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Transfer of hexabromocyclododecane flame retardant isomers from captive American kestrel eggs to feathers and their association with thyroid hormones and growth

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23 **Abstract**

24 Feathers are useful for monitoring contaminants in wild birds and are increasingly
25 used to determine persistent organic pollutants. However, few studies have been
26 conducted on birds with known exposure levels. We aimed to determine how well
27 nestling feather concentrations reflect *in ovo* exposure to hexabromocyclododecane (α-,
28 β- and γ-HBCDD), and to determine if feather concentrations are related to physiological
29 biomarkers. Captive kestrels (n=11) were exposed *in ovo* to maternally transferred
30 HBCDD-isomers at concentrations of 127, 12 and 2 ng/g wet weight of α-, β- and γ-
31 HBCDD (measured in sibling eggs), respectively, and compared to controls (n=6).
32 Nestling growth was monitored at 5 d intervals and circulating thyroid hormone
33 concentrations assessed at d 20. Tail feathers were collected prior to the first molt and
34 analyzed for HBCDD isomers. The mean ΣHBCDD concentration in feathers was 2405
35 pg/g dry weight (in exposed birds) and α-, β- and γ-HBCDD made up 32%, 13%, and
36 55%, respectively of the ΣHBCDD concentrations. This isomer distribution deviated
37 from the typical dominance of α-HBCDD reported in vertebrate samples. Exposed chicks
38 had significantly higher feather concentrations of β- and γ-HBCDD compared with
39 controls (p=0.007 and p=0.001 respectively), while α-HBCDD concentrations did not
40 differ between the two groups. Feather concentrations of α-HBCDD were best explained
41 by egg concentrations of β- or γ-HBCDD concentrations (w_i=0.50, 0.30 respectively),
42 while feather concentrations of β- and γ-HBCDD were influenced by growth parameters
43 (rectrix length: w_i=0.61; tibiotarsus length: w_i=0.28). These results suggest that feather *α*-
44 HBCDD concentrations may reflect internal body burdens, whereas β- and γ-HBCDD
45 may be subject to selective uptake. The *α*-HBCDD concentrations in the feathers were

46 negatively associated with the ratio of plasma free triiodothyronine to free thyroxine
47 (T_3:T_4; p=0.020), demonstrating for the first time that feather concentrations may be used
48 to model the effect of body burdens on physiological endpoints.

49

50 ***Keywords:*** HBCD; Feathers; Thyroid hormones; Growth; Bird

51

52 **Capsule Abstract:**

53 Feathers of kestrels exposed to 90% α-HBCDD *in ovo* were dominated by γ-HBCDD; α-
54 HBCDD in feathers was correlated with the circulating thyroid hormone ratio

55

56 **Introduction**

57 Birds of prey are valuable as indicators for the impact of organohalogen
58 compounds on wildlife and human health [1]. However, employing them as
59 environmental sentinels is accompanied by ethical and logistic sampling challenges. As
60 feathers grow they are connected to the blood supply, and as a result, contaminants are
61 deposited during this time. Consequently, feathers can act as an archive of contaminant
62 exposure for the individual bird during the time of feather growth [2-4]. Feathers are thus
63 an attractive body compartment for examining and monitoring contaminant exposure in
64 raptors for several reasons. Perhaps most importantly, feathers allow for non-destructive
65 sampling, which is useful for wildlife, particularly protected species. They are also easy
66 to collect and transport, can be collected from carcasses, do not have special storage
67 requirements [5], and only one flight feather is required (for larger birds such as raptors
68 [6, 7]), which reduces many of the logistical restraints of fieldwork.

69 Feathers have been used to monitor contamination in birds for decades,
70 particularly heavy metals [2-4]. The use of feathers for monitoring anthropogenic
71 persistent organic pollutants (POPs) is more recent, but is also a confirmed strategy that is
72 becoming more widely used. Feathers have been successfully analyzed for several
73 organochlorines (OCs), including polychlorinated biphenyls (PCBs) and other
74 organochlorine pesticides (OCPs) [6-8]), and more recent contaminants such as poly- and
75 perfluoroalkyl substances (PFASs) [9], and the brominated flame retardants (BFRs),
76 polybrominated diphenyl ethers (PBDEs) [6, 7], hexabromocyclododecane (HBCDD)
77 [10] and others [10]. For current-use contaminants, which are generally present at

78 considerably lower concentrations than for OCs (e.g. [7]), this is particularly interesting
79 because these small concentrations can still be detected in feather samples.

80 While the usefulness of feathers for assessing contaminant exposure has been well
81 established, few studies have determined how well feathers reflect and model internal
82 body burdens and environmental exposure concentrations of bioaccumulative
83 contaminants. European starlings (*Sturnus vulgaris*) exposed to CB-153 by silastic
84 implants showed significant correlations between feather concentrations of CB-153 in
85 feathers and other body compartments (muscle, liver, brain, and blood) [8]. Additionally,
86 a few studies on wild birds have demonstrated that some chlorinated and brominated
87 contaminant levels detected in feathers are related to concentrations in tissues used more
88 frequently for biomonitoring including liver and plasma in adult [6-8, 11] and nestling
89 birds [10, 12, 13]. These studies confirm that feathers can be useful indicators of
90 exposure to POPs, but more research would be beneficial.

91 Studies reporting how feather concentrations relate to standard physiological
92 biomarkers such as hormone concentrations, as well as reproductive and developmental
93 parameters, are fewer still. Reductions in immune and growth biomarkers have been
94 linked to heavy metal concentrations in feathers of two heron species [14, 15], suggesting
95 that they may be as useful for this purpose as conventionally used tissues. Additionally,
96 feather heavy metal concentrations were positively associated with feather corticosterone
97 concentrations in urban dwelling common blackbirds (*Turdus merula*) [16]. However,
98 considerably more research is needed particularly for xenobiotics, and information on the
99 associations between feather contaminant levels and other biomarkers is required.
100 Controlled experiments where contaminant exposure concentrations are known are

101 critical to further our understanding of how POPs are taken up or deposited in bird
102 feathers, and how these concentrations may relate to internal health biomarkers.
103 However, to our knowledge, no studies have yet examined these questions.

104 HBCDD, as a high-production volume additive BFR, is a xenobiotic contaminant
105 that is detectable in bird feathers [10]. It is globally distributed in the environment and
106 wildlife (reviewed in [17]), including birds of prey [10], where relative to all other biota,
107 maximal levels were reported in peregrine falcon (*Falco peregrinus*) eggs in Canada [18].
108 HBCDD demonstrates endocrine disrupting potential *in vitro* since it binds to estrogen,
109 androgen and progesterone receptors causing inhibition, and binds to the thyroid receptor
110 causing potentiation, of the ensuing pathways [19]. In captive American kestrels (*Falco*
111 *sparverius*), *in vivo* exposure to the technical mixture of HBCDD (HBCDD-TM) causes
112 changes in reproduction compared to controls including earlier lay dates and lighter eggs
113 [20], and reductions in courtship and parental behaviors [21]. Compared to controls,
114 adult male kestrels exposed to HBCDD demonstrated increased testicular mass and
115 changes in histology as well as moderate increase in circulating testosterone and
116 reductions in thyroxine, supporting the *in vitro* endocrine disrupting potential of HBCDD
117 [22].

118 In the present study, we measured the concentrations of the major α-, β- and γ-
119 HBCDD isomers in feathers of captive American kestrel juveniles exposed *in ovo*
120 through direct maternal transfer from females exposed by diet to environmentally
121 relevant levels of the HBCDD-TM [20]. The behavioral, reproductive and physiological
122 effects of exposure to HBCDD have been previously reported for the parents of these
123 birds [20-22], as has its uptake, distribution and depletion in adult kestrel tissues [23].

