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In ovo transformation of two emerging flame retardants in Japanese quail (*Coturnix japonica*)

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22 **Abstract**

23 Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) and Dechlorane Plus (DP) are two
24 chlorinated, alternative flame retardants that have been found in wild birds and bird eggs. Little
25 is known about the fate and effect of these compounds in birds, especially during the vulnerable
26 stages of embryonic development. To investigate the ability of birds to biotransform these
27 compounds, an *in ovo* exposure experiment with Japanese quail eggs was performed. Quail
28 eggs were injected in the yolk sac with 1000 ng/g egg of TDCIPP (2.3 nmol/g ww), DP (1.5
29 nmol/g ww) or a mixture of both and were then incubated at 37.5 °C for 17 days. To get a time-
30 integrated understanding of the *in ovo* transformation of the compounds, one egg per treatment
31 was removed from the incubator every day and analyzed for TDCIPP and its metabolite bis(1,3-
32 dichloro-2-propyl) phosphate (BDCIPP) and/or for DP. By the end of the incubation period,
33 TDCIPP was completely metabolized, while simultaneously BDCIPP was formed. The
34 conversion of the parent compound into the metabolite did not occur proportionally and the
35 concentration of BDCIPP showed a tendency to decrease when TDCIPP became depleted, both
36 indicating that BDCIPP was further transformed into compounds not targeted for analysis.
37 Further untargeted investigations did not show the presence of other metabolites, possibly due
38 to the volatility of the metabolites. On the other hand, the DP concentration did not decrease
39 during egg incubation. This study indicates that within the incubation period, avian embryos
40 are able to biotransform TDCIPP, but not DP.

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42 Keywords: biotransformation, *in ovo,* tris(1,3-dichloro-2-propyl) phosphate, Dechlorane Plus,
43 flame retardants, Japanese quail

1. Introduction

Consumer products, such as textiles, building materials, electric and electronic equipment and furniture, consist largely of different types of polymers. Most of these polymers are petroleum-based, which renders them flammable (Alaee et al., 2003). To comply with the increasingly strict fire safety standards, flame retardants (FRs) are added to these products. Flame retardants are chemicals used to prevent ignition and slow down combustion in case of fire. However, these compounds are not always chemically bound to the material (additive FRs) and can therefore easily leach out into the environment during any point of the product's life cycle. In recent decades, it has been shown that some FRs are persistent and exert toxic effects in biota (Ezechiáš et al., 2014; Guigueno and Fernie, 2017; van der Veen and de Boer, 2012). This has created a paradox between fire safety regulations and environmental and health safety regulations. The strict regulations on the use of some brominated FRs by the Stockholm Convention (UNEP, 2017) and other directives have caused an increase in the production and usage of new and unrestricted alternatives, such as chlorinated and phosphorous FRs. Two alternative FRs that replace restricted compounds are tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) and Dechlorane Plus (DP). TDCIPP (Fig. 1a) is a chlorinated organophosphate ester (OPE) that has been produced since the 1970s in order to replace tris(2,3-dibromo-propyl) phosphate (or Tris) in textiles, after the latter was shown to have mutagenic properties (Blum and Ames, 1977). TDCIPP is mainly used in polyurethane foams and is one of the most commonly detected FRs in residential furniture (Stapleton et al., 2012). Multiple common and trade names (Fyrol FR-2, TDCP, TDCPP, etc.) have been used (van der Veen and de Boer, 2012) but for consistency, the acronym TDCIPP will be used throughout the present article. DP is a chlorinated FR that is produced since the mid-60s as a substitute for Dechlorane (or Mirex). It is used among others in electrical hard plastic connectors and cable coatings in

68 televisions and computers (Feo et al., 2012). The commercial mixture of DP consists of two
69 stereoisomers, *syn* and *anti* (Fig. 1b and c) in a 35:65 ratio (Oxychem, 2013).

