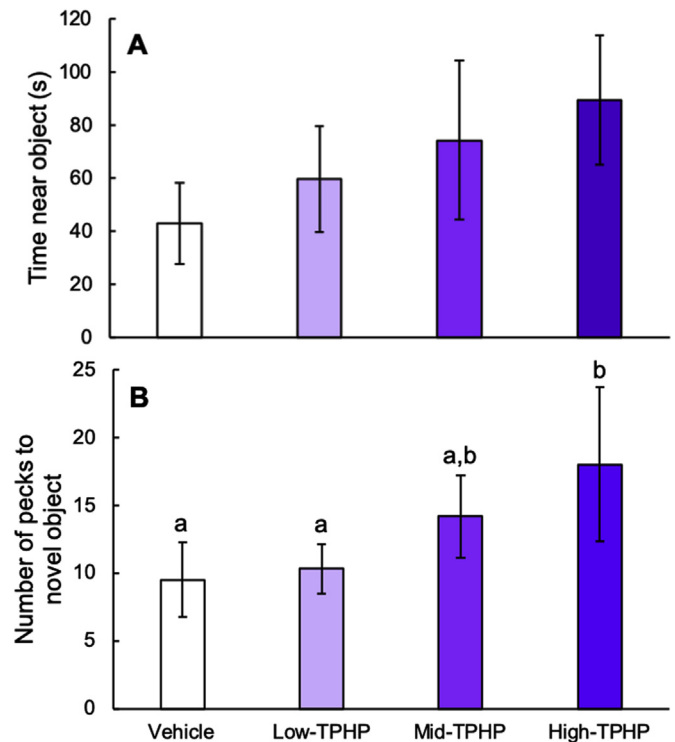

Fig. 2. Measures of neophobia in triphenyl phosphate (TPHP)-exposed Japanese quail chicks presented with a novel object (mirror ornament). The chicks were tested in social groups consisting of one individual from each of the four treatments; vehicle control, Low-TPHP, Mid-TPHP, and High-TPHP (see Table 1 for doses). The sample size for each treatment group was n = 6. Means (±SE) are shown for time spent near novel object (A), and number of pecks to novel object (B). Letters represent results of Tukey-Kramer post-hoc tests with adjusted p-values.

$p = 0.04$).

3.5. Activity level

Chicks traveled between 123 and 506 cm over the course of the 2 min activity trial. On average they spent 46 s immobile. There was no significant difference among treatments in the total distance covered ($F_{3,20} = 0.42$, $p = 0.74$), nor in the time spent immobile ($F_{3,20} = 0.25$, $p = 0.86$) (Table 2).

