In Ovo Effects of Two Organophosphate Flame Retardants—TCPP and TDCPP—on Pipping Success, Development, mRNA Expression, and Thyroid Hormone Levels in Chicken Embryos

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Received March 4, 2013; accepted April 18, 2013

Tris(1-chloro-2-propyl) phosphate (TCPP) and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) are organic flame retardants detected in the environment and biota for which avian toxicological data are limited. In this study, domestic chicken eggs were injected with TCPP or TDCPP (maximum dose = 51,600 and 45,000 ng/g egg, respectively) to determine dose-dependent effects on pipping success, development, hepatic messenger RNA (mRNA) expression levels of genes associated with xenobiotic metabolism and the thyroid hormone (TH) pathway, and TH levels following 20–22 days of incubation. Neither compound reduced pipping success; however, TCPP significantly delayed pipping at 9240 and 51,600 ng/g and reduced tarsus length at 51,600 ng/g. TDCPP exposure resulted in significant decreases in head plus bill length, embryo mass, and gallbladder size at 45,000 ng/g and reduced plasma free T₄ levels at 7640 ng/g. Type I deiodinase, liver fatty acid-binding protein, and cytochrome P450 (CYP) 3A37 mRNA levels were significantly induced by TCPP, whereas TDCPP induced CYP3A37 and CYP2H1. Chemical analysis of egg contents at incubation days 0, 5, 11, 18, and 19 revealed that > 92% of the injected TCPP or TDCPP concentration was detectable up to day 5; however, < 1% was detected by day 19. The observed phenotypic responses to TCPP and TDCPP exposure may be associated with disruption of the TH axis, which is critical for normal growth and development in birds. The effects of TDCPP on the gallbladder indicate that the disturbance of lipid metabolism is a likely mechanism of toxicity.

Key Words: tris(1-chloro-2-propyl) phosphate; tris(1,3-dichloro-2-propyl) phosphate; organophosphate flame retardant; chicken; mRNA expression; thyroid hormone.

Tris(1-chloro-2-propyl) phosphate (TCPP) and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) are high production volume organophosphate flame retardants (OPFRs) added to a variety of consumer products to prevent flame ignition. TCPP is used primarily in rigid polyurethane foam (PUF) for construction, whereas TDCPP is mainly used in flexible PUF for automotive upholstery (Shaw et al., 2010). In 2000, European production of TCPP reached 36,000 tonnes/year (European Union, 2008a), whereas TDCPP production was about 10,000 tonnes/year (European Union, 2008b); production of these flame retardants (FRs) has since grown as they have replaced other commonly used FRs, such as tris(chloroethyl) phosphate and polybrominated diphenyl ethers, due to health concerns (IPCS, 2004; Stapleton et al., 2009). Because these FRs are not chemically bound to the product to which they are added, they can leach into the environment at ambient conditions; 40% of the added TCPP may be released throughout a product’s lifetime, whereas 10% of TDCPP is available for release (European Union, 2008b). Because neither compound is readily degraded in water or soil (European Union, 2008a,b), they tend to persist and accumulate in the environment, posing potential exposure risks for humans and wildlife.

The rapid metabolism of TCPP and TDCPP in fish and rats suggests that they have little potential for bioaccumulation (European Union, 2008a,b); however, both compounds have been detected across the globe in various environmental media, including biota. Significant sources of wildlife exposure to these FRs include surface water, river water, sediment, and landfill leachate (European Union, 2008a,b). Aquatic wildlife, including mussels and fishes, had concentrations of TCPP and TDCPP up to 1300 and 140 ng/g lipid weight, respectively (Sundkvist et al., 2010; van der Veen and de Boer, 2012). TCPP was detected in various Norwegian bird species and in Great Lakes herring gull eggs at concentrations of 10 and 4.1 ng/g wet weight (ww), respectively (Chen et al., 2012; Leardons et al., 2011). TDCPP concentrations of 1.9 and 0.17 ng/g ww were measured in Norwegian great black-backed gull eggs and Great Lakes herring gull eggs, respectively (Chen et al., 2012; Leardons et al., 2011).
Although TCPP is produced in greater volumes and is often detected at higher environmental concentrations relative to TDCPP, there are fewer studies characterizing its potential toxicity. TCPP has been suggested to cause reproductive toxicity in female mice (European Union, 2008b); however, data are limited on its potential carcinogenicity, embryotoxicity, teratogenicity, and immunotoxicity. TDCPP was recently classified as a chemical carcinogen (OEHHHA, 2011) and has neurotoxic properties in PC12 cells (Dishaw et al., 2011). In addition, overt neurotoxic effects of TDCPP in chickens, such as leg/wing weakness, have been observed at doses > 1.2 mg/g (Ulsamer et al., 1980). TDCPP was cytotoxic in chicken embryonic hepatocytes (CEH) and human adrenal cells (Crump et al., 2012; Liu et al., 2012) and both TCPP and TDCPP upregulated phase I and II xenobiotic-metabolizing enzymes in CEH and zebrafish (Crump et al., 2012; Liu et al., 2012). Furthermore, TDCPP is suspected of being an endocrine disrupting chemical as it has been correlated with decreased circulating thyroid hormone (TH) levels in humans (Meeker and Stapleton, 2010), disrupted sex hormone levels in zebrafish (Liu et al., 2012), and altered messenger RNA (mRNA) levels of TH-responsive genes in CEH (Crump et al., 2012).

