Can variability in corticosterone levels be related to POPs and OPEs in feathers from nestling cinereous vultures (*Aegypius monachus*)?

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Abstract

Persistent organic pollutants (POPs) are still globally distributed and some of have been shown to interact with the endocrine system of birds. However, the relationship between POPs and the stress response mediated by the hypothalamic-pituitary-adrenal (HPA) axis is still poorly understood. Raising concerns are now focused on the toxic properties of emergent organophosphate ester flame retardants (OPEs), but whether OPEs interact with the HPA axis response has not yet been investigated. We measured corticosterone concentrations in feathers (CORTf) as a long-term biomarker of the bird HPA axis response and we investigated their relationship with POP and OPE concentrations in down feathers of nestling cinereous vultures (*Aegypius monachus*). We also examined whether high contaminant burden and high CORTf concentrations impacted the duration of chick development. The most predominant compounds were the following: *p,p'*-DDE (3.28 ± 0.26 ng g^{-1} dw) > *γ*-HCH (0.78 ± 0.09 ng g^{-1} dw) > BDE-99 (0.73 ± 0.09 ng g^{-1} dw) > CB-153 (0.67 ± 0.04 ng g^{-1} dw). The most persistent POP compounds (CB-170, -177, -180, -183, -187, -194 and *p,p'*-DDE) were associated with high concentrations of CORTf (range: 0.55-6.09 pg mm^{-1}) (P=0.02), while no relationship was found when OPEs were tested (P>0.05). Later egg-laying was positively associated to high levels of CORTf (P=0.02) and reduced duration of chick development (P<0.001), suggesting a beneficial effect of the HPA axis response on the growth of the chicks. In addition, males with high concentrations of the most persistent POP compounds tend to show a reduced duration of the nestling period (P=0.05) and an equal fledging success than chicks with lower levels. These findings suggest that POPs, but not OPEs, may interact with the HPA axis response of chicks, although levels were not high enough to cause detrimental consequences.

Keywords: down feathers, raptors, POPs, OPEs, stress response, HPA

1. Introduction

Apex predators such as birds of prey have been extensively used as sentinel species to monitor anthropogenic pollution (Furness, 1993; Jaspers et al., 2006). Persistent organic pollutants (POPs) [i.e. polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs)] are among the most studied contaminants due to their global distribution, high persistence in the environment, and their bioaccumulation and biomagnification properties (Chen and Hale, 2010; Elliott et al., 2009; Espín et al., 2010; Thomas et al., 2006). Since regulations were applied, concentrations of POPs in different matrices have decreased (Gómez-Ramírez et al., 2014; Miller et al., 2015). However, progress on POPs remediation are still insufficient in Europe (e.g. PCBs; Law and Jepson, 2017) and high POP levels can still be found in top predators, such as in marine mammals (Jepson et al., 2016) and birds of prey in particular (Elliott et al., 2015; Espín et al., 2016). Recent studies on birds of prey have reported the potential of several POPs to impair bird physiology, behavior, and reproduction (Bustnes et al., 2015; Goutte et al., 2014; Fernie et al., 2015; Verreault et al., 2008; see also the review of Letcher et al., 2010). Moreover, while concerns about exposure to POPs continue, a more recent conservation issue has arisen regarding contamination by the emergent organophosphate ester flame retardants (OPEs). These compounds are currently and extensively used in the industry as fire retardants (American Chemistry Council, 2014), being constantly released into the environment by volatilization, leaching or abrasion (Marklund et al., 2005). Increasing levels of OPEs have been detected in indoor and outdoor environments (Andresen et al., 2004; Marklund et al., 2003; Van den Eede et al., 2011) and have been highly measured in biological matrices, including bird eggs (Barón et al., 2014; Greaves and Letcher, 2014; Sundkvist et al., 2010) and feathers (Eulaers et al., 2014a; Monclús et al., 2018a). In addition, in the recent years, it has been shown that some OPEs can bioaccumulate in biota (Greaves and Letcher, 2014) and exert toxic effects (Chen et al., 2012; Guigueno and Fernie, 2017; Kim et al., 2011; Sundkvist et al., 2010).