70 Both TDCIPP and DP have been detected in the environment and biota, including birds (Chen
71 et al., 2013; Eulaers et al., 2014; Greaves and Letcher, 2014; Marteinson et al., 2016) and bird
72 eggs (Barón et al., 2014; Champoux et al., 2017; Chen et al., 2012; Greaves and Letcher, 2014;
73 Guerra et al., 2011; Muñoz-Arnanz et al., 2011, 2012; Su et al., 2015; Vorkamp et al., 2015).
74 Their presence in bird eggs provides evidence for maternal transfer and may pose a risk to
75 embryonic development and/or later developmental stages. This raises questions on the
76 metabolizing capacity of avian embryos. OPEs have been shown to be rapidly metabolized in
77 mammals by phase I and II metabolism (Hou et al., 2016). DP on the other hand, has shown
78 not to metabolize easily in biota (Meeker et al., 2013; Tomy et al., 2008; Xian et al., 2011).
79 Degradation products of DP have been detected in bird eggs (Guerra et al., 2011; Muñoz-
80 Arnanz et al., 2011, 2012; Zheng et al., 2014a), but some studies suggest they are formed
81 through biotic or abiotic processes prior to uptake or even through analytical impurities (Sverko
82 et al., 2008, 2010; Tomy et al., 2008; Zheng et al., 2010, 2014b). Until now, information on
83 exposure and metabolism of TDCIPP and DP during the embryonic development of birds is
84 very scarce. Farhat et al. (2013) previously showed a decrease of TDCIPP *in ovo* during
85 incubation and Zheng et al. (2014a) observed no change in DP concentrations in eggs from
86 three different time points. However, neither the major metabolite of TDCIPP, bis(1,3-
87 dichloro-2-propyl) phosphate (BDCIPP), nor the general kinetics of the metabolism have been
88 investigated in eggs prior to the present study. Other studies on the metabolism of these
89 compounds had an *in vitro* approach (Chabot-Giguère et al., 2013; Greaves et al., 2016) or
90 were focused on mammals (Lynn et al., 1981; Meeker et al., 2013; Van den Eede et al., 2013).

91 The objective of this study was to investigate the ability of avian embryos to biotransform two
92 alternative flame retardants, TDCIPP and DP, by means of an *in ovo* experiment. An *in ovo*

93 experiment offers the advantage of mimicking exposure through maternal transfer and allows
94 us to study the effect of a compound or a mixture of compounds on the development of the
95 bird. Here, the Japanese quail (*Coturnix japonica*) was used as an avian model species. Because
96 of its short developmental period and small egg size, this precocial species is very suitable for
97 toxicokinetic studies in the early development (Huss et al., 2008; Jaspers, 2015).

98 **2. Materials and methods**

99 *2.1 Chemicals and standards*

100 Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP, CAS no. 13674-87-8; 100 µg/mL in toluene)
101 and Dechlorane Plus *syn*- (*syn*-DP, CAS no. 135821-03-3; 50 µg/mL in toluene) and *anti*- (*anti*-
102 DP, CAS no. 135821-74-8; 50 µg/mL in toluene) isomers used for egg injections were
103 purchased from AccuStandard (New Haven, CT, USA). Lecithin and peanut oil were purchased
104 from Merck (Darmstadt, Germany). Ethanol was purchased from VWR International LLC
105 (Radnor, PA, USA).

106 Individual standards of TDCIPP, BDCIPP, *syn*- and *anti*-DP and the corresponding labelled
107 internal (TDCIPP-d_{15}, BDCIPP-d_{10}, ^{13}C-*syn*-DP and ^{13}C-*anti*-DP) and recovery (triphenyl
108 phosphate-d_{15} / TPhP-d_{15}, 2,2',3,3',4,4',5,6,6'-nonachlorobiphenyl / CB207, β-
109 hexabromocyclododecane-d_{18} / β-HBCDD-d_{18} and tris(2-chloroethyl) phosphate-d_{12} / TCEP-
110 d_{12}) standards for targeted and untargeted chemical analysis were obtained from Wellington
111 Laboratories (Guelph, Ontario, Canada). The recovery standard 1,3-dichloro-2-propanol-d_5
112 (1,3-DCP-d_5) for untargeted chemical analysis was purchased from Sigma-Aldrich (St. Louis,
113 MO, USA). All solvents used (*n*-hexane, acetone acetic acid, ammonia, methanol,
114 dichloromethane / DCM, sulfuric acid, *iso*-octane, cyclohexane and ethyl acetate) were of
115 SupraSolv (Merck, Darmstadt, Germany) or Picograde (LGC Promochem, Wesel, Germany)

116 grade. Sodium sulfate (Na_2SO_4, Merck) and silica gel (0.063 - 0.200 mm, Merck) were pre-
117 washed with *n*-hexane and heated overnight at 150 °C before use.