In the present study, we injected fertilized chicken (Gallus gallus domesticus) eggs with TCPP or TDCPP to determine their effects on (1) pipping success, (2) embryonic growth and development, (3) chemical accumulation in whole egg contents and specific tissues, (4) hepatic mRNA expression levels of genes associated with xenobiotic metabolism, the TH pathway, lipid metabolism, and growth, and (5) thyroid hormone (T₃) and triiodothyronine (T₄) levels in plasma and thyroid glands. This study assessed numerous levels of biological organization with the goal of linking potential molecular/biochemical changes to overt phenotypic effects.

MATERIALS AND METHODS

**Chemicals and solutions.** TCPP (CAS no. 13674-87-8; >95% purity) was purchased from TCI America (Portland, OR). Stock solutions and serial dilutions were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON). Working solutions were prepared to yield nominal doses of 10, 100, 1000, 10,000, and 50,000 ng TCPP/g egg and 10, 1000, and 50,000 ng TDCPP/g egg. Chemical analysis of the working solutions (described below) indicated that the actual dosing concentrations were close to the desired concentrations; they ranged from 12 to 51,600 ng TCPP/g egg and 9 to 45,000 ng TDCPP/g egg (Table 1).

The internal standard for low chromatography-electrospray ionization (+)-tandem quadrupole mass spectroscopy (LC-ESI(+)-MS-MS), d₁₃-trityl phosphate (d₁₃-TBP), was purchased from Cambridge Isotope Laboratories (Andover, Cambridge, MA). Dichloromethane and n-hexane were purchased from Caledon Laboratories (Georgetown, ON) and high-performance liquid chromatography (HPLC) grade methanol was purchased from Sigma-Aldrich.

**Egg injection and tissue collection.** Three egg injection studies were conducted following protocols approved by the Animal Care Committee at the National Wildlife Research Centre (Environment Canada) using fertilized, unincubated, white Leghorn chicken eggs obtained from the Canadian Food Inspection Agency (Ottawa, ON). Studies 1 and 2 determined the concentration-dependent effects of TCPP and TDCPP on pipping success, embryonic development, tissue-specific accumulation, hepatic mRNA expression, and TH levels. Study 1 included the following groups: DMSO vehicle control (n = 23), 12 ng TCPP/g egg; (n = 21), 90 ng TCPP/g egg (n = 21), 928 ng TCPP/g egg (n = 21), 9240 ng TCPP/g egg (n = 21), and 51,600 ng TCPP/g egg (n = 21). Treatment groups for study 2 were DMSO vehicle control (n = 20), 9 ng TDCPP/g egg (n = 17), 7640 ng TDCPP/g egg (n = 19), and 45,000 ng TDCPP/g egg (n = 41). Study 3 was conducted to determine the concentration of TCPP and TDCPP in whole egg contents throughout incubation following injection of either 51,600 ng TCPP/g egg (n = 5) or 50,222 ng TDCPP/g egg (n = 5); the contents of a random egg from each treatment group were collected on days 0 (unincubated), 5, 11, 18, and 19 postinjection and stored at −20°C until chemical analysis.

Egg injections were performed as previously described (Cassone et al., 2012). For studies 1 and 2, embryos that pipped (days 20–22 of incubation) were euthanized by decapitation and the following measurements were recorded: time to pip, embryo mass, yolk sac mass, liver mass, tarsus length, gallbladder length, and head plus bill length (head + bill: back of head to tip of bill). Pipping success was calculated by dividing the number of embryos that pipped (by day 22) by the total number of fertile eggs. Embryos that did not make a pipping star by the end of day 22 were considered unfit to hatch and were included in the pool of dead embryos, which were excluded from all downstream analyses. Significant differences in morphological/developmental endpoints among groups were determined using a one-way ANOVA followed by a Bonferroni’s t-test for multiple comparisons versus the vehicle control (GraphPad Prism v. 5.02). Changes were considered statistically significant if p < 0.05.

Blood samples for studies 1 and 2 were collected from every individual and mixed with 10 µl of heparin (0.2 mg/ml) and centrifuged (14,000 rpm, 10 min) to separate plasma from red blood cells. Plasma samples were stored at −20°C until subsequent free T₃ and T₄ determination. The left lobe of the liver was collected from the first eight individuals to pip in each group, flash-frozen in liquid nitrogen, and stored at −80°C for RNA extraction. The yolk sac, right lobe of the liver, and left cerebral hemisphere were collected from the same eight individuals and stored at 4°C; yolk sacs and subsamples of liver and cerebral hemisphere were pooled for chemical analysis of TCPP and TDCPP. Finally, both thyroid glands were collected from the same eight individuals and stored at −20°C until subsequent TH determination.