Several studies have illustrated that specific POPs can interact with the endocrine system of birds, i.e. the thyroid system (Cesh et al., 2010; Fernie and Marteinson, 2016; Nøst et al., 2012; Rogstad et al., 2017; Van den Steen et al., 2010) and the sex steroids (Nossen et al., 2016; Rogstad et al., 2017; Van den Steen et al., 2010; Verboven et al., 2008). Less research has been done regarding the effects of POPs on the hypothalamic-pituitary-adrenal (HPA) axis and mixed results have been published [e.g., Tartu et al., 2014, 2015; Monclús et al., 2018b; but also Love et al., 2003 (summarized in Table A1 in Appendices)]; probably because of the different matrixes used and the different species and field/lab conditions tested. The HPA axis is one of the most important regulatory pathways to deal with stressors by releasing corticosterone (CORT) into bloodstream (Romero, 2004; Sapolsky, 2000). Although short-term elevations of CORT are imperative for bird homeostasis and survival (Angelier et al., 2007; Sapolsky et al., 2000; Wingfield, 2003), chronic elevations can negatively impact bird growth, cognitive ability, immune defense, body condition, reproduction and survival (Angelier et al., 2010; Harms et al., 2010; Koren et al., 2012; Lodjak et al., 2015; Monclús et al., 2017a). Some POPs, such as PCBs and DDTs , have been observed to be adrenal disruptors affecting the HPA axis activity, either by interfering with hormone receptors or by modulating the hormone synthesis, metabolism, transport and degradation (reviewed in Hampl et al., 2016; Maqbool et al., 2015). POPs may also increase the allostatic load of organisms causing important energetic challenges (Bustnes et al., 2001; Nordstad et al., 2012). Monitoring the effects of these contaminants is therefore justifiable. In addition, despite the increasing evidence for OPEs disrupting the thyroid endocrine system (Farhat et al., 2013; Wang et al., 2013), it remains unknown whether these compounds affect the HPA axis activity.

Taking into account the above, the present study aimed to assess whether POPs (including PCBs, OCPs and PBDEs) and OPEs are associated with CORT concentrations measured in feathers of nestling cinereous vultures (*Aegypius monachus*). Feathers have become the matrix of preference when biomonitoring environmental pollution in birds of prey (Espín et al., 2016).

This nondestructive and minimally-invasive matrix integrates circulating contaminants (Burger, 1993; Jaspers et al., 2007, 2006) and CORT (Aharon-Rotman et al., 2015; Bortolotti et al., 2009; Jenni-Eiermann et al., 2015) concentrations proportionately to blood levels during the growth phase of the feathers. Therefore, feathers provide a relevant measure of the retrospective long-term HPA axis activity and the internal state of contamination during this period of time. For the first time in the literature, this study explores the relationship between contaminants and CORT concentrations in down feathers of nestling birds of prey. Due to nestlings are restricted to the nest, they are less exposed to possible environmental confounding factors compared to free-roaming adults. In addition, nestling feathers allow to delimit the time of CORT deposition.

2. Material and Methods

2.1 Study birds and data collection

The cinereous vulture, catalogued as Near Threatened by the IUCN (BirdLife International, 2017), is a scavenger species situated at the top of the food chain, and is mainly feeding on medium-size carcasses of livestock and wild ungulates (Del Moral and De la Puente, 2017). The breeding season takes place between February and September in colonies of low density, and the clutch size is only one egg (Donázar, 1993). The nests are typically built in large trees in forested mountainous areas (Hiraldo, 1977).