124 Our objectives in the current study were twofold: first to determine how well nestling
125 kestrel feather concentrations reflect *in ovo* exposure concentrations of HBCDD isomers,
126 and second to determine if feather concentrations may be related to the growth and
127 thyroid hormones (which are affected by exposure to HBCDD in kestrels [22]). To the
128 best of our knowledge, this is the first report of feather concentrations in nestling birds
129 experimentally exposed to any xenobiotic, and the first time feather contaminant
130 concentrations have been examined in conjunction with detailed growth and endocrine
131 data collected during the time of feather growth and contaminant deposition.
132
133 **Materials and methods**
134 *Animal treatment and exposure protocol*
135 Captive American kestrels from McGill University (Montreal, QC, Canada) were
136 used for this experiment. Breeding pairs were housed in separate enclosures and the
137 present study subjects were reared naturally by their parents in an attached nest box.
138 Twenty-one of the initial 30 pairs (N = 20 HBCDD-exposed, 10 control) hatched and
139 raised nestlings: 16 pairs from the HBCDD-exposed group (from which 11 were
140 randomly selected for the present study due to logistical restraints) and 6 from the control
141 group [20]. All experimental procedures, protocols and care of the birds followed
142 Canadian Counsel on Animal Care guidelines and were approved by the Animal Care
143 Committee of McGill University.
144 Throughout the study, parent birds were fed day-old frozen-thawed cockerels
145 (*Gallus domesticus*) under *ad libitum* conditions. For the exposure group, the HBCDD-
146 TM was injected into the cockerel feed daily, immediately prior to feeding. The exact

147 exposure procedures are detailed elsewhere [20, 21]. Briefly, the kestrel parents were

148 exposed to 0.52 μg HBCDD-TM/g kestrel/d for the four weeks preceding pairing through

149 until 2 days before the chicks hatched (~ 75 days). Comparisons were made to control

150 pairs exposed to safflower oil vehicle only. Egg HBCDD concentrations resulting from

151 direct maternal transfer from the above exposure regime were determined for the first-

152 laid egg of each brood: means ± standard error about the means (SEM) were 163.5 ± 75.1

153 ng/g wet weight (ww) for α-HBCDD, 13.9 ± 5.6 ng/ g^{-1} ww for β-HBCDD/g ww and 2.5

154 ± 3.8 ng/g ww for γ -HBCDD in eggs of exposed pairs, and 0.3 ± 0.4 ng/g ww, 0.2 ± 0.1

155 ng/g ww and 0.2 ±0.1 ng/g ww for the same isomers in controls, respectively. These egg

156 concentrations of the exposed pairs were similar to those measured in wild free-ranging

157 peregrine falcon eggs from Canada [18]. Since the dosing of the parent birds ceased 2

158 days prior to their chicks hatching, the nestlings, including the present study subjects,

159 were not exposed to HBCDD by dietary input, but only by maternal transfer of HBCDD

160 isomers into the eggs (*in ovo*). The measured *in ovo* concentrations of HBCDD isomers

161 from the first egg were used as an estimate of exposure for the 1-4 sibling nestlings that

162 hatched within the same brood.

163

164 *Feather collection*

165 The levels of HBCDD isomers deposited in feathers were assessed in 11

166 HBCDD-exposed birds, each of which had been the oldest chick from their randomly

167 selected brood (9 males, 2 females). The feathers were collected from these nestlings

168 following their euthanization at 9 months of age when it was determined that no further *in*

169 *vivo* studies would be conducted. This occurred before the first set of feathers was

170 molted, allowing for the testing of HBCDD isomers that would have been deposited in
171 them between d 10-12 and 35 of the nestling phase when feathers grow. Following
172 euthanasia the kestrels were frozen at -20 °C for preservation until the time of analysis.
173 Before analysis, carcasses were thawed and one tail feather (right central rectrix) was
174 collected. Unlike the HBCDD-exposed kestrels, control birds (n=6) were not euthanized
175 at the end of the study as these individuals were saved for future research and propagation
176 within the colony. Thus, control feathers were gathered opportunistically from control
177 birds hatched in the same year that died of natural causes prior to their first feather molt
178 (2-11 months of age). Three of these birds were control young from the HBCDD
179 exposure study exposed *in ovo* to safflower oil vehicle only, and for which *in ovo*
180 exposure concentrations were measured (1 male, 2 females). Three other controls were
181 young produced in another study (not relating to chemical exposure) at the same facility,
182 which were fed an identical diet (though not exposed to vehicle and HBCDDs were not
183 tested in sibling eggs) [24]; thus they were not expected to demonstrate differing HBCDD
184 concentrations from the other controls.

185

186 *HBCDD isomer analysis and quality control of eggs*

187 The first egg of each clutch was analyzed for α-, β- and γ-HBCDD in
188 Letcher/Organic Contaminants Research Lab (Environment and Climate Change Canada)
189 by methods that have been fully reported elsewhere. [20, 23]. Briefly, injection stock
190 solution and tissue samples were taken and spiked with 10 ng of three internal standards
191 ($^{13}C_{12}$-labeled surrogates of all three HBCD isomers; Wellington Laboratories, Guelph,
192 ON, Canada). Subsequently, samples were subjected to an accelerated solvent extraction

193 (ASE200; Dionex) using dichloromethane:hexane (50:50, *v:v* i.e. *vol:vol*). Sulfuric acid
194 silica (50%) was used to concentrate and clean up the extract. The target compound-
195 containing eluant was collected and concentrated, and finally solvent-exchanged to
196 methanol to prepare it for analysis. HBCDD isomer analysis was conducted using high-
197 performance liquid chromatography–tandem quadrupole mass spectrometry using
198 electrospray ionization in the negative ion mode (LC-ESI(-)–MS/MS). The neat HBCDD-
199 TM solid and safflower oil solutions were also analyzed for α-, β- and γ-HBCDD isomers
200 to determine the original isomer compositions and whether any isomerization had
201 occurred during the preparation process. Contaminant levels in the cockerel diet are
202 reflected by those in the eggs laid by control females. The mean method detection limit
203 for all three isomers was 0.01 ng/g ww and recovery efficiencies were 89%, 98%, and
204 101%, for α-, β- and γ-HBCDD, respectively. A blank sample and in-house reference
205 material (egg homogenates of double-crested cormorant (*Phalacrocorax auritus*; DCCO)
206 were analyzed with batches of 8 samples.
207
208 *Chemical analysis and quality control of feathers*

209 The analysis for individual α-, β- and γ-HBCDD isomers in tail feathers was
210 conducted in the Toxicological Centre at the University of Antwerp and was adapted
211 from an earlier described protocol [10]. Briefly, individual tail feathers were thoroughly
212 rinsed with deionised water, and dried overnight at ambient temperatures while being
213 covered with standard laboratory paper in order to prevent dust deposition. The dried
214 feathers, from which the calami (base of feather shaft) were omitted, were cut in ~1 mm
215 pieces, were spiked with ^{13}C-labelled internal standards for all three targeted HBCDD

216 isomers and incubated overnight at 45 °C with 5 mL of HCl (4 M) and 5 mL of a mixture
217 of hexane:dichloromethane (4:1, *v:v*). After liquid-liquid extraction using
218 hexane:dichloromethane (4:1, *v:v*), the extracts were cleaned-up with acidified silica (800
219 mg; 44% H_2SO_4), topped with anhydrous Na_2SO_4 (400 mg), and the analytes eluted with
220 10 mL of hexane:dichloromethane (4:1, *v:v*). The cleaned eluates were consequently
221 fractionated on silica SPE cartridges (3 mL/500 mg; Varian, Palo Alto, CA, USA). A first
222 fraction was eluted with 6 mL of hexane and then discarded, while the second fraction,
223 containing HBCDD isomers, was eluted with 8 mL of dichloromethane. The eluates were
224 concentrated under a gentle N_2 flow until dryness, and finally reconstituted in methanol.
225 The three isomers were quantified using tandem mass spectrometry (Agilent 6410, Palo
226 Alto, CA, USA) coupled to liquid chromatography (Agilent LC 1100 series) using a Luna
227 C18(2) reversed phase column (150 mm x 2 mm; 3 μm particle size; Phenomenex,
228 Utrecht, the Netherlands). With every 23rd sample, a procedural blank was analyzed. All
229 analyte concentrations were blank-corrected using the average procedural blank values.
230 The method limit of quantification (LOQ) was specific to each analyte and set at 3*SD of
231 the values found in the procedural blanks. LOQs for the α-, β- and γ-HBCDD isomers
232 were 0.40, 0.15 and 0.30 ng/g dry weight (dw), respectively.
233
234 *Nestling growth and physiology*
235 The date that eggs were laid and their fresh mass was recorded and have been
236 previously reported for all pairs in this project [20]. In the larger sample of the overall
237 project, eggs of HBCDD-exposed pairs were smaller compared with controls, and the
238 Julian lay dates of exposed pairs were on average 6 days earlier than controls [20]. The