**Determination of TCPP and TDCPP concentrations.** The method for quantification of organophosphate triesters described by Chen and colleagues (2012) was used to determine tissue concentrations of TCPP and TDCPP. In brief, ~0.5 g of tissue was homogenized with diatomaceous earth (J.T. Baker, NJ) and spiked with 5 ng of internal standard d₁₃-TBP. TCPP and TDCPP were extracted from the sample (Dionex ASE 200, Sunnyvale, CA) with 50:50 dichloromethane/hexane. The extract was cleaned and separated on a 1-g ISOLUTE amionpropyl silica gel SPE column (Biotage, Charlotte, NC) packed into a 6-ml Supelclean glass cartridge (Sigma-Aldrich). The eluant was dried, suspended in 200 µl methanol, and filtered through a centrifugal filter (0.2-µm Nylon membrane, 500 µl; VWR, Mississauga, ON). A 10-µl aliquot of the resulting filtrate was used for instrumental analysis. The resulting filtrates for samples from study 3 were diluted 100–250 times prior to chemical quantification of TCPP and TDCPP.

TCPP and TDCPP were determined in sample fractions using a Waters 2695 HPLC system coupled to a Waters Quattro Ultima tandem quadrupole mass spectrometer (Milford, MA) and equipped with a Waters Xterra C₁₈ column (2.1 x 100 mm, 3.5-µm particle size). The mobile phases consisted of water (A) and methanol (B), both spiked with 0.1% formic acid (vol/vol). The elution gradient was as follows: 5% B increased linearly to 70% B over 3 min, then to 80% B over 12 min, followed by a linear increase to 95% B over 3 min and held for 12 min. Finally, it was reduced to 5% B within 1 min and held for 15 min. The MS system was equipped with an electrospray ionization probe operated in positive mode. The detection and quantification of analytes were performed in selected reaction monitoring mode using the most abundant parent and daughter ions for each FR.
Procedural blanks containing only diatomaceous earth spiked with d_{13}-TBP (n = 3) were included to monitor for background contamination. TCPP and TDCPP levels in the blanks were subtracted from tissue sample fractions to yield the reported tissue concentrations. The method limits of quantification in egg samples were previously determined to be 0.20 ng/g ww for TCPP and 0.06 ng/g ww for TDCPP (Chen et al., 2012).

**RNA isolation and cDNA synthesis.** Total RNA was isolated from ~30 mg of the left lobe of the liver (n = 8 per treatment group) using Qiagen RNeasy mini kits according to the manufacturer’s instructions. The concentration and purity of extracted RNA was quantified by determining the A260/A280 absorbance ratio for all samples that had UV absorbance values within the range of 1.8 to 2.1. RNA samples with an A260/A280 ratio > 2.0 were pooled from eight individuals prior to chemical analysis. Concentrations of working solutions and tissue concentrations were determined by HPLC-MS/MS. Pipping success is the number of embryos that pipped by day 22 of incubation divided by the total number of fertile embryos.

**Real-time RT-PCR.** All real-time RT-PCR assays were performed using Brilliant Multiplex QPCR Mastermix kits (Agilent Technologies, no. 600553) and run on a Stratagene MX3000P PCR instrument (La Jolla, CA). Approximately 200 ng of total RNA was DNase treated using DNA-free kits as per the manufacturer’s instructions (Ambion, Austin, TX). Samples with an A260/A280 absorbance ratio above 1.7 were reverse transcribed to cDNA using SuperScript II and random hexamer primers as described by the manufacturer (Invitrogen, Burlington, ON). A 1:5 dilution of cDNA was prepared with diethylylpyrocarbonate (DEPC)-treated water and stored at −80°C for subsequent real-time reverse transcription PCR (real-time RT-PCR).

**Table 1**

Concentrations of TCPP and TDCPP in Liver, Cerebral Hemisphere, and Yolk Sac Sampled from Chicken Embryos 20–22 Days Postinjection and the Effects of Exposure on Pipping Success