118 *2.2 Vehicle preparation*

119 An emulsion of peanut oil and water, using lecithin as an emulsifier, was used as a vehicle to
120 dissolve the compounds in the egg yolk. The emulsion was prepared in accordance to
121 Brunström and Örberg (1982) by dissolving lecithin (L-α-phosphatidylcholine from egg yolk)
122 in DCM and peanut oil. Then DCM was evaporated under a stream of clean air, using a heating
123 plate at 35 °C and a stirring magnet. The compounds in solution were then added to the
124 lecithin/peanut oil mixture and the toluene was evaporated using a rotary evaporator (RV 10
125 digital, IKA) at 150 mbar and approximately 50 °C. In the framework of a larger experiment
126 where the effect of mixture toxicity was assessed, we also investigated the biotransformation
127 of the two compounds when occurring in a mixture. Therefore, the final emulsions contained
128 TDCIPP or DP (*syn*- and *anti*-isomer, nominal proportion 30:70) singly or in an equal mixture
129 of the two, in a concentration of 500 µg/mL. Subsequent chemical analysis indicated that the
130 actual proportions of *syn*- and *anti*-DP in the emulsion ranged between 31:69 and 34:66. After
131 autoclave sterilization (25 min, 120 °C), two parts (1.6 mL) of emulsion were mixed with three
132 parts (2.4 mL) of sterile distilled water to mimic the lipid:water proportion of egg yolk
133 (Brunström and Örberg, 1982). The emulsions were sonicated (Ultrasonic Cleaner, VWR)
134 during 30 seconds after which they were injected. Chemical analysis of the control emulsions
135 did not show any peaks.

136 *2.3 Egg injection and incubation*

137 The egg experiment was performed in the animal laboratory facilities at the Department of
138 Biology at NTNU, Norway. Fertilized Japanese quail eggs were obtained from a breeder
139 (Birkeland, Norway) and were stored, for a maximum of two days, in a dark refrigerated room

140 at a temperature of 13 °C. Fifty eggs were divided into three injection treatments: 1000 ng/g
141 egg of TDCIPP (*n* = 16), 1000 ng/g egg of DP (*n* = 17) and an equal mixture of 1000 ng/g egg
142 TDCIPP and DP (*n* = 17). The maximum injected volume was 2 μL/g egg, therefore this
143 volume was adjusted according to the individual egg mass (mean ± SD: 14.3 ± 1.5 g). Eggs
144 were injected in the yolk sac at embryonic day (ED) zero, so before the start of incubation.
145 Prior to injection, the blunt segment of each egg was cleaned with ethanol (70 % *v/v*). Using a
146 round shaped dentist drill bit mounted on an electrical drill (Robust 140W 9922 (GS), Hong
147 Kong, P.R. China), a 0.6 mm hole was made in the eggshell until visibility of the inner shell
148 membrane. The solution was injected using a Hamilton syringe (50 μL) and disposable needles
149 (0.5 × 25 mm) and the hole was sealed with paraffin. Eggs were incubated for 16 - 17 days at
150 37.5 °C and 50 - 70 % humidity and were randomly divided over two incubators (wooden
151 cabinet incubator, type 180 - 220 V ~ 300 W; Hemel, Verl, Germany) equipped with an
152 automatic egg-turning device.

153 Each day, one egg per treatment was taken randomly from the incubator (see Table S1 for exact
154 sampling intervals) and stored at -20 ºC until analysis. Before sample preparation, TDCIPP-
155 exposed eggs were opened, the developmental status of the embryo was verified
156 macroscopically and undeveloped eggs could be appointed (see Table S1). Development of
157 DP- and mixture-exposed eggs was only verified after egg homogenization. The developmental
158 status of mixture-exposed eggs from ED9-11 (*n* = 4) was uncertain and concentrations in these
159 eggs should therefore be considered with care. Concentrations of the eggs sampled after 24
160 hours (ED1) were used to confirm the actual injected concentrations.

161 *2.4 Targeted chemical analysis*

162 Targeted chemical analyses were performed at the Toxicological Centre at the University of
163 Antwerp, Belgium. Aliquots of the homogenized quail egg content (0.25 g) were ground to a
164 powder with 2 g anhydrous Na_2SO_4 and transferred to a 15 mL polypropylene Falcon tube.