4. Discussion

Behaviour is considered to be a useful endpoint for ecotoxicological research because it integrates information across multiple levels of biological information and therefore has direct and intuitive relevance to ecologically important outcomes such as growth, reproduction, and survival. It is also considered to be an early warning indicator, being 10-1,000 times more sensitive than more acute endpoints such as mortality (Hellou, 2011; Peterson et al., 2017). A recent review exploring effects of FRs in birds suggested that behavioural endpoints may be more sensitive than physiological or molecular endpoints, with a higher proportion of behavioural studies reporting significant effects (Guigueno and Fernie, 2017). In the current study, we used a Japanese quail model to explore behaviour in early life stage birds exposed to TPHP. This study builds upon previous findings suggesting that TPHP is a potent neurotoxicant and teratogen (Greaves and Letcher, 2014; Noyes et al., 2015) that has been detected in the eggs of wild birds (Greaves and Letcher, 2014; Greaves et al., 2016). Here, the focus was on behaviours associated with fear, boldness, aggression,
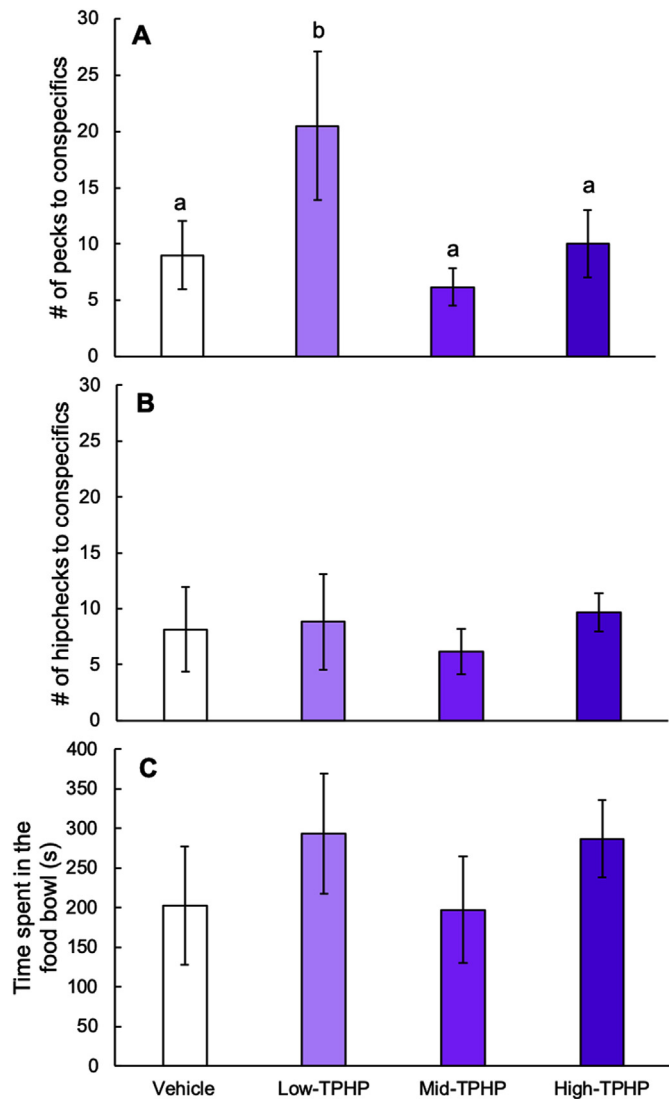
Fig. 3. Measures of aggressive behaviour in triphenyl phosphate (TPHP)-exposed Japanese quail chicks presented with food after a 1-h food deprivation. The chicks were tested in social groups consisting of one individual from each of the four treatments; vehicle control, Low-TPHP, Mid-TPHP, and High-TPHP (see Table 1 for doses). The sample size for each treatment group was n = 6. Means (±SE) are shown for number of pecks directed to conspecifics (A), number of hip checks to conspecifics (B), and time spent inside the food bowl (C). Letters represent results of Tukey-Kramer post-hoc tests with adjusted *p*-values.

and activity, that place individuals on a spectrum of bold to shy (Fraser et al., 2001). The results suggest that exposure to TPHP can increase boldness (expressed as an absence of neophobia), and aggression (expressed in terms of behaviour towards conspecifics) in birds. No associations were found between TPHP exposure and behaviour for an extreme fear response (tonic immobility) or locomotor activity (Table 2).

4.1. Tonic immobility

The tonic immobility test (also referred to as the 'righting response', 'thanatosis' or 'animal hypnosis') is a common behavioural test that can be used for a variety of species including birds (Watsky and Gruber, 1990). It refers to a period of immobility and lack of responsiveness following exposure to a stressful stimulus. Tonic immobility is an unlearned fear response associated with the

autonomic nervous system (Quinn, 2012). In birds, tonic immobility may protect individuals from sustained attack by a predator (Thompson et al., 1981). A propensity for freezing, and longer periods of immobility once frozen, are presumed to be associated with a higher level of fear. Increases in these two endpoints may be considered adaptive or maladaptive depending on the circumstances.

We assessed tonic immobility in Japanese quail chicks at 2 days of age, and found no significant differences across treatment groups for two endpoints; number of attempts to first freeze, and time spent freezing (Table 2). Most individuals performed well at this test; 94% (32/34) of the chicks displayed freeze behaviour within 5 attempts. Among quail chicks, freeze times varied dramatically from 1.7 to 99.4 s with an average freeze duration in the vehicle control group of 21.0 ± 3.3 s. This is within the same range, but lower than the average durations obtained by Launay et al. (1993) and Calandreau et al. (2011), who observed average freeze durations by control Japanese quail of 51.3 and 113.8 s respectively. The difference could be due to the older ages of the chicks; both groups of chicks in the aforementioned studies were 7 days old whereas the chicks in our study were 2 days old.

