First evaluation of the use of down feathers for monitoring persistent organic pollutants and organophosphate ester flame retardants: a pilot study using nestlings of the endangered Cinereous Vulture (*Aegypius monachus*)

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Abstract

Raptor feathers have been increasingly used to assess pollutants in ecotoxicological monitoring studies. However, the suitability of down feathers to detect pollutants has not yet been investigated. In this study, concentrations of persistent organic pollutants (POPs) and organophosphate ester flame retardants (OPEs) were assessed in down and juvenile contour feathers of Spanish cinereous vulture (*Aegypius monachus*) nestlings (circa 73 days old) and contaminant concentrations were compared between both types of feathers from the same individuals. Concentrations of polychlorinated biphenyls (PCBs: 1.30-6.16 ng g^{-1} dw feather), polybrominated diphenyl ethers (PBDEs: 0.23-1.35 ng g^{-1} dw feather), *p,p'*-dichlorodiphenyldichloroethylene (*pp*-DDE: 0.09-6.10 ng g^{-1} dw feather) and tris (1-chloro-2-propyl) phosphate (TCiPP: 0.86-48.96 ng g^{-1} dw feather) were significantly higher in down than in contour feathers. In contrast, contour feathers showed higher levels of the more volatile POP, lindane (0.25-3.12 ng g^{-1} dw feather). Concentrations of hexachlorobenzene (HCB) and OPEs (except TCiPP) were similar between the two types of feathers. By showing high accumulation of the most persistent POPs investigated, down feathers presented a contamination profile similar to that previously described in raptor eggs. As these feathers grow during the first days of a vulture chicks life, they probably reflect the contaminant burden of the chick due to maternal transfer to the egg. Overall, the present study provides the first indication that down feathers may be useful for biomonitoring studies. Further research is needed to confirm whether nestling down feathers reflect the concentrations in the egg.

Keywords

Down feathers; contour feathers; nestlings; POPs; OPEs

Capsule

Nestling down feathers can be useful for contaminant monitoring.

Introduction

The assessment of organic pollutants in feathers is a recently developed technique that has been increasingly used in ecotoxicological monitoring studies (Abbasi et al., 2016a; Pollack et al., 2017). The most common contaminants measured in feathers have been the persistent organic pollutants (POPs) including the polychlorinated biphenyls (PCBs) and the organochlorine pesticides (OCPs), and more recently the polybrominated diphenyl ethers (PBDEs) (Eulaers et al., 2014a; Jaspers et al., 2011, 2009). PCBs and OCPs were banned several decades ago, and PBDEs were recently restricted (Directive EEC, 2003). However, they are still widespread in the environment (Lohmann et al., 2007; Thomas et al., 2006) causing adverse effects on biota, particularly affecting high-trophic level wildlife, such as predatory bird species (Eulaers et al., 2011a). Along with PBDEs, the organophosphate ester flame retardants (OPEs) have gained increasing attention due to their current extensive use and their ubiquitous, persistent and potentially toxic proprieties (Guigueno and Fernie, 2017). Yet, very few studies have used feathers as bioindicators for the presence of OPE traces in the environment (Eulaers et al., 2014a, 2014b).

Feathers have become a preferred method when nondestructive and noninvasive sampling is required (García-Fernández et al., 2013). They can provide a valuable assessment of internal body burdens of contaminants (Eulaers et al., 2014b, 2011a; Jaspers et al., 2006; Jaspers et al., 2007). Feathers grow during a limited period of time during which they accumulate circulating pollutants proportionally to blood levels (Burger, 1993). Thus, sampling grown feathers allows retrospective assessment of long-term contaminant exposure during the period of feather growth. Some recent studies have emphasized the importance of sampling the appropriate type of feather (Abbasi et al., 2016b; García-Fernández et al., 2013; Jaspers et al., 2011), which would depend on the bird species and the aim of the study. For instance, in raptors, wing feathers (mainly primary and secondary feathers) show an asynchronous molt that can last far longer than one season (Forsman, 1999), overlap with breeding and migratory seasons (Rohwer et al., 2009; Rohwer and Rohwer, 2013), and show a higher influence of external contamination (Jaspers et al., 2011). Whereas, body feathers seem to be advantageous (Eulaers et al., 2014b; Jaspers et al., 2011), as they molt over a more fixed and defined period of time (Gill, 2007; Hardy et al., 2006), offering more control of the temporal exposure (Jaspers et al., 2011). Other factors, such as age, gender or spatial factors, including habitat or migratory strategies, have also been detected to influence levels of pollutants (García-Fernández et al., 2013). In this sense, sampling at the nestling stage may offer several advantages such as mitigating the age-confounding effect, providing a small-scale geographical accuracy, and reducing the time gap between feather growth and feather sampling (Eulaers et al., 2011b).

The current study investigates levels of organic pollutants (PCBs, OCPs, PBDEs, and OPEs) in two types of feathers of nestling cinereous vultures (*Aegypius monachus*). For the first time, we investigated the suitability of down feathers in comparison to contour feathers (feathers with a vane), which have been used in all previous studies. We expected to find differences in the contaminant burdens between the different types of feathers, as they are growing at different times during the nestling stage.