The present study was performed within the framework of a monitoring program of cinereous vultures implemented during the breeding period of this species established since 1997 in Sierra Guadarrama Madrid (Spain). 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). The feeding area covered a circular area with a radius of about 100 Km, with the vulture colony at the center. Vultures of the studied colony feed primarily on native carrion, as no feeding stations can be found in the surrounding area. Lately, vultures have been observed to also feed on rubbish dumps close to Madrid city and remains of plastic bags have been frequently found in their nest (personal observations). In this study, we could not quantify the food acquired from native carrion or rubbish dumps.

Ninety-nine (99) nests were monitored and visited during the breeding season of 2016 (from 15th of February to 15th of September; see Del Moral and De la Puente, 2005) in order to determine breeding activity (i.e. incubation behavior), egg-laying and breeding output (i.e. fledging success). Laying and hatching dates were determined from nests observations by backdating, visiting the nests once a week and determining the average day between the last day of visit when the bird was up (with no incubation behavior) or when was incubating the egg and the first day the bird was incubating or when a chick appear at nest, thus achieving an interval of 3.5 days for both laying and hatching dates (De la Puente, 2006). In addition, hatching date was confirmed by observational data of the estimated age of chicks at banding (i.e. by assessing plumage pattern and chick size). From mid-February to early-April egg-laying was determined and in late-April to early-June hatching date was recorded. Then, nests were monitored until nestlings fledged to determine fledging success (same method as above). Fifty-seven (57) nests produced nestlings successfully and all of these nestlings were captured and sampled from late-July to late-August before their anticipated fledging dates (mean age: 57 d, range: 35-89 d). Laying date was categorized in three groups as it follows: L1 (15_{th}-29_{th} February); L2 (1_{st}-15_{th} March); and L3 (16_{th} March-4_{th} April). The duration of chick development (in days) was calculated per each individual from hatching to fledging. Nestlings were carefully lowered from the nest in duffel bags. Second natal down feathers (referred to here as down feathers) were gently pulled from the scapular region. Only forty-two (42) individuals presented enough feather mass to perform the contaminant and hormonal analysis (see section 2.2 and 2.3 for feather mass requirements). Down feathers grow from 15 to 25 days post-hatching and replace the first white natal down that covered the nestling since its hatching (Bernis, 1966; De la Puente, *in press*). Therefore, down feathers were completely grown when sampled. After sampling, feathers were stored in paper envelopes at room temperature until analysis. Blood was collected for sex determination using the PCR protocol developed by Griffiths et al. (1998) (n=22 females, n=17 males, n=3 not available). After sampling, the nestlings were returned to their nest. The nests were monitored in the subsequent weeks and all the nestlings fledged from the nests without any problems. Descriptive statistics for biological variables are provided in Table A2.

2.2 Contaminant analysis

From the 42 samples collected, 16 were utilized in a previous study (Monclús et al., 2018a) to test the suitability of down feathers to analyze POP and OPE compounds. Analytical procedures for feathers were similar to the methods described previously (Monclús et al., 2018a) and followed those from Eulaers et al. (2014b; see Appendix A1). Analysis was done using a gas chromatograph coupled with a mass spectrometer (GC/MS; see Appendix A1).

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), *p,p´*-DDT and metabolites (*p,p´*-DDD, *p,p ´*-DDE), hexachlorobenzene (HCB), hexachlorocyclohexanes (HCHs: *α*-, *β*- and *γ*- HCHs) and chlordanes (cis-nonachlor, trans-nonachlor and oxychlordane). Only 22 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-dichloroiso-propyl) phosphate (TDCiPP).

2.3 Hormone analysis

All individuals (n=42) were assayed for feather CORT (CORTf) levels. For each individual, a mean number of 25 (ranging from 18 to 36) unwashed down feathers were pooled, measured (mm) with a calliper to the nearest 0.1 mm (mean ± SD: 26.5 ± 7.03 mm) and weighted (mg) with a precision scale to the nearest 0.1 mg (mean ± SD: 41.3 ± 15.4 mg). An optimized protocol for extracting CORT from feathers was used (Monclús et al., 2017b; see Appendix 2). Following Bortolotti et al. (2008), all feather CORT values were expressed as a function of feather length (pg mm^{-1}). However, concentrations for feather mass (pg mg^{-1}) were also calculated (Lattin et al. 2011). Concentrations of CORTf in mg were strong and significantly correlated to concentrations of CORTf in mm (Pearson's Correlation: r=0.82, P<0.001).