<table>
<thead>
<tr>
<th>Flame retardant (FR)</th>
<th>Injected [FR] (ng/g egg)</th>
<th>Liver [FR] (ng/g ww)</th>
<th>Cerebral hemisphere [FR] (ng/g ww)</th>
<th>Yolk sac [FR] (ng/g ww)</th>
<th>Pipping success</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCPP</td>
<td>&lt; 0.2</td>
<td>&lt; 0.2</td>
<td>2.3</td>
<td>&lt; 0.2</td>
<td>20/23 87</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>&lt; 0.2</td>
<td>5.8</td>
<td>2.6</td>
<td>19/21 90</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>1.1</td>
<td>3.5</td>
<td>10.0</td>
<td>17/18 94</td>
</tr>
<tr>
<td></td>
<td>928</td>
<td>2.8</td>
<td>1.5</td>
<td>8.7</td>
<td>18/20 90</td>
</tr>
<tr>
<td></td>
<td>9240</td>
<td>1.1</td>
<td>1.5</td>
<td>6.2</td>
<td>16/20 80</td>
</tr>
<tr>
<td></td>
<td>51,600</td>
<td>4.8</td>
<td>0.7</td>
<td>3.6</td>
<td>16/19 84</td>
</tr>
<tr>
<td>TDCPP</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>&lt; 0.06</td>
<td>0.9</td>
<td>17/19 89</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.6</td>
<td>0.6</td>
<td>1.0</td>
<td>15/17 88</td>
</tr>
<tr>
<td></td>
<td>7640</td>
<td>1.4</td>
<td>8.1</td>
<td>54.0</td>
<td>14/17 82</td>
</tr>
<tr>
<td></td>
<td>45,000</td>
<td>2.0</td>
<td>15.0</td>
<td>100.0</td>
<td>31/40 78</td>
</tr>
</tbody>
</table>

*Ratio %

Note. Tissue samples were pooled from eight individuals prior to chemical analysis. Concentrations of working solutions and tissue concentrations were determined by HPLC-MS/MS. Pipping success is the number of embryos that pipped by day 22 of incubation divided by the total number of fertile embryos.

β-Actin expression was consistent across all treatment groups and was therefore an effective control gene to which all cycle threshold (Ct) data were normalized. The fold change in target gene mRNA expression for treatment groups compared with the DMSO control was calculated using the 2^{ΔΔCt} equation (Schmittgen and Livak, 2008). Significant differences in mRNA expression were determined using a one-way ANOVA of 2^{ΔΔCt}-transformed data followed by a Bonferroni correction for multiple comparisons to identify concentrations that elicited a significant effect relative to the control (p < 0.05). In cases where the assumption of equal variance was violated (Bartlett’s test: p < 0.05; CYP3A37, CYP2H1, and L-FABP), a Kruskal-Wallis test followed by a Dunn’s multiple comparisons test was performed instead of the ANOVA (GraphPad Prism v. 5.02).

**Determination of TH concentrations.** Plasma free T_{3} levels were determined using AccuBind Free T_{3} kits (Monobind Inc., Lake Forest, CA) as per the manufacturer’s instructions. The same eight individuals per group that were analyzed by real-time RT-PCR were selected for free T_{3} determination; any further additions to the sample size were chosen at random. The TCPP dose groups included: DMSO control (n = 9), 12 ng/g egg (n = 9), 90 ng/g egg (n = 9), 928 ng/g egg (n = 10), 9240 ng/g egg (n = 9), and 51,600 ng/g egg (n = 10). The TDCPP dose groups assessed were: DMSO control (n = 15), 9 ng/g egg (n = 13), 7640 ng/g egg (n = 12), and 45,000 ng/g egg (n = 28). Free T_{3} concentrations (ng/dl) were calculated from a standard curve generated using the six reference samples (fit with a four-parameter variable slope model; GraphPad Prism v. 5.02). The limit of detection (LOD) was 0.05 ng/dl; values equal or less than this were assigned a value of 0.035 (LOD/√2). A one-way ANOVA followed by a Bonferroni correction was used to determine significant differences in free T_{3} concentrations between the treatment groups (p < 0.05).

Free T_{3} levels in plasma were determined using AccuBind Free T_{3} kits (Monobind Inc.) as per the manufacturer’s instructions, and analyzed as described above for T_{3}. The dose groups for TCPP were: DMSO control (n = 9), 12 ng/g egg (n = 8), 90 ng/g egg (n = 8), 928 ng/g egg (n = 9), 9240 ng/g egg (n = 8), and 51,600 ng/g egg (n = 10). For TDCPP, the following groups were included for T_{3} determination: DMSO control (n = 8), 9 ng/g egg (n = 7), 7640 ng/g egg (n = 6), and 45,000 ng/g egg (n = 8).

Extraction of THs from thyroid glands was performed using a method modified from McNabb et al. (2004). Briefly, thyroid glands were homogenized (Retsch MM301 Mixer Mill, Newtown, PA) in 100 μl of digestion medium (0.605 g Tris base, 40 μg ura, 1 ml Triton X-100, and 100 ml H₂O titrated to pH 8.0) containing 7 mg of Pronase E (Sigma-Aldrich, St Louis, MO) per gram of thyroid and incubated at 37°C for 24 h. Extraction was continued by adding
1 ml of absolute ethanol and incubating at −20°C for 24 h. Samples were then centrifuged and the supernatant was collected. Presumably all bound T4 became free so TH measurement was of total T4 in thyroid. Prior to total T4 determination (AccuBind Free T4 kit; described above), samples were diluted 10× to 50× depending on the thyroid mass. A total of eight individuals were included for all dose groups with the exception of the DMSO (n = 7) and 12 ng/g (n = 7) dose groups from study 1.