Material and Methods

The cinereous vulture is a species catalogued as Near Threatened on a worldwide scale (BirdLife International, 2017). This species is suffering an ongoing decline in Asian countries. Because of reintroductions into the wild and conservation actions, the population of cinereous vultures is currently increasing in Europe, especially in Spain (BirdLife International, 2017). The cinereous vulture is a scavenger raptor that mainly feeds on carcasses, selecting medium-sized carcasses such as rabbit, livestock and big game (Del Moral and De la Puente, 2017). This species breeds in colonies of low density, typically building its nest in large trees in forested areas of mountainous zones (Hiraldo, 1977). Their clutch size is always one egg, so, in successful nests there is only one nestling (Donázar, 1993). The present study was performed within the framework of a monitoring program implemented during the breeding period (February to September) established since 1997. The feeding area for the study colony was about 100 Km in the surroundings. Lately, vultures of the studied colony have been observed to feed on a rubbish dump close to Madrid city and very often remains of plastic bags have been found in their nests (personal observations). Permission to work in the area was granted by national park authorities (Consejería de Medio Ambiente, Administración Local y Ordenación del Territorio de la Comunidad de Madrid, Spain).

During the breeding season of 2016, 99 nests were monitored in Sierra Guadarrama Madrid (Madrid province, Central Spain), of which 57 produced nestlings. Shortly before their anticipated fledging dates, nestlings were carefully lowered down from the nest in duffel bags and were banded for identification. Different biological samples were obtained within the framework of long-term conservation and monitoring studies on this population (De la Puente et al., 2011). For the purpose of the current study, we sampled down and juvenile contour feathers from the nestling during the same sampling effort. The two types of feathers are grown at different points in time (Fig 1). As described by Bernis (1966) and De la Puente (*in press*), second natal down feathers (referred here as down feathers) grow from 15 to 25 days post-hatching and replace the first white natal down that covered the nestling since hatching. The second natal down has a grey color instead of the white first natal down. At this age, pin feather development of the remiges and rectrices is apparent. From 30 days post-hatching, juvenile

black scapular and wing covert feathers start to be visible due to their contrasting color against the natal down (Bernis, 1966; De la Puente, *in press*). These feathers constitute the juvenile plumage that grows over a longer time (during weeks) and lasts until they start the molt to subadult plumage, one year later (Forsman, 1999). Samples from only 16 nestlings (mean age: 73 d, range: 65 - 89 d) could be used for the contaminant analysis, due to feather mass requirements. Down feathers (mean feather mass: 0.14 g; range 0.09 - 0.20 g) were gently pulled from the flanks under the wings. Contour feathers from the interscapular zone were still developing at that time as they were still (partly) in the shaft. Three to five contour feathers (mean feather mass: 0.22 g; range 0.15 - 0.31 g) were gently pulled and possible blood remains were removed from the shaft. Feathers were stored in paper envelopes at room temperature until analysis. After sampling, the nestlings were returned to their nest. The nests were monitored in the subsequent weeks and all the nestlings successfully fledged from their nests.

Analytical procedures for feathers were similar to the method described by Dauwe et al. (2005) and Eulaers et al. (2014a). Briefly, feathers were thoroughly rinsed with distilled water and barbs were carefully separated using tweezers to remove exogenous dust particles and other unwanted deposition (Jaspers et al., 2011, 2008). After washing, feathers were covered with standard laboratory paper and dried overnight at room temperature. Dried feathers were cut into pieces of ~1 mm^2 with scissors, weighted (juvenile: mean 0.22 g, range 0.15 - 0.31 g; down feathers: mean 0.14 g, range 0.09 - 0.20 g) and transferred to analytical glass recipient. Feather samples were then spiked with 100 µL of internal POPs standard containing 200 pg μL^{-1} CB143; 25 pg μL^{-1} ε-HCL; 25 pg μL^{-1} BDE77 and 25 µL of internal OPEs standard containing 1 ng μL^{-1} TCEP-d12; 1 ng μL^{-1} TDCiPP-d15; 1 ng μL^{-1} TPHP-d15; 1 ng μL^{-1} TAP; 2 ng μL^{-1} TBOEP-d6. After overnight incubation at 45 °C in 5 mL of HCl (4M) and 6.5 mL of hexane/dichloromethane (4:1, v:v), the analytes were liquid-liquid extracted using 5 mL hexane/dichloromethane (4:1, v:v). Cleanup of the resulting extracts was performed on Florisil® cartridges (Supelco®) topped with anhydrous Na_2SO_4 (200 mg). The cartridges were prewashed with 6 mL of ethyl acetate and 6 mL of hexane and analytes (POPs) were eluted with 10 mL of hexane:dichloromethane (1:1, v:v) to obtained the first fraction (F1) that was concentrated to ~200 µL using a gentle flow of nitrogen gas. OPEs were eluted with 10 mL of ethyl acetate to obtain the second fraction (F2) that was concentrated to near dryness using a gentle flow of nitrogen gas. A second cleanup was performed for F1 on acidified silica (500 mg; 44 % H_2SO_4) topped with anhydrous Na_2SO_4 (500 mg). Cartridges were previously washed with 6 mL of hexane and analytes were eluted with 10 mL hexane:dichloromethane (1:1, v:v). The F1 second cleanup extracts were concentrated to near dryness using a gentle flow of nitrogen gas. Finally, F1 and F2 extracts were reconstituted with 100 µL of isooctane. Five samples of

each batch were reconstituted with 100 µL of recovery standard CB207 (50 pg μL^{-1} in isooctane-toluene 9:1, v:v). Fraction F1 was analyzed for POPs and fraction F2 for OPEs.