239 Julian hatch date was recorded for each nestling. All nestlings were weighed and the
240 tibiotarsus length was measured to the nearest mm (using digital calipers) on the day they
241 hatched, as well as every 5 days thereafter based on the age of the oldest chick in the
242 brood to minimize disturbance and cannibalism; hatching within a brood is largely
243 synchronous. Tail feather pins emerged between day 10 and day 15 of nestling age, and
244 the length of the feather was measured to the nearest mm at 5-day intervals thereafter
245 until 30 days of age using a metal ruler.

246 Blood was collected from nestlings at 20 d of age and assessed for plasma
247 concentrations of free thyroxine (FT_4) and free triiodothyronine (FT_3). Blood was
248 collected between 9:00 AM and 12:00 PM to minimize any diurnal effect on thyroid
249 hormone concentrations. Hormone analyses were conducted identically to Fernie and
250 Marteinson [25] using $^{125}I\text{-}T_3/T_4$ solid phase radioimmunoassays kits (Coat-A-Count;
251 Siemens Medical Solutions Diagnostics, previously Diagnostic Products Corporation,
252 Canada). Thyroid hormones were assessed in duplicate in one or two nestlings per brood
253 in the larger overall project. Because broods were chosen at random for feather analysis
254 in the present study, hormone concentrations are not available for all of the selected 11
255 treatment birds for which HBCDD isomer concentrations were assessed in their feathers;
256 sample sizes were as follows: FT_4 n=8, FT_3 n=7. Concentrations were above the detection
257 limit in all cases, and the % Coefficients of Variability between replicates was always
258 below 5%.

259

260 *Statistics*

261 HBCDD values below the method LOQ were assigned a value equal to LOQ/2 for

262 all statistics and calculations of means (max 2 out of 11 exposed birds). Concentrations of
263 individual and total HBCDD isomers were compared between both groups using t-tests
264 (data were normally distributed). Proportions of HBCDD isomers in feathers of exposed
265 and control kestrels were compared using Mann-Whitney U tests. Proportions of HBCDD
266 isomers were compared between feather and sibling eggs using Mann-Whitney U tests
267 for HBCDD-exposed birds only.

268 To determine which factors best explained the observed variation in feather
269 concentrations of HBCDD-isomers (α-, β- or γ-HBCD), a series of Generalized Linear
270 Models (GLZs) were conducted, one for each isomer (α, β, γ) and ranked using Akaike's
271 Information Criterion corrected for small sample sizes (AIC_c) [26, 27]. Generalized linear
272 models were chosen because they are linear regression models that do not require
273 response variables to have a normal distribution [28]. Models included variables that may
274 affect feather HBCDD concentrations and included *in ovo* concentrations of α-, β- and
275 γ-HBCDD, and sum (Σ) HBCDD, Julian lay and hatch dates, fresh egg mass, nestling
276 body mass (d 10 and 25), tibiotarsus length (d 10 and 30), length of the right central
277 rectrix feather (d 10 and 30), mass (d 25, i.e. maximum) as well as the growth rate (for
278 body mass or feather length). Only single variable models were used due to the small
279 sample size; the null model was assessed for comparison, and only models for which the
280 AIC_c was lower than that of the null model (and thus more highly ranked) were
281 examined. For each variable, the ΔAIC_c, and weight (w_i) were calculated [26]. Linear
282 regressions were also conducted for all models ranked better than the null model to
283 generate the associated adjusted R^2 values. These statistics were conducted using IBM
284 SPSS 21®.

285 We additionally aimed to determine the effect of growth dilution on HBCDD
286 isomer concentrations in the studied birds, first calculating growth curve parameters
287 following the methods used by Fernie et al. [29]: the growth data were fitted to a logistic
288 growth model [30] using nonlinear regression to estimate the growth rate constant (K),
289 the asymptotic size at d 21 (A), and the inflection point (I; day when maximal growth
290 begins), for each individual nestling. Then, two main assumptions were employed: 1) that
291 nestling HBCDD body burdens at hatching resulted from complete assimilation of
292 measured *in ovo* concentrations, (from direct maternal transfer) and 2) that additional
293 dietary input of HBCDD isomers during the nestling stage were negligible as exposure to
294 their parents ceased before chicks hatched (see methods). Under these assumptions, three
295 concurrent processes were assumed to alter the maternally transferred HBCDD isomers
296 over the nestling stage, i.e., growth dilution [31], feather sequestration [10, 12, 13], and
297 metabolism [23]. Differences in growth and concentration parameters between
298 experimental treatments, days, and their interactions were tested using Analysis of
299 Variance (ANOVA) on general linear models. The statistics were performed using birds
300 for which *in ovo* concentrations of HBCDD isomers were available (n = 14), including
301 the 11 *in ovo* exposed and 3 control birds from the original HBCDD study only. Data
302 were tested for normality, homoscedasticity, and to determine if there were any outliers,
303 using procedures suggested by Zuur et al [32]. HBCDD isomer concentrations were
304 consequently *log*-transformed. These statistics were performed using R version 3.2.2 (R
305 Core Team 2015).
306 To determine if feather concentrations of HBCDD isomers were associated with
307 physical size and endocrine biomarkers, Spearman's Rank Correlation analyses (due to

308 small sample size) were conducted between feather concentrations of α-, β-, γ- and
309 ΣHBCDDs in feathers and plasma concentrations of FT_4 and FT_3, as well as the FT_3/FT_4
310 ratio. These statistics were conducted using IBM SPSS 21. A significance level of α =
311 0.05 was employed throughout when applicable; all means are depicted with SEM.
312

313 **Results**
314

315 *HBCDD* in ovo *exposure, uptake and deposition into feathers*

316 The mean concentrations of the HBCDD isomers in the sibling eggs for the
317 present HBCDD-exposed kestrels (n = 11) were 127 ± 21 ng/g ww, 12 ± 2 ng/g ww and
318 2 ± 0.5 ng/g ww for mean α-, β- and γ-HBCDD concentrations, respectively,, making up
319 90%, 9%, and 1% of the ΣHBCDD concentrations each (Fig. 1). These concentrations of
320 the three isomers, and the mean ΣHBCDDs concentration of 141 ± 23 ng/g, were similar
321 to those previously published for the overall sample of birds including the current
322 individuals [20].

323 In the current study, all three isomers of HBCDD were detected in the tail feathers
324 of the exposed kestrels and the raw data for each individual is presented in Table 1.
325 Uptake of HBCDD isomers occurred in the tail feathers of all of the exposed individuals
326 (mean ± SE: ΣHBCDD: 2405 ± 267 pg/g dw). In these feathers, γ-HBCDD was the
327 dominant congener making up 55% of ΣHBCDDs and was found in all exposed
328 individuals (n = 11), followed by α-HBCDD (32%; n = 10 kestrels) and then β-HBCDD
329 (13%; n = 9, Figs. 1, 2). At least one HBCDD isomer was detected in 5 out of 6 control
330 individuals, although only one individual had uptake and deposition of all three isomers
331 in their central rectrix feather (Table 1).