165 For TDCIPP/BDCIPP analysis, samples were spiked with a 50 µL mixture of internal standards
166 (2 ng/µL TDCIPP-d_{15} and 2.5 ng/µL BDCIPP-d_{10}). Egg homogenates were extracted with 5
167 mL of a hexane:acetone (1:1, *v/v*) mixture containing 1 % acetic acid and by successively using
168 vortexing (1 min), ultra-sonication (5 min) and centrifugation (2500 *g*, 3 min). This extraction
169 step was performed twice and each time the supernatant was transferred to a clean tube. The
170 obtained extract was evaporated until dryness under a gentle nitrogen stream and further
171 reconstituted in 1 mL ultrapure water (18.2 MΩ, Elga LabWater, Veolia Water Technologies),
172 further added with 0.5 mL sodium acetate buffer (pH 4.5, 1 M) and vortexed shortly. The
173 extract for TDCIPP/BDCIPP was cleaned-up on a methanol pre-washed Oasis® WAX cartridge
174 (3 mL, 60 mg, Waters). TDCIPP/BDCIPP were eluted with 4 mL of 5 % NH_4OH in methanol,
175 evaporated to dryness and reconstituted in 50 µL TPhP-d_{15} (in methanol, 1 ng/µL) and 50 µL
176 of ultrapure water. Quantification of TDCIPP/BDCIPP was performed with an Agilent 1100
177 series liquid chromatograph coupled with an Agilent 6410 triple quadrupole mass spectrometer
178 system (LC-MS/MS). The LC was equipped with a Kinetex® Biphenyl column (50 mm × 2.1
179 mm, 1.7 µm, Phenomenex) and the MS was operated in electrospray ionization mode (Cooper
180 et al., 2011; Malarvannan et al., 2015; Poma et al., 2017). Further details on the instrumental
181 analysis are provided in the supplementary material.

182 For Dechlorane Plus analysis, samples were spiked with a 100 µL mixture of internal standards
183 (200 pg/µL ^{13}C-*syn*-DP, 200 pg/µL ^{13}C-*anti*-DP). Egg homogenates were extracted similarly
184 to TDCIPP and BDCIPP but with a hexane:DCM mixture (1:1, *v/v*). The obtained extract was
185 evaporated until dryness under a gentle nitrogen stream and further reconstituted in 1 mL of

186 hexane:DCM. The extract for DP analysis was then cleaned up using a hexane pre-washed
187 polypropylene cartridge (6 mL, Supelco), containing silica acidified with 44 % H_2SO_4 and
188 topped with 0.5 g anhydrous Na_2SO_4. DP was eluted with 8 mL of hexane, then evaporated to
189 dryness and reconstituted in 150 µL CB207 (50 pg/µL in *iso*-octane). Quantification of DP was
190 performed using an Agilent 6890-5973 gas chromatograph coupled with a mass spectrometer
191 system (GC-MS). The GC was equipped with a 15 m × 0.25 mm × 0.10 µm DB-5ms capillary
192 column (J&W Scientific, USA) and the MS was operated in electron capture negative
193 ionization mode (Dodson et al., 2017; Sales et al., 2017). Further details on the instrumental
194 analysis are provided in the supplementary material.

195 Retention times (RT), ion chromatograms and relative abundance of the monitored ions were
196 used as identification criteria. A deviation of ion abundance ratios within 15 % of the mean
197 values for calibration standards was considered acceptable. Quantification was based on five-
198 point calibration curves for each compound. The peaks were positively identified as target
199 compounds if: (1) the RT matched that of the standard compound within ± 0.1 min and (2) the
200 signal-to-noise ratio (S/N) was higher than 3:1. Procedural blanks were analyzed
201 simultaneously with every batch of seven samples to check for interferences or contamination
202 from solvent and glassware. Procedural blanks were consistent (RSD < 30 %) and therefore
203 the mean value was calculated for each compound and subtracted from the values in the
204 samples. The limit of quantification (LOQ) was calculated as three times the standard deviation
205 of the mean of the blank measurements and was 2 and 4 ng/g wet weight (ww) for TDCIPP
206 and BDCIPP, respectively and 0.1 ng/g ww for DP.

207 For estimating recoveries, chicken egg was spiked with TDCIPP and BDCIPP or with *syn*-DP
208 and *anti*-DP (as control samples). The experiment was done in ten replicates for
209 TDCIPP/BDCIPP and in triplicate for DP and the control samples were processed as the real

210 exposed samples (see above). Mean ± SD recoveries were 95.6 ± 3.6 %, 87.6 ± 3.0 %, 102.5 ±
211 1.6 % and 100.8 ± 0.9 % for BDCIPP, TDCIPP, *syn*-DP and *anti*-DP, respectively.