RESULTS

Pipping Success

Neither TCPP nor TDCPP affected the pipping success of chicken embryos up to the highest administered dose (HD = 51,600 ng TCPP/g or 45,000 ng TDCPP/g; Table 1). The minimum pipping success in the HD TDCPP group was 78%, which falls within the range of values previously observed for DMSO-treated chicken embryos (Cassone et al., 2012; Crump et al., 2011; O’Brien et al., 2009). TCPP significantly delayed pipping time at the two highest doses tested. The average DMSO-treated embryo required 495 h (20.6 days) to pip, whereas embryos exposed to 9240 or 51,600 ng TCPP/g required an additional 13 or 17 h, respectively (Fig. 1). TDCPP-exposed embryos also showed a trend toward delayed pipping with increasing treatment, with a maximum 10-h delay at 7640 ng/g; however, this delay was not statistically significant (Fig. 1).

Embryonic Development

Both TCPP and TDCPP had an effect on at least one of the morphological endpoints measured. HD TCPP caused a significant reduction in tarsus length (24.2 vs. 25.4 mm) and a dose-dependent increase in liver somatic index (LSI) (Figs. 2A and B). Embryos exposed to HD TDCPP had a significantly reduced mass (26.8 vs. 28.7 g) and shorter head + bill (26.3 vs. 27.6 mm) compared with the DMSO group (Figs. 2C and D). There was also a significant reduction in gallbladder size of embryos treated with HD TDCPP (Fig. 3A); 4 out of 31 embryos in the HD group did not have a gallbladder (determined by visual inspection). Where measurements could be made, the gallbladder of a HD TDCPP-treated embryo was, on average, 42% of the size of a gallbladder from the DMSO group. There was a significant positive correlation between gallbladder size and embryo mass (Fig. 3B; Pearson correlation: p < 0.05).

TCPP and TDCPP Concentrations

Liver, cerebral hemisphere, and yolk sac (studies 1 and 2). TCPP was detected in the liver, cerebral hemisphere, and yolk sac for all dose groups except the 12 ng/g group; however, tissue concentrations were not correlated with the injected concentrations (Table 1). There was a positive correlation between injected TDCPP concentrations and tissue concentrations (Table 1), but this was not statistically significant; concentrations increased in a dose-dependent manner for all tissues reaching a maximum of 2.0, 15, and 100 ng/g ww for the liver, cerebral hemisphere, and yolk sac, respectively, in the highest

### Table 2

List of Transcripts Assessed by Real-Time RT-PCR in Liver Tissue of Chicken Embryos Exposed to TCPP and TDCPP In Ovo

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type III iodothyronine 5′-deiodinase (D3)</td>
<td>NM_001122648</td>
<td>Yongphachan et al. (2011)</td>
</tr>
<tr>
<td>Type II iodothyronine 5′-deiodinase (D2)</td>
<td>NM_204114</td>
<td>Crump et al. (2010)</td>
</tr>
<tr>
<td>Insulin-like growth factor-1 (IGF-1)</td>
<td>NM_001004384</td>
<td></td>
</tr>
<tr>
<td>β-Actin (BA)</td>
<td>X00182</td>
<td></td>
</tr>
<tr>
<td>Type I iodothyronine 5′-deiodinase (D1)</td>
<td>NM_001097614</td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450 2H1 (CYP2H1)</td>
<td>NM_001001616</td>
<td>Crump et al. (2009)</td>
</tr>
<tr>
<td>Cytochrome P450 3A37 (CYP3A37)</td>
<td>NM_001001751</td>
<td>Crump et al. (2008)</td>
</tr>
<tr>
<td>Uridine 5′-diphospho-glucuronosyltransferase 1A9 (UGT1A9)</td>
<td>XM_421883</td>
<td></td>
</tr>
<tr>
<td>Transthyretin (TTR)</td>
<td>NM_205335</td>
<td></td>
</tr>
<tr>
<td>Liver fatty acid-binding protein (L-FABP)</td>
<td>AF380998</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Effects of in ovo TCPP and TDCPP exposure on pipping time of chicken embryos. Pipping time is the number of hours of incubation required by the embryo to form a pipping star. Significant delays are indicated for TCPP relative to the DMSO control. Error bars represent the SEM (n = 16–20; **p < 0.01, ***p < 0.001).
dose group. These values are 22,500, 3000, and 450 times lower than the injected dose of 45,000 ng/g.

Egg contents (study 3). The contents of chicken eggs that were injected with 51,600 ng TCPP/g egg or 50,222 ng TDCPP/g egg contained 64,876 ng TCPP/g or 46,589 ng TDCPP/g on day 0 (> 92% of the injected concentrations; Fig. 4). TCPP and TDCPP concentrations decreased to 26,713 and 18,951 ng/g by day 11 and were further reduced to 1 and 5 ng/g by day 19 (< 1% of the injected concentrations).