332 The mean ΣHBCDDs concentration in feathers of controls was 860 ± 310 pg/g dw
333 in which, similar to exposed birds, γ-HBCDD made up the largest proportion (46%; n =
334 4) followed by α-HBCDD (31%) and β-HBCDD (6%; n = 2; Fig. 2). HBCDD-exposed
335 birds had significantly higher levels of γ-HBCDD (t_{17} = -4.13, p = 0.001), β-HBCDD
336 (t_{17} = -3.10, p = 0.007) and ΣHBCDDs (t_{17} = -3.53, p = 0.003) compared to control
337 individuals. In contrast, feather concentrations of α-HBCDD were similar between
338 exposed and control birds (p = 0.416). The proportions of HBCDD isomers in the
339 feathers were statistically similar between the HBCDD-exposed and control kestrels ($p \geq$
340 0.216).

341 Feather concentrations of α-HBCDD were on average 203 ± 38 times lower than
342 the *in ovo* sibling egg concentrations of the same isomer for exposed birds. Differences
343 between the *in ovo* and feather concentrations of the γ- or β-HBCDD isomers were much
344 smaller in comparison to the differences in α-HBCDD: γ-HBCDD was 2 ± 1 times
345 greater, and β-HBCDD was 54 ± 18 times higher, in eggs compared to feathers. The
346 ΣHBCDDs concentrations measured in feathers were 72 ± 16 times lower than those
347 measured in sibling eggs of exposed birds. The proportions of the three isomers differed
348 between feathers and eggs for individual isomers ($-4.26 < Z < -2.94$, $0.000 < p < 0.002$).
349 The isomer profile in the feathers of the kestrels was dominated by γ-HBCDD (55%; Fig.
350 1) while *in ovo* concentrations were highly dominated by α-HBCDD (90%; Fig. 1).

351

352 *Estimated impact of growth on HBCDD isomer body burdens*

353 The kestrels grew considerably over the nestling stage gaining significantly in
354 mass ($F_{1,94}$ = 375.36; p < 0.01). Their rapidly increasing body mass has likely resulted in

355 the dilution of the HBCDD burden at hatching. Assuming assimilation of the HBCDD
356 deposited in the entire egg, and that there were no differences in concentrations relating
357 to egg-laying order within the brood, we estimated that the body burdens of the *in ovo*
358 exposed birds contained median ΣHBCDDs concentrations of 2,000 pg ww (range: 1,100
359 - 3,900 pg) at hatching (Table 2). Considerable weight gain over the nestling stage
360 resulted in the dilution of the initial maternal egg yolk burden and subsequent estimated
361 body burdens at hatching by a median factor of 11 (range: 9.5 - 12) after 20-30 days of
362 age (Fig. 3). Assuming minimal metabolic capability and in the absence of excretion and
363 dietary input, such growth dilution resulted in body burdens of fledglings (d 30) having a
364 median ΣHBCDDs concentration of 160 pg ww (range: 84 - 360 pg) (Table 2).
365
366 *Ranking of factors affecting feather HBCDD concentrations*
367 The concentrations of α-HBCDD in tail feathers were best explained by the *in ovo*
368 concentrations of β- or γ-HBCDD (w_i = 0.50, 0.30 respectively), each of which explained
369 61% and 57% of the variation in α-HBCDD in the feathers (Table 3; Fig. 4). All other
370 models had Akaike weights considerably lower than the two top models (w_i = 0.02 –
371 0.07). Among these two top models, both the rectrix feather length at d 10 (R^2 = 0.43)
372 and the length of the tibiotarsus bone on d 30 (R^2 = 0.31), accounted for a considerable
373 amount of variation in the α-HBCDD feather concentrations. In contrast, feather
374 concentrations of β- and γ-HBCDD were only influenced by variables related to body or
375 feather size and growth, and not the *in ovo* concentrations of any of the three HBCDD
376 isomers. Feather β-HBCDD concentrations were best explained first by rectrix length on
377 d 30 of age (w_i = 0.61) followed by tibiotarsus length on d 10 of age (w_i = 0.28) each

378 accounting for approximately half of the variation in feather β-HBCDD concentrations
379 (Table 3). Feather γ-HBCDD concentrations were best explained by the growth
380 asymptote (w_i = 0.70) followed by body mass at d 25 of age (w_i = 0.30). However, these
381 variables only accounted for 31% and 24% of the variation respectively (Table 3).
382
383 *Associations between feather HBCDD concentrations and thyroid hormone levels*
384 The mean concentrations of FT_3 and FT_4 in the 20 d old exposed nestlings were
385 2.24 ± 0.19 pg/mL and 5.51 ± 0.91 pg/mL and their mean FT_3:FT_4 ratio was 0.52 ± 0.08.
386 The feather concentrations of α-HBCDD only, were negatively associated with the
387 FT_3:FT_4 ratio (r = -0.82, p = 0.020; Fig. 5). There were no associations between the other
388 two HBCDD isomer concentrations, or ΣHBCDDs concentrations, in the feathers and the
389 circulating concentrations of FT_3 or FT_4 measured in nestlings.
390
391 **Discussion**
392 This research demonstrates that HBCDD isomers can be transferred into the
393 feathers grown by American kestrels following their *in ovo* exposure. This confirms a
394 previous report that detected all three HBCDD isomers in the feathers (primary, tail and
395 body) of wild barn owls (*Tyto alba*) from Belgium and France [10]. As expected,
396 concentrations of HBCDD isomers in the present kestrel feathers were lower than the
397 initial *in ovo* exposure concentrations, by 2 orders of magnitude for α-HBCDD, and one
398 order of magnitude for β-HBCDD (γ-HBCDD was only 2 times lower in feathers). This
399 is consistent with most reports showing that feather concentrations of contaminants,
400 including HBCDD [10], are usually low compared with other tissues (e.g. [6, 8, 9] but see

401 [13]). This study provides the first examination of HBCDD uptake and deposition into
402 feathers of nestling birds for which *in ovo* exposure concentrations prior to feather growth
403 are known, and is only the second laboratory exposure study to evaluate feather uptake
404 and deposition of POPs.

405 First, we want to address the possible impact of external contamination of feathers
406 on their HBCDD profile, which is a recurring concern for the assessment of feather POP
407 concentrations from wild birds [33, 34]. We believe that this is unlikely to have occurred
408 in this captive study because exposure to HBCDD of the parents of these nestlings ceased
409 two days prior to the projected hatch date. Birds were fed *ad libitum* and typically
410 consumed most of the prey, however, some remains may have been left during the initial
411 days of the hatchling period, leaving a slim chance for the presence of small amounts of
412 HBCDD-TM to be present in the pens although American kestrels are not scavengers.
413 However, this is additionally highly unlikely since the control nestling kestrels
414 demonstrated the same high proportions of γ-HBCDD compared to α-HBCDD in their
415 feather rectrices and the control birds would have had no possibility of external exposure
416 to the technical mixture in their pens having received their background exposure via their
417 cockerel feed. The possibility of exposure to appreciable HBCDD-TM from the indoor
418 environment is also unlikely because birds were housed in wood barns without insulation,
419 electronics or upholstry (products that contain HBCDD-TM). Hence with this
420 experiment, we are able to address uptake and metabolism of *in ovo* exposure to
421 HBCDD, and demonstrate that it can be deposited in feathers.
422
423 *HBCDD isomer profile in nestling kestrel feathers*

424 In the present kestrels, the change in the proportions of HBCDD isomers from
425 90% α-HBCDD dominating the *in ovo* concentrations to 55% γ-HBCDD dominating the
426 feather concentrations (followed by α-HBCDD at 32%), is an unexpected and novel
427 finding. This profile of HBCDD isomers in the nestling feathers is in contrast to findings
428 in the majority of other studies. Though environmental compartments, similar to the
429 commercial mixture, are dominated by γ-HBCDD (70-80%), α-HBCDD makes up 80-
430 95% of ΣHBCDDs concentrations in tissues and eggs of vertebrates (reviewed in: [17,
431 35]). The HBCDD isomer profile in the present kestrel nestling feathers is also in contrast
432 to the profile seen in the diet-exposed adult kestrels (similarly exposed to the parents of
433 the current study subjects), in which plasma (after the depuration period), liver and
434 adipose tissue were dominated by α-HBCDD that comprised ~70-80% of ΣHBCDDs
435 concentrations [23], as well as the feathers of barn owls in which α-HBCDD comprised
436 ~60-80% of ΣHBCDD concentrations [10]. This latter study shows that the different
437 HBCDD isomer profile dominated by γ-HBCDD in the present kestrel nestling feathers
438 cannot be simply attributed to the matrix. Other factors must have affected the change in
439 HBCDD isomer profile from α-HBCDD dominating the *in ovo* exposure concentrations
440 to γ-HBCDD dominating in feathers. We hypothesize that the age and/or rapid growth of
441 the nestlings may be at the basis for the 'anomalous' feather HBCDD profile. A number
442 of possible explanations may exist based on whether or not the feathers reflected internal
443 proportions of HBCDD isomers or not.