212 *2.5 Untargeted chemical analysis*

213 At present, only standards for TDCIPP and the metabolite BDCIPP are commercially available.
214 Therefore, other potential halogenated metabolites were screened based on a previously
215 described untargeted and qualitative approach (Cariou et al., 2016). This untargeted chemical
216 analysis was performed at LABERCA, France. The eggs exposed to TDCIPP only (*n* = 16),
217 along with four procedural blanks, were extracted according to the method described for
218 targeted analyses. 1,3-DCP-d_5, TDCIPP-d_{15} and BDCIPP-d_{10} (100 ng each) were added as
219 internal standards. Minimum purification was performed to remove lipids. Extracts
220 reconstituted in a mixture of cyclohexane:ethyl acetate (1:1, *v/v*) were subjected to Gel
221 Permeation Chromatography (58 cm × 24.4 mm). SX3 Biobeads (Bio-Rad, Philadelphia, PA)
222 were used as stationary phase and a mixture of cyclohexane:ethyl acetate (1:1, *v/v*) as mobile
223 phase at 5 mL/min. Recovery standards (β-HBCDD-d_{18} and TCEP-d_{12}) were added in collected
224 fractions (22 - 69 min). Extracts were reconstituted in 50 µL of a mixture of methanol:H_2O
225 (1:1, *v/v*), centrifuged and 10 µL analyzed by liquid chromatography - high resolution mass
226 spectrometry coupling fitted with an electrospray ionization source (LC-ESI-HRMS) on a
227 Q-Exactive instrument (Thermo Fischer Scientific) in the negative and positive modes. The LC
228 was equipped with a Hypersil Gold column (100 × 2.1 mm, Thermo). Data were acquired in
229 the full scan mode over the 120 - 700 *m/z* range at a resolution of 140,000 full width at half
230 maximum at *m/z* 200. Chromatographic features were extracted and aligned using the *centWave*
231 and *retcor* functions (*xcms* package, R version 3.2.0) and halogenated clusters were paired
232 according to Cariou et al. (2016). Manual investigations were then performed on selected
233 filtered chlorinated clusters according to suspect screening of known potential metabolites,
234 intensity and change according to time of incubation, with the visual help of a H/Cl-scale mass

235 defect plot. More details regarding post-acquisition data treatment are provided in the
236 supplementary material.

237 Observed mass deviation along the sequence was lower than 2 ppm. Even though recovery
238 yields were not estimated, TCDIPP-d_{15} appeared quite stable and BCDIPP-d_{10} more variable,
239 possibly due to volatilization. 1,3-DCP-d_5 was not recovered, possibly due to volatilization as
240 well as irrelevance of ESI, in accordance with literature (Racamonde et al., 2011; Kim et al.,
241 2015).

242 *2.6 Data treatment and computational analysis*

243 Concentrations were converted from ng/g ww to nmol/g ww to control for the difference in
244 molecular weight between the compounds. During the development of the embryo, the weight
245 of the egg decreases due to evaporation of water, gas exchange, oxidation of fats etc (Romanoff,
246 1967). Whole egg weight (including egg shell) after incubation was not recorded and therefore
247 eggs were not normalized for egg weight. Although the pattern of degradation and formation
248 of the compounds remains the same (as confirmed by calculations using the egg content weight
249 excl. shell), concentrations might be overestimated and metabolization might actually occur
250 faster than presented here. Eggs that were found to be undeveloped, were excluded from the
251 data (TDCIPP: $n = 1$, DP: $n = 6$, mixture: $n = 4$). See Table S1 for an overview of the sample
252 dataset. Changes in concentrations were fitted using a self-starting nonlinear least squares (nls)
253 3-parameter logistic model (SSlogis). BDCIPP concentrations were fitted excluding the last
254 time point (ED17). Graphs were made in R (version 3.4.0; R Core Team, 2017) using the
255 package *ggplot2* (Wickham, 2009).

256 **3. Results**

257 *3.1 TDCIPP and its metabolite BDCIPP*

258 During the development of the Japanese quail embryos, TDCIPP concentrations in the single-
259 exposed eggs decreased from 2.08 nmol/g ww to a concentration below the limit of
260 quantification (< 2.0 ng/g or 0.005 nmol/g ww). The average rate of decrease was 0.14 nmol/g
261 ww per day (max. rate: 0.38 nmol/g ww per day at ED10, Fig. 2). At the same time,
262 concentrations of BDCIPP increased from < LOQ (< 4.0 ng/g or 0.01 nmol/g ww) to 0.64
263 nmol/g ww at ED14. The average rate of increase was 0.015 nmol/g ww per day (max. rate:
264 0.073 nmol/g ww per day at ED9, Fig. 2). This increase of the metabolite was thus slower than
265 the decrease of the parent compound, TDCIPP. At the last time point, ED16, the concentration
266 of the di-ester BDCIPP finally decreased to 0.24 nmol/g ww. The egg at ED15 was found to
267 be undeveloped after 15 days of incubation and is therefore not included in Figure 2 (see also
268 Table S1). In this egg (ED15), TDCIPP and BDCIPP concentrations were found to be 2.03 and
269 0.37 ng/g ww, respectively.