4.2. Neophobia

Neophobia tests are designed to examine boldness in the context of novel stimuli. Depending on the study design, these tests can be used to provide information on a range of behaviours, such as anxiety and fear of predators (Greggor and Clayton, 2016). Our results suggest that high-TPHP chicks exhibited behaviour consistent with increased boldness, by delivering a significantly higher number of pecks to a novel object. A related measure, time spent near the novel object, was also elevated in a pattern that matched the number of pecks, but this change was not statistically significant (Fig. 2A). To our knowledge, TPHP has not been previously evaluated in the context of neophobia, but one study suggests that TPHP may reduce exploratory behaviour in adult fish (Oliveri et al., 2015). This seems to contrast with the increase in boldness of the high-TPHP Japanese quail chicks in the present study, but many experimental details differ between the two studies (e.g. age, species, exposure method).

4.3. Aggression

Aggression is an antagonistic social interaction. In ecology, aggression is measured in several contexts including sibling rivalry (Morandini and Ferrer, 2015) and competition for food (Enquist et al., 1985). While the effects of TPHP on avian behaviour were previously unstudied, increased aggression was observed in adult male American kestrels during courtship and brood rearing when the birds were exposed to the FR, β-1,2-dibromo-4-(1,2-dibromoethyl)-cyclohexane (β-DBE-DBCH) (Marteinson et al., 2015). The low-TPHP quail chicks in the present study pecked conspecifics more than chicks from any other group, indicating a higher level of aggression (Fig. 3). However, the low-TPHP chicks did not spend more time in the food bowl than birds from other treatment groups. Although we are not aware of other laboratory-based studies that have examined the effects of TPHP on aggression, a human epidemiological study found that *in utero* exposure to OPEs impacts behavioural development in children. Levels of DPHP (a metabolite of TPHP), and bis(1,3-dichloro-2-propyl phosphate) (a metabolite of TDCIPP) in the urine of pregnant mothers were associated with changes in several behaviours of their children, including aggression and hyperactivity (Doherty et al., 2019). It is difficult to compare directly between the former and present studies, but the collective results provide evidence that TPHP and

other FRs can disrupt normal behavioural development, specifically relating to aggression.

TPHP was not observed to affect other aspects of aggression in the present study, such as the time that quail chicks spent in the food bowl or the number of hip checks delivered to conspecifics (Fig. 3). This may suggest that the number of pecks delivered did not have a meaningful effect on the amount of food consumed by each individual. Nevertheless, our findings relating to aggression are in general agreement with the literature suggesting that TPHP can affect behavioural outcomes in vertebrates.

4.4. Activity

Activity level is reflective of mechanical energy expended by an animal, and is frequently correlated with metabolic rate (Wolf and Weissing 2012). However, although we previously reported a significant negative effect of TPHP on resting metabolic rate in Japanese quail chicks (Guigueno et al., 2019), TPHP did not alter activity level in the present study. The absence of a change in activity level in the quail is in contrast with a large number of studies that have associated waterborne exposure to TPHP and other OPEs with hypoactivity in larval zebrafish (Alzualde et al., 2018; Dishaw et al., 2014; Jarema et al., 2015; Noyes et al., 2015; Shi et al., 2018) and medaka (Sun et al., 2016). The concentrations of test chemical used in these studies varied, but in the majority, the effect on activity was observed at concentrations that did not cause direct toxicity (i.e., mortality or teratogenicity). One study with larval zebrafish reported a different response; exposure to the OPE, tris(1,3-dichloroisopropyl)phosphate (TDCIPP), was associated with hyperactivity, and no effect was observed with TPHP (Oliveri et al., 2015). Activity levels of other birds, notably breeding adult American kestrels (*Falco sparverius*), was also influenced by exposure to various FRs, with increased activity reported in birds exposed to β-DBE-DBCH (Marteinson et al., 2015) or the technical PBDE mixture, DE-71 (Fernie et al., 2008), and decreased activity with exposure to HBCD (Marteinson et al., 2012). Embryonic exposure to DE-71 also caused decreased activity (Marteinson et al., 2010).