Hepatic mRNA Expression

Of the nine mRNA transcripts assessed, only three were significantly affected by TCPP treatment (Fig. 5). There was a dose-dependent increase in D1 mRNA expression following TCPP exposure, with a significant twofold induction at the HD. Hepatic L-FABP mRNA expression was significantly induced 2.7-fold and CYP3A37 was upregulated 4.5-fold in the TCPP HD treatment group; no significant effects were observed at the lower treatment concentrations. Two phase I metabolizing enzymes, CYP3A37 and CYP2H1, were significantly induced 7.9-fold and 2.1-fold, respectively, in embryos treated with HD TDCPP (Fig. 6). No significant changes in hepatic mRNA expression were observed for the other transcripts (D2, D3, TTR, and UGT1A9) in chicken embryos exposed to TCPP or TDCPP (data not shown).

TH Status

TDCPP-exposed chicken embryos had lower plasma free T4 levels relative to controls at all concentrations tested; however, only the decrease at the 7640 ng/g dose was statistically significant (Fig. 7). There were no significant effects on plasma free T3 levels of TCPP-exposed embryos. Neither FR caused significant changes in plasma free T3 nor thyroid gland total T4 concentrations in any of the dose groups assessed (data not shown).

DISCUSSION

To our knowledge, this is the first study to investigate the effects of embryonic exposure to TCPP or TDCPP in an avian species. We determined the effects of in ovo exposure on pipping success, morphological growth parameters, hepatic mRNA expression, and TH levels in chicken embryos at a wide range of doses; the lowest dose was similar to concentrations detected in wild avian species (Chen et al., 2012; Leonards et al., 2011).
Due to the rise in production volume of these OPFRs (Stapleton et al., 2009; van der Veen and de Boer, 2012) and their persistence in the environment and biota, it is important to understand the biological and toxicological implications of exposure.

Neither TCPP nor TDCPP elicited a lethal response to treatment following injections up to 51.6 and 45 µg/g egg, respectively. These maximal injected concentrations were lower than the minimum acute LD50 values previously determined in rats: 1017 µg TCPP/g body weight (bw) and 2250 µg TDCPP/g bw (IPCS, 2004). The only avian studies available for these FRs investigated neurotoxic potential and were based on posthatch exposures (IPCS, 2004). Exposure of hens to two separate doses of 13,200 µg TCPP/g bw was not lethal (Sprague et al., 1981), whereas a 5-day oral exposure to 4800 µg TDCPP/g/day bw in chickens caused 100% mortality (Ulsamer et al., 1980). No mortality was observed however after a 90-day oral exposure up to 100 µg TDCPP/g/day (IPCS, 2004).

In the present study, delays in pipping up to 17 and 10 h were observed following TCPP and TDCPP exposure, respectively. This finding could have variable consequences based on the parent-young relationship at hatching. Precocial birds, such as chickens, tend to hatch synchronously and leave the nest soon after hatching (Nice, 1962). Delayed pipping could result in a late hatchling being (1) abandoned and/or (2) unable to compete for resources due to its smaller size. It is well established that THs peak in the perihatch period, stimulating metabolic and developmental processes necessary for pipping (McNabb, 2007), and numerous studies have demonstrated the correlation between hypothyroidism and delayed hatching (Balaban and Hill, 1971; Decuypere and Kûhn, 1988; Haba et al., 2011).

Both TCPP and TDCPP affected at least one of the morphological endpoints measured. The tarsus length of embryos exposed to HD TCPP was significantly reduced relative to controls. King and May (1984) emphasized the importance of THs in the final 4 days of incubation on chicken embryo growth; exposure to goitrogens late in incubation caused a 35% decrease in leg growth (King and Delfiner, 1974). HD TCPP also increased LSI, which is a general indicator of metabolic energy demands, is sensitive to environmental contamination (Adams et al., 1993), and is viewed as an adaptive response to increase the detoxification capacity of the liver (Goede and Barton, 1990). TDCPP treatment led to a significant reduction of head + bill length and embryo mass. Comparable results have been observed for TDCPP in other species; prenatal exposure in rats caused an increase in fetal death and a decrease in maternal body weight at 400 µg/g/day (Tanaka et al., 1981), and exposure to ≥ 50 µg/l decreased body weight in zebrafish (Wang et al., 2013).

The most pronounced effect of TDCPP exposure was the reduction in gallbladder size of HD-treated embryos. Four out of 31 embryos did not develop a gallbladder, and those that had gallbladders large enough to be measured were ~42% the size of those from the DMSO control. Only one incidence of