270 In the mixture-exposed eggs, the TDCIPP concentration decreased from 2.4 nmol/g ww to
271 0.0087 nmol/g ww after 17 days of incubation. The average rate of decrease of TDCIPP was
272 0.15 nmol/g ww per day (max. rate: 0.19 nmol/g ww per day at ED10, Fig. 3). Simultaneously,
273 0.39 nmol BDCIPP/g egg was formed after 17 days of incubation, with a maximal
274 concentration of 0.61 nmol/g ww at ED15. This increase occurred on average at 0.023 nmol/g
275 ww per day (max. rate: 0.057 nmol/g ww per day at ED8, Fig. 3). Undeveloped mixture-
276 exposed eggs contained BDCIPP concentrations ranging from 0.26 - 0.43 nmol/g ww and
277 TDCIPP concentrations of 0.98 - 2.00 nmol/g ww.

278 Although not quantitative, the untargeted approach in single-exposed eggs confirmed the
279 changes regarding TDCIPP and BDCIPP over the time of incubation obtained through the

280 targeted approach. The sensitivity was also similar. No TDCIPP with oxidized side chains, nor
281 mono(1,3-dichloro-2-propyl) phosphate (MDCIPP) were observed in the dataset, even though
282 it was expected that signals would arise from their presence in the negative mode. Regarding
283 other signals, no investigated chlorinated cluster appeared as intense as TDCIPP. Some
284 increasing and decreasing changes in intensities over the time of incubation were observed but
285 considering the experimental design, no clear correlation to the TDCIPP injection could be
286 concluded. A hexachlorinated cluster was observed at *m/z* 629.052 and a RT slightly after
287 TCDIPP, with a kinetic fitting the one of TCDIPP. Possible formula for this ion could be
288 $[C_{21}H_{38}O_6PCl_6]^-$ (1.7 ppm deviation; see Figure S3).

289 *3.2 Dechlorane Plus*

290 Concentrations of DP were found to be stable around the injected dose of 1.5 nmol/g ww during
291 the entire embryonic development (Fig. 4). The mean ± SE concentrations found in the single-
292 and mixture-exposed eggs were 1.43 ± 0.06 and 1.59 ± 0.09 nmol/g ww, respectively. The
293 isomer ratio of DP (f_{anti}) was stable over the incubation time. The mean ± SE f_{anti} value 0.67 ±
294 0.001 and 0.67 ± 0.008 for DP- and mixture-exposed eggs, respectively.

295 **4. Discussion**

296 *4.1 TDCIPP and its metabolite BDCIPP*

297 During the incubation period of *C. japonica,* TDCIPP was rapidly transformed to BDCIPP *in*
298 *ovo.* This rapid transformation of TDCIPP is in accordance with a previous study with *in ovo*-
299 injected chicken eggs, in which less than 1 % of the injected TDCIPP (50 µg/g egg) was
300 detected by ED19 (Farhat et al., 2013).

301 The time series of eggs (Fig. 2 and 3) show that TDCIPP decreased faster than BDCIPP
302 increased. The concentration of the parent compound and its metabolite does not equal the
303 injected TDCIPP concentration ($[TDCIPP+BDCIPP]_{detected}/[TDCIPP]_{injected} \neq 1$) meaning that

304 the molar conversion of TDCIPP into BDCIPP is not complete. This suggests 1) further
305 metabolization of BDCIPP and/or 2) metabolization of TDCIPP into other compounds than
306 BDCIPP. In contrast, a complete molar conversion of TDCIPP into BDCIPP within 36 h was
307 previously reported in chicken embryonic hepatocytes and therefore no further metabolization
308 was assumed (Farhat et al., 2014). In the present study, the molar conversion in the TDCIPP-
309 exposed eggs was practically complete (ratio ~ 1) at the initiation of embryonic development
310 (until ED5), as well as in the undeveloped egg. But in the eggs thereafter (from ED6), an
311 embryo was visible and the ratio decreased to 0.10 at ED16. This leads to the hypothesis that
312 after ED6, the embryo further biotransformed BDCIPP into a metabolite that was not targeted
313 for analysis. The liver of chicken embryos differentiates at ED5 (corresponding to ED5 in
314 quails; Ainsworth et al., 2010) and shows xenobiotic metabolism very early in the
315 development. Hamilton et al. (1983) f.e., found that aryl hydrocarbon hydroxylase, shown to
316 be involved in TDCIPP metabolism (Nomeir et al., 1981), is activated between ED6 and 7 in
317 chicken (corresponding to early ED6 in quails; Ainsworth et al., 2010). In the mixture-exposed
318 eggs, the same pattern could be observed, but with a slower, more gradual biotransformation
319 compared to the single-exposed eggs. It is unclear if this is related to the mixed exposure or to
320 unidentified undeveloped eggs in the mixture dataset (possibly ED9-.

321 Formation of BDCIPP prior to ED6 and in the undeveloped eggs indicates spontaneous
322 degradation in addition to biotransformation by the embryo (Fig. 2 and 3), in contrast to what
323 was previously found in chicken embryonic hepatocytes (Farhat et al., 2014). Spontaneous
324 chemical reactions are not unlikely since the eggs were kept for over two weeks at a relatively
325 high temperature (37.5 °C). Nevertheless, the transformation of TDCIPP to BDCIPP was found
326 to be more extensive when an embryo became visible (ED6).

327 The further biotransformation is also supported by the sudden decrease in BDCIPP in the last
328 embryonic stage of the single-exposed eggs (ED16; 0.24 nmol/g ww, Fig. 2). Due to the

329 depletion of TDCIPP between ED15 and ED16, BDCIPP stopped increasing. Therefore, this
330 decrease at ED16 can be interpreted as potential further metabolization. In the mixture-exposed
331 eggs, the same trend could be observed at ED17. These data (n = 2) were not included in the
332 model since this decrease was only seen on the last day of the time series. Therefore, any further
333 interpretation regarding this hypothesis should be carefully considered.

334 In comparison to the *in vitro* assays performed with hepatic microsomes (Greaves et al., 2016)
335 or hepatocytes (Farhat et al., 2014), it seems that biotransformation *in ovo* is much slower. One
336 reason for this is probably the time it takes for the embryo to develop functional organs and
337 ingest the exposed yolk sac (Romanoff, 1967), compared to the readily available and directly
338 exposed liver cells. In addition, *in vitro* studies expose specialized, isolated liver cells that are
339 known to be metabolically active due to the high abundance of enzymes (De Oliveira et al.,
340 2008). The present *in ovo* study is analyzing the whole egg content, including less
341 metabolically active tissues, which can slow down the overall net metabolism.

342 Previous studies have also investigated biotransformation of TDCIPP in mammals. BDCIPP
343 (Figure S1a) was found to be the major metabolite of TDCIPP in urine of rats after intravenous
344 dosing (Lynn et al., 1981; Nomeir et al., 1981). The di-ester was already detectable in most
345 tissues five minutes after the intravenous administration. To a lesser extent, MDCIPP and 1,3-
346 DCP were also identified (Lynn et al., 1981; Figure S1b and c). It was shown that rats mainly
347 eliminate TDCIPP by metabolization into BDCIPP, while BDCIPP was eliminated primarily
348 by excretion through urine and feces (Lynn et al., 1981; Nomeir et al., 1981). In addition to the
349 latter metabolites and a glutathione conjugate, Nomeir et al. (1981) also detected 3-chloro-1,2-
350 propanediol (3-MCPD; Figure S1d) in rat liver microsomes after *in vitro* exposure, and
351 suggested it was formed as a metabolization product from 1,3-DCP. In human liver fractions,
352 BDCIPP was the major metabolite as well, followed by the glutathione conjugate of TDCIPP
353 (Van den Eede et al., 2013). Other detected metabolites in that study were hydroxylated forms

354 of both TDCIPP and BDCIPP, a hydrocarboxylated TDCIPP, resulting from phase I
355 metabolism and a glutathione conjugate of TDCIPP formed by phase II metabolism (Van den
356 Eede et al., 2013).

357 The reasons for the lack of detection of metabolites by the untargeted analysis in the current
358 study could be manifold. TDCIPP with oxidized side chains was not detected, even though
359 Lynn et al. (1981) and Nomeir et al. (1981) indicated extensive oxidation of the parent
360 compound when it was observed that 20 % of the injected TDCIPP in rats was expired as CO_2.
361 However, gaseous exchange (O_2-uptake and CO_2-release) by the embryo occurs through the
362 eggshell and steadily increases with age and size (Romanoff, 1967). The lack of detection of
363 3-MCPD and 1,3-DCP as metabolites in the present study can thus potentially also be attributed
364 to biotransformation into CO_2 and consequent release through the eggshell. In addition, 1,3-
365 DCP and MDCIPP were possibly not detected due to volatilization as well as irrelevance of
366 electrospray ionization in the case of 1,3-DCP (Kim et al., 2015; Racamonde et al., 2011).
367 Naturally, differences in metabolic capacity between birds and mammals could also be a reason
368 for why the metabolites weren't detected in this study.

369 Further, a hexachlorinated cluster, exhibiting the same kinetic pattern as TDCIPP, indicates
370 that TDCIPP possibly binds to the fatty acids in the emulsion (peanut oil, lecithin) or in the egg
371 itself.