4.5. Effects of TPHP and other stressors on measures of boldness

Overall, our results suggest an increase in boldness in high-TPHP chicks (expressed as decreased neophobia) and low-TPHP chicks (expressed as increased aggression towards conspecifics). Boldness is a measure of risk-taking behaviour which is assessed on a bold-shy continuum (Fraser et al., 2001) and can encompass traits such as aggressiveness, neophobia, and exploratory behaviour. As aspects of personality, these behavioural traits are often inter-related and consistent within individuals. For example, individuals that are more aggressive also tend to experience less neophobia and an increased willingness to explore new environments (Grunst et al., 2019). Consistent with the literature, we found that TPHP-exposed chicks in general were bolder and more aggressive, although different doses had different effects on these two endpoints.

Previous research exploring the behavioural effects of TPHP has concentrated on aquatic organisms. In contrast to our finding that development exposure to TPHP resulted in bolder chicks, Oliveri et al. (2015) found that adult zebrafish exposed to TPHP are less likely to explore new environments. However, fish treated with TDCIPP fled less frequently when faced with a simulated predator, perhaps indicating an increase in boldness. Other aspects of our study, for example the lack of effect of TPHP on activity level, are also inconsistent with the zebrafish literature where multiple studies (Alzualde et al., 2018; Dishaw et al., 2014; Jarema et al., 2015; Noyes et al., 2015; Shi et al., 2018; Sun et al., 2016)

associate TPHP with hypoactivity, although there are exceptions (Oliveri et al., 2015; Shi et al., 2019). It is not surprising that there are differences between these studies and ours, given that organisms from different vertebrate classes, different exposure regimes, and different life stages were used. Moreover, behavioural tests are sensitive to small differences in methodology.

Ours is the first behavioural study of the effects of OPEs in birds, but previous research suggests that other contaminants, such as mercury and lead, can be associated with decreases in boldness in birds (assessed as increased neophobia and/or more risk aversion) (Megan et al., 2015; Grunst et al., 2018). Additionally, certain urban populations of greater tits are shifted towards the 'bold' end of the bold-shy continuum compared to non-urban populations (Grunst et al., 2019). These studies were completed with wild birds in natural settings. The laboratory-based approached used here could help to elucidate the mechanism of action of some of these effects in relation to particular contaminants.

In the present study, there were significant effects of TPHP on boldness and aggression in Japanese quail chicks. Changes in these two behavioural measures could have important ecological ramifications, although what these might be is difficult to precisely determine (Peterson et al., 2017). For example, behavioural changes such as increased aggression can be considered to be adaptive or maladaptive depending on the context (e.g. Wolf and Weissing 2012; Müller et al., 2011). Regardless of the direction of change, disrupting the normal composition of shy-bold behavioural types in a population can have important ecological ramifications. For example, shoals of Trinidadian guppies (*Poecilia reticulata*) containing a mix of shy and bold individuals provide the most benefits to all individuals involved when approaching a novel food source, compared to groups of only shy or only bold individuals (Dyer et al., 2009).

Interestingly, the most dramatic effects on behaviour in the present study appear to have resulted from the small and unintentional increase in incubation temperature experienced by the batch 1 and 2 quail embryos. Batch 1 and 2 individuals were excluded from the analysis of the effects of TPHP that are presented in the results section of this paper, because of a strong effect of the nominal heat stress on behaviour (see section 2.5.1. 'consideration of batch effects'). These results highlight the importance of properly regulating temperature in avian incubation experiments, and also the sensitivity of the avian embryos to perturbation by a variety of environmental stressors.

5. Conclusion

To our knowledge, the present study provides the first evidence that TPHP can alter behaviour in birds, as has previously been shown for fish and mammals. The results demonstrate that exposure to TPHP can increase boldness and aggression in Japanese quail chicks at concentrations that may be experienced by wild birds (Marteinson et al., 2019). The ecological implications of these changes remain to be determined. This research builds on our understanding that TPHP can disrupt several systems in Japanese quail, including growth, resting metabolic rate, thyroid function, and now, behaviour.

CRediT authorship contribution statement

Ashley K. Hanas: Formal analysis, Investigation, Writing - original draft. **Mélanie F. Guigueno:** Conceptualization, Formal analysis, Investigation, Writing - review & editing, Visualization. **Kim J. Fernie:** Conceptualization, Investigation, Funding acquisition. **Robert J. Letcher:** Conceptualization, Investigation. **François Ste-Marie Chamberland:** Formal analysis, Investigation. **Jessica A.**

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113695.

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