FIG. 3. Effects of TDCPP exposure on the gallbladder size of developing chicken embryos. (A) The reduction in gallbladder size with increasing TDCPP treatment. Error bars represent the SEM (n = 8–11; ***p < 0.001). (B) The correlation between gallbladder size and embryo mass (significant Pearson correlation: p < 0.05). The solid line depicts a linear regression with an R² = 0.27.
gallbladder agenesis has been reported in an avian species and was caused by the potent carcinogen diethylnitrosamine (Williams et al., 2011). Studies on mice did not report any effect of TDCPP on the gallbladder at doses well above those tested in this study (Kamata et al., 1989). Bile acids, which are stored and concentrated in the gallbladder, aid in the digestion and absorption of fatty acids (Schmidt and Ivy, 1937). A reduction in bile flow could help explain the depressed growth in TDCPP-treated chicken embryos; the inability to utilize the available lipid resources in the yolk efficiently would reduce the embryo’s energy supply, thereby hindering its growth. Gallbladder size was positively correlated with embryo mass in this study (Fig. 3) and embryos without gallbladders had some of the lowest body weights. This finding is consistent with a previous study that associated reductions in chicken embryo weight and tarsus length with a reduced yolk lipid uptake (Feast et al., 1998). Further research on the mechanism(s) of gallbladder development in birds is warranted in order to understand the nature of the disruption caused by TDCPP exposure.

Concentrations of TCPP and TDCPP in liver, cerebral hemisphere, and yolk sac were surprisingly low relative to injected concentrations. The lack of correlation between TCPP tissue concentrations and injected concentrations can be attributed, in part, to TCPP contamination associated with sample preparation. For example, the concentration of TCPP in the DMSO dosing solution was below the LOD, whereas the concentration in cerebral hemispheres from the DMSO group was 2.3 ng/g (Table 1). This indicates that the TCPP detected in cerebral tissue did not originate from the DMSO dosing solution but through the sample preparation (e.g.,

![FIG. 5. Hepatic mRNA expression of TH-responsive genes (D1, L-FABP) and a xenobiotic-metabolizing enzyme (CYP3A37) in chicken embryos exposed to TCPP. Injected concentrations are in ng TCPP/g egg. Fold changes are presented relative to the DMSO vehicle control, as are significant changes in expression. Error bars represent the SEM (n = 8; *p < 0.05, **p < 0.01, ***p < 0.001).](image1)

![FIG. 6. Effect of TDCPP exposure on hepatic mRNA expression of two xenobiotic-metabolizing enzymes, CYP2H1 and CYP3A37, in chicken embryos. Fold changes are presented relative to the DMSO vehicle control, as are significant changes in expression. Error bars represent the SEM (n = 8; *p < 0.05, **p < 0.01).](image2)

![FIG. 7. Free plasma thyroxine (T4) levels of chicken embryos exposed to increasing concentrations of TDCPP. Significant changes are indicated relative to the DMSO control. Error bars represent the SEM (n = 12–28; **p < 0.01).](image3)
solvents used during extraction). Furthermore, because residual tissue concentrations of TCPP were so low, they effectively fell within the background levels of TCPP, further obscuring the relationship between injected concentrations and tissue concentrations. A time course study (study 3) was carried out to determine whether the low tissue concentrations at pipping were due to (1) rapid metabolism of the parent compounds during development and/or (2) preferential accumulation in tissues that were not examined. More than 92% of the injected FR was detected in the egg content on day 0 of injection; however, by day 19, < 1% of the parent compound was detected. The metabolic function of the liver is established in chicken embryos by day 7 of incubation (Sandström and Westman, 1971). This supports the likelihood that enzyme-mediated metabolism of TCPP and TDCPP occurred between days 5 and 19 of incubation leading to extremely low tissue residue concentrations relative to the injected doses in studies 1 and 2. It is remarkable that the developing chicken embryo was able to metabolize/eliminate almost 2.5 mg of FR (50,000 ng FR/g egg; average egg = 50 g), especially considering that TCPP and TDCPP have been detected in wild avian eggs (Chen et al., 2012; Leonards et al., 2011) that were likely exposed to far lower quantities through maternal transfer. It is possible that the enzymes necessary for complete biotransformation are only activated above a certain exposure threshold that is not reached at current environmental levels. The rapid metabolism of these FRs in chicken embryos agrees with studies performed in rats in which > 95% of TCPP or TDCPP was eliminated within 7 days of treatment (Lynn et al., 1981; Minegishi et al., 1988).

In addition to the determination of phenotypic alterations caused by TCPP or TDCPP exposure, we assessed effects on hepatic mRNA levels of genes associated with xenobiotic metabolism, TH homeostasis, and lipid metabolism; these genes have previously been identified as responsive to FR exposure (Crump et al., 2008, 2010, 2011). Furthermore, developmental effects observed in this study and endocrine effects, observed in previous studies on TDCPP (Liu et al., 2012; Meeker and Stapleton, 2010; Wang et al., 2013), supported the determination of TH levels in plasma and thyroid gland.

CYP3A37 and CYP2H1 are phase I xenobiotic-metabolizing enzymes regulated by the chicken xenobiotic receptor (Podvinec et al., 2002). The mRNA levels of CYP3A37 and CYP2H1 were increased after TDCPP exposure, whereas TCPP only induced CYP3A37. Our findings are consistent with a recent avian in vitro study, which demonstrated that CYP3A37 was the most responsive gene to TCPP and TDCPP exposure in CEH (Crump et al., 2012). Induction of these CYPs is associated with enhanced biotransformation of xenobiotic compounds (Goriya et al., 2005) and is likely what led to the almost complete elimination of TCPP and TDCPP. Ideally, CYP3A37 enzyme activity would be monitored throughout incubation to confirm this hypothesis, but an avian CYP3A37 activity assay has not yet been developed in our laboratory. An increase in LSI has also been associated with activation of CYPs (Huuskonen and George, 1995), an observation that is consistent with TCPP-exposed embryos in this study.