In all feathers, we analyzed 23 PCBs congeners (CB-28, 49, 52, 74, 95, 99, 101, 105, 110, 118, 138, 149, 153, 156, 170, 171, 177, 180, 183, 187, 194, 206 and 209), 7 PBDEs congeners (BDE- 28, 47, 99, 100, 153, 154 and 183), dichlorodiphenyltrichloroethane (*p,p´*-DDT) and metabolites (*p,p´*-DDD, *p,p´*-DDE), hexachlorobenzene (HCB), hexachlorocyclohexanes (α-, β-, γ-HCHs) and chlordanes (cis-nonachlor, trans-nonachlor and oxychlordane). Only 7 individuals could be analyzed for OPEs due to significant sample loss in the first batch because of a malfunctioning of the oven overnight. We analyzed tris(2-chloroethyl) phosphate (TCEP), tris (1-chloro-2-propyl) phosphate (TCiPP), tri-phenyl phosphate (TPhP) and tris (1,3-dichloro-2-propyl) phosphate (TDCiPP).

Analysis of POPs was done via gas chromatography (GC; Agilent GC 6890, Palo Alto, CA, USA) coupled to electron capture negative ionisation mass spectrometry (MS; Agilent MS 5973). A DB-5 capillary column (30 m x 0.22 mm x 0.25 µm) was used. Analysis of OPEs was done via gas chromatography (GC; Agilent GC 6890, Palo Alto, CA, USA) coupled to electron impact ionisation mass spectrometry (MS; Agilent MS 5973). A HT-8 capillary column (25 m x 0.22 mm x 0.25 µm) was used. Pesticide-grade solvents were obtained from Merck KGaA Chemicals (Darmstadt, Germany) and Acros Organics (Geel, Belgium). A procedural blank was analysed every 10^{th} sample. Recovery of internal standards was on average 76 ± 8%. All compounds were blank-subtracted using the average procedural blank values. The limit of quantification (LOQ) was set at 3 * SD of the procedural blanks, or, for analytes not detectable in blanks, calculated from a 10:1 signal to noise ratio. Concentrations of pollutants are expressed in ng g^{-1} dry weight (dw). LOQs were 0.005 ng g^{-1} dw for PCBs, HCB and HCHs, 0.1 ng g^{-1} dw for *p,p'*-DDE and *p,p'*-DDD, 0.003 ng g^{-1} dw for PBDEs and 1.0 ng g^{-1} dw for OPEs.

All statistical analyses were performed using R software version 3.2.2 (R-project, R Development Core Team, University of Auckland, New Zealand). Samples with levels below the LOQ were assigned a value of DF x LOQ, with DF the proportion of measurements with levels above the LOQ, or the detection frequency (Voorspoels et al., 2002) as has been done in most previous studies on feathers. A different DF value was calculated for each type of feather. Compounds detected less than 50% in both types of feathers were excluded (CB-28, 49, 52, 74, 95, 99, 101, 118, 149, 206, 209; α-HCH, β-HCH, cis-nonachlor, trans-nonachlor and oxychlordane; BDE-28, 100, 153, 154, 183; TCEP). Due to detection frequencies being different between the two types of feathers, for some compounds one type of feather showed a DF of 100% while the other showed ~40% (i.e. CB-105, lindane); therefore those compounds were included in the analysis.

Data were not normally distributed (Shapiro-Wilk's test>0.05), thus were log-transformed (log10(x+1)) to meet normal distribution requirements. The level of significance was set at α=0.05. We used a Repeated Measures two-way ANOVA to test differences of contamination levels and profiles between types of feathers (down feathers vs. contour feathers). Parametric Pearson Correlations were calculated between concentrations of compounds in both types of feathers. In addition, profiles were investigated by Principal Component Analysis (PCA) in order to better visualize differences between types of feathers using a biplot for the first two principal components (PC1 and PC2) and plotting both factor loadings and factor scores.

Results and Discussion

Accumulation levels

Amounts of ~150 mg of down feathers were sufficient to quantify 14 different POPs and 4 OPEs, suggesting that down feathers may be useful for contaminant monitoring. Table 1 shows mean concentrations, range, and detection frequencies for POPs and OPEs in down and juvenile contour feathers. The highest differences in the detection frequencies between types of feathers were recorded for PCBs. Twelve PCB compounds (CB-105, 118, 138, 153, 156, 170, 171, 177, 180, 183, 187, and 194) were quantified above LOQ in more than 50% of all down feather samples, in contrast to seven compounds (CB-105, 118, 138, 153, 170, 180, and 187) quantified in juvenile contour feathers. All seven PCBs showed higher detectability in down feathers, except for CB-153 and CB-180 that were detected in all analyzed samples. Some OCPs and PBDEs also showed different detection frequencies between the two types of feathers, with γ-HCH showing the highest difference. In contrast, *p,p´*-DDE, and BDE-99 were detected at similar rates in both types of feathers and *p,p´*-DDE in particular was detected in all samples, as previously reported in other types of feathers (Abbasi et al., 2016a). The congeners BDE-100, -153 and -154 were only detected in down feathers and showed very low detectability, far below the threshold of 50%. This prohibits any conclusion concerning the higher brominated PBDEs in accordance to other studies (Jaspers et al., 2009). Among OPEs, TCiPP, TPhP and TDCiPP were detected above LOQ in > 50% of the samples in both types of feathers at similar detection frequencies.