444 Few studies have compared feather concentrations of contaminants with internal
445 body compartments, however feather concentrations of POPs, which are deposited from
446 blood during their growth, often well reflect concentrations in rapidly perfused tissues

447 (e.g. liver and plasma). Feather concentrations of PCBs, DDTs, and PBDEs in the
448 nestlings of three other raptor species largely correlated with plasma concentrations [12],
449 and European starlings exposed to CB-153 (via silastic implants) had feather
450 concentrations that correlated with plasma and liver concentrations [8]. In relation to
451 HBCDD specifically, feather concentrations of α-HBCDD were correlated with liver
452 concentrations of the same isomer in adult barn owls [10]. If the feather HBCDD profile
453 from the present nestling kestrels reflected their circulating profile, then this study
454 provides evidence of a change from α-HBCDD dominating in eggs (and thus exposure to
455 the chick) to γ-HBCDD dominating by the end of the nestling period. If this were the
456 case, metabolism or isomerization would likely have had to take place in these kestrel
457 nestlings since the entire egg yolk is absorbed by the chick within 3-5 d of hatching, and
458 we can assume complete assimilation of *in ovo* contaminants (90% α-HBCDD) by then
459 or earlier during embryonic development. While we cannot rule out the explanation that
460 feather HBCDD proportions reflect the internal body burden-blood profile without
461 knowing the HBCDD concentrations from other tissues during growth and at the time of
462 feather completion, it may be unlikely for two reasons. First, because of the
463 overwhelming evidence for the dominance of α-HBCDD in animal tissues including
464 several bird species (reviewed in: [17]) as well as in adult kestrels exposed to the same
465 HBCDD-TM mixture [23]. Second, because to date, α-HBCDD does not appear to be
466 isomerized or metabolized *in vivo,* although only two studies have examined this
467 potential [36, 37]. The dominance of α-HBCDD in animal tissue has been attributed to its
468 longer half-life and thus greater persistence in the body relative to β- and γ-HBCDD
469 (reviewed in: [23]). There is some evidence for isomerization of γ-HBCDD into α-

470 HBCDD in laboratory rodents [38]. There is also some evidence for the metabolism of β-
471 and γ-HBCDD by CYP enzymes (into, e.g., OH-HBCDDs: [37]), which would contribute
472 to their shorter half-life. Conversely, α-HBCDD does not appear to be isomerized in the
473 reverse direction [36], nor metabolized by liver enzymes in laboratory rodents [37].
474 However, though the weight of evidence suggests that this HBCDD isomer profile in the
475 kestrel feathers is highly unlikely to reflect the internal profile, there is not yet enough
476 research on the subject. Indeed, the similarity in feather α-HBCDD concentrations
477 between control and exposed birds may suggest that some isomerization of α-HBCDD
478 did occur in these birds, although the detection rate in controls was much lower (50%
479 compared to 90% in exposed birds). It is possible that HBCDD metabolism could be
480 different in developing nestlings. To date, studies reporting HBCDD concentrations in
481 nestling birds have all published low ΣHBCDD concentrations or concentrations below
482 the detection limit [39-41], thus it is not known if nestling birds show a different HBCDD
483 isomer profile compared to adults in any other species nor in the wild, and further
484 research would be beneficial.

485 The next possible explanation for the change in HBCDD isomer profiles between
486 sibling eggs and feathers in the present kestrels is that the feather profile of HBCDD
487 isomers did not reflect internal proportions, which has been previously documented in
488 some studies [12, 42]. In such a case, selective uptake or deposition of the different
489 isomers into tail feathers may have occurred, ultimately causing an increase of β- and γ-
490 HBCDD relative to α-HBCDD. Interestingly, the profile of HBCDD isomers in the
491 cerebral cortex of chicken (*Gallus domesticus*) embryos exposed *in ovo* to the same
492 HBCDD-TM as the parents of the present kestrels, did not differ from the dosing solution

493 at pipping (i.e. fully developed) [43]. The authors attributed this to the lower activity of
494 CYP enzymes in the brain relative to liver and possibly other tissues as well [43].
495 However, it is possible that there may have also been some form of selective passage of
496 β- and γ-HBCDD though the blood-brain-barrier, which could have been related to their
497 chemistry or other processes.

498 The fact that adult wild barn owls showed α-HBCDD dominance in feathers [10]
499 may seem like evidence against the selective uptake of β- and γ-HBCDD into feathers
500 based on their chemical properties alone, and indicates that the profile of HBCDD
501 isomers seen in the kestrel feathers does not always present itself. It is possible that
502 deposition of POPs into feathers may be different for young and/or growing birds
503 compared to adults. The fact that the β- and γ-HBCDD concentrations in feathers
504 depended on body size measures or growth parameters, and not on *in ovo* exposure (in
505 AIC model selection), suggests that their uptake into feathers may have been affected by
506 the growth phase. We demonstrated a very rapid and steep effect of growth dilution on
507 HBCDD body burdens and this could have impacted the deposition of HBCDD isomers
508 into the feathers. Though very similar HBCDD profiles were seen in primary, tail and
509 body feathers in wild barn owls [10], adults of most species, molt their feathers
510 individually in sequence over 1-2 months [44]. Conversely nestlings grow their entire
511 first set of feathers at once, which may have impacted the distribution and/or deposition
512 of contaminants into the feathers, which could also have differed across feathers in
513 different parts of the body. Eulaers and colleagues have made a similar suggestion, i.e.,
514 that the age of the nestlings may have impacted feather concentrations following their
515 study of various chlorinated and brominated POPs in nestling raptor feathers [13]. For

516 nestlings sampled between 1 and 6 weeks of age, when feathers were still connected to
517 the circulating blood stream and growth was ongoing, feather concentrations correlated
518 significantly with plasma concentrations of most POPs [12]. However, in 9 week-old
519 white-tailed eaglets (*Haliaeetus albicilla*), when growth was complete and feathers were
520 atrophied from the blood stream (similar to the present study birds), single POP
521 contaminant concentrations were not or were less strongly correlated with plasma
522 concentrations [13]. The authors suggested that in addition to feather atrophy, transitional
523 physiological characteristics of fledglings may explain the lack of relationship between
524 feather and plasma POP concentrations in older nestlings [13]. These included
525 differential preening behavior (and thus contribution of contaminants from preen oil) and
526 metabolic changes [13] which are also hypotheses worth investigating in future studies on
527 feather deposition of POPs in young birds. One final possible factor for the unusual
528 dominance of γ-HBCD in the kestrel feather profile versus α-HBCDD in avian tissues in
529 other studies (reviewed in: [17, 35]) is that the current exposed kestrels were subject to an
530 ever-diminishing body burden of HBCDD (both α-HBCDD and ΣHBCDD) over the
531 course of the 28-d nestling period that originated from their embryonic exposure via
532 maternal transfer. In comparison, wild birds could be continuously ingesting
533 contaminated food and hence exposed to HBCDD that would maintain their body
534 burdens of α-HBCDD.

535 Ultimately, a combination of these different hypotheses may explain the HBCDD
536 profiles in the nestling kestrel feathers. The α-HBCDD feather concentrations were best
537 explained by the *in ovo* concentrations of β- and γ-HBCDD, suggesting the concentration
538 of this isomer may reflect internal concentrations, whereas in contrast, those for β- and γ-

539 HBCDD were unrelated to *in ovo* concentrations but were explained by growth and size
540 parameters, thus may have been subject to some form of selective uptake influenced by
541 growth. Regardless, the present study corroborates the cautionary conclusion of Eulaers
542 and colleagues [13]: that nestling tail feathers may not always accurately indicate internal
543 concentrations of POPs and/or exposure levels (body feathers may be better). This
544 appears to be the case particularly for older nestling or juvenile birds, although more
545 studies are needed in general. Further research on concentrations of POPs, including
546 HBCDD, in feathers in conjunction with those in other body compartments of nestlings
547 throughout the growth period is needed. In addition, if some HBCDD isomers were
548 subject to selective uptake in the kestrel feathers, the growth dilutions calculated herein
549 must be interpreted with caution as it was assumed that feather concentrations reflected
550 body burdens in the modeling.

551

552 *Use of feather concentrations as indicators of exposure*

553 Many studies now demonstrate that a wide variety of contaminants can be
554 measured in bird feathers, and that they are thus useful for biomonitoring purposes. A
555 number of studies further show that feather concentrations correlate with internal
556 exposure concentrations [8, 9, 12] and the present results indicate that feather
557 concentrations can also reflect *in ovo* exposure concentrations (for α-HBCDD). The next
558 step is to begin using feather concentrations to determine relationships between
559 contaminants and physiological endpoints that may be affected by exposure. This line of
560 research is limited by the fact that feathers represent exposure concentrations at the time
561 of feather growth, and tissue and other data collection cannot always be collected

562 simultaneously in the field. However, the use of feathers in this manner is highly
563 attractive for studies on wild birds because they can provide a minimally invasive and
564 non-destructive means for determining contaminant body burdens. To the best of our
565 knowledge, this has not yet been attempted with respect to POPs in any species in
566 captivity or in the wild.

567 Our second objective was thus to determine if feather concentrations may be
568 related to physiological endpoints known to be affected by HBCDD, to determine if they
569 may be useful for this purpose. *In vitro* studies identified HBCDD isomers as strong to
570 medium thyroid disruptors [19, 45], which is supported by *in vivo* studies. Reductions of
571 circulating T_4 following exposure to HBCDD have been noted in rats [46-48], rainbow
572 trout (*Onorhynchus mykiss*) [49] and adult male kestrels [22], with disruption of thyroid
573 function observed in kestrel nestlings exposed to HBCDD from a larger sample including
574 the present individuals (K. Fernie, unpublished data). Additionally, wild beluga whales
575 (*Delphinapterus leucas*) with greater exposure to HBCDD demonstrated lower
576 concentrations of thyroid hormones in plasma [50]. In the current study, we show that α-
577 HBCDD concentrations are negatively correlated with the FT_3:FT_4 ratio in plasma
578 collected when nestlings were 20 d of age. This suggests that those nestlings having
579 higher feather HBCDD concentrations also had higher plasma concentrations of T_3, the
580 biologically active thyroid hormone converted from T_4, which could indicate changes in
581 thyroid gland function and/or metabolism of these hormones [51]. Interestingly, however,
582 neither β- nor γ-HBCDD feather concentrations were correlated with this thyroid ratio,
583 which may be because they were not directly related to *in ovo* exposure concentrations
584 and may have been subject to selective uptake into feathers. Similarly, none of the three

585 isomers were associated with the individual plasma concentrations of the two thyroid
586 hormones. Though our sample size in the present study is small, and these results should
587 be considered preliminary, the use of feathers, here to determine concentrations of α-
588 HBCDD which were directly correlated with *in ovo* exposure concentrations, show
589 promise in determining how contaminant body burdens may relate to physiological
590 effects of exposure.
591
592 *Conclusions*
593 This study demonstrates that HBCDD isomers can be deposited and measured in
594 the feathers of nestling kestrels exposed only *in ovo* to maternally transferred HBCDDs.
595 Concentrations of HBCDDs in feathers were much lower than *in ovo* sibling egg
596 concentrations, as expected, however, a shift from an α-HBCDD to a γ-HBCDD
597 dominated isomer profile represents a novel finding. We hypothesize that feather
598 concentrations of α-HBCDD may reflect the internal body burden, while β- and γ-
599 HBCDD may have undergone selective uptake into the tail feathers. We suspect that this
600 may have been influenced by the rapid growth of these kestrel nestlings. Further studies
601 on how POPs are deposited into feathers in conjunction with internal concentrations in
602 growing birds, as well as in adult birds in which toxicodynamics may differ, is thus
603 important. Additionally, feather α-HBCDD was positively correlated with the plasma
604 FT_3:FT_4 ratio in this small group of kestrels. While these could be considered preliminary
605 results, our findings demonstrate for the first time that when feathers accurately reflect
606 exposure regimes, as was the case for α-HBCDD, they can be used as a non-destructive
607 means to assess the possible effects of contaminant exposure on physiology in wild birds.

608

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618 the University of Antwerp and FWO Flanders (to A. Covaci and M. Eens).

619

620 **Table 1**: Mean concentrations (±SEM) of HBCDD isomers in the right central rectrix

621 feathers of captive American kestrels exposed *in ovo* to mean concentrations of 127 ± 21

622 ng/g ww, 12 ± 2 ng/g ww and 2 ± 0.5 ng/g ww of α-, β- and γ-HBCDD concentrations,

623 respectively.

624

		Concentration (pg/g dw)				% of Σ HBCDD detected:		
	ID	**α-HBCDD**	**β-HBCDD**	**γ-HBCDD**	**Σ HBCDD**	**α-HBCDD**	**β-HBCDD**	**γ-HBCDD**
Exposed	4412	533	368	2785	3685	14.45	9.98	75.57
(*n*=11)	4420	1002	400	1666	3069	32.66	13.05	54.29
	4431	636	512	1300	2447	25.97	20.90	53.13
	4441	524	*<150*	317	841	62.33	0.00	37.67
	4446	1033	466	695	2195	47.09	21.22	31.69
	4453	*<400*	400	2094	2493	0.00	16.03	83.97
	4462	574	301	1009	1885	30.45	15.98	53.57
	4466	503	*<150*	494	996	50.45	0.00	49.55
	4471	1257	430	1238	2925	42.97	14.71	42.31
	4478	773	556	1366	2695	28.70	20.62	50.68
	4491	606	424	2198	3228	18.76	13.14	68.09
		694.64	**364.27**	**1378.36**	**2405.36**	**32.17**	**13.24**	**54.59**
		90.14	**47.74**	**229.29**	**267.42**	**5.36**	**2.25**	**4.76**
Control	4414	*<400*	*<150*	385	385	0.00	0.00	100.00
(*n*=6)	4434	1333	297	500	2130	62.60	13.94	23.46
	4488	703	245	*<300*	948	74.20	25.80	0.00
	4496	625	*<150*	617	1242	50.32	0.00	49.68
	4504	*<400*	*<150*	*<300*	*n/a*	0.00	0.00	0.00
	4507	*<400*	*<150*	458	458	0.00	0.00	100.00
		443.65	**90.23**	**326.56**	**860.44**	**31.19**	**6.62**	**45.52**
		222.29	**57.47**	**107.75**	**310.58**	**14.28**	**4.46**	**18.78**

625 The limits of quantification were 400, 150, and 300 pg/g dw for α-, β- and γ-HBCDD
626 respectively and non detects are indicated as <400, <150 and <300. Isomer proportions
627 are calculated using values above the detection limit only.
628

629
630 **Table 2.** HBCDD body burden concentrations (pg/g ww) of *in ovo* exposed kestrel
631 nestlings (n = 11) at hatching (day 1) and fledging (day 30) estimated by statistical
632 modelling of the growth dilution of the initial *in ovo* exposure.
633

	day 1	**day 30**
α-HBCDD	1800 (940 - 3700)	160 (84 - 360)
β-HBCDD	190 (140 - 320)	17 (12 - 32)
γ-HBCDD	22 (5.4 - 96)	2.0 (0.44 - 9.7)
ΣHBCDD	2000 (1100 - 3900)	180 (100 - 400)

634
635

636 **Table 3:** Factors affecting the deposition of α-, β- and γ-HBCDD concentrations in the

637 right central rectrix feathers of American kestrels exposed *in ovo* to known HBCDD

638 concentrations (127 ± 21 ng/g ww, 12 ± 2 ng/g ww and 2 ± 0.5 ng/g ww for mean α-, β-

639 and γ-HBCDD concentrations, respectively).

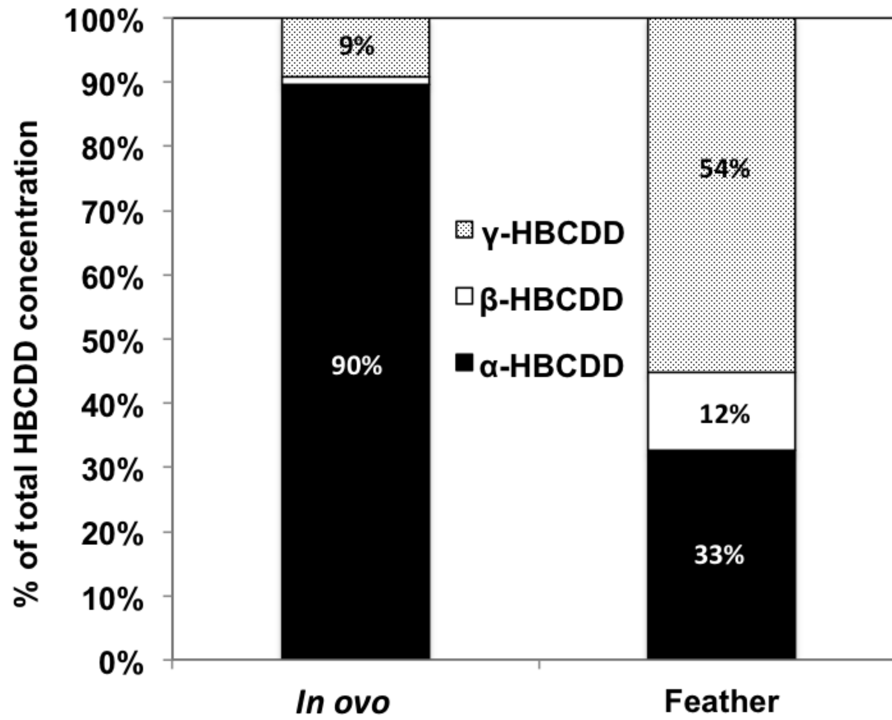
640

	B	ΔAIC_c	w_i	R^2
Feather α-HBCDD				
in ovo β-HBCD	80.31	0.00	0.50	0.61
in ovo γ-HBCD	139.29	1.03	0.30	0.57
tibiotarsus d 10	115.38	4.08	0.07	0.43
in ovo ΣHBCD	3.07	4.67	0.05	0.40
retrix length d 10	137.35	5.36	0.03	0.36
in ovo α-HBCD	3.06	5.46	0.03	0.36
tibiotarsus d 30	128.88	6.22	0.02	0.31
Null		7.54		
Feather β-HBCDD				
retrix length d 30	34.96	0	0.61	0.53
tibiotarsus d 10	62.49	1.56	0.28	0.46
retrix length d 10	66.88	4.53	0.06	0.29
Mass d 10	10.91	4.89	0.05	0.27
null		5.51		
Feather γ-HBCDD				
growth asymptote	83.03	0	0.70	0.31
mass d 25	81.31	1.72	0.30	0.24
null		1.90		

641

642 Statistics from the Generalized Linear Models: B = partial regression coefficient from the
643 parameter estimate (when positive, it indicates a positive relationship and vice versa),
644 ΔAIC_c = difference in Akaike's Information Criterion corrected for small sample sizes, w_i
645 = Akaike weight. Statistic from linear regression: R^2 = coefficient of determination.
646

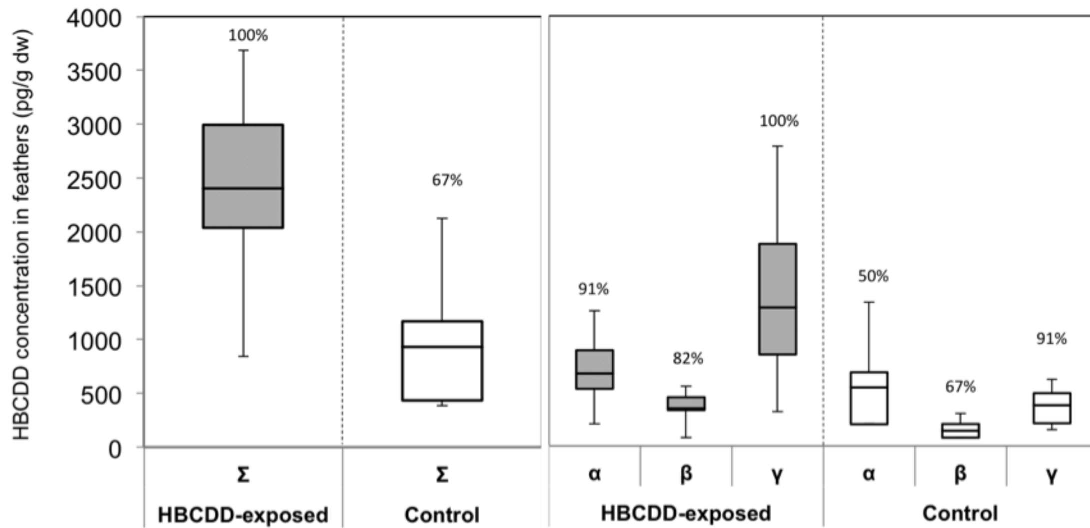
647
648 **Figure 1:** The composition of the HBCDD isomer profile (mean percentages) in sibling
649 eggs and right central rectrices of American kestrels exposed *in ovo* to known HBCDD
650 isomer concentrations (n=11).



651
652

653 **Figure 2:** Concentrations of HBCDD isomers deposited in the right central rectrix
654 feathers of captive American kestrels exposed *in ovo* by maternal transfer to
655 environmentally relevant levels of HBCDD isomers (n=11) and of control kestrels (n =
656 6). The detection frequency is depicted as the percentage of individuals with measurable
657 concentrations of HBCDD isomers.

658



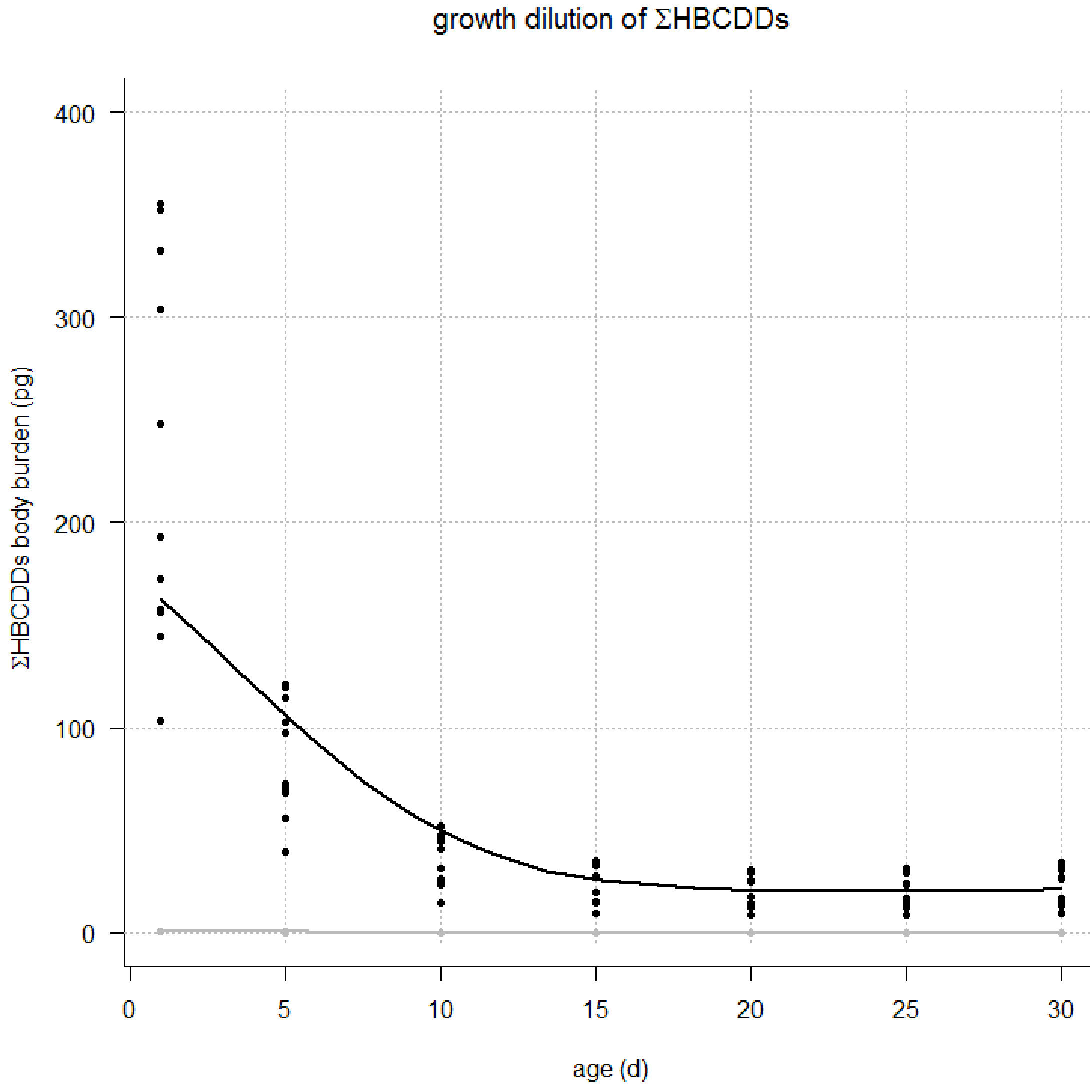
659

660

661 **Figure 3: Estimated** dilution of ΣHBCDDs throughout growth of American kestrel

662 nestlings exposed *in ovo* by maternal transfer to environmentally relevant levels of

663 HBCDD isomers (n=11) generated by a logistic growth model.



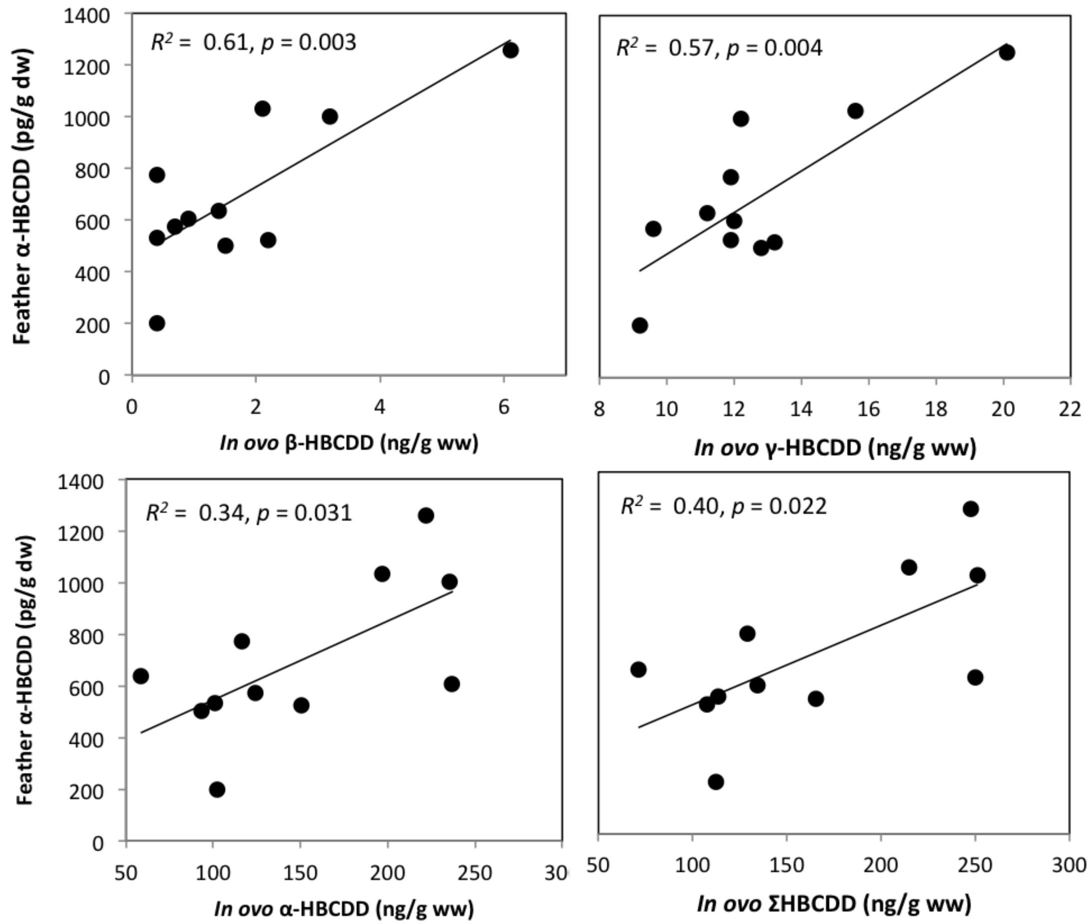
664

665

666 **Figure 4**: Associations between concentrations of α-HBCDD in right central rectrices of

667 exposed American kestrels (n=11)and the *in ovo* concentrations of HBCDD isomers in

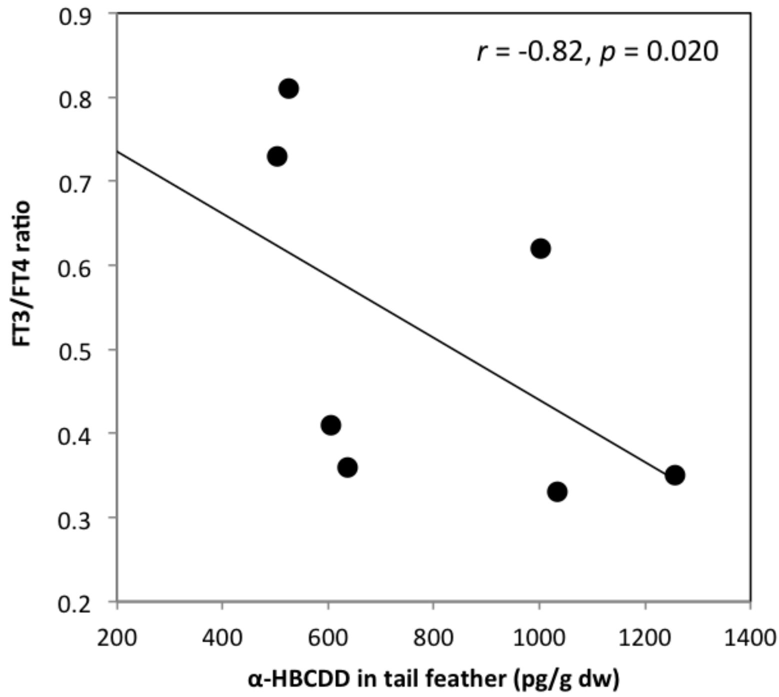
668 sibling eggs.



669

670

671 **Figure 5**: The ratios of free triiodothyroinine to free thyroxine (FT_3:FT_4) measured in
672 plasma of kestrel nestlings exposed *in ovo* to HBCDD isomers by direct maternal transfer
673 (n=7), in relation to feather concentrations of α-HBCDD.



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