D1, one of three deiodinase enzymes assessed, was upregulated in response to TCPP treatment. Induction of hepatic D1 often indicates an increased conversion of T3 to T4 to maintain circulating TH levels (Darras et al., 2006). In rats, a hypothyroid state is associated with decreased hepatic D1 activity (Santini et al., 1993). The TH-deiodinase relationship in birds however is less clear; some have observed transient increases in expression of D1 with reduced plasma TH levels (Beck et al., 2006), whereas others have seen a marked decrease in D1 expression (Gould et al., 1999). Finally, L-FABP, a lipid-binding protein involved in fatty acid transport, uptake, and metabolism (Wang et al., 2006), was induced by TCPP treatment. L-FABP mRNA levels were responsive to T3 administration in hypothyroid rats (Iwen et al., 2001) and were higher in hyperthyroid versus hypothyroid chickens (Cogburn et al., 2003). The disruption in expression of TH-responsive genes and genes involved in metabolism may explain the observed delay in pipping of TCPP-exposed embryos; Willemesen et al. (2011) associated changes in metabolic rate during late incubation to delays in hatching.

The TH pathway serves numerous functions, including metabolic maintenance and pipping initiation, and is essential for normal growth and development in birds (McNabb, 2007). TCPP did not affect plasma or glandular THs at pipping in this study; however, disruption in TH levels is not always apparent at external pipping (first break in the shell). For example, chicken embryos exposed to polychlorinated biphenyl-77 had reduced plasma TH levels and were delayed in pipping by 12 h (Roelens et al., 2005); plasma T3 levels were significantly reduced at internal pipping (penetration of the inner membrane) but were restored to normal by external pipping (12–24 h later). It is possible that the internal pipping stage of TCPP-exposed embryos was prolonged due to a disruption of the TH pathway; however, TH levels were only measured at external pipping, thereby potentially overlooking earlier disruption.

Conversely, a significant reduction in circulating free T3 levels was observed in TDCPP-treated embryos, albeit only at 7640 ng/g. The slight increase at 45,000 ng/g, compared with 7640 ng/g, might be considered nonmonotonic, which would not be unusual among endocrine disrupting chemicals (Vandenberg et al., 2012); however, the T3 level at the HD remains well below the DMSO control and does not likely reflect a reverse in trend. The depleted T3 levels may have been associated with the observed reduction in mass and head + bill length, and because numerous organ systems depend on THs for tissue-specific differentiation (McNabb, 2007), it may have also hindered gallbladder development. Furthermore, studies have shown hypothyroidism to alter bile composition due to effects on lipid metabolism (Andreini et al., 1994; Day et al., 1989) and reduce bile flow in mammals (Laukkarinen et al., 2002, 2003), which could have led to the reduced gallbladder size. The endocrine disrupting
potential of TDCPP has previously been observed in humans and in fish. Meeker and Stapleton (2010) associated TDCPP concentrations in dust with an increase in serum prolactin levels and a decrease in free plasma T₄ levels of the household residents. A recent study on zebrafish (Liu et al., 2012) found that TDCPP disrupted the balance of sex hormones such as 17β-estradiol and testosterone and effected the expression of vitellogenin. Our observations of reduced growth and free T₄ levels in TDCPP-exposed chicken embryos support the existing pool of evidence that marks TDCPP as a potential endocrine disrupting chemical.

In conclusion, no adverse morphological or developmental effects were observed at environmentally relevant doses of TCPP (9 ng/g egg) or TDCPP (12 ng/g egg). However, at injected concentrations, three orders of magnitude higher, TCPP increased LSI, delayed pipping time, reduced tarsus length, and altered gene expression associated with xenobiotic metabolism, the TH axis, and lipid metabolism. TDCPP exposure impaired embryo growth, gallbladder development, and plasma T₄ levels and affected the mRNA levels of phase I metabolizing enzymes. Furthermore, we showed that even at the highest administered concentration, TCPP and TDCPP were almost completely depleted in ovo by day 19 of incubation. To further understand the link between the observed molecular/biochemical disturbances and adverse phenotypic outcomes, a genome-wide microarray expression study is currently underway for these two FRs. Although the present study suggests that current environmental levels are unlikely to cause adverse effects to avian embryo development, the endocrine disrupting potential of these compounds warrants further investigation.

FUNDING

Funds from three sources within Environment Canada: Chemicals Management Plan; Strategic Technology Applications of Genomics for the Environment; Ecotoxicology and Wildlife Health Division.

ACKNOWLEDGMENTS

The authors declare that there are no conflicts of interest.

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