In general, concentrations of most POP compounds were higher in down feathers compared to juvenile contour feathers (Table 1). *p,p´*-DDE reached the highest concentration in both types of feathers followed by γ-HCH, CB-153 and CB-138 in juvenile contour feathers and by CB-153, CB-180 and CB-138 in down feathers (Table 1). These findings are in line with the previous study of Goutner et al. (2011), which showed that *p,p´*-DDE was the dominant compound in adults and nestlings of cinereous and Eurasian griffon vultures (*Gyps fulvus*). Concentrations of

p,p´-DDE and PBDE were significantly higher in down feathers than in juvenile contour feathers, and were similar to concentrations reported previously on contour feathers in White-tailed eagle (*Haliaeetus albicilla*) nestlings (Eulaers et al., 2014a). Concentrations of most PCBs were also higher in down feathers, but were one to two orders of magnitude lower than reported previously on contour feathers in several raptor species (Abbasi et al., 2016b; Eulaers et al., 2013). γ-HCH was significantly higher in juvenile contour feathers than in down feathers. This insecticide has a shorter environmental half-life (2 years approximately; Blus and Henny, 1985) compared to most OCPs and it is relatively rapidly metabolized and excreted in organisms (i.e. biomagnification factor < 0.4 vs. 1.9 for α-HCH and 7.3 for β-HCH in black guillemots; see Moisey et al., 2001). Thus, the significant presence of γ-HCH in juvenile contour feathers reflects most likely a recent exposure of birds to lindane. The high persistent compounds BDE-47 and BDE-99 were more predominant in down feathers. These compounds have been highly detected in feathers, eggs and other tissues samples of several bird species (Chen and Hale, 2010; Eulaers et al., 2014b; Morales et al., 2012) and their presence likely indicates a long historic exposure, rather than a recent exposure (i.e. reflecting the exposure of the mother transferred through the egg). Concentrations of HCB were similar between the two types of feathers. This compound is more volatile than other POPs, and variations in its levels have been more related to inter-annual fluctuations rather than to ecological factors (Eulaers et al., 2013).

Concentrations of OPEs were more similar between down and juvenile contour feathers than POP compounds, except TCiPP which was significantly higher in down feathers (Table 1). In general, OPE concentrations were one order of magnitude higher than POPs and were similar to the concentrations described in contour feathers of White-tailed eagle nestlings (Eulaers et al., 2014a). However, in contrast to that study, TCEP showed a lower detection frequency (<50%) and was found at lower concentrations, probably because of the small sample size.

Accumulation profiles

Fig 2 illustrates the different accumulation profiles between the two types of feathers. CB-153 was the predominant congener in both types of feathers. It was followed by CB-180 and CB-138 in down feathers and by CB-138 and CB-180 in juvenile contour feathers. The importance of the high chlorinated PCB compounds in both types of feathers is in concordance with previous studies performed in feathers of several raptor species (Abbasi et al., 2016b; Eulaers et al., 2013; Jaspers et al., 2007). The PCB profiles were different between the two types of feathers for almost all compounds (all $P<0.01$) except CB-105 which showed no significant difference ($F_{1.15}=0.40$; $P=0.54$) and CB-180 which only showed a tendency ($F_{1.15}=3.83$, $F=0.07^{T}$). A different OCP profile could also be observed between the two feather types. The most important OCP was *p,p'*-DDE in the two types, representing ~50% of the total sum of

OCPs in juvenile contour feathers and more than 60% in down feathers ($F_{1.15}$=3.11; P=0.08). *γ*-HCH was the second OCP more predominant in both types of feathers, however, while it represented ~40% in juvenile contour feathers, it only represented ~10% in down feathers ($F_{1.15}$=24.18; P<0.001). HCB showed no differences in the contribution profile (all P > 0.05) and represented less than 10%. BDE-99 represented ~50% of the total sum of PBDEs in down feathers and ~60% in juvenile contour feathers ($F_{1.15}$=3.61; P=0.08), being closely followed by BDE-47 (< 50%) that did not show any difference between the two types of feathers ($F_{1.15}$=2.13; P=0.16).

The different contribution trends of POPs between the two types of feathers are shown in Fig 3a. Factor loadings indicated that PC1 discriminated between the most persistent PCBs, *p,p'*-DDE and HCB on one hand, and CB-105, CB-118, CB-138 and *γ*-HCH on the other hand. Here, down feathers could be distinguished from juvenile contour feathers by showing higher presence of almost all of the most persistent compounds. In contrast, juvenile contour feathers showed a higher presence of the most volatile POPs, such as *γ*-HCH, and the lower chlorinated PCBs. Although PC2 discriminated between BDE-47 and BDE-99, it did not explain the variation between the two types of feathers.

When evaluating OPE profiles, an inverse pattern was found between the two types of feathers with significant differences in the contribution of TCiPP ($F_{1.6}$=6.48; P=0.04) and TPhP ($F_{1.6}$=7.16; P=0.04). Specifically, while TCiPP contributed significantly more in down feathers, TPhP was the predominant OPE in juvenile contour feathers (Fig 2). Therefore, the second most predominant compound was TPhP in down feathers and TCiPP in juvenile contour feathers (Fig 2). The contribution of TDCiPP was statistically identical in both types of feathers (less than 20%; P>0.05) (Fig 2).

As shown in Fig 3b, PC1 mainly discriminated between TPhP on one hand and TCiPP on the other, while PC2 discriminated between TDCiPP from the rest. As such, the biplot showed that down feathers could be distinguished from juvenile contour feathers by showing a profile with higher TCiPP representation, while TPhP were more important in juvenile contour feathers. However, as mentioned above, only TCiPP was significantly different between the two types of feathers (Table 1), with down feathers displaying the highest concentrations.