372 *4.2 Dechlorane Plus*

373 Neither single- nor mixture-exposed eggs showed breakdown of DP. The absence of a
374 transformation process is in accordance with previous *in vitro* and *in vivo* studies on birds and
375 other biota (Chabot-Giguère et al., 2013; Tomy et al., 2008; Zheng et al., 2014a). Therefore,
376 the detection of degradation products of DP in bird eggs in earlier studies rather suggests
377 bioaccumulation and maternal transfer of these compounds. Degradation products that have

378 been identified in bird eggs were two dechlorinated products,
379 undecachloropentacyclooctadecadiene (*anti*-Cl11DP; Guerra et al., 2011; Muñoz-Arnanz et
380 al., 2012, 2011; Figure S2a) and decachloropentacyclooctadecadiene (*anti*-Cl10DP; Guerra et
381 al., 2011; Figure S2b) and the monoadduct of DP (1,5-DPMA; Guerra et al., 2011; Figure S2c).
382 Because of the detection of these degradation products in sediments (Sverko et al., 2008, 2010),
383 it remains unclear if these compounds bioaccumulate from the environment or biotransform *in*
384 *vivo* (Rjabova et al., 2016). *Anti*-Cl10DP and *anti*-Cl11DP could also not be evidenced
385 unequivocally in the samples of the present study.

386 **5. Conclusions**

387 Based on this *in ovo* experiment with Japanese quail, we can conclude that *C. japonica* can
388 readily metabolize TDCIPP during the embryonic development. By the end of the incubation
389 period, TDCIPP was completely degraded. Since the transformation of TDCIPP into BDCIPP
390 was not proportional, we hypothesize that the parent compound is transformed into other
391 metabolites, either directly from TDCIPP or derived from BDCIPP. Since the toxicity of some
392 compounds might be induced following metabolism (metabolic activation, e.g. the
393 procarcinogen benzo[*a*]pyrene), we recommend that metabolites of organophosphate tri-esters
394 (and DP) should be analyzed whenever possible.

395 Further untargeted investigations on metabolites of TDCIPP did not show the presence of
396 known oxidized metabolites. This can probably be attributed to the sample preparation and
397 analytical difficulties, as well as potential expiration of smaller metabolites as CO_2 through the
398 eggshell. The analysis did highlight the presence of several chlorinated compounds, possibly
399 related to TDCIPP injection.

400 Corresponding to other studies in birds, avian embryos in this study did not show the capacity
401 to biotransform DP. DP might therefore pose a risk to the health of developing and fully
402 developed birds, which warrants further investigation.

403 **Funding**

404 This work is part of the NEWRAPTOR project, which is supported by the Norwegian Research
405 Council [project number 230465/F20] and the Norwegian University of Science and
406 Technology (NTNU); Dr. G. Malarvannan, Dr. G. Poma, and Dr. A. Covaci are supported by
407 the University of Antwerp and A. Léon by the "Région Pays de la Loire" and the IFREMER.

408 **Acknowledgements**

409 The authors acknowledge the help of Olga Novillo Sanjuan for assisting with the egg injections.
410 Ariane Flamend is acknowledged for the help with the graphical abstract.

411 **Supporting Information**

412 Details on the targeted instrumental analysis of TDCIPP/BDCIPP and DP as well as details on
413 post-acquisition data treatment related to the untargeted chemical analysis are described in the
414 supplementary material. In addition, figures are provided on potential metabolic products,
415 illustration of results obtained by untargeted chemical analysis and a table with details on the
416 samples included in statistical analysis. Supporting Information is freely available online at
417 xxx.

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595

596 **Figure captions**

597 **Figure 1:** Chemical structure of a) TDCIPP (CAS: 13674-87-8), b) *syn*-DP (CAS: 135821-03-
598 3) and c) *anti*-DP (CAS: 135821-74-8).

599 **Figure 2:** Change in egg concentrations (nmol/g ww) of TDCIPP (black ▲) and its metabolite
600 BDCIPP (grey ●) in TDCIPP-exposed eggs over the time of incubation. The model was fitted
601 excluding the data point for BDCIPP at ED16 (○). The dashed line shows an extrapolation
602 according to the fitted model.

603 **Figure 3:** Change in egg concentrations (nmol/g ww) of TDCIPP (black ▲) and its metabolite
604 BDCIPP (grey ●) in mixture-exposed eggs over the time of incubation. The model was fitted
605 excluding the data point for BDCIPP at ED17 (○). The dashed line shows an extrapolation
606 according to the fitted model.

607 **Figure 4:** Egg concentrations (nmol/g ww) of DP during the time of incubation. Both values
608 for single- (●) and mixture- (○) exposed eggs are shown and remain stable around the injected
609 dose (dashed line).