2.4 Data analysis

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 limit of quantification (LOQ) were assigned a value of DF x LOQ, with DF the proportion of measurements with levels above the LOQ (Voorspoels et al., 2002). POP compounds with DF below 60% of individuals detected ≥ LOQ were omitted for further statistics (CB- 28, 49, 52, 74, 95, 99, 101, 110, 149, 156, 170, 171, 180, 206, 209; BDE- 28, 100, 153, 154, 183; cis-nonachlor, trans-nonachlor and oxychlordane). In the case of OPEs, the threshold was set at 50% due to the smaller sample size and two compounds were excluded (TCEP and TDCiPP). Since not all variables were normally distributed (Shapiro-Wilk tests, P < 0.05), data were log10 transformed to meet parametric assumptions. Significance levels were set at P<0.05 (*), 0.01 (**) and 0.001 (***). A P-value between <0.1 and ≥0.5 was considered a trend (^{T}).

We used linear models to test compound-specifically whether sex influenced contaminant concentrations. Then, we examined whether contaminants influenced CORTf concentrations. In order to reduce the dimensionality of the data set and to avoid intercolinearity among contaminants, principal component analysis (PCA) was used. We extracted two principal components (PCs) based on the log10 transformed concentrations of contaminants for POPs (PCs-POPs; n=42) and for POPs and OPEs altogether (PCs-all; n=22). In both cases, compounds explaining little variation (loadings < 0.3 in both PC1 and PC2 lists) were eliminated in a backward stepwise procedure (HCB, *β*-HCH, *γ*-HCH and BDE-47 in the case of PCs-POPs and HCB, *α*-HCH, *γ*-HCH, BDE-47 and TCiPP in the case of PCs-all). Fig A1 illustrates the PCs and Table A3 and A4 provide factor scores for PCs-POPs and PCs-all, respectively. Here, we referred the PC lists as "PC1-POPs", "PC2-POPs", "PC1-all" and "PC2-all". We then specifically explored the influence of each list, as well as sex and egg-laying on the CORTf concentrations. Post-hoc Tukey HSD Test were made *a posteriori*. Finally, a generalized linear model with Poisson error distribution was used to test whether PC-POPs lists, PC-all lists, sex, CORTf concentrations and egg-laying influence the duration of chick development. Because this was the first study exploring the effects of OPEs on CORTf concentrations, we also explored TCiPP and TPhP on separate models, in addition to the PC-all lists. However, because results were the same than the PC-all lists, we do not include this information on this manuscript and we further refer to the results of PC-all lists.

In all cases, the initial models contained all the main effects and the possible interactions. Akaike's Information Criterion (AIC) and Akaike weight (Wi) (likelihood that a given model is the best among all candidate models) were used to rank models in each set (Burnham and Anderson, 2002). The model with the greatest Wi and lowest AIC value indicated the most parsimonious model. Models with ΔAIC<2 units from the best-supported were also considered (Burnham and Anderson, 2002). Tables A5 and A6 reporting the best-supported and null models are provided in Appendices.

3. Results

3.1 Contaminants concentrations

Out of the 40 targeted POP compounds, 27 could be detected but only ten PCBs (CB- 105, 118, 138, 153, 170, 177, 180, 183, 187, 194), five OCPs (*p,p'*-DDE, HCB, *α*-HCH, *β*-HCH, *γ*-HCH) and two PBDEs (BDE- 47, 99) were quantified above LOQ in more than 60% of the individuals and thus were included in further statistics. The most abundant compound was *p,p'*-DDE (mean ± SE: 3.28 ± 0.26 ng g^{-1} dw), followed by *γ*-HCH (0.78 ± 0.09 ng g^{-1} dw), BDE-99 (0.73 ± 0.09 ng g^{-1} dw), CB-153 (0.67 ± 0.04 ng g^{-1} dw) and CB-180 (0.60 ± 0.06 ng g^{-1} dw) (Table A7). Regarding OPEs, the four compounds analyzed (TCEP, TCiPP, TPhP, TDCiPP) could be detected but only two (TCiPP and TPhP) were quantified above LOQ in more than 50% of the individuals. The most abundant compound was TPhP (10.27 ± 2.31 ng g^{-1} dw) followed by TCiPP (9.74 ± 3.46 ng g^{-1} dw) (Table A7). Males showed significantly lower concentrations of the sum of HCHs than females and a similar trend was observed in concentrations of CB-153, CB-177 and *γ*-HCH (Table 1). No sex-relation was found for the rest of compounds (all P>0.05).

3.2 Relationships between contaminants and CORTf levels according to egg-laying date

The top set candidate models explaining CORTf variation included sex, egg-laying, PC1-POPs, PC2-POPs and PC2-all, whereas PC1-all was not included (Table A5). There was a positive and significant relationship between concentrations of CORTf and PC1-POPs (Table 2; Fig. 1a), whereas CORTf was not related to PC2-POPs (Table 2; Fig 1b) nor to PC2-all (Table A5). A positive and significant relationship between CORTf concentrations and egg-laying was found in all POP models (Table 2), indicating that chicks with later egg-laying date showed higher concentrations of CORT deposited in feathers grown during the third week of life. When explored with Tukey post hoc, we found that chicks with latest date of egg-laying showed higher CORTf concentrations (L3; mean ± SE= 3.76 ± 0.48 pg CORT mm^{-1} feather) than those chicks with earlier egg-laying date (L1: mean ± SE= 2.30 ± 0.24 pg CORT mm^{-1} feather, Tukey post hoc: P<0.01; L2: mean ± SE= 2.88 ± 0.37 pg CORT mm^{-1} feather, Tukey post hoc: P=0.05) (Fig 2a). No significant difference was observed between L1 and L2 groups (Tukey post hoc: P=0.43) (Fig 2a). Egg-laying date was not significant in the models containing OPE compounds

(PC1-all, PC2-all; P>0.05), probably because the smaller sample size (n=22) and the smaller distribution of the variable.

3.3 Influence of contaminants and corticosterone concentrations on the duration of chick development

The best-supported model explaining the duration of chick development included CORTf concentrations, sex, egg-laying date and the different contaminants tested (PC1-POPs, PC2-POPs, PC1-all, PC2-all) (Table A6). The duration of chick development was negatively influenced by the date of egg-laying (Table 3). When explored with Tukey post hoc, chicks with the earliest date (L1) showed a longer period of chick development (mean ± SE: 124.3 ± 3.1 days) than chicks with later egg-laying date, either from L2 group (mean ± SE: 113.8 ± 2.4 days; Tukey post hoc: P=0.04) or from L3 group (mean ± SE: 106.5 ± 4.6 days; Tukey post hoc: P<0.01) (Fig 2b). No significant difference was observed between L2 and L3 groups (Tukey post hoc: P=0.14) (Fig 2b). Although this relationship was very significant in the POP models, it was no significant in the OPEs model (PC1-all and PC2-all) (Table 3), again probably because of the smaller sample size and the smaller distribution of the variable. Lastly, the significant interaction "PC1-POPs x sex" indicated that the duration of chick development were shorter in males with high PC1-POPs concentrations (Table 3). Concentrations of PC2-POPs, PC1-all, PC2-all and CORTf did not influence the duration of chick development (Table 3).

4. Discussion

4.1 Contaminants accumulation in nestling cinereous vultures

Few data on POPs and emerging organic pollutants exist in vultures (Gómara et al., 2004; Goutner et al., 2011; Hernández et al., 2018; Van Drooge et al., 2008) and are particularly scarce when monitoring these contaminants in feathers. Only one study has been published reporting concentrations of PCBs and OCPs in feathers of adult individuals of Asian Indian vultures (*Gyps indicus*) and white-rumped vultures (*Gyps bangalensis*) (Abbasi et al., 2016). In addition, a preliminary study comparing levels between down and juvenile feathers was recently done for nestling cinereous vultures (Monclús et al., 2018a). In the latter study, down feathers were reported as a suitable matrix to biomonitor contamination in nestling vultures; further, down feathers were suggested to reflect concentrations transferred by the mother to the egg. The nestling cinereous vultures studied here showed concentrations similar to those previously reported for the 16 nestlings also included in Monclús et al. (2018a), except for TPhP and TCiPP that were 1.5 to 2 times lower respectively in the current study (Table A6). In comparison to other literature, our nestling cinereous vultures showed almost 3-fold higher PCB levels than those reported in Indian vultures and 8-fold higher than those of white-rumped vultures (Abbasi et al., 2016). However, in comparison to adults of other raptor species (Abbasi et al., 2016; Eulaers et al., 2013; Jaspers et al., 2007) and nestlings of white-tailed eagles (*Haliaeetus albicilla*) (Eulaers et al., 2014b), the present nestlings showed far lower PCB levels (~2 to 16 times lower). In general, low PCB levels have been noted in vulture species in comparison to other raptor species, either in plasma (Gómara et al., 2004; Goutner et al., 2011) or in internal tissues (Jaspers et al., 2006; Van Drooge et al., 2008). These findings are probably explained by the opportunistic diet of vultures, that feed on mammals (Hiraldo, 1977), thereby presenting higher capacity to metabolize POP compounds in comparison to specialist species (Fossi et al., 1995; Walker et al., 1987) or raptors that feed on birds (Van Drooge et al., 2008). The observed predominance of high-chlorinated PCB congeners (CB-138, -153 and -180) is in concordance with previous studies on vultures (Gómara et al., 2004; Goutner et al., 2011) and also on other raptor species (Eulaers et al., 2013; Jaspers et al., 2007). However, in the study of Abbasi et al. (2016), the congeners CB-180 and CB-138 were not detected in feathers of Indian vultures and their concentrations were very low in white-rumped vultures. The different spatial exposure, characteristics of their habitats and migratory patterns between the Asian vulture species and the studied Spanish vulture probably explain the above-mentioned PCB differences. In addition, the different feather types analyzed, with different growth times, could also bring variations as they are exposed to different conditions (Abbasi et al., 2016; García-Fernández et al., 2013; Jaspers et al., 2011). Indeed, our earlier findings (Monclús et al., 2018a) illustrated a clear difference in the contaminant burden between down and contour feathers in nestlings, with down feathers showing the highest amount of persistent contaminants.

Of special relevance is the contribution of the *p,p'*-DDE congener that was detected in almost all samples and exhibited the highest concentrations among targeted compounds. The congeners γ-HCH and BDE-99 followed *p,p'*-DDE in terms of concentrations. Similar findings were previously reported in vultures (Goutner et al., 2011), denoting the importance of the foraging activity of these species in agricultural lands and rubbish dumps (Goutner et al., 2011; Pérez-López et al., 2016). Here, the non-detection of *p,p'*-DDT indicates a declining use of this pesticide in Spain following the ban (Orden de 4 de Febrero, 1994). In the present nestling vultures, levels of *p,p'*-DDE were similar to those reported in adult Indian vultures from Pakistan (Abbasi et al., 2016) and nestling white-tailed eagles from Norway (Eulaers et al., 2014b). Levels of BDE-99 were also found similar to those of Eulaers et al. (2014b). Regarding HCHs, lindane and *β*-HCH were the most frequent HCH isomers, but the concentrations of lindane were higher than those of *β*-HCH, as already observed in our preliminary study (Monclús et al., 2018a). It is possible than lindane is still in use in some Spanish crops in spite of its prohibition (Decision, 2000/801/EC).

In comparison to nestling white-tailed eagles (Eulaers et al., 2014b), the present vulture nestlings showed lower concentrations of TCiPP and similar levels of TPhP. In addition, TPhP concentrations in down feathers were higher than TCiPP. So far, TPhP has been found the most dominant OPE measured in biota samples (Van der Veen and De Boer, 2012). However, we expected higher concentrations of TCiPP in relation to TPhP following our preliminary results

(Monclús et al., 2018a) and the findings reported by Greaves and Letcher (2014). The latter study reported on the preferential transfer of TCiPP *in ovo*, while showing low affinity of TPhP for the yolk of the egg. Thus, considering that down feathers probably reflect the contaminant burden transferred by the mother via the egg, one could expect higher levels of TCiPP. The contrary pattern observed in the current study is probably explained by the external deposition of high atmospheric concentrations of TPhP onto the surface of feathers increasing thus its concentrations (Kucharska et al., 2015).

4.2 Relationships between contaminants and CORT levels in feathers according to egg-laying date and duration of chick development

The current study showed three main results: 1) chicks with later date of egg-laying showed higher CORTf levels; 2) high concentrations of the most persistent POPs were positively associated to CORTf levels; and 3) the duration of the nestling period varied between chicks [ranging from 86 to 142 days; in agreement with Del Moral and De la Puente (2017)] and was negatively related to the date of egg-laying and, in males, to high concentrations of PC1-POPs. Differences in CORTf levels may result from stress when the nestlings were still growing and developing their feathers at the nest. On one hand, nestlings with later date of egg-laying could have had an urgent need to accelerate their growth to catch up those chicks with earlier laying dates. In that context, the stress response may be beneficial for the development of the nestlings, either by reallocating the available energy (Müller et al., 2009) or by improving the energy intake through begging or food-caching behavior (Kitaysky et al., 2003; Pravosudov 2003). On the other hand, it may also be possible that later egg-laying produced low quality chicks which, at the same time, displayed higher levels of CORT (Love et al., 2008; Saino et al., 2005). In addition, although vulture nestlings are dependent on their parents during early life, being protected and restricted to their nest, they can also be exposed to environmental variation that in turn may affect their CORT levels. For instance, Fairhurst et al. (2012) found that microclimate at nest boxes influenced CORT levels in nestling tree swallows (*Tachycineta bicolor*). Diet quality and nutritional status affected by parental quality, e.g., foraging activity feeding on native carrion or rubbish dumps, may also influence CORT levels (see Will et al., 2015), although, in this study we could not disentangle the percentage of the diet obtained from these two sources. Overall, chicks in our study that presented later egg-laying and higher CORT levels fledged successfully and did not show any negative effects during their development. However, increased CORT levels could have long-term consequences on the juvenile or adulthood period (Saino et al., 2005); calling thus for further research.

It should also be considered that in large raptor species as vultures, new parents may present delayed laying in comparison to older parents (Blas et al. 2009; Margalida et al., 2003; 2012) and may suffer more stress during the breeding season. Considering that mothers can transmit their steroid hormones to their offspring via the yolk of the egg (Janczak et al., 2006; Rubolini et al., 2005), it is possible that high CORT levels measured in down feathers in chicks may result from maternal stress. In this study we were not able to identify the parents and thus we could not rule out this hypothesis. However, the fact that those chicks with later date of egg-laying showed shorter duration of development during the nestling period probably indicates an energy investment by the chick to catch up. Therefore, the higher CORTf levels could be rather related to the HPA axis activity of the nestlings than the levels inherited by maternal transfer.

Moreover, results of this study suggest a potential role of contaminants interacting with the HPA axis activity of chicks, being associated to high CORTf levels. Considering that contaminant concentrations in down feathers probably reflect the contaminant burden transferred by the mother via the egg (Monclús et al., 2018a), high CORTf levels would reflect the endocrine response of the chick to the inherited contaminants. Chicks have limited capacity to metabolize the compounds at an early stage, thus the energy costs related (i.e. detoxification or biotransformation of POPs; Pottinger, 2003) might elevate the secretion of CORT. However, due to the correlative nature of this study, we cannot confirm a direct or causally link between POPs and CORT which should be further addressed in future work.

In particular, we found a significant and positive association between PC1-POPs (reflecting the most persistent contaminants) and CORTf levels but no significant effect was found for PC2-POPs (reflecting the less persistent contaminants), PC1-all and PC2-all (including the OPEs). This is the first investigation exploring whether OPEs relate to the HPA axis activity. Despite our findings indicate no significant influence, further research is recommended given the lack of validation for the washing protocol and removing external deposition onto the feather in the OPE analysis (Eulaers et al., 2014a). Another possible confounding effect could be the smaller sample size used in the OPEs analysis (n=22) in comparison to the POPs analysis (n=42). However, the strong and positive relationship between the most persistent POP compounds and the HPA axis activity and the lack of significance for the less persistent and for the OPE compounds are suggestive of a different behavior of these compounds. In fact, in the literature, different noxious effects depending on the type of contaminants have been reported (Cesh et al., 2010; Nordstad et al., 2012). Indeed, already in the 70's, Fyfe et al. (1976) suggest not only that DDE and PCBs showed different effects (i.e. DDE, but not PCBs, reduced egg productivity while both were related to abnormalities in territory defense behavior), but also that the effects could be species-specific [i.e. Praire falcons (*Falco mexicanus*) were more sensitive to DDE than merlins (*Falco columbarius richardsonii*)]. In our study, the positive relationship between the most persistent POPs and CORTf concentrations is in agreement with previous reports in glaucous gulls (*Larus hyperboreus*) (Verboven et al., 2010) and also in black-legged kittiwakes (*Rissa tridactyla*) (Nordstad et al., 2012), although in the latter only PCBs were related to CORT concentrations. These two previous studies suggested that high concentrations of several POPs may increase the environmental stress burden and compromise the ability of birds to adapt to a changing environment. However, a more recent study (Monclús et al., 2018b) has observed that high POP levels may not solely be positively correlated to high CORT levels but also to increasing levels of dehydroepiandrostendione (DHEA), a protective adrenal hormone with "anti-CORT" properties, thus suggesting an adaptive response of the HPA axis. In the current study, high concentrations of the most persistent POPs were positively associated to CORTf and only impacted the duration of chick development in males. Surprisingly, the effect was not negative and males with high concentrations of PC1-POPs showed a shorter period of chick development and not longer, as it would be expected if the concentrations of POPs were high enough to cause negative consequences on the growth of the chicks. Overall, these findings may indicate a compensatory response of the chicks rather than a means of chronic stress (see Dickens and Romero, 2013), in agreement with the previous findings of Monclús et al. (2018b).

5. Conclusions

The results of the present study showed that persistent POP compounds, but not OPEs, were associated with high concentrations of CORT in feathers grown during the third week of life in nestling cinereous vultures. This is the first study investigating this relationship in nestlings and more concretely using down feathers. Despite this study being correlative, and the exact impact of contaminants on the CORT regulation cannot be established, we provide evidence for a strong association with the most persistent POPs and the CORT secretion, while no association was found for the OPE compounds. However, although some POPs may interact with the stress response ,being associated with high CORTf concentrations, they were not observed to impact the growth rate of the chicks. Contrary, those chicks with high levels of CORTf and males with high concentrations of the most persistent POP compounds, showed rapid development and equal fledging success. In addition, a delayed egg-laying was related to increased CORTf levels and reduced duration of the nestling period. Overall, the current study highlights the plasticity of CORT in the framework of benefits towards deleterious effects and suggests that contaminant levels were not high enough to cause detrimental consequences. However, because higher contaminant burdens could compromise HPA axis functioning and may have farther-reaching consequences than the one observed for the growth and development of the chicks, further research should elucidate the contaminant burden when the triggering stress response could turn into prejudicial.

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Notes

The authors declare no competing financial interest.

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