Correlations between down and contour feathers

When exploring correlations, we found that almost all PCBs, HCB, *p,p´*-DDE and BDE-47 showed positive correlations between concentrations in down and juvenile contour feathers, although this was not always significant (Table 2). Correlation coefficients ranged from 0.43 to 0.79 (Table 2), indicating that chicks reaching higher POP concentrations in down feathers also

297 reach higher concentrations in juvenile contour feathers, though the latter showing numerically
298 lower concentrations. Contrastingly, γ-HCH showed a negative, although not a significant,
299 correlation (r=-0.43, P=0.09; Table 2), suggesting that higher γ-HCH levels tend to accumulate
300 with advancing age. Finally, none of the OPE compounds showed any significant correlation. It
301 is possible that OPE levels fluctuated highly due to the potential influence of external
302 contamination (see section "Differences in accumulation between down and contour feathers").

303 **Differences in accumulation between down and contour feathers**

304 Bourgeon et al. (2013) hypothesized that the accumulation of the most persistent pollutants in
305 chicks of a top predator seabird was mainly through maternal transfer via the eggs rather than by
306 food intake, while the least persistent were acquired by dietary transfer. Our results provided
307 support for this hypothesis. We analyzed different feather types that reflect different periods
308 during the nestling development. Since juvenile contour feathers show a longer period of
309 growth than down feathers and only start to grow with advancing nestling age (see Material and
310 Methods section), juvenile contour feathers are exposed to more volatile and less persistent
311 pollutants present in the environment, and may thus reflect recent changes in their
312 concentrations (i.e. the increasing levels of *γ*-HCH found in juvenile contour feathers).
313 Alternatively, down feathers grow during the first days of chick life and probably reflect the
314 contaminant burden of the chick by maternal transfer through the egg. High concentrations of
315 highly chlorinated PCBs, PBDEs and *p,p'*-DDE were found in down feathers, in concordance
316 with previous studies describing similar contaminant patterns in eggs (Chen and Hale, 2010;
317 Gómara et al., 2008; Jiménez et al., 2007; Morales et al., 2012).

318 In addition, the only OPE compound that showed differences between the two types of feathers
319 was TCiPP, with down feathers having the highest concentrations. TCiPP was also the
320 compound that showed the highest concentrations of all OPEs. Greaves and Letcher (2014)
321 reported that TCiPP was the OPE with a higher accumulation burden in the yolk of eggs.
322 Findings of that study indicated a preferential transfer of TCiPP to the egg rather than
323 distribution in maternal tissues (i.e. fat, liver or blood). In the studied nestling vultures, the high
324 concentrations of TCiPP in down feathers may reflect maternal transfer, while the significant
325 difference between down and juvenile contour feathers probably reflects a rapid metabolism of
326 this compound in the chick, as previously suggested in adult gulls (*Larus argentatus*) (Greaves
327 and Letcher, 2014). However, information on how OPEs distribute in the body is lacking and
328 further research should be carried out. In addition, it is worth mentioning that only few studies
329 have measured OPEs in feathers (Eulaers et al., 2014a, 2014b) and their uptake in keratinous
330 matrices is not studied. In fact, the effectiveness of the washing protocol based on distilled
331 water, validated for the determination of POPs in feathers (Espín et al., 2010; Jaspers, 2008), is

still unclear for OPEs (Eulaers et al., 2014a). It is possible that concentrations of OPEs in feathers may be due to airborne particle deposition onto the feather surface reflecting more the atmospheric levels, as previously suggested for human hair (Kucharska et al., 2015) and bird feathers (Eulaers et al., 2014a), rather than the internal burdens.

Conclusions

The present study shows for the first time that POPs and OPEs can be measured in down feathers of cinereous vulture nestlings and that generally higher detectability and higher concentrations are found in down feathers in comparison to juvenile contour feathers. Findings of this pilot study suggest that down feathers seem to reflect rather the concentrations transferred by the mother to the egg and juvenile contour feathers reflect recent exposure from the diet during the nestling stage. This should be further investigated in the future. Further research is also needed to elucidate the deposition of OPEs onto feathers.

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Cinereous vulture nestlings at different ages

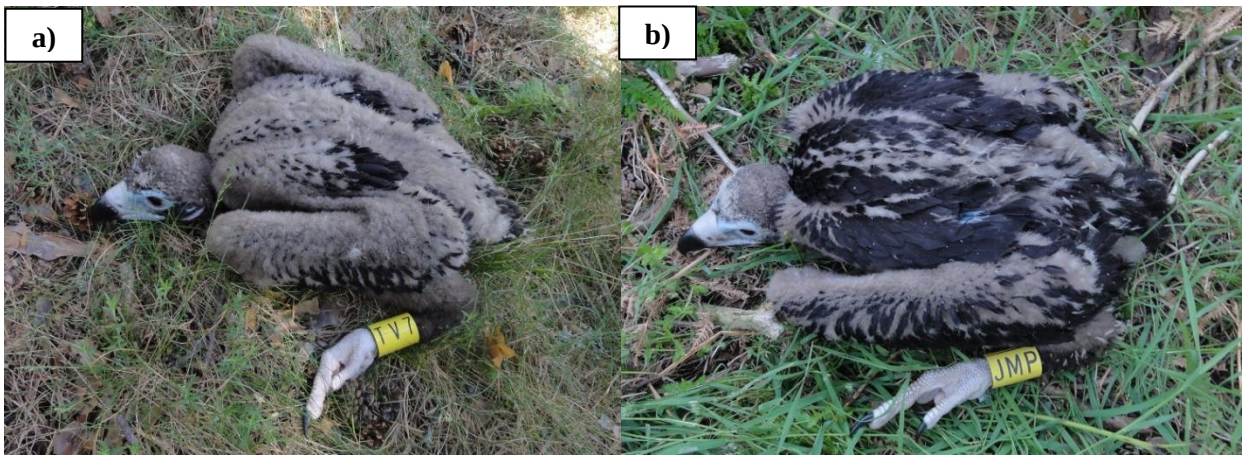

Fig 1 Nestlings of cinereous vulture (*Aegypius monachus*) at different ages showing different plumage. **a)** Nestlings at approximately 30 d post-hatching covered with the second natal down, which has a grey appearance. Pin feather development mainly of the scapulars, remiges and rectrices is apparent. **b)** Nestlings at approximately 40 d showing black juvenile scapular and wing covert feathers that contrast against the grey second down. In both cases remains of the whitish natal down can be seen only on the forehead near the bill.

Table 1 Concentrations (mean ± SE; median; min-max) and detection frequency (DF) (%) of PCB, OCP, PBDE and OPE compounds quantified in contour and down feathers (ng g^{-1} dw feather) of nestling cinereous vultures (*Aegypius monachus*). ANOVA analyses between types of feathers, along with significances (P<0.05)* (P<0.01)** (P<0.001)*** are shown.

	Contour feathers					Down feathers					Test for significance	
	n	DF %	Mean ± SE ng g^{-1} dw	Median ng g^{-1} dw	Min - Max ng g^{-1} dw	n	DF %	Mean ± SE ng g^{-1} dw	Median ng g^{-1} dw	Min - Max ng g^{-1} dw	F	P
CB-105	16	38	0.04 ± 0.01	0.02	0.02 - 0.15	16	100	0.11 ± 0.01	0.10	0.05 - 0.20	34.25	<0.001***
CB-118	16	88	0.10 ± 0.02	0.10	0.05 - 0.35	16	100	0.19 ± 0.02	0.17	0.09 - 0.29	48.52	<0.001***
CB-138	16	94	0.22 ± 0.05	0.18	0.05 - 0.92	16	100	0.49 ± 0.06	0.43	0.23 - 0.94	37.64	<0.001***
CB-153	16	100	0.25 ± 0.05	0.20	0.09 - 1.00	16	100	0.62 ± 0.06	0.60	0.32 - 1.12	69.32	<0.001***
CB-156	16	6	0.01 ± <0.01	<0.01	<0.01 - 0.06	16	38	0.04 ± 0.01	0.02	<0.01- 0.15	-	-
CB-170	16	50	0.08 ± 0.03	0.03	0.02 - 0.48	16	100	0.28 ± 0.05	0.20	0.09 - 0.67	156.03	<0.001***
CB-171	16	6	0.01 ± 0.01	<0.01	<0.01- 0.16	16	38	0.05 ± 0.01	0.20	0.02 - 0.13	-	-
CB-177	16	6	0.02 ± 0.01	<0.01	<0.01- 0.21	16	69	0.10 ± 0.02	0.08	0.03 - 0.30	-	-
CB-180	16	100	0.19 ± 0.07	0.11	0.07 - 1.17	16	100	0.61 ± 0.09	0.49	0.18 - 1.49	96.23	<0.001***
CB-183	16	6	0.01 ± 0.07	<0.01	<0.01- 0.20	16	81	0.10 ± 0.02	0.08	0.03 - 0.28	-	-
CB-187	16	56	0.08 ± 0.02	0.05	0.05 - 0.41	16	100	0.26 ± 0.04	0.22	0.10 - 0.78	101.87	<0.001***
CB-194	16	13	0.03 ± 0.02	0.01	<0.01- 0.27	16	88	0.14 ± 0.03	0.08	0.04 - 0.44	-	-
∑PCBs	16		0.96 ± 0.24	0.66	0.32 - 4.48	16		2.99 ± 1.50	2.47	1.30 - 6.16	108.86	<0.001***
HCB	16	100	0.10 ± 0.01	0.09	0.06 - 0.18	16	81	0.14 ± 0.04	0.11	0.04 - 0.69	1.68	0.21
α-HCH	16	38	0.07 ± 0.02	0.02	0.02 - 0.34	16	38	0.15 ± 0.14	0.02	0.02 - 1.31	-	-
β-HCH	16	44	0.08 ± 0.02	0.03	0.03 - 0.20	16	38	0.13 ± 0.06	0.02	0.02 - 0.34	-	-
γ-HCH	16	100	1.00 ± 0.21	0.83	0.25 - 3.12	16	44	0.53 ± 0.25	0.02	0.02 - 2.14	12.43	<0.01**
p,p'-DDE	16	100	1.17 ± 0.15	1.32	0.42 - 2.77	16	100	2.49 ± 0.42	2.69	0.09 - 6.10	12.51	<0.01**
p,p'-DDT	16	6	0.02 ± 0.02	<0.01	<0.01- 0.23	16	ND	-	-	-	-	-
BDE 47	16	94	0.12 ± 0.02	0.11	0.03 - 0.30	16	100	0.30 ± 0.06	0.24	0.09 - 0.89	16.95	<0.001***
BDE 99	16	94	0.17 ± 0.03	0.14	0.03 - 0.60	16	94	0.32 ± 0.07	0.25	0.03 - 1.08	4.20	0.05^{T}
BDE 100	16	6	<0.01 ± <0.01	<0.01	<0.01- 0.03	16	13	0.02 ± 0.01	<0.01	<0.01- 0.11	-	-
BDE 153	16	ND	-	-	-	16	6	0.01 ± 0.01	<0.01	<0.01- 0.09	-	-
BDE 154	16	ND	-	-	-	16	6	0.01 ± <0.01	<0.01	<0.01- 0.06	-	-
∑PBDEs	16		0.29 ± 0.04	0.27	0.06 - 0.70	16		0.61 ± 0.09	0.51	0.23 - 1.35	11.45	<0.01**
OxC	16	6	0.02 ± 0.01	<0.01	<0.01- 0.21	16	ND	-	-	-	-	-
TCEP	7	43	6.04 ± 2.89	0.43	0.43 - 18.2	7	43	2.41 ± 1.40	0.43	0.43 - 10.6	-	-
TCiPP	7	71	6.23 ± 2.88	2.26	0.71 - 18.53	7	86	18.17 ± 5.72	13.64	0.86 - 48.96	8.26	0.03*
TPhP	7	100	13.00 ± 2.31	10.51	6.34 - 24.68	7	86	12.67 ± 5.06	7.96	0.86 - 41.33	0.80	0.41
TDCiPP	7	57	1.36 ± 0.31	1.48	0.58 - 2.40	7	57	2.25 ± 0.93	1.72	0.58 - 7.50	0.37	0.57
∑OPEs	7		20.59 ± 4.63	16.43	7.63 - 41.10	7		33.09 ± 8.83	20.60	14.78 - 72.32	2.59	0.16

ND= Not detected

Fig 2 Contribution profile (% mean ± SE) of **a)** PCBs (n=16), **b)** OCPs (n=16), **c)** PBDEs (n=16) and **d)** OPEs (n=7) in contour and down feathers of nestling cinereous vultures (*Aegypius monachus*). Significant differences (P<0.05)* along with tendencies (0.1>P≤0.5)^{T} are shown. "na" represent compounds not analyzed in further statistics due to low detectability.

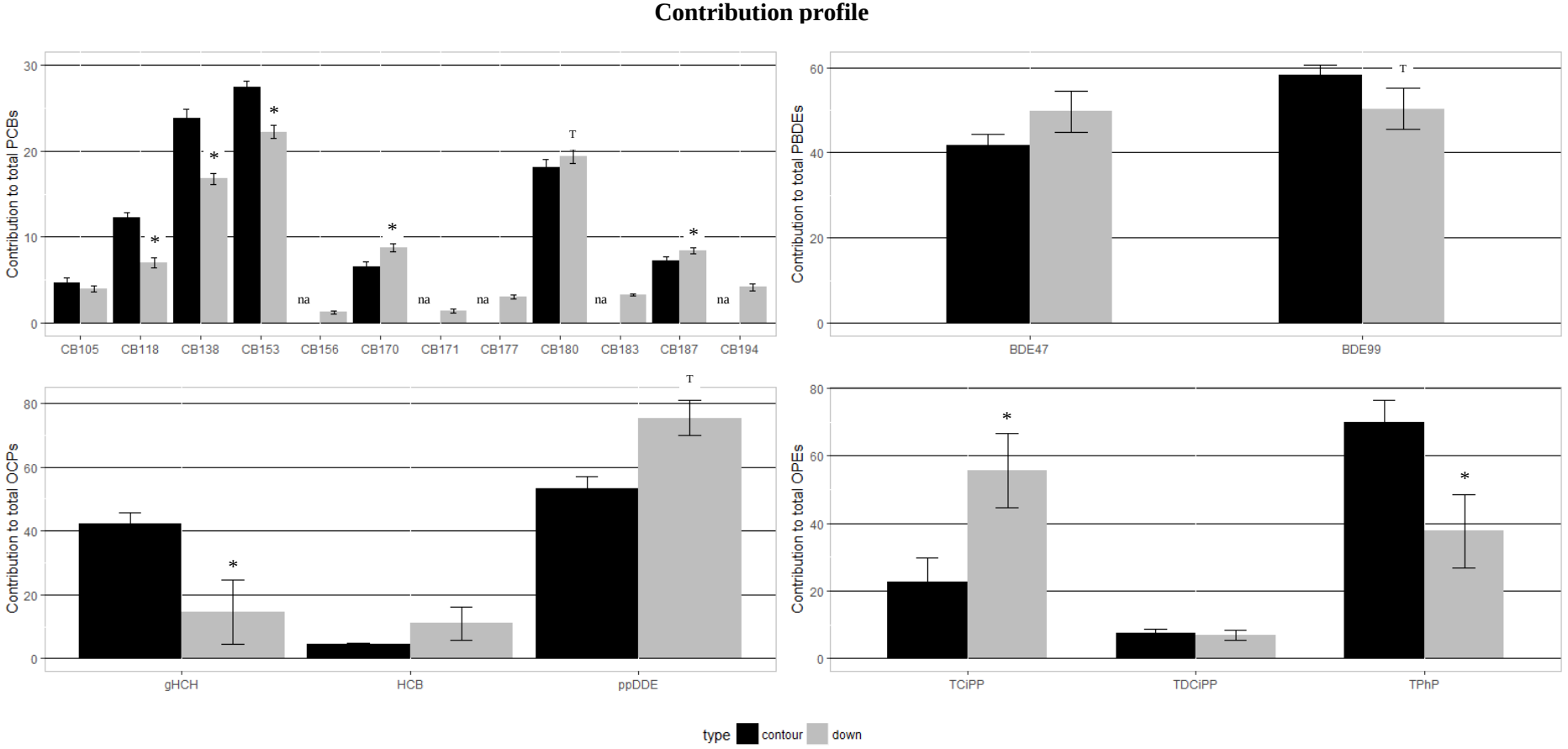

Fig 3 Correlation biplots between two types of feathers of cinereous vultures (*Aegypius monachus*) (down vs. contour feathers) comparing levels of **a)** POPs (n=16) and **b)** OPEs (n=7). Percentage of variation explained by each principal component is given in brackets on each axis. Note that circles represent contour feathers while triangles represent down feathers. The big circle and the big triangle represent the mean for contour and down feathers respectively.

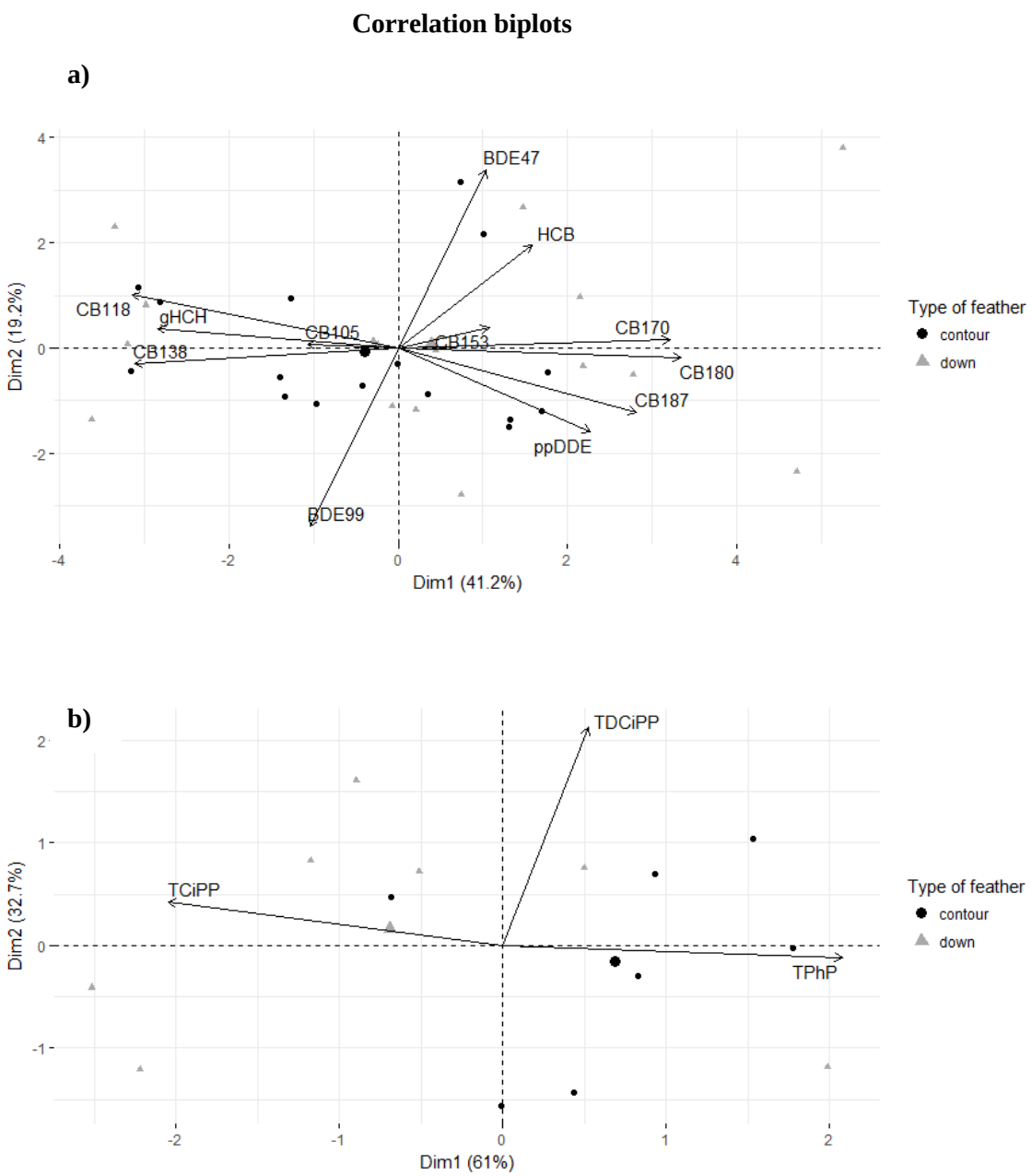

Table 2 Pearson correlation coefficients (r) between concentrations of PCBs (n=16), OCPs (n=16), PBDEs (n=16) and OPEs (n=7) in different feathers types (down vs. contour feathers) (ng g^{-1} dw feather) of nestling cinereous vultures (*Aegypius monachus*). Significances (P<0.05)* (P<0.01)** (P<0.001)*** and tendencies ($0.1<P\geq0.5$)^{T} are shown.

	r	P
CB-105	0.09	0.75
CB-118	**0.64**	**<0.01****
CB-138	0.45	0.08^{T}
CB-153	0.43	0.09^{T}
CB-170	**0.79**	**<0.001*****
CB-180	**0.66**	**<0.01****
CB-187	**0.65**	**<0.01****
∑PCBs	**0.60**	**0.02***
HCB	0.47	0.06^{T}
γ-HCH	-0.43	0.09^{T}
BDE-47	0.46	0.07^{T}
BDE-99	0.14	0.60
∑PBDEs	-0.13	0.62
p,p´-DDE	**0.55**	**0.03***
TCiPP	0.52	0.23
TPhP	-0.01	0.99
TDCiPP	0.21	0.65
∑OPEs	0.31	0.50