Universitat Autònoma de Barcelona

FEATHERS AS A MATRIX TO ASSESS STRESS RESPONSE IN BIRDS AND BIOMONITOR ENVIRONMENTAL POLLUTANTS: AN INTEGRATIVE APPROACH

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PhD Thesis

DISSERTATION TO OBTAIN THE DEGREE OF DOCTOR
IN ANIMAL MEDICINE AND HEALTH:
Laura Monclús Anglada

UNDER THE DIRECTION OF:
Dr. Manel López Béjar

PRESENTED TO THE DEPARTMENT OF ANIMAL HEALTH AND ANATOMY,
VETERINARY FACULTY, UNIVERSITAT AUTÒNOMA DE BARCELONA

Bellaterra, 2018

FACULTAT DE VETERINÀRIA DE BARCELONA

FUNDACIÓN TATIANA PÉREZ DE GUZMÁN EL BUENO

Manel López Béjar, professor titular d'universitat del Departament de Sanitat i Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

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Que la memòria titulada "**Feathers as a matrix to assess stress response in birds and biomonitor environmental pollutants: an integrative approach**", presentada per Laura Monclús Anglada amb la finalitat d'optar al grau de Doctor amb Menció Internacional en Medicina i Sanitat Animals, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritza la seva presentació perquè sigui jutjada per la comissió corresponent.

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La doctoranda Laura Monclús Anglada ha podido realizar esta tesis doctoral gracias a la concesión de una beca predoctoral en el área de Medioambiente de la **Fundación Tatiana Pérez de Guzmán el Bueno** (convocatoria 2014), Madrid (España), que además ha financiado una estancia de tres meses en el extranjero para la obtención de la mención Internacional.

Laura Monclús Anglada was supported by a Doctoral Scholarship in Environmental Sciences from the **Fundación Tatiana Pérez de Guzmán el Bueno** (call 2014), Madrid (Spain), which has also financed an International research stay of three months to obtain the International Doctoral Research Component.

"La felicidad de la abeja y la del delfin es existir.
La del hombre es descubrir esto y maravillarse por ello"

Jacques Costeau

Acknowledgements/Agraïments

Doncs bé, ja la tenim aquí. La tesi llesta i apunt. Només falta escriure la meva part preferida, la que he esperat fins al final per poder-la disfrutar i la que us he de dir que em fa més il·lusió de totes (després d'acabar les conclusions, és clar!). Aquesta tesi no hauria sigut possible sense l'ajuda, la motivació i l'escalf que tantíssima gent m'ha brindat durant aquests tres anys.

En primer lloc, vull agrair-li al meu director, al **Manel**, l'oportunitat que em va donar per formar part del món de la ciència i confiar en mi des del primer moment. Perquè quan el vaig escriure ara farà cinc anys explicant-li les meves motivacions, em va orientar des del principi, proposant-me treballs de final de màster i obrint-me les portes per formar part del seu grup de recerca. Gràcies a ell, més tard, vaig aconseguir la beca que em va permetre fer el doctorat. Durant tot aquest temps m'ha donat llibertat per pensar, investigar i inventar. Gràcies Manel per l'oportunitat i per guiar-me en tot el procés!

En segundo lugar, quería agradecer a la **Fundación Tatiana Pérez de Guzmán el Bueno** por concederme una beca pre-doctoral en medioambiente. Esta tesis no habría sido posible sin esta magnífica oportunidad. Muchísimas gracias por confiar en mí!

I un cop assentada en el grup de recerca, està l'equip. I quin equip! Aquests anys de tesi no haurien estat els mateixos si no hagués sigut pels meus companys i amics de despatx i laboratori. Pels millors moments viscuts amb les "*umites*" **Oriol** i **Annaïs**, que són totalment incomptables. Per fer del dia a dia una magnífica aventura. Per les discussions científiques, les hores de laboratori compartides, els congressos i les nostres aventures en els mostrejos. Per formar part de més enllà de la tesi. Per haver-me arrencat somriures en els pitjors dies i acompanyar-me en els millors! També li vull agrair a la **Maria**. Tot i que vam compartir només mig camí, sempre ha estat per qualsevol cosa que pogués necessitar. Els seus consells, la seva llibreta-bíblia... Que hauria fet sense ella? Gràcies Maria! A la **Meritxell**, per les xerrades al laboratori, els super menús vegetarians i els xupitos de tequila! Al **Mateo** i al **Jaume**. Tot i que heu aparegut al final d'aquesta etapa, hem compartit molts bons moments. Em fa especial il·lusió la incorporació del Jaume. Encara et recordo amb bata i a les pràctiques com a estudiant! I de l'equip, peça clau, l'**Encarna**! Gràcies Encarna per donar-me un cop de mà i estar als ELISES, per tota la pau i serenor que aportes!

També voldria donar les gràcies al **Carlos**, per la seva complicitat, pels dinars de reflexions i de jocs de paraules, per estar sempre present i disponible (gràcies per la teva ajuda a les pràctiques!). I als **estudiants de grau** que han estat amb mi aquest temps. Als de treball de final de grau i als que han vingut a donar un cop de mà al laboratori (ells ja saben qui són :)). Gràcies nois! He disfrutat moltísim amb tots vosaltres, gràcies per la vostra ajuda en processar mostres i per compartir tantes estones! I no em voldria oblidar dels **becaris** (amb qui hem compartit cursos, consells molt valuosos, i hem amenitzat el doctorat amb uns quants soparets). Entre ells, el **Sergi**. Gràcies pels teus magnífics consells estadístics!

I voldria seguir donant les gràcies a la **Silvia Lacorte,** peça clau en el desenvolupament de la meva tesi. Gràcies Silvia per acollir-me i fer-me sentir una més en el teu grup d'investigació. Per la teva paciència i ajuda per introduir-me en un món nou per mi (el dels contaminants).

Gràcies per tot el feedback i per trobar sempre un temps per mi! També al **Rafa Mateo**, pels seus consells, gràcies! I si parlo del IDAEA-CSIC, no puc sinó agrair al **Miguel**, al **Rubèn,** al **Pablo** i a l'**Helena** per fer-me un espai en el seu grup, delectar-me amb cançons mentre extraiem els contaminants o mentre intentem pensar com podem participar en ONGs per evitar la contaminació global (o simplement per reciclar el paper de plata). En especial, gràcies Rubèn, per compartir campana, explicar-me una i altra vegada com funciona la GC/MS i per compartir diumenges xD. Al Miguel, i al seu pare, el **Carlos Velázquez**, per aconseguir-me el dibuix més bonic del món (el de la portada!). Gràcies.

También quería agradecer a **Rafa Molina** del Centro de Torreferrussa, a **Miguel Ángel Quevedo** del ZooBotáncio de Jerez, a **María Cortés** del Centro de Recuperación de Alfranca y a **Javier De la Puente** de la SEO-Montícola. Ellos han sido mi nexo con los animales y con su biología, y me han ayudado a entender los mecanismos biológicos. Muchísimas gracias a todos! En especial, quería agradecerle a Javier por haberme dejado formar parte del grupo de captura y muestreo de los milanos. Esa parte fue una de mis preferidas de toda la tesis, así que muchísimas gracias, la disfruté muchísimo.

I am especially grateful to **Veerle Jaspers**. When I was looking for a research stay and I contacted her, she responded to me automatically and, very kindly, offered me the possibility to form part of her research group. This experience was a great step forward for me, personally and professionally. Thanks Veerle for your great help, moral support and for teaching me so many things! It has been a pleasure to join you and your group in Trondheim, and having worked with you remotely. Thank you so much! Also, I would like to thank **Grethe** for her great help at lab. And to **Adrian Covaci** for his valuos input and good tips in analyzing data! Thank you! A very special thanks to my friends in Trondheim, **Jose**, **Mari**, **Courtney**, **Alex**... and, especially, **Maria** and **Nathalie.** Thanks guys for making me feel like home!

També vull agrair tot el suport que he rebut de la **Marina, Rosa**, **Albes** (x2), **Iris** i **Raquel**. Gràcies per interessar-vos pel que feia i dir-me que jo podia amb això i molt més! A la **Laura,** per recordar sempre dates importants. I en especial, a la **Gemma**. M'ha encantat compartir el mateix camí aquests anys i que haguem pogut fer cafès sencers donant-nos suport (finalment és possible!).

Deixo pel final a la meva família, tot i que són els impulsors que hagi arribat fins aquí. Al meu pare **Carlos**, a la meva mare **Marcel·la** i al meu germà **Adrià**. Per ser els ciments que aguanten i donar-me les forces i el suport per seguir. Per haver-me ajudat fins i tot en els mostrejos! Gràcies. I, a la Dana, que no podia faltar! Per recodar-me perquè em vaig fer veterinària. Gràcies també a la resta de la família, sobretot als avis, i en especial record a la meva àvia, per haver-me recolzat sempre.

I, finalment, a la **Marta**, perquè aquesta tesi va dedicada a tu. Estic segura que avui no estaria aquí si no fos per tu. Gràcies per fer-me creure que era possible i per ser-hi sempre. Per donar-me suport de tantísimes maneres. Per aprendre de tu i amb tu. Per compartir tantes coses i per lluitar per un món millor.

I, com va sent tradició... per fi ha arribat el moment de dir: *Thesis the end!!!*

SUMMARY

Exposure to environmental pollution has been one of the major threats for ecosystems and wildlife populations. Pollution stress can cause important alterations to wildlife, especially to top predators such as birds of prey, which have been widely used as important sentinels of their ecosystem. Understanding the stress-copping mechanism of organisms is crucial for species conservation. To this purpose, stress hormones (i.e. corticosterone in birds) have been used as biomarkers of challenging or stressful environments. Corticosterone is the endpoint of the hormonal cascade along the Hypothalamic-Pituitary-Adrenal (HPA) axis, which is activated under the presence of some stressors. The HPA axis is one of the main regulatory pathways birds use to deal with changes in the environment, and its effective functioning is imperative for maintaining homeostasis. Thus, detecting alterations in the HPA axis activity (i.e. chronic stress) can be used as a biomarker of populations at risk and offer valuable insights regarding population health and fitness. Feathers are the unique matrix able to provide long-term levels of corticosterone as an assessment of long-term adrenal activity with a retrospective insight. Furthermore, feathers can reflect the internal state of contamination, providing a valuable tool for biomonitoring environmental pollutants. However, as a relatively new matrix, feathers still present some methodological issues that need to be addressed for a proper interpretation of data. In addition, very little information exists on the influence of pollutants on the HPA axis activity.

The present thesis aimed to evaluate, on one hand the use of feather corticosterone as a biomarker of bird fitness, addressing some methodological issues of this matrix, and on the other, evaluate the applicability of feathers to biomonitor environmental pollution exploring the effects of pollutants on birds adrenal stress response. First, we explored the suitability of different types of feathers to measure corticosterone and environmental pollutants. Corticosterone concentrations were compared between body feathers and flight feathers, which had been used in almost all previous studies. By showing lower variability, body feathers were found to be a more suitable type of feather to sample due to they provide more specific information in time and minimize confounding factors. In addition, nestling down feathers were described as a new non-invasive method for biomonitoring contaminants. Second, we demonstrated consistency and stability of feather corticosterone concentrations in body feathers over the same feather generation, while we found that levels differ from year to year indicating individual flexibility. Furthermore, we validated an optimized protocol for extracting corticosterone from feathers in a more timesaving and ecological way. Third, we observed that high concentrations of feather corticosterone predict mortality rate and reproductive failure the following seasons, demonstrating the potential utility of this metric in bird management programs. Fourth, we showed that the most persistent pollutants influenced the HPA axis activity of free-living birds, either adults or nestlings, but not captive birds. Interestingly, although a positive association was found between these pollutants and high adrenal activity, they were not observed to negatively affect growth development in nestlings, and through assessing dehydroepiandrosterone in feathers, we observed an adaptive response of the HPA axis in adults. Finally, we explored different potential biological and methodological confounding factors. Overall, this thesis provides important evidence for the robustness of body feathers to assess long-term levels of corticosterone and its usefulness as a biomarker of bird fitness, as well as a step forward for understanding the effects of environmental pollution on the adrenal stress response in birds of prey.

RESUM

L'exposició a la contaminació ambiental és una de les principals amenaces per a la salut dels ecosistemes i les poblacions silvestres. L'estrès produït per la contaminació pot causar importants alteracions a la fauna, especialment als depredadors com les aus rapinyaires que, d'altra banda, s'utilitzen com espècies sentinelles del seu ecosistema. Comprendre el mecanisme mitjançant el qual els organismes fan front a l'estrès ambiental i s'adapten a un ambient canviant és crucial per a la seva conservació. Amb aquest propòsit, les hormones d'estrès (corticosterona en aus) s'han utilitzat com a biomarcadors d'estrès ambiental. La corticosterona és l'hormona resultant de l'activació de l'eix hipotalàmic-hipofisari-adrenal (HPA), element clau de la resposta d'estrès. L'activació d'aquest eix i el seu correcte funcionament permet als vertebrats mantenir l'homeòstasi i fer front a les pertorbacions del seu ambient. Per tant, detectar alteracions en el funcionament d'aquest eix (com és l'estrès crònic) pot ser utilitzar com un biomarcador de poblacions en risc i oferir informació valuosa sobre l'estat de l'animal i la seva eficàcia biològica. Les plomes són la única matriu capaç de proporcionar una mesura a llarg termini de corticosterona, i per tant de reflexar l'activitat de l'eix HPA, de manera retrospectiva i integrada en el temps. A més, aquesta matriu pot reflectir l'estat intern de contaminació d'una au, essent una eina molt útil per a la biomonitorització de contaminació ambiental. No obstant, al ser una matriu relativament nova, encara existeixen moltes incògnites metodològiques. A més, tot i que els contaminants tenen el potencial d'alterar l'activitat de l'eix HPA, hi ha molt poca informació al respecte.

L'objectiu principal d'aquesta tesi és, per una banda, avaluar l'ús de les plomes per mesurar corticosterona com a biomarcador de la salut i l'eficàcia biològica de les aus, explorant alguns aspectes metodològics d'aquesta matriu, i per l'altra, avaluar l'aplicabilitat de les plomes per biomonitoritzar contaminació ambiental estudiant els efectes dels contaminants en la resposta adrenal d'estrès de les aus. Primer, vam explorar la idoneïtat de diferents tipus de plomes per mesurar corticosterona i contaminants ambientals. Vam comparar nivells de corticosterona entre plomes corporals i plomes primàries de vol, que són les més utilitzades en gairebé tots els estudis previs. Les plomes corporals van mostrar una menor variabilitat en els nivells de corticosterona, indicant que aquest tipus de ploma és més adequat per obtenir informació més específica en el temps i minimitzar factors de confusió. A més, es va descriure per primer cop que les plomes natals dels pollets són útils per biomonitoritzar contaminants, essent un nou mètode de mostreig no invasiu. En segon lloc, vam demostrar que els nivells de corticosterona en plomes cobertores són consistents i estables al llarg de la generació de la ploma, mentre que varien d'un any a l'altre indicant flexibilitat individual. A més, es va validar un protocol optimitzat per extreure corticosterona de les plomes de manera més ecològica i ràpida. En tercer lloc, vam observar que concentracions de corticosterona en ploma podien predir mortalitat i fallada reproductiva en el següent període, demostrant la utilitat potencial d'aquesta eina en programes de maneig d'aus. En quart lloc, es va mostrar com els contaminants més persistents influeixen l'activitat de l'eix HPA en aus de vida lliure, adultes o pollets, però no en aus en captivitat. Tot i la relació positiva entre contaminants i l'activitat adrenal, no es va observar que els contaminants afectessin el creixement dels pollets i, mitjançant l'avaluació de la dehidroepiandrosterona en plomes, vam observar una resposta adaptativa de l'eix HPA en adults. Finalment, es van explorar diferents factors fisiològics i metodològics que potencialment podien crear confusió. En general, aquesta tesi proporciona una evidència important de la solidesa i utilitat de les plomes corporales per avaluar nivells de corticosterona a llarg termini i la seva utilitat com a biomarcador de l'eficàcia biològica en aus, així com un avanç per comprendre els efectes de la contaminació ambiental sobre la resposta adrenal d'estrès en les aus rapinyaires.

RESUMEN

La exposición a la contaminación ambiental es una de las principales amenazas para la salud de los ecosistemas y las poblaciones silvestres. El estrés producido por la contaminación puede causar importantes alteraciones en la fauna, especialmente en los depredadores como las aves rapaces, que además son utilizadas como especies centinelas de su ecosistema. Comprender el mecanismo mediante el cual los organismos hacen frente al estrés ambiental y se adaptan a un medioambiente cambiante es crucial para su conservación. Con este propósito, las hormonas de estrés (en aves la corticosterona) se han utilizado como biomarcadores de estrés ambiental. La corticosterona es la hormona resultante de la activación del eje hipotalámico-hipofisario-adrenal (HPA), elemento clave de la respuesta de estrés. La activación de este eje y su buen funcionamiento permite a los vertebrados mantener la homeóstasis y hacer frente a las perturbaciones ambientales. Por lo tanto, detectar alteraciones en el funcionamiento de este eje (como es el estrés crónico) puede ser utilizado como biomarcador de poblaciones en riesgo y ofrecer información valiosa sobre el estado de salud y eficacia biológica de los animales. Las plumas son la única matriz capaz de proporcionar una medición a largo plazo de corticosterona, y por tanto de reflejar la actividad del eje HPA, de manera retrospectiva e integrada en el tiempo. Además, esta matriz puede reflejar el estado interno de contaminación de una ave, siendo una herramienta muy útil para biomonitorizar contaminación ambiental. No obstante, al ser una matriz relativamente nueva, aún existen algunas incógnitas metodológicas. Además, aunque los contaminantes tienen el potencial de alterar la actividad del eje HPA, hay muy poca información al respecto.

El objetivo principal de esta tesis es, por un lado evaluar el uso de las plumas para cuantificar corticosterona como biomarcador de la salud y eficacia biológica de las aves, explorando algunos aspectos metodológicos de esta matriz, y por el otro, evaluar la aplicabilidad de las plumas para biomonitorizar contaminación ambiental estudiando los efectos de los contaminantes en la respuesta adrenal del estrés de las aves. Primero, exploramos la idoneidad de diferentes tipos de plumas para medir corticosterona y contaminantes ambientales. Las concentraciones de corticosterona se compararon entre plumas corporales y plumas primarias de vuelo, que son las más utilizadas en casi todos los estudios previos. Las plumas corporales mostraron una menor variabilidad en los niveles de corticosterona, indicando que este tipo de pluma es más adecuado para obtener información específica en el tiempo y minimizar factores de confusión. Además, se describió por primera vez que el plumón natal de los pollos es útil para biomonitorizar contaminantes, siendo un nuevo método de muestreo no invasivo. En segundo lugar, demostramos que los niveles de corticosterona en plumas corporales son consistentes y estables a lo largo de la generación de la pluma, mientras que varían de un año al otro indicando flexibilidad individual. Además, se validó un protocolo optimizado para extraer corticosterona de las plumas de una forma más ecológica y rápida. En tercer lugar, observamos que concentraciones de corticosterona en pluma pueden predecir mortalidad y fallo reproductivo en el siguiente período, demostrando la utilidad potencial de esta herramienta en programas de manejo de aves. En cuarto lugar, mostramos que los contaminantes más persistentes influyen en la actividad del eje HPA en aves de vida libre, adultos o pollos, pero no en aves en cautiverio. Aunque se encontró una relación positiva entre contaminantes y una alta actividad adrenal, no se observó que los contaminantes afectaran el crecimiento de los pollos y, mediante la evaluación de la dehidroepiandrosterona en plumas, observamos una respuesta adaptativa del eje HPA en adultos. Finalmente, se exploraron diferentes factores fisiológicos y metodológicos que potencialmente podían crear confusión. En general, esta tesis proporciona una evidencia importante de la solidez y utilidad de las plumas corporales para evaluar niveles de corticosterona a largo plazo y su utilidad

como biomarcador de la eficacia biológica en aves, así como un avance para comprender los efectos de la contaminación ambiental sobre la respuesta adrenal del estrés en las aves rapaces.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropine hormone
AIC	akaike's information criterion
AICc	akaike's information criterion corrected for small sample size
ANOVA	analysis of variance
AVP	arginine vasopressin
AVT	arginine vasotocin
BC	body condition
BFR	brominated flame retardants
CBG	corticosteroid-binding globulin
CORT	corticosterone
CORTf	feather corticosterone
CNS	central nervous system
CRF	corticotropin releasing factor
CRH	corticotropin-releasing hormone
CV	coefficient of variation
CYP	cytochrome
DDD	dichlorodiphenyldichloroethane
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DF	detection frequency
DHEA	dehydroepiandrosterone
DHEAf	feather dehydroepiandrosterone
DW	dry weight
EDC	endocrine disrupting chemical
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
FCC	feather corticosterone concentration
FR	flame retardants
GAS	general adaptation syndrome
GC	glucocorticoid
GC/MS	gas chromatography / mass spectrometry
GR	glucocorticoid receptor
HCB	hexachlorobenzene
HCBD	hexachlorobutadiene
HCH	hexachlorocychlohexane
HPA	hypothalamic-pituitary-adrenal
ICC	intra-class correlation coefficient
IPM	interacting protein modification
LOQ	limit of quantification
MR	mineralocorticoid receptor
OCP	organochlorine pesticide
OPE	organophosphate ester flame retardant
NBI	northern bald ibis
REML	restricted maximum-likelihood method
PCA	principal component analysis
PBDE	polybrominated diphenyl ether
PC	principal component
PCB	polychlorinated biphenyl

PeCB	pentachlorobenzene
POP	persistent organic pollutant
PTM	post-translational modification
RIA	radioimmunoassay
SD	standard deviation
SE	standard
SNS	sympathetic nervous systhem
TCEP	tris(2-chloroethyl) phosphate
TCiPP	tris(1-chloro-2-propyl) phosphate
TDCiPP	tris(1,3-dichloro-iso-propyl) phosphate
TPhP	tri-phenyl phosphate
VI	variable's relative importance
Wi	akaike weight

LIST OF SPECIES MENTIONED

Common name	Scientific name
American kestrel	*Falco sparverius*
Bald eagle	*Haliaeetus leucocephalus*
Black kites	*Milvus migrans*
Black-legged kittiwakes	*Rissa tridactyla*
Black guillemots	*Cepphus grylle*
Broiler	*Gallus gallus domesticus*
Cinereous vulture	*Aegypius monachus*
Common buzzards	*Buteo buteo*
Common eiders	*Somateria mollissima borealis*
Common magpies	*Pica pica*
Cory's shearwater	*Calonectris diomedea*
Eastern bluebirds	*Sialis sialis*
Egyptian vulture	*Neophron percnopterus*
European eel	*Anguilla anguilla*
Eurasian griffon vulture	*Gyps fulvus*
European starling	*Sturnus vulgaris*
Eurasian sparrowhawk	*Accipiter nisus*
Glaucous gulls	*Larus hyperboreus*
Golden eagle	*Aquila chrysaetos*
Great tits	*Parus major*
Greater snow geese	*Chen caerulescens atlantica*
House sparrow	*Passer domesticus*
Humans	*Homo sapiens sapiens*
India vulture	*Gyps indicus*
Magellanic penguin	*Spheniscus magellanicus*
Northern bald ibis	*Geronticus eremita*
Peregrine falcon	*Falco peregrinus*
Razorbills	*Alca torda*
Red kites	*Milvus milvus*
Red-legged partridges	*Alectoris rufa*
Red-winged blackbird	*Agelaius phoeniceus*
Rhinoceros auklet	*Cerorhinca moncerata*
Tree swallow	*Tachycineta bicolor*
White-tailed eagle	*Haliaeetus albicilla*
White-rumped vulture	*Gyps bangalensis*

INTRODUCTION

Animals live in complex environments that are currently subjected to the negative effects of human population growth and its expanding activities. In the past 100 years, the world has changed dramatically in the form of habitat loss or alteration, climate change, spread of exotic species and accumulation of persistent pollutants, provoking biodiversity loss (Sih et al., 2011). Predicting the consequences of global environment change is a complex task. However, understanding whether and how an organism can adapt to a changing environment is crucial for species conservation (Wingfield, 2008). Exposure to environmental pollutants such as persistent organic pollutants has been one of the major threats for ecosystems and wildlife health due to the high persistence of these compounds in the environment, their ubiquity, and bioaccumulation and biomagnification properties (Elliott et al., 2009). In addition, emerging pollutants (i.e. organophosphate ester flame retardants) are of increasing concern due to their extensive use in industry and potential toxic effects (Guigueno and Fernie, 2017). Birds, and particularly raptors, are considered important sentinels of their ecosystem (Helander et al., 2008), being early warning systems of potential impacts of contaminants in the ecosystem (Furness, 1993; Gómez-Ramírez et al., 2014) and have widely been used in biomonitor studies of environmental pollution (Espín et al., 2016; Helander et al., 2008).

On the other hand, stress hormones (i.e. corticosterone in birds) have been widely used as biomarkers of challenging or stressful environments (Bortolotti et al., 2008a; Fairhurst et al., 2012; Legagneux et al., 2013). Corticosterone is the endpoint of the hormonal cascade along the Hypothalamic-Pituitary-Adrenal (HPA) axis, the pivotal element of the stress response, which is activated in presence of some stressors (Bortolotti et al., 2008a). This stress response shifts energy investment away from non-vital activities and redirects it towards survival, with the aim to restore homeostasis, overcome the stress status and return to initial conditions (Wingfield et al., 1998). The presumed benefits of an acute activation of the HPA axis contrasts with the deleterious effects of a chronic activation. When the stressor persists in time or in frequency, the animal becomes chronically-stressed (Wingfield and Romero, 2001), potentially driving to pathological conditions: affecting cognitive ability, growth, immune defense, reproduction and survival (Romero et al., 2009; Sapolsky et al., 2000; Wingfield and Sapolsky, 2003). Assessing chronic stress can be used as an integrator of an individual's internal and external environmental quality. When applied in a population level, detecting chronic stress could be used as a biomarker of population at risk, due to for example anthropogenic contamination, and offer valuable insights regarding population health and fitness (Cooke and O'Connor, 2010; Madliger et al., 2015).

So far, different matrixes have been developed (i.e. blood, faeces, eggs) to assess stress response in birds, but feathers are the unique matrix able to provide a retrospective long-term measure of the HPA axis activity (Bortolotti et al., 2008a; Romero and Fairhurst, 2016). Feathers accumulate corticosterone during their growth via diffusion from the blood quill and feather corticosterone concentrations are described to be correlated to stressors experienced by the bird during the period of feather growth (Bortolotti et al., 2008a; Jenni-Eiermann et al., 2015). In addition, feathers accumulate environmental pollutants (i.e. persistent organic pollutants and emerging pollutants such as flame retardants) during their growth, reflecting internal state of contamination (Dauwe et al., 2005; Eulaers et al., 2011a; Jaspers et al., 2007, 2006). This means that feathers not only provide an assessment of the adrenal stress response, but also reflect contaminant accumulation patterns, with the potential to integrate the monitoring of long-term adrenal activity and environmental pollution and enable us to explore how pollutants impact bird populations (Espín et al., 2016). In addition to this, feathers can be minimally- or non-invasive collected, with easy storage and transport requirements, offering practical advantages for their use in field conditions. However, and despite their promising capacity as a useful matrix, feathers still present some methodological issues that need to be addressed for a proper interpretation of data (García-Fernández et al., 2013; Romero and Fairhurst, 2016). In addition, very little information exist investigating the influence of persistent organic pollutants and emerging pollutants on the HPA axis activity (Bourgeon et al., 2012; Nordstad et al., 2012; Verboven et al., 2010) and no previous studies existed investigating this relationship by using feathers.

LITERATURE REVIEW

1. STRESS

Stress has become extraordinarily important to our society today. "I am stressed out" is a sentence everyone says and everyone knows (or thinks to know) what it means. Actually, this concept is not easy to define and multiple definitions, preconceptions and implications have been made. Curiously the general conception is that stress is prejudicial and damaging for organisms. What is usually forgotten is the adaptive and beneficial aspects of stress for a changing environment: "*Stress is part of how animals cope with a changing environment*" (Romero and Wingfield, 2016).

But, what stress exactly means?

1.1. Stress definition and the stress models

Defining stress has a long trajectory in human history. Aristotle, Hippocrates and the other Ancients were aware of stress (Fink, 2009). However, it was not until the 20th century that the research of stress took power. Hans Helye (born in 1907) defined stress as "*the nonspecific response to the body to any demand*" and proposed the General Adaptation Syndrome (GAS), currently recognized as the Flight-or-Fight response, as the unique vertebrate response to stress. The concept and definition of Helye was controversial for some scientists as they considered it was too general and did not include cognitive and physiological factors (Fink, 2009). In fact, the GAS theory turned to be incorrect. However, Helye put the concept of stress on the map and he is considered the "father" of stress.

Since then, uncountable attempts to define stress have been made, often with vague results (Romero and Wingfield, 2016). Much of the dissatisfaction in defining rigorously the term "stress" originates from its multidimensionality nature. Stress includes at least three components that interact between each other (Levine, 2005; Le Moal, 2007; Romero et al., 2015): (1) the stress stimuli or "stressor" (both internal and external) that causes stress; (2) the processing systems (the perception of the stressor); and (3) the emergency physiological and behavioral responses, or "stress response", activated in response to the stressor. In this context, an over-stimulation of the emergency response, known as "chronic stress", may provoke pathological consequences for the organism. Notwithstanding, understanding when or which stress stimuli becomes stressor (noxious) or what behavioral and physiological responses are stress response remain ambiguous (Romero et al., 2009). A forth (4) component should be then included to understand the stress concept: "homeostasis" (Levine, 2005;

Romero et al., 2009; Romero and Wingfield, 2016). Homeostasis refers to the stability of physiological systems that maintain life through change in both internal and external environments, whereas "allostasis" is the process of maintaining homeostasis (McEwen and Wingfield, 2003). Thus, one could think that a stress stimulus that disrupts or represents a threat for maintaining homeostasis could be considered as a stressor. However, this is not as simple as that.

In fact, McEwen and Wingfield (2003) proposed the "Allostasis Model" replacing the traditional conception of stress by the concepts of homeostasis and allostasis and included new concepts such as "allostatic load" and "allostatic overload". A brief review of this model is following:

Allostasis is the process that accounts for daily and seasonal adjustments (called "allostatic state") and allows to maintain physiological parameters, for example blood glucose levels or cortisol rhythm. Under environmental changes, such as storms or winter conditions, the allostatic state of the individuals increases and, in parallel, it raises the necessity of the individual to work harder to maintain stability of the physiological parameters. The cumulative result of an allostatic state, when different environmental and life-history challenges meet together, is called allostatic load. It is important to consider that an animal may encounter, apart from the daily and seasonal routines to obtain food and survive, the extra need of energy to migrate, reproduce or, in the case of birds, molt. Under this scenario, when unpredictable or additional energetic challenges occur, the allostatic load can increase and the animal can get into physiological trouble (allostatic overload).

In general, this model was a great step forward in understanding how individuals cope with stress, although some weakness were identified (reviewed in Romero et al., 2009). In an attempt to integrate the Allostasis Model to the classical notion of stress, Romero et al. (2009) proposed the "Reactive Scope Model". This model incorporates a framework that enables insight into how an individual respond in terms of physiological mediators to stress. Different names have been given to this process, varying from "homeostatic mediators" (McEwen, 2003), "allostatic mediators" (McEwen and Wingfield, 2003) to "stress response" (Sapolsky et al., 2000). In this thesis, I use the term "stress response", explained in more detail in section 1.3. The Reactive Scope Model differed from the Allostasis Model in the conception of allostatic overload. However, as this discussion is still ongoing in the literature and it is not essential for the comprehension of the present thesis, we do not go deeper in these differences. The lector can find more information in Romero et al. (2009), McEwen and Wingfield (2010) and Romero and Wingfield (2016).

1.2. Stress stimulus or stressor

In the literature, the terms stress stimuli and stressor have often been used as synonyms, however stress stimuli may not represent a threat to the animal, so stress stimuli would not always be a stressor (Cockrem, 2007). Instead, stressor is defined as a noxious or unpredictable stimuli that initiates a cascade of events referred to as the stress response (Romero, 2004) (Fig. 1). Therefore, stressors may only be identified through the detection of the stress response.

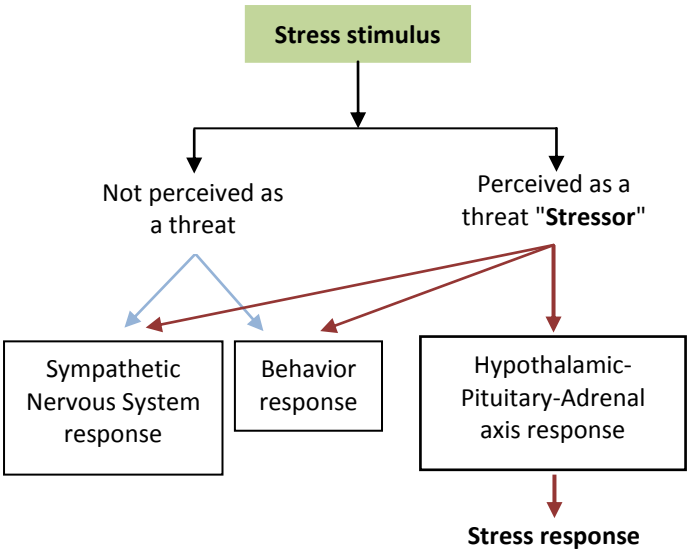


Fig. 1 Responses of animals to a stimulus not perceived or perceived as a threat. Source: adapted from Cockrem, 2007.

Some work has been done trying to identify which stimulus are or are not a stressor (Day, 2005). However, the description of the stressor is connected with individual perception because it is the central nervous system (CNS) which gives to a stress stimuli the status of stressor, which can be physical, physiological or both (Charmandari et al., 2005; Reeder and Kramer, 2005). In general, stress stimuli becomes stressor when an animal is faced with uncertainty, lack of information and/or lack of control (Levine and Usin, 1991, Koolhaas et al., 2011). Importantly, the severity or intensity of the stress stimuli is not as important as the perception of the threat by the own individual (Levine, 2005, Moberg, 2000). This is why physiological stressors can be so devastating (Buchanan, 2000) and explains the importance of genetic, temperament, past experience and age as key factors affecting the perception of a threat as a stressor (Moberg, 2000).

Many stressors, especially the physiological, require a cognitive assessment of the stimulus to determine whether it is a stressor. Different circuits in the brain are activated when exposed to a stimulus but it is thought that the amygdala and the hippocampus, both in the limbic system, are the two main structures involved in interpreting stimuli (Charmandari et al., 2005, Fink, 2007). Once these two structures decide that a stimulus is a stressor, a neuronal sign is sent to the hypothalamus and the stress response triggers.

1.3. The stress response

The stress response is mediated by the stress system, which has both CNS and peripheral components (Chrousos, 2009). The CNS includes the hypothalamic hormones arginine vasopressin (AVP) [in birds the arginine vasotocin (AVT) (Carsia et al., 1987)] and the corticotropin-releasing hormone (CRH) as the main effectors. The main peripheral components are the glucocorticoids, regulated by the Hypothalamic-Pituitary-Adrenal (HPA) axis, and the chalecolamines, norepinephrine and epinephrine, regulated by the systemic and adrenomedullary sympathetic nervous systems (SNS) (Chrousos, 2009). The activation of the HPA axis is called the stress response, while the stimulation of the SNS is called the Flight-or-Fight response (Sapolsky et al., 2000).

The most quick response is the Flight-or-Fight response that appears few seconds after the perception of a stressor (McEwen and Wingfield, 2010). This response is the typical, for example, in an attack by a predator, and comprises both physiological and behavioral responses. After a neuroendocrinological cascade, catecholamines are released by both the adrenal medulla and the nerve terminals of the SNS. This response is designed to increase heart rate, respiration rate and rapid mobilization of glucose from glycogen, among others, that help the animal to survive (Wingfield, 2003).

The response mediated by the HPA axis is less rapid and is the latter presented. Although it starts at the time the stressor is perceived, it generally takes 3 to 5 minutes to result in measurable glucocorticoid (GC) concentrations (Sapolsky et al., 2000; Sheriff et al., 2011). This stress response is highly conserved across all the vertebrate taxa and it results in a hormonal cascade that finally triggers GC hormones in blood circulation. Depending of the species, the predominant GC secreted is cortisol (in mammals and fish) or corticosterone (in birds, amphibians, reptiles and rodents). In this thesis we make reference of corticosterone as we focus on birds. See Box 1 for the main components of the HPA axis.

1.4. Life-history stages and the emergency stage

All vertebrates have a series of life-history stages that form part of their life cycle. For example, the temporal sequence of life-history for a typical non-migrating bird would be the following: (1) wintering (non-breeding stage) → (2) breeding stage (gonadal maturation, courtship, territorial behavior, etc.) → (3) molting stage (feather loss, feather growth) → come back to (1) wintering (Wingfield and Kitaysky, 2002). This sequence is unidirectional and predictable,

being determined by environmental factors such as photoperiod or seasonality. In this context, some stressors such as a predatory attack, severe storms, human disturbance and so on, known as "labile perturbation factors" (Wingfield et al., 1998), superpose to the original cycle. When the perturbation is severe enough, it provokes an activation of the "emergency stage" (Wingfield, 2003; Wingfield et al., 1998).

Box 1. The main components of the Hypothalamic-Pituitary-Adrenal (HPA) axis

When an animal perceived a stress stimulus as a stressor, or as a response of an input of the circadian system, the hypothalamic paraventricular nucleus (PVN) is stimulated, causing the secretion of CRH and AVT (in birds) and its analogues, into the hypophyseal portal system that connects the hypothalamus and the anterior pituitary gland. The anterior pituitary gland is then stimulated to synthesize adrenocorticotropine hormone (ACTH) and release it into the bloodstream where it stimulates the adrenal cortex. Circulating ACTH is the regulator of GC secretion in the adrenal cortex (Sheriff et al., 2011).

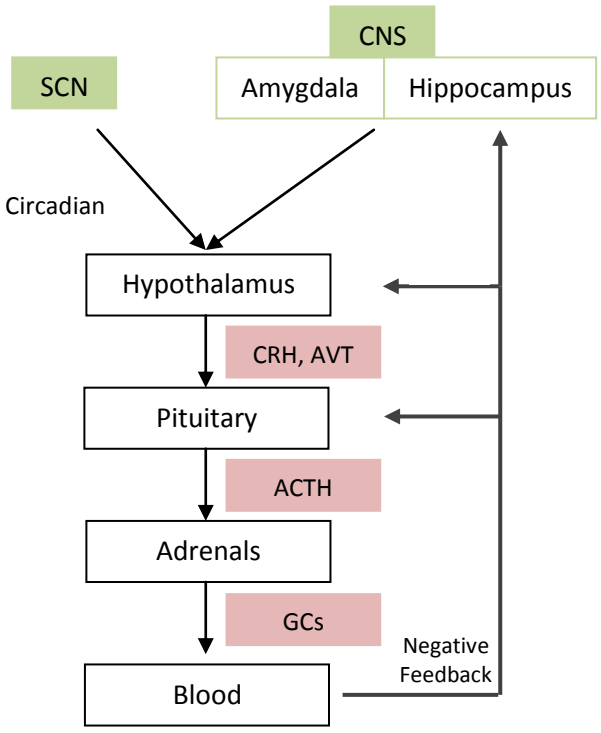


Fig. adapted from Lightman and Conway-Campbell, 2010; Sheriff et al., 2011; and Romero and Wingfield, 2016.

Usually, the peak levels for the GCs are at 15-30 min after the stressor. Then, GCs exert a rapid inhibitory effect on the ATCH secretion, as well as interact with GC receptors in the hypothalamus and hippocampus allowing levels return to baseline concentrations within 60-90 min (Kloet et al., 2005). However, this self-control mechanism of GCs depends on the severity of the stressor. Under an acute stressor, the mechanism operates efficiently and the system rapidly returns to normality. Under a more chronic or severe stressor, the negative feedback is weaker and the systems remains activated for longer periods (Romero, 2004; Sapolsky et al. 2000; Sheriff et al. 2011).

It is during the emergency stage when the HPA axis triggers high GCs into blood circulation; promoting changes in physiology and behavior (see Table 1). Under this situation, the individual enters a coping state in which priories allocation of resources towards self-maintenance and restores homeostasis at the expense of temporary suppression of non-vital activities (Wingfield and Romero, 2001; Wingfield et al., 1998). For example, it has been observed that elevated GC levels can suppress reproductive behavior to allow the animal to survive (Romero, 2004). This stage is nonetheless transitory and facilitates the ability of the animal to cope with the perturbation and overcome the adverse situation. Once the perturbation factor passes, the individual can return to its original life-history stages (Wingfield and Kitaysky, 2002; Wingfield et al., 1998).

The lector should note that there are different components of the emergency life-history stage and different types of labile perturbation factors that are not further discussed in this thesis. For an extended information the author recommend the reading of Wingfield (2003).

Table 1. Effects of Corticosterone in an Emergency life-history stage

Acute **Short-term: minutes or hours**	**Chronic** **Long-term: days to weeks**
Suppress reproductive behavior	Inhibit reproductive behavior
Regulate immune system	Suppress immune system
Increase gluconeogenesis	Promote severe protein loss
Increase foraging behavior	Disrupt second messenger systems
Promote escape behavior (day)	Damage neuronal cells
Promote restfulness behavior (night)	Suppress growth and development
Promote recovery on return to normal life-history stage	

Source: Wingfield and Romero, 2001

1.5. Stress-system disorders

The presumed benefits of an acute activation of the HPA axis contrast with the deleterious effects of a chronic activation. Under a persistent stressor or when a stressor occurs at regular frequent intervals, the entire functioning of the HPA axis changes (Charmandari et al., 2005). As illustrated before in Box 1, there is a hyperactivation of the stress response system resulting in an increased and/or prolonged secretion of GCs in blood circulation. In general, the more intense the stressor, the greater the amount of GC released (Dallman et al., 1987). In addition, under high or prolonged secretion of GCs, the receptors decrease in blood, thereby altering the GC levels available in the tissues (Breuner and Orchinik, 2002) and increasing the impact of GCs. This chronic activation of the HPA axis, "chronic stress", causes a host of negative consequences impairing biological functions and animal well-being (summarized in Table 1 and

reviewed in more detailed in Sapolsky et al., 2000). Chronic stress is defined as the physiological state in which individuals are no longer functioning within an adaptive capacity and the mediators of stress (GCs) provoke more pathophysiological effects than protection and no longer restore homeostasis (McEwen and Wingfield, 2010). The development and severity of these conditions are individual-dependent and factors such as genetic, epigenetic and resilience of the individuals become crucial to deal with stress (Chrousos, 2009).

Assessing chronic stress can be used as an integrator of an individual's internal and external environmental quality (Madliger et al., 2015). When applied in a population level, detecting chronic stress could be used as a biomarker of populations at risk, due to for example anthropogenic disturbances, and offer valuable insights regarding population health and fitness. However, distinguish between the beneficial aspects of acute stress and the detrimental aspects of chronic stress is still challenging for ecologists, and the direction towards which GC levels change under some disturbance has not always been consistent with the general assumption that levels increase (Dickens and Romero, 2013; Madliger and Love, 2014). In addition, very little is known about how free-living animals deal with chronic stress in the wild. It is important to consider that chronic stress in the natural world commonly occurs, with animals being evolved in developing mechanisms to cope with it and are able to maintain an equilibrium (Boonstra, 2013). However, with the apparition of anthropogenic disturbances (e.g. habitat loss, pollution and climate change, among others) that have high permanency in time and in intensity, the coping mechanism may become insufficient or more challenging (Romero and Wingfield, 2016).

1.6. Acclimation or habituation

Romero (2004) defined acclimation when the animal no longer perceived the stressor to be as noxious as it did before and reduces its GC response. Acclimation is a specific response to a repeated stimulus, so exposure to a new stimulus should result in the stress response functioning normally (Cyr and Romero, 2009). Habituation is a term that has been extendedly used in the literature as a synonym for acclimation (Cyr and Romero, 2009; Lynn et al., 2010; Romero and Wingfield, 2016) and in the present thesis it will be used instead of acclimation.

Habituation has been widely studied in birds for many years now. For example, in the 90s a study in Magellanic penguins (*Spheniscus magellanicus*) associated a decrease in GC levels with habituation to the presence of humans (Fowler, 1999). Similarly, several experimental studies demonstrated in captive [e.g. great tits (*Parus major*) (Cockrem and Silverin, 2002) and

American kestrels (*Falco sparverius*) (Love et al., 2003)] and in free-living [e.g. eastern bluebirds (*Sialis sialis*) (Lynn et al., 2010)] birds that individuals exposed to repeated stress stimulus showed a progressively decrease of GC levels. However, habituation not always occurs for all stressors, especially for those that are relatively severe (Dallman and Bhatnagar, 2001). In the worse of the cases, there is a chronically diminish functioning of the HPA axis that becomes incapable to mounting an appropriate response (Romero, 2004), also called exhaustion (Cyr and Romero, 2009). In this situation, the individual is so fatigued that it is unable to maintain a stress response and show very low GC values. In addition, there are two other alternatives of low GC response: a different perception of the stressor depending on the season or life-history stage, or a desensitization of the physiological response without habituation to the stressor (Cyr and Romero, 2009). Overall, the four situations (habituation, exhaustion, perception and desensitization) are difficult to differentiate, especially in free-living birds and, despite different criteria have been suggested (reviewed in Cyr and Romero, 2009), defining habituation in the field is not always possible. Importantly, the physiological response should continue to function normally when perceiving novels stressors. If not, the animal has not habituated to the repeated stressor and instead, is suffering an exhaustion or desensitization.

2. STRESS ASSESSMENT

As explained in the first section of this introduction, GCs are the main mediators of the stress response. Understanding the main functions of GCs, determining when GCs can be appropriate biomarkers of animal health and using the most suitable technique to measure GCs are crucial factors to assess stress.

2.1. Glucocorticoids

Since first studied in 1930s by the father of stress, Hans Selye, GCs have become a major topic for endocrinologists. These steroid hormones are the last step of the HPA axis cascade and are responsible of most of effects associated to HPA axis activity. The physiological action of GCs is to mobilize energy stores throughout the body with the objective to re-establish homeostasis (Ralph and Tilbrook, 2016). These hormones influence the expression of approximately the 10% of the genome and control metabolism, growth, repair, reproduction and the management of resource allocation (Ralph and Tilbrook, 2016). Once the HPA axis has been stimulated, GCs secreted in blood circulation bring the major physiological changes of the stress response. These physiological changes are extensively detailed in Sapolsky et al. (2000)

and include: (1) mobilization of energy; (2) enhancement of the substrate delivery to muscle; (3) stimulation of the immune function; (4) inhibition of the reproductive physiology; (5) decrease of feeding and appetite; (6) sharpening of cognition and increase of cerebral perfusion rates. During stress, GCs have two different main actions: modulation actions that alter organism's response to stressor, and preparative actions that prepare organism's response to a subsequent stressor or aid in adapting to a chronic stressor (Sapolsky et al., 2000). However, the HPA axis is also activated by physiological factors such as circadian rhythm, and for example, GCs secretion increase after eating or have peaks of secretion in the morning in diurnal species and in the evening in nocturnal species (Mormède et al., 2007). In fact, GCs have crucial functions necessary for body maintenance, growth and repair (Ralph and Tilbrook, 2016). Considering the complexity of GC actions and the innate variations of the HPA axis functioning within individuals (Mormède et al., 2007), careful study the biology and physiology of each species, in context with its environment, is crucial to assess stress in animals (Touma and Palme, 2005).

2.2. Use of glucocorticoids as biomarkers

Concentrations of GCs have been increasingly used in studies as indices of animal well-being (Busch and Hayward, 2009) and as biomarkers of how environmental disturbances (i.e. climate, habitat or anthropogenic disturbances) impact populations (Meillère et al., 2016; Nordstad et al., 2012; Treen et al., 2015;). Measuring GCs can help to understand how animals deal with perturbations. However, to be effective diagnostic tools, GCs should change with environmental variables and correlate with fitness parameters, especially with the most relevant, survival and reproductive success (Busch and Hayward, 2009; Cooke and O'Connor, 2010). Also, controlling for appropriate covariates, such as body condition, sex or life-history stage, is also essential (Madliger et al., 2015). However, the current understanding of the relationship between GCs and fitness is far from complete (Bonier et al., 2009). A key factor for using GCs as biomarkers is the method of quantification (section 2.3).

2.3. Evaluation of glucocorticoids

Blood has been the most used matrix to measure GCs in animal stress studies (Mormède et al., 2007; Möstl and Palme, 2002). However, endocrinologists have developed protocols for assessing GCs non-invasively or less-invasively in other matrixes, for example in saliva, milk, urine or faeces (Mormède et al., 2007; Möstl and Palme, 2002; Sheriff et al., 2011) and, more recently, in keratinous matrixes such as hair, feathers or even skin sheds of reptiles (Berkvens

et al., 2013; Bortolotti et al., 2008a; Raul et al., 2004; Sheriff et al., 2011). Understanding the metabolism and excretion of GCs is essential for seeking matrixes to measure these hormones. Box 2 illustrates the main roads of secretion, metabolism and excretion of GCs and the main matrixes to assess GCs.

Box 2. Metabolism and excretion of GCs. Matrixes to assess GCs

Adrenal glands secrete GCs directly to bloodstream (Charmandari et al., 2005; Sapolsky, 2000), being blood the most widely used matrix to detect stress (Mormède et al., 2007). Free blood GCs are filtered by the salivary gland and diffused into the saliva (Sheriff et al., 2011). Also, free blood GCs are metabolized in the liver (Möstl and Palme, 2002) and eliminated via urine or excreted to the duodenum by the bile duct where is metabolized and, finally, metabolites of GCs are excreted in faeces (Mormède et al., 2007). It is important to consider that in birds, urine and faeces are mixed together in the cloaca prior to excretion. Thus, the sample matrix to measure the metabolites of GCs in birds is excreta, rather than faeces (Cook, 2012).

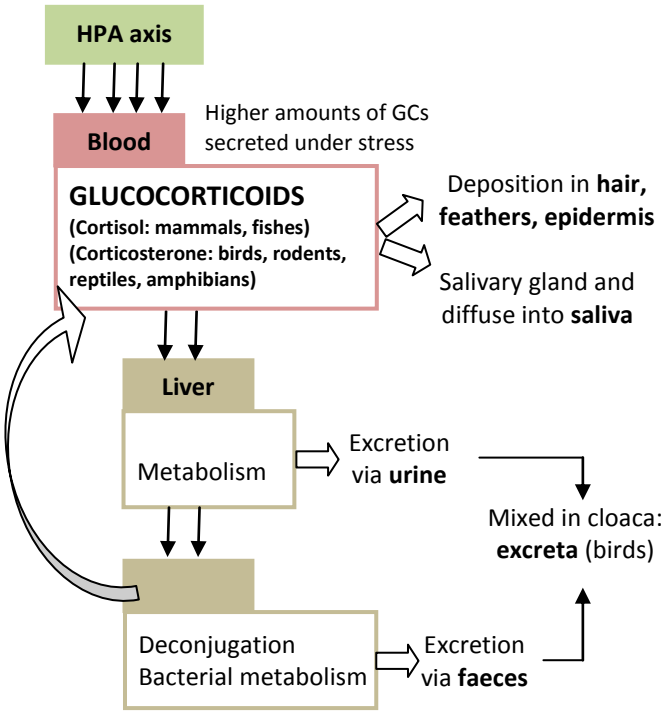


Fig. adapted from Möstl and Palme, 2002.

In addition, keratinous matrixes such as hair in mammals, feathers in birds or shed skin in reptiles accumulate GCs from blood diffusion during all their growth (Berkvens et al., 2013; Bortolotti et al., 2008a; Raul et al., 2004).

2.4. Types of glucocorticoids

The most important and biologically relevant GCs are cortisol and corticosterone (Palme et al., 2005) (Fig 2). Despite both hormones exist in all animals, cortisol is more predominant in mammals and fishes, while corticosterone (CORT) is the predominant GC in birds, reptiles,

amphibians and rodents (Von Holst, 1998). Given that the present thesis is focused on birds, CORT will be the GC studied.

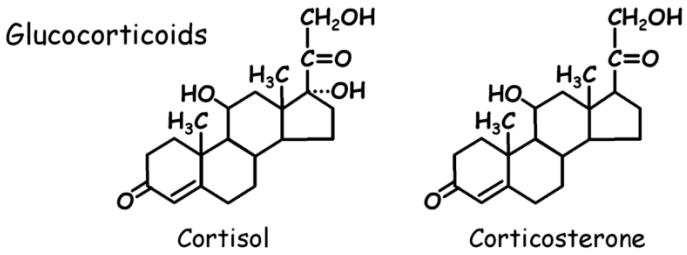


Fig. 2 Chemical structure of the main GCs. Source: Palme et al., 2005

2.5. Matrixes to assess glucocorticoids in birds

Traditionally, GCs have been measured in blood samples that provide a measure of circulating plasma steroid concentrations. However, this method has some drawbacks (described below) and alternative non-invasive or minimally-invasive matrixes have been developed. In birds, the alternative matrixes are eggs, excreta and feathers and, similarly to mammals (Tallo-Parra, 2016), can be classified in three groups depending on the time-integrated information they provide (Table 2). They all have pros and cons and, depending on the objective of the study, the species and the field conditions, their sampling is more or less suitable. The different matrixes for birds are described below.

Table 2. Classification of matrixes

Matrixes	Mammals[1]	Birds[2]
Single-point matrixes	Blood Saliva	Blood
Intermediate matrixes	Urine Faeces Milk	Excreta Eggs
Cumulative matrixes	Hair	Feathers

Source: [1] Tallo-Parra, 2016; [2] Author

2.5.1. Single-point matrixes

Blood provides a "snap shot" picture of the HPA axis activity at the time the sample is taken (Cook, 2012), thus representing the HPA axis activity of a single moment allowing to detect acute stress. In fact, blood is the matrix most directly connected to the HPA axis activity as adrenal glands secrete CORT directly to bloodstream (Charmandari et al., 2005; Sapolsky et al., 2000). Consequently, the delay existing between the reactivity of the HPA axis and the secretion of GCs into bloodstream is practically inexistent.

Despite blood has been the most widely sample used to assay CORT in birds, several confounding factors have been associated to this matrix. One of the main problems is the large variation of CORT levels throughout the day due to circadian rhythm and also across months

due to seasonality (Mormède et al., 2007; Palme et al., 2005). In addition, CORT secretion is very sensitive to many environmental factors and can rapidly rise in response of, for example, catching and handling of birds and the mere extraction of blood; thus masking the real blood CORT levels existing before sampling (Romero, 2002; Wingfield and Romero, 2001). To solve this problem, endocrinologists have employed the technique of extracting blood samples within 2-3 minutes of catching the bird, before the adrenal cortex has been activated (Romero and Reed, 2005). However, this is not always possible in field conditions. Another fact that should be considered is that concentrations of CORT assessed in serum include both protein-bounded and free CORT (Breuner et al., 2006; Hammond et al., 1991). Any change in levels of CORT-binging globulins may affect the total serum CORT measured, without being related to stress (Mormède et al., 2007; Sheriff et al., 2011). Finally, blood sampling is the most invasive technique to assess CORT. It implies capturing and handling the bird and the risk of create a stressful perturbation than can interfere with the well-being of the animal, being especially important in free-living animals.

2.5.2. Intermediate matrixes

Similar to mammals, CORT metabolites in excreta represent a longer period of time than blood samples as they accumulate circulating CORT for some time (Cook, 2012; Möstl et al., 2005). In fact, the time lag they represent is the passage time of food through the gut, containing the pooled amount of excreted hormones allocated to one dropping and representing, in that way, an integral measure of hormone metabolites (Goymann, 2005). In addition, their collection is non-invasive and avoid stress generated by blood extraction (Cook, 2012; Mormède et al., 2007; Sheriff et al., 2011). Faeces have been extensively used and validated in mammals (Möstl and Palme, 2002). Some work have also been done in birds (Möstl et al., 2005), however the dual nature of excreta in birds (i.e. urine, faeces) can bring some complications in the interpretation of data. In addition, the CORT content of excreta can be affected by the composition of the diet (Goymann, 2005) and, in fact, the CORT metabolites in excreta can change in time depending on exposure to the environment (Cook, 2012). Other confounding factors are the method of sample preservation and storage. A delay between excreta collection and the store of samples in appropriate conditions is crucial, with significant alterations in the CORT concentrations if samples are not frozen immediately (Buchanan and Goldsmith, 2004; Mormède et al., 2007).

Measuring CORT in eggs, concretely in egg yolk and albumin, have been studied as a biomarker of maternal HPA axis activity and its influence on the stress coping qualities of the offspring

(Cook, 2012). These matrixes represent different time-integrated periods. While yolk represents a time of 10-11 d in laying hens, the albumin represents approximately 6 h (Cook, 2012; Von Engelhardt and Groothuis, 2005). In addition, different studies observed that albumin and yolk concentrations of CORT were not sensitive to relatively short or acute increases of CORT in blood (Cook et al., 2009; Love et al., 2008). While it seems that the yolk of the egg provides an integrated measure of plasma CORT over many hours or days, concentrations measured in the albumin seem to be more related to daily adrenocortical output of CORT (Cook et al., 2009; Von Engelhardt and Groothuis, 2005). Despite the non-invasiveness of this technique and their great potential to assess maternal stress, these matrixes are not the preferred one when studying stress in birds, probably because of the difficulty of their validation (Larsen et al., 2015).

2.5.3. Cumulative matrixes

The cumulative matrixes have recently gained much attention due to their unique long-term, integrated and retrospective insight into the HPA axis activity of vertebrates and have been extensively used to assess chronic stress. Hair in mammals (Raul et al., 2004), shed skins in reptiles (Berkvens et al., 2013) and feathers in birds (Bortolotti et al., 2008a) are the main cumulative matrixes utilized. All of them are keratinous tissues, which gives them great stability in time in front of degradation, thus they do not require especial storage or transport conditions (Sheriff et al., 2011). Feathers are explained in more detailed in section 5.

2.6. Glucocorticoids detection methodologies

2.6.1. Methods for analyzing glucocorticoids

The common method for analyzing GCs is immunoassay and two versions have been widely used: radioimmunoassay (RIA) and enzyme immunoassay (EIA). Both methods are competitive binding assays and highly sensitive (Sheriff et al., 2011). RIA is a highly specific and precise method that has become the method of election for a growing number of studies (i.e. Angelier et al., 2007; Bortolotti et al., 2008a; Fairhurst et al., 2011). However, this method has the disadvantage of using radioisotopes, including health hazards, and needs for a costly radioisotope license (Sink et al., 2008). Alternatively, EIAs have been validated for many species. As a more economic method, and without having any health risk associated, EIAs are readily available from many manufacturers (Sink et al., 2008). Notwithstanding, the absence of specific EIAs require validation of the methodology for the target hormone in each matrix and

species (Buchanan and Goldsmith, 2004). In the present thesis, EIA is the method of choice for determining CORT in feathers. Thus, especial focus is done for the validation tests.

2.6.2. Validation tests for enzyme immunoassays

The main requirements necessary to validate an immunoassay, described below, are based on Mccallister and Smith (1996), Buchanan and Goldsmith (2004) and Sheriff et al. (2011).

(i) Specificity: assessment of the absence of cross-reactive substances other than the target hormone intended to be measured in the extracted medium with the antibody. The cross-reactivity percentage is often provided by the kit manufacturer. Parallelism test of diluted samples is usually used as a complementary tool to assess specificity.

(ii) Linearity or dilution: demonstration of the ability to obtain results which are directly proportional to the concentration of analyte in the sample. The dilution test is used to confirm that the target hormone extracted from feathers interacts with the assay antibody in a dose-dependent manner.

(iii) Accuracy: measure of the exactness of the analytical method. Usually the spike-and-recovery tests is used with the aim to measure how efficient the extraction method is at separating the target hormone from the matrix and examining possible interference of components within the extract with antibody binding.

(iv) Precision, blanks and sensitivity: assessment of the reproducibility of results using the coefficients of variation, demonstrating blanks and determination of the detection and quantification limit. The sensitivity is defined as the smallest value that can be reliably discriminated from zero values with a 95% of probabilities.

3. CONTAMINANTS AS ENVIRONMENTAL STRESSORS

Animals live in complex environments that can change in any time. In addition to natural stress, wildlife is now facing anthropogenic stress. In the past 100 years, urbanization has spread all over the world, covering hundreds of kilometers and increasing. Currently, more than 50% of the Earth's surface is being inhabited by urban areas or has been modified by human activities, such as agriculture, deforestation and desertification (Hooke et al., 2012). Among the major anthropogenic disturbances impacting wildlife species are environmental pollution, including pesticides and industrial chemicals, climate change and habitat alteration (Jenssen, 2006). Pollution stress can be additive to the existing natural stress causing

important alterations to wildlife; ranging from devastating effects (i.e. raptor population decline; Newton et al., 1993) to subtle or sub-lethal effects on the endocrine system (called "endocrine disruption"; Colborn et al., 1993).

3.1. Persistent Organic pollutants

Persistent Organic Pollutants (POPs) were identified as one of the major groups of environmental contaminants. POPs were extensively used in various industrial and agricultural processes until their prohibition, at the end of the 20th century. These contaminants were detected to impact either aquatic and terrestrial environments (Letcher et al., 2010), being of especially concern for the health of ecosystems. Their main characteristics are:

(i) POPs are highly lipophilic substances, potentially bioaccumulate in animal tissues and biomagnify through food chains (Borga et al., 2011; Henny et al., 2003), reaching very high concentrations in top predators (Letcher et al., 2010; Sonne, 2010).

(ii) POPs are highly persistent in the environment and ecosystems are still highly exposed to POPs (Espín et al., 2010; Lohmann et al., 2007; Orton et al., 2013; Thomas et al., 2006), although legal mitigations were undertaken years ago.

(iii) POPs are subjected to long-range atmospheric and hydrologic transport (Lohmann et al., 2007). As a consequence, zones with very low production and utilization of these chemicals (i.e. circumpolar Arctic) reach very high concentrations (AMAP, 2011; UNEP, 2011).

Initially, twelve POP compounds were considered as priority pollutants by the Stockholm convention (UNEP, 2001), including industrial chemicals [polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB)], organochlorine pesticides [OCPs; dichlorodiphenyltrichloroethanes (DDTs), aldrin, chlordane, dieldrin, endrin, heptachlor, HCB, mirex and toxaphene] and secondary products from unintentionally industrial processes (polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans). Some years later, polybrominated diphenyl ethers (PBDEs) were added in the Stockholm list (UNEP, 2009) and their analyses, along with PCBs and OCPs, were recommended for global monitoring programs (UNEP, 2011). These compounds are termed "legacy POPs". "Emerging pollutants" refer to compounds still in used, such as flame retardants (FRs), surfactants, steroids, hormones and pharmaceuticals, among others. These latter compounds can be found in the environment and may have potential toxic proprieties (Li et al., 2014; Sauvé and Desrosiers, 2014). Albeit, their use is still not restricted by law.

3.1.1. Polychlorinated biphenyls (PCBs)

PCBs are a group of chlorinated aromatic structures of up to 209 individual congeners with different chlorination and substitution pattern (WHO, 1993; Fig 3). The chlorination pattern of PCBs determines the toxicity of the substance. Lower chlorinated congeners are more mobile, less persistent and more degradable, while high chlorinated are more stable and persistent (De March et al., 1998; WHO, 1993). Because PCBs are chemically stable and heat resistant compounds, they were highly used as diffusion pump oils, extenders for pesticides, hydraulic and heat-exchange fluids, transformed and capacitor oils, lubricating oils and as plasticizers in the industry for nearly 50 years (from 1930s to 1970s) (De March et al., 1998; Yu, 2014). PCBs were first detected and reported to accumulate in wildlife and humans in the 1960s and were associated to toxic effects (Jensen, 1972). After that, in the 1970s these compounds were banned in almost all developed countries (in Spain they were banned in the 1990s; Orden de 4 de Febrero de 1994). Since then, levels have progressively decreased and are on the way to disappear from the global environment (Elliott and Elliott, 2013). However, levels still remain sufficiently high to cause adverse effects on living beings (Hamlin and Guillette, 2010; Schettgen et al., 2015), especially on top predators (Bourgeon et al., 2013; Luzardo et al., 2014; Sletten et al., 2016).

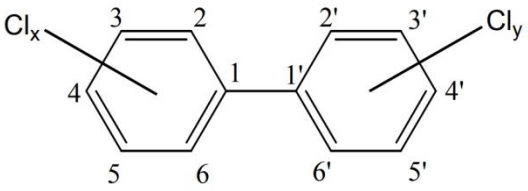
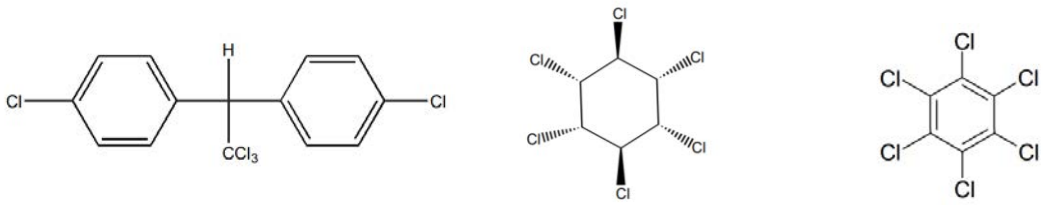


Fig. 3 General chemical structure of polychlorinated biphenyls. Source: Agency for Toxic Substances & Disease Registry (ATSDR)

3.1.2. Organochlorine pesticides (OCPs)

In this chemical class, five major groups can be distinguished: DDT and its metabolites [dichlorodiphenyldichloroethylene (*p,p'*-DDE); and dichlorodiphenyldichloroethane (*p,p'*-DDD)]; hexachlorocychlohexanes [HCHs; *α*-, *β*- , *γ*- (lindane) and *δ*- HCH]; cyclodienes (dieldrin, endrin, etc.); toxaphene and chlordanes (i.e. HCB); and mirex. This thesis will focus mainly on DDTs, HCHs and HCB (Fig 4).



DDT γ-HCH HCB

Fig. 4 General chemical structure of dichlorodiphenyltrichloroethanes (DDT), γ-hexachlorocychlohexanes (*γ*-HCH) and hexachlorobenzene (HCB). Source: Agency for Toxic Substances & Disease Registry (ATSDR)

While the contamination by PCB was not detected until the 1960s, the accumulation of DDT in nature was first recognized in 1950s (Jensen, 1972). When DDT was sprayed on crops, the birds living disappeared (Borg, 1958). Ratcliffe (1970) proved the disappearance of peregrine falcon (*Falco peregrinus*) in Britain to the accumulation of *p,p'*-DDE. Cyclodines were introduced as common pesticides in the 50s and were also found to be toxic for mammals and birds as well (Furness, 1993). All these OCPs were banned in 1970s along with PCBs (in 1990s in Spain). Lindane was the alternative pesticide to heptachlor (Blus and Henny, 1985) and was extensively used until the 2000s when it was definitively banned in Spain, one of the 10 European countries with the highest lindane usage (PETI, 2016). Similarly to PCBs, high levels of OCPs still remain in the environment, for instance the main metabolite of DDT, *p,p'*-DDE (Rattner et al., 2000), causing important adverse effects on biota (Cesh et al., 2010; Espín et al., 2010; Martínez-López et al., 2015; Van Drooge et al., 2008a). In addition, DDT is still in used in some developing countries as the unique and most effective method against malaria (Lohmann et al., 2007).

3.1.3. Polybrominated diphenyl ethers (PBDEs)

Polybrominated diphenyl ethers (PBDEs) are aromatic compounds that are structurally related to the PCBs, but with bromine substitution instead of chlorine (De March et al., 1998; Fig 5). They form part of the family of brominated flame retardants (BFRs) with 209 possible congeners of varying degrees of halogenation (WHO, 1994). Since 1960s, BFRs were incorporated in consumption products, such as plastics, textiles, insulation materials and electrical and electronic equipment, in order to increase their fire resistance (De Wit, 2002; WHO, 1994), reaching maximum production in the 1980s. There were three important commercial PBDE mixtures in the global market (i.e. Penta-, Octa-, and Deca-BDEs) (De Wit, 2002). As PBDEs are not chemically bound to the polymers that contain them, a fraction are released into the air, water and soil, during their production, use or disposal (Chen and Hale,

2010). As a consequence, increasing levels of PBDEs have been detected in the environment since the start of their use (twenty years ago), leading up to the 2000s. However, as a consequence of restrictions in their use (European Court of Justice, 2008), levels are now progressively declining in the environment (Elliott et al., 2015; Fernie and Letcher, 2010; Miller et al., 2015, 2014). While the bioaccumulation proprieties of PBDEs have been demonstrated, some authors question the degree to which PBDEs bioaccumulate through food chains (Elliott et al., 2009). Yet, high concentrations have been reported in terrestrial birds (Chen and Hale, 2010) and the highest concentrations have been found in the apex predators wild birds of prey (Elliott et al., 2015; Fernie and Letcher, 2010; Guerra et al., 2015).

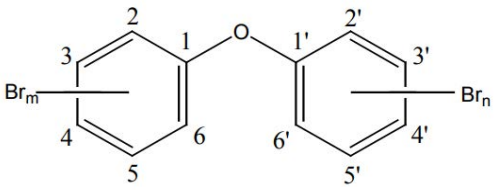


Fig. 5 General chemical structure of polybrominated diphenyl ethers. Source: Agency for Toxic Substances & Disease Registry (ATSDR)

3.2. Emerging pollutants

As a result of the recent legislation restricting the use of PBDEs (European Court of Justice, 2008) and some other BFRs (UNEP, 2017), the production of alternative FRs such as organophosphate ester flame retardants (OPEs) has increased in the last years. Currently, OPEs can be found in multiple industrial products, such as furniture, plastics, electronics and textiles to enhance fire safety (American Chemistry Council, 2014). Similarly to PBDEs, OPEs are not chemically bound to the material and can therefore leach out in the environment during any point of the product's life cycle (Marklund et al., 2005), being highly detected in the environment (Andresen et al., 2004; Marklund et al., 2003; Van den Eede et al., 2011). The most frequently used OPEs are: tris(2-chloroethyl) phosphate (TCEP), tris(1-chloro-2-propyl) phosphate (TCiPP) and tris(1,3-dichloro-iso-propyl) phosphate (TDCiPP) that are used as replacements for Penta-BDE (Dodson et al. 2012; Fig 6).

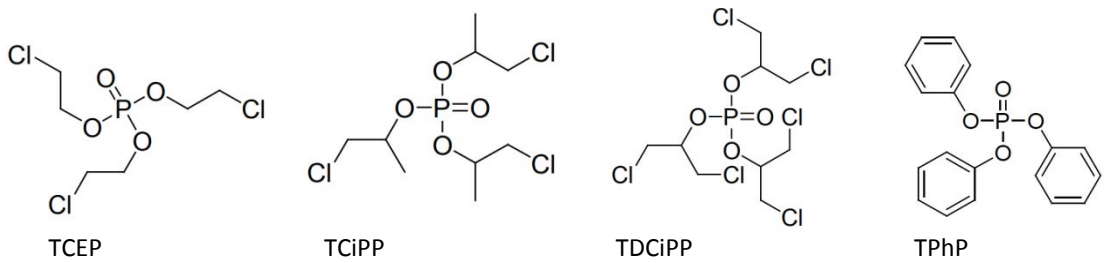


Fig. 6 General chemical structure of tris(2-chloroethyl) phosphate (TCEP), tris(1-chloro-2-propyl) phosphate (TCiPP), tris(1,3-dichloro-iso-propyl) phosphate (TDCiPP) and tri-phenyl phosphate (TPhP). Source: United States Environmental Protection Agency (EPA)

In addition, non-halogenated OPEs, such as tri-phenyl phosphate (TPhP), have also being highly utilized (Van der Veen and De Boer, 2012; Fig. 6). The knowledge about OPEs toxicity is limited, but neurotoxic proprieties, skin irritation, hemolysis and carcinogenic effects have been described (WHO, 1998). In addition, considering the propensity of OPEs to accumulate in biota (Barón et al., 2014; Briels et al., 2018; Greaves and Letcher, 2014; Sundkvist et al., 2010), concerns about their potential impact on wildlife populations are now increasing.

3.3. Effects of pollutants on birds

Several studies have investigated how pollutants impact on birds. The main adverse effects are the following (reviewed in Letcher et al., 2010):

(i) Endocrine system disruption. Contaminant exposure can impair the CORT stress response (Nordstad et al., 2012; Verboven et al., 2010); thyroid function (Fernie and Marteinson, 2016); and induce oxidative stress (Abbasi et al., 2017).

(ii) Alteration of phenotypic features; e.g. alteration of iris pigmentation or other important social signs (Bortolotti et al., 2003).

(iii) Impairment of reproduction; e.g. delayed egg-laying, thinner eggshells, weight loss during embryonic development, reduce fertility and reproductive success (Fernie et al., 2009; Jiménez et al., 2007).

(iv) Impairment of immune system. Dysfunction in the immune system has mostly been associated with heavy metals (Lewis et al., 2013; Vallverdú-Coll et al., 2015), however immunological effects have also been related to high POP levels (see Letcher et al., 2010).

(v) Changes in behavior; e.g. altered incubation period, aggression during courtship interactions, clutch abandonment, cracked eggs (Bustnes et al., 2008; Fernie et al., 2007a).

(vi) Others: impairment of growth and development (Fernie et al., 2007b; Ortiz-Santaliestra et al., 2015); neurotoxic effects (Eng et al., 2012); alteration of biochemical parameters (Ortiz-Santaliestra et al., 2015; Van den Steen et al., 2010); carcinogenic effects (WHO, 1998; 1994; 1993).

Given that the present thesis focuses on evaluating the impact of contaminants on the endocrine system, particularly on the stress response system, further information is focus on this regard.

Contaminant compounds such as POPs are considered Endocrine Disrupting Chemicals (EDCs) (Bergman et al., 2012). One of the first compound to be recognized as a EDC was DDT (Ottinger et al., 2013). Other components are also included in the EDC list, such as fungicides, herbicides or other pesticides (i.e. streptomycin, atrazine, endosulfan, and carbofuran, among others). Alternatively, PFRs are still not classified as EDCs, although their potential (Guigueno and Fernie, 2017). EDCs can affect hormone sensitive neural systems, organs and tissues impairing reproduction, metabolic and immune functioning (Kavlock et al., 2012) and extended information exist on the effects of EDCs on sex hormones, thyroid function and oxidative stress induction (Abbasi et al., 2017; Cesh et al., 2010; Fernie and Marteinson, 2016; Ortiz-Santaliestra et al., 2015; Rogstad et al., 2017; Van den Steen et al., 2010). However, very little information is available regarding the effect of EDCs on the adrenal hormone secretion (Bergman et al., 2012; Hinson, 2006) despite the increasing evidence suggesting adrenal function as an important target for EDCs (Hampl et al., 2016; Maqbool et al., 2015).

Generally, although the concrete mechanism of action is still unknown, EDCs are thought to interfere in the hormonal function in various sites; either mimicking the CORT molecule or directly modifying a given target or multiple targets (see Box 3). Thus, depending on the target modified or organ involved, EDCs may affect different physiological processes that are regulated by CORT.

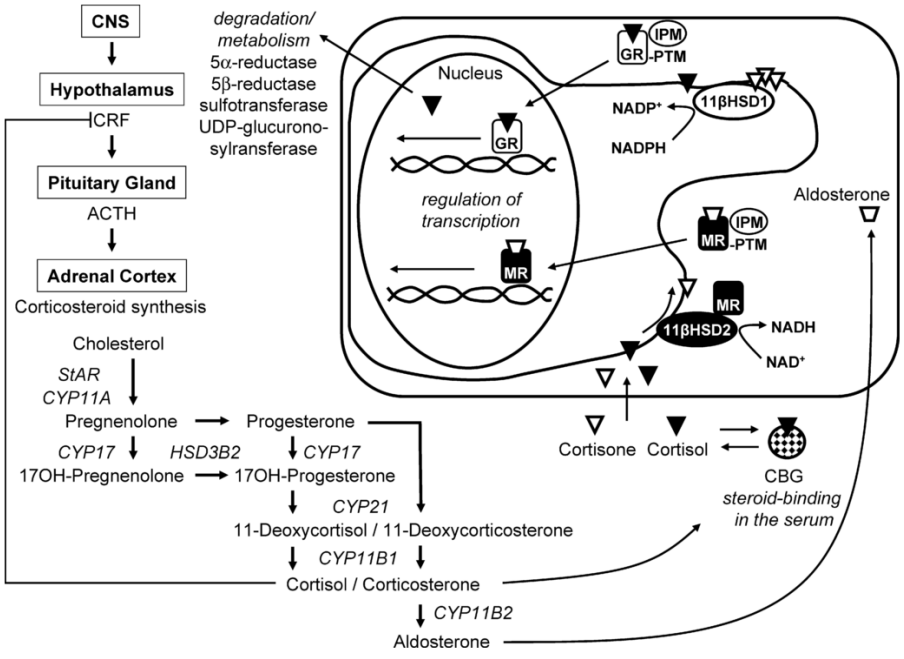
Several persistent environmental pollutants are thought to reduce hormone secretion by inhibiting steroidogenic enzymes, while others induce mRNA synthesis and hormone secretion. For instance, cytochrome P450 (CYP450) has a fundamental role in steroid hormone biosynthesis and it is regulated by different organs such as adrenal gland, testis, ovary, brain, placenta and adipose tissues (Maqbool et al., 2015). Different EDCs have been observed to inhibit CYP450 or CYP1A1. Some derivates of DDT are adrenotoxic in dogs and derivates of DDE and PCBs potentially inhibit CYP11B1 in the adrenocortical cells, and thus inhibiting the synthesis of CORT (reviewed in Maqbool et al., 2015). Metabolites of PCBs and HCB may also interfere with the glucocorticoid receptors (GR), decreasing the GR activation (Johansson et al., 2005; Lelli et al., 2007). In the laboratory, *p,p'*-DDE has been established to be a relatively potent antagonist of the androgen receptor (reviewed in Sanderson, 2006). Some other EDCs have also been observed to impair the enzyme 11β-HSD Type 2, whose action is to protect the kidney and also the testicular Leyding cells from an excess of GCs (Hampl et al., 2016).

The study of GC disruptors is an emergency field of research and identifying the mechanisms of toxicity remains a major challenge. For example, it is still not clear the threshold from which

various EDCs can have a "cocktail effect", potentially increasing their impact on the endocrine system (Ottinger et al., 2013). In fact, ECDs may interfere in different endocrine steps in parallel, leading to incredible diversity and complexity of ECDs action (Hampl et al., 2016). This thesis is not going further in the study and discussion of the mechanism of action of ECDs on the endocrine system. For further information refer to: Sanderson, 2006; Odermatt and Gumy, 2008; Bergman et al., 2012; Maqbool et al., 2015; Hampl et al., 2016.

Box 3. Disturbances of EDCs on the regulation of corticosterone hormone

The potential disturbances of EDCs on the regulation of CORT physiological processes are the following: **(i)** Affecting the hormone **biosynthesis**, release of stimulatory or inhibitory hormones at its endocrine source (disruption of the HPA axis); **(ii)** Affecting the **metabolism** (i.e. affecting the biotransformation of hormones by inducing inhibition of steroidogenic enzyme catalytic activity); **(iii)** Interfering with the **transport** (i.e. affecting the concentrations or functioning of serum-binding proteins); and finally **(iv)** Impairing the **mechanism of action** on both receptor and post-receptor levels (Bergman et al., 2012; Guillette and Gunderson, 2001; Hampl et al., 2016).



Source: Odermatt and Gumy, 2008

Abbreviations: CNS: central nervous system; CRF: corticotrophin releasing factor; ACTH: adrenocorticotrophic hormone; CBG: corticosteroid-binding globulin; IPM: interacting protein modification; PTM: post-translational modification; GR: glucocorticoid receptors; MR: mineralocorticoid receptors.

4. BIOMONITORING OF CONTAMINANTS

Contaminants are widespread in the environment, in the air, water, sediments and organism living therein. However, only the measurement of contaminant concentrations in tissues, fluids and products derived from the organism living can reveal the bioability and impact of toxic compounds on the organisms and on the ecosystems (Furness, 1993). Species of choice has to be representative of the entire ecosystem to be analyzed (Burger, 1993; Furness, 1993). Species or ecological communities can be used as biomonitors of their environment if they: (1) respond in observable ways to anthropogenic disturbances; (2) enable us to assess the levels of environmental contamination; (3) estimate human health risks; (4) identify contamination of the food-chain; and (5) reflect spatial or temporal trends in contaminant levels (Gómez-Ramírez et al., 2014; Pollack et al., 2017).

4.1. Types of samples used

Birds have been widely used as important biomonitors of environmental pollution (Gómez-Ramírez et al., 2014; Newton et al., 1993; Smits and Fernie, 2013). Most pollution studies on birds have been conducted using internal tissues (liver, muscle, adipose tissue, kidney) (Jaspers et al., 2006; Van Drooge et al., 2008b; Voorspoels et al., 2006, 2002). However, because practical and ethical reasons inhibit the sacrifice of free-living animals, methods for non-destructive biomonitoring have been developed. Several studies have used different non-destructive matrixes to biomonitor contaminants: blood (Gómara et al., 2004; Ortiz-Santaliestra et al., 2015; Tartu et al., 2015); faeces (Martinez-Haro et al., 2013; Sánchez-Virosta et al., 2015); regurgitated pellets (Berny, 2007; Lopes et al., 2009); eggs (Gómez-Ramírez et al., 2012; Mañosa et al., 2003; Morales et al., 2012); preen oil (Eulaers et al., 2011a; Jaspers et al., 2008); and feathers (Dauwe et al., 2005; Eulaers et al., 2011b; Jaspers et al., 2007, 2006).

Blood provides a measure of recent exposure of contaminants (Eulaers et al., 2011a; Martínez-López et al., 2009). Similarly than in hormones, this matrix cannot provide an intermediate or long-term measure. In spite of this, different studies have showed correlations between blood and internal tissues concentrations (García-Fernández et al., 1996; Henriksen et al., 1998). However, these correlations can be disrupted by shorts mobilization of contaminants, for instance during egg laying, molting or migration (Espín et al., 2016), or by recent feeding (Olsson et al., 2000). In addition, blood is considered an invasive matrix and samples need special storage and transport conditions (reviewed in Espín et al. 2016).

Contaminant concentrations in preen oil has also been reported to closely reflect the internal state of contamination. For example Eulaers et al. (2011a) found a strong correlation between OCPs in blood and preen oil in nestling white-tailed eagles (*Haliaeetus albicilla*). Another study (Jaspers et al., 2011) reported strong correlations in levels between preen oil and internal muscle and feathers in different Belgian bird species. However, the compound profile between preen oil and internal tissues may be different (Jaspers et al., 2013). Preen oil is an oily secretion from the uropygial gland, that birds expand to their feathers to protect and waterproof them. Collecting preen oil may be difficult, as it is necessarily to press the gland softly to extract the oil, but sampling this matrix is considered minimally-invasive. However, preen oil is rarely utilized in pollution studies (reviewed in Espín et al., 2016).

Other matrices, such faeces or regurgitations, have also been explored. Both matrixes provide information about spatial-temporal variation in diet (Espín et al., 2016). In addition, their collection is totally non-invasive and a large number of samples can be collected. Pellets have been mostly utilized for analyzing lethal poisoning by anticoagulants, carbamates or similar (Berny, 2007). A large number of studies have utilized faeces for analyzing contaminants, mostly heavy metals (Martinez-Haro et al., 2013; Sánchez-Virosta et al., 2015). However, levels measured in faeces have been observed to not correlate to internal contamination levels; therefore it appears that faecal measurements may not be suitable as an indicator of accumulation (Espín et al., 2016).

Eggs and feathers are the most widely collected samples (Espín et al., 2016; Gómez-Ramírez et al., 2014). These are the most practical matrixes to biomonitor contamination in birds. Both are non-invasive (failed or deserted eggs and molted feathers) or minimally-invasive (plucked feathers) matrixes; non-destructive; easily collected (proactively or opportunistically), stored and transported; and enable us to biomonitor either live and dead birds. Eggs has a long trajectory in the study of contamination (Gómez-Ramírez et al., 2012; Mañosa et al., 2003; Morales et al., 2012). This homogeneous matrix provides a contaminant exposure that can be directly related to reproductive effects and success (Crosse et al., 2012; Pereira et al., 2009) or hatching success and chick development (Harris and Elliott, 2011), indicating the load of contaminants the nestling is exposed during embryonic development (García-Fernández et al., 2008). However, eggs are not indicative of all individuals of one population (i.e. males and juveniles birds). In addition, only failed or deserted eggs can be collected; thus becoming a non-random matrix, as they can only represent breeding failure (Espín et al., 2016). Lastly, eggs can only be sampled during the breeding season, limiting the spatio-temporal framework of

the study. On the other side, feathers allow to monitoring all individuals, offering a global perspective of the contamination exposure, and enable us to relate contaminant exposure to stress. Further detailed information regarding feathers is provided in Section 5.

The following table (Table 3) summarizes the characteristics of the main samples utilized in birds to biomonitoring contaminants.

Table 3 Characteristics of the main samples utilized in birds to biomonitoring contaminants

	Internal tissues	Blood	Eggs	Feathers
Window in time	Present measure	"Snap -shot" (only recent exposure)	During breeding season[1]	Retrospective and long-term measure
Reflect internal load	Direct measure	Correlation with internal levels[2]	Correlation with internal levels	Correlation with internal levels[3]
Sampling	Post-mortem (possible collection from rehabilitation centers)	In field	In field	In field; museums specimens
Type of matrix	Destructive	Non-destructive	Non-destructive	Non-destructive
Invasiveness	Invasive / non-invasive (if specimen is already dead)	Invasive	Non-invasive (deserted or failed egg) / invasive (viable egg)	Non- invasive (molted) or minimally-invasive (plucked)
Confounding factors	Non-random sampling (bias by the cause of death or illness)	Susceptible to recent ingestion; high fluctuation among seasons	Non-random sampling (failed or deserted eggs); bias by egg-laying sequence; bias by decomposition	External contamination[4]; type of feather sampled; molt period
Limiting factors	Inherent difficulties in sampling (especially in endangered sp.)	Storage and transport requirements	Only breeding females; limited number of samples; limited spatial-temporal	Temporal limitation (only reflect growth period); bias by preen oil[4]

Source: Author (revised from Espín et al., 2016; Gómara-Ramírez et al., 2014; García-Fernández et al., 2008)
Notes: [1] Only in income breeders species (i.e. raptors) that use recently ingested food to cover the energetic cost of breeding, thus layed eggs reflect contaminant exposure within breeding territories. Instead, capital breeders accumulate lipophilic contaminants during winter and migration and mobilize and transfer the contamination load to the eggs during the breeding season (see Espín et al., 2016). [2] But see (Espín et al., 2016). [3] But see (Jaspers et al., 2007, 2006). [4] But see (Eulaers et al., 2011a; Jaspers et al., 2011, 2008).

4.2. Raptors as key species to biomonitor environmental pollutants

Raptors are interesting sentinels for ecotoxicological monitoring studies. These animals occupy high positions on the food chain, being highly susceptible to the accumulation and

biomagnification of contaminants (Furness, 1993). In addition, predatory birds have relatively long lifespan over which to accumulate contaminants, show large geographical areas of distribution, and are especially sensitive to environmental changes (Furness, 1993). These species were the first wildlife birds known to be affected by anthropogenic pollution. During the 20th century, dramatic declines in raptor populations were observed in North America [e.g. bald eagle (*Haliaeetus leucocephalus)*; Bowerman et al., 1995] and in Europe [e.g. peregrine falcon (*Falco peregrinus),* Eurasian sparrowhawk (*Accipiter nisus),* golden eagle (*Aquila chrysaetos*); Ratcliffe, 1970], raising awareness of the consequences of anthropogenic pollution and driving to a range of International policies and legislative instruments restricting their use (i.e. Stockholm Convention). As a result, raptors were considered important sentinels of their ecosystem (Helander et al., 2008) and have been widely used in biomonitoring programs as an early warning system of potential impacts in the ecosystem and in humans (Espín et al., 2016; Gómez-Ramírez et al., 2014). For example, exposure to lead bullets were observed to impact red kites (*Milvus milvus*) (Pain et al., 2007) and human (Pain et al., 2010) health in UK. In addition, raptors are species of public interest worldwide ("flagship species") and are positively associated with high levels of diversity of other taxa ("umbrella species") (Burfield, 2008; Burgas et al., 2014). Thus, their conservation and study can deliver broader biodiversity benefits (Sergio et al., 2005).

However, some drawbacks are associated with the use of raptors to biomonitor contaminants. First, most birds of prey are classified as Endangered or Under Concern by the IUCN, and thus are protected species. Lethal sampling is then not permissible and capturing and sampling non-destructive samples in free-living specimens is often limited. Second, most of the species are migratory, thus making difficult to relate contaminant levels in the bird to a particular geographical area. Third, various confounding factors, such as different feeding habits, sex, age, body condition and type of sample used, may trigger large fluctuations in contaminant levels in birds.

5. THE USE OF FEATHERS TO MONITOR STRESS AND ENVIRONMENTAL POLLUTION

Feathers are the most recently introduced technique to measure CORT levels (Bortolotti et al., 2008a) and contaminants (Dauwe et al., 2005; Jaspers et al., 2006) in birds. This matrix has become the method of choice when non-destructive and non-invasive sampling is required (García-Fernández et al., 2013; Romero and Fairhurst, 2016). Feathers can provide an integrated and retrospective assessment of the long-term HPA axis activity of birds and biomonitor pollutant exposure. Below, are the main characteristics of feathers, which should be taken into account for a proper interpretation of results.

5.1. Characteristics of feathers

Feathers are extraordinarily diverse and complex structures. Feathers are branched, or pinnate, epidermal derivative composed of a matrix of keratin (Prum and Williamson, 2001; Fig 7). A typical contour of the feather is the calamus, which extends into the rachis, the central shaft of the feather vane. The primary branches of the rachis are the barbs and the secondary are the barbules. The shaft of the barb is the ramus, and the rami supports the barbules. The afterfeather (aftervane and aftershaft) are typical of contour feathers.

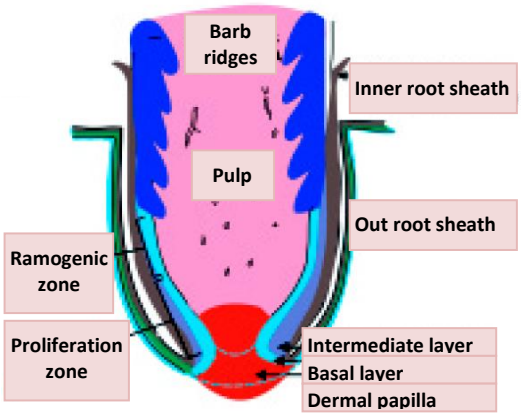


Fig. 7 Feather morphology. Source: Prum and Williamson, 2001

5.1.1. Deposition of elements in feathers

Like hair, feathers grow from their bases (Prum, 1999). Feathers are cylindrical structures that grow and elongate through mitotic cell division and subsequent keratinization along the radial circumference of the feather (Prum and Williamson, 2001), growing in "a radial fashion" form (Bortolotti et al., 2008b). Only during growth, the highly vascularized cells in the feather are connected to the bloodstream (Bortolotti et al., 2009). Nutrients and carotenoid pigments are supplied to the feather from the pulp, the highly vascularized center of the blood quill around which the developing of the feathers is wrapped (Yu et al., 2004; Fig 8).

Functional elements (nutrients and carotenoids) are incorporated into the feather matrix in a mass dependent way, contrary to non-functional compounds (e.g. heavy metals, contaminant traces, CORT) that are rather incorporated as a function of time (Bortolotti et al., 2008a; Dauwe et al., 2003). Once the feather is fully grown, the pulp of the feather atrophies and the vascularization ends, thereby feathers become inert structures (Maderson et al., 2009).

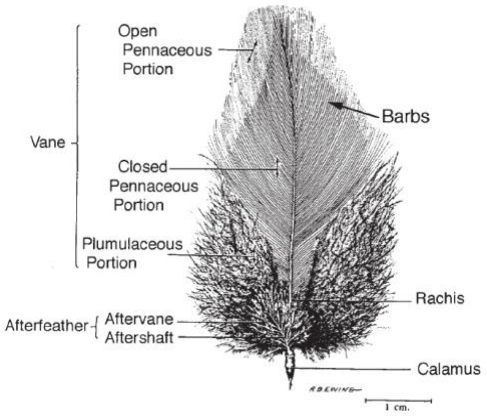


Fig. 8 The basis of feather. Source: Yu et al., 2004

In mammals, steroid hormones are incorporated into the hair from blood via diffusion but also from analogous systems such as sebaceous and sweat gland secretions (Thieme et al., 2003); therefore is of special importance the washing protocol of hair prior the hormone extraction (Davenport et al., 2006). In birds, the incorporation of CORT in feathers is thought to occur during the cell differentiation phase of feather growth, happening mainly in the blood quill (Jenni-Eiermann et al., 2015) and there is no evidence for analogous systems (Taves et al., 2011). In addition, preen oil apparently does not contain detectable levels of CORT (Lattin et al., 2011), although a very recently report suggested that CORT can also bind into the surface of the feather (Jenni-Eiermann et al., 2015). Notwithstanding, washing the feathers was observed to have no effect on feather CORT concentrations (Bortolotti et al., 2008b; Jenni-Eiermann et al., 2015), at least when CORT levels are normal or low (Jenni-Eiermann et al., 2015), and thus the current protocols do not incorporate this step.

When analyzing contaminants, washing the feathers is a crucial step as it allows to remove exogenous sources (atmospheric dirt and dust particles) deposited onto the surface of the feathers (Jaspers et al., 2011, 2008). In fact, external contamination onto the feathers has extensively been investigated for POPs (Jaspers et al., 2011, 2008). Further information is detailed below (section 5.3).

5.1.2. The importance of molt

Unlike hair, which is grown and replaced continuously (Stalder and Kirschbaum, 2012), the growth and replacement of feathers occur at frequent intervals in the year, determined by the molt strategy of each species in particular (Bortolotti, 2010; Hardy et al., 2006; Hedenström, 2006). Feathers have to be replaced regularly because physical abrasion and ultraviolet light

rapidly degrade their structure (Rohwer et al., 2009). Among the most important feathers are the flight feathers of the wing (primary and secondary) and the rectrices feathers of the tail that provide most of the bird's flight surface. Body feathers are also vital as they maintain body temperature and provide protection (Forsman, 1999; Gill, 2007). Since CORT and contaminants are deposited into feathers during the growth phase of feathers, it is crucial to identify and determine the growth period of each type of feather sampled for a proper interpretation of data.

5.2. Detection of corticosterone in feathers

By accumulating CORT during all its growth, feathers provide the longest-term measure of CORT and is the unique matrix that gives a retrospective insight into the HPA axis activity with a single sampling (Romero and Fairhurst, 2016). Bortolotti et al. (2009, 2008a) were the pioneer authors using this technique and established that the amount of CORT deposited in feathers correlated to stressors experimented by the bird during feather growth. More recently, other authors (Aharon-Rotman et al., 2015; Horak et al., 2013; Jenni-Eiermann et al., 2015) gave support to this assumption, suggesting that feather CORT (CORTf) levels may reflect average circulating levels throughout the period of feather growth (Bortolotti et al., 2009). In addition, daily variations in baseline CORT levels or short raises due to acute stressors have been proven to be insignificant to the global CORTf value (Fairhurst et al., 2013).

Since the first apparition in the literature, CORTf has generated increasing interest and a growing body of studies have used this technique to monitor chronic stress, measure energy consumption or predict carry-over effects (Romero and Fairhurst, 2016). The sampling of this matrix can be totally non-invasive, when molted feathers are collected in the field (in this case the presence of the bird is not needed) or minimally-invasive, when feathers are plucked from the bird. Thus, sampling feathers has practical advantages in the field and can provide crucial information about the stages of the annual cycle when birds are inaccessible for sampling (Kouwenberg et al., 2013). In addition, the minimal requirements for collecting, storing and transporting feathers add flexibility to the field and lab work.

However, measuring CORTf is a complex technique and some weaknesses have been detected (reviewed in Romero and Fairhurst, 2016). For example, mixed results have been published regarding the proportionately between baseline CORT levels (not stress-induced) and CORT integrated in feathers (Bortolotti et al., 2008a; Fairhurst et al., 2013; Jenni-Eiermann et al., 2015; Lattin et al., 2011). Other critical limitations of this matrix are related to the type of

feather sampled (i.e. the often unknown synchronization of molt within populations), the lab protocol used for extracting and measuring CORT, and the influence of potential biological confounding factors (Table 4).

5.3. Detection of pollutants in feathers

Feathers were first used to biomonitor contaminants than steroid hormones (Bortolotti et al., 2009, 2008a) and the first contaminants studied were the heavy metals (Burger, 1993; Dauwe et al., 2003). Since then, feathers have extensively been used to biomonitor metal contamination (Borghesi et al., 2017; Dolan et al., 2017; Espín et al., 2016; García-Fernández et al., 2013) and all other types of contaminants, except rodenticides (reviewed in Espín et al., 2016). Persistent organic pollutants have also extensively been monitored in feathers (Abbasi et al., 2016a; Dauwe et al., 2005; Eulaers et al., 2011b; Jaspers et al., 2011, 2006) to the point that feathers have become the preferable matrix to their biomonitoring (Espín et al., 2016; Gómez-Ramírez et al., 2014).

Similarly than the CORTf measurement, the use of feathers allows to obtain with a single sampling a global measure of contaminant exposure, coming along with easy collection, storage and transport requirements. In addition, strong and significant correlations have been reported between contaminant concentrations in feathers and in internal tissues (Eulaers et al., 2011b; Jaspers et al., 2011, 2007, 2006; Van den Steen et al., 2007). This significant correlation was a crucial point for the validation of this matrix because the principal aim of using feathers was to assess the internal body burden of birds in a non-destructive way. However, and despite this matrix has been widely used since then, some studies have showed discrepancies in regards to the correlation feather-internal burdens (Espín et al., 2012, 2010). Possibly, these discrepancies are explained by the time gap between feather growth and the collection of internal tissues (due to blood atrophy of fully grown feathers) (García-Fernández et al., 2013). An alternative explanation may be the influence of external contamination, particularly by preen oil. This oil originates from internal levels and may contribute to a more strongest correlation between feathers-internal tissues (Jaspers et al., 2008). Thus, some authors have suggested preen oil as a beneficial factor. Notwithstanding, the exact contribution of preen oil is still not well known. Some other confounding factors that should be considered are: the molt period, the collection time of the feather, the type of the feather collected and biological factors such as age, sex, food and migratory habits (Table 4).

Table 4. Some limitations for the use of feathers to monitor CORT and contaminants

	CORT	Contaminants
Methodology	▪ Possible variation along the feather length [1] ▪ Mixed results in washing or not the feathers [2,3] ▪ Efficiency of methanol extracting CORT [4] ▪ Differences in the immunoassays among labs (especially EIAs) [5]	▪ Possible interference of the structure of feathers (barb, rachis, vane) [14] ▪ POPs: Possible bias of external contamination through preen oil [15,16] ▪ OPEs: Lack of validation for the washing protocol for extracting atmospheric deposition (either by preen oil or atmospheric dust and particles) [17]
Matrix	▪ Stability of CORT in feathers [6] ▪ Mixed results in the proportionately between baseline CORT and feather CORT (in no stress-induced situations) [7-9] ▪ Deposition rate: measuring in mm or mg [7,10] ▪ Deposition dynamics of CORT in feathers after growth [2] ▪ Small sample artifact [4,9]	▪ Stability of compounds in feathers [14] ▪ Mixed results in the correlations between contaminants in feathers and in internal tissues [18,19] ▪ Deposition rate: measuring in mm or mg [10,14]
Interpretation of data	▪ Inter-specific confounding effects: age, sex, body condition [6,11] ▪ Fitness-related seasonal interactions [12] ▪ Influence of molt and differences between types of feathers [12] ▪ Internal *vs.* external quality measurement [11,13]	▪ Inter-specific confounding effects: age, sex, body condition [14,18,20,21] ▪ Intra-specific confounding effects: migratory patterns and diet [22-24] ▪ Influence of molt and differences between types of feathers [14,18,25]

Source: [1]Romero and Fairhurst, 2016; [2]Jenni-Eiermann et al., 2015; [3]Bortolotti et al., 2008b; [4]Berk et al., 2016; [5]Tate and Ward, 2004; [6]Romero and Fairhurst, 2016; [7]Bortolotti et al., 2008a; [8]Fairhurst et al., 2013; [9]Lattin et al., 2011; [10]Bortolotti et al., 2010; [11]Harms et al., 2015; [12]Boves et al., 2016; [13]Legagneux et al., 2013; [14]García-Fernández et al., 2013; [15]Eulaers et al., 2011a; [16]Jaspers et al., 2008; [17]Eulaers et al., 2014; [18]Jaspers et al., 2007; [19]Jaspers et al., 2006; [20]Dauwe et al., 2005; [21]Jaspers et al., 2011; [22]Behrooz Dahmardeh et al., 2009; [23]Eulaers et al., 2013; [24]Bustnes et al., 2008; [25]Espín et al., 2016.

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OBJECTIVES

The **main purpose** of this doctoral thesis is to evaluate and optimize the use of feathers to measure long-term levels of corticosterone and biomonitor environmental pollutants, exploring their effects on birds.

The **specific objectives** of this thesis can be summarized as follows:

1) To test the suitability of different types of feathers to measure corticosterone and monitor environmental pollutants.

2) To determine individual consistency, stability and repeatability in feather corticosterone levels over time and validate a new protocol for the extraction of feather corticosterone.

3) To examine the potential uses of feather corticosterone as a biomarker of bird fitness (survival and reproduction).

4) To evaluate the usefulness of feathers to biomonitor environmental pollutants and investigate potential relationships with adrenal hormones.

5) To examine potential confounding factors in relation to corticosterone and contaminant measurements in feathers.

With the aim to reach each specific objective, this thesis was structured on two main parts:

The first part entitled "Feather corticosterone: methodological considerations and biological relevance" was focused on the study of feather corticosterone as an assessment of long-term adrenal activity in birds and included both methodological and biological studies. In this part, three different studies were performed.

The first study (**Chapter I**): "Relationship between feather corticosterone and subsequent health status and survival in wild Eurasian sparrowhawk (*Accipiter nisus*)" explored the objectives 1 and 3.

The second study (**Chapter II**): "Corticosterone levels in feathers predict reproductive success in a captive population of Northern bald ibis (*Geronticus eremita*)" explored the objective 2 and 3.

The third study of this part (**Chapter III**): "Validation of a new protocol for corticosterone extraction from feathers of a raptor species" achieved part of the objective 2 and was the base to conduct the chapters V and VI.

The second part entitled: "The use of feathers to biomonitor environmental pollution and explore their relationship with adrenal hormones" was focused on the study of feathers as a tool to biomonitor environmental pollution and explore the relationship with adrenal stress hormones. Three different studies were also conducted.

The fourth study of the present thesis (**Chapter IV**): "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*)" allowed to achieve the objective 1.

The fifth study (**Chapter V**): "High levels of corticosterone are related to persistent organic pollutants, but not to organophosphate ester flame retardants, in feathers of nestling Cinereous vultures (*Aegypius monachus*)" explored the objective 4.

Finally, the sixth study of the present thesis (**Chapter VI**): "Persistent organic pollutants affect the endocrine response of free-living, but not captive, red kites (*Milvus milvus*)" assessed the objective 4.

Chapter I, II, V and VI explored the objective 5.

PART I:

Feather Corticosterone: Methodological Considerations and Biological Relevance

CHAPTER I

Relationship between feather corticosterone and subsequent health status and survival in wild Eurasian sparrowhawk

Photo ®: Rafa A. Molina López; Laura Monclús

Relationship between feather corticosterone and subsequent health status and survival in wild Eurasian sparrowhawk

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Published in: J Ornithol (2017) 158:773-783. DOI 10.1007/s10336-016-1424-5

1. Abstract

In birds, integrated levels of corticosterone (CORT) measured in feathers (CORTf) allow us to make inferences on past levels of stress demands. It has been suggested that levels of CORTf track carry-over effects across seasons. Nevertheless, our understanding of how this measure can be used to assess future health status is far from complete. In this study, we aimed to investigate whether CORT deposited in feathers over the molting period was related to subsequent mortality and health status in wild raptors admitted to rehabilitation centers. Thirty-four Eurasian Sparrowhawks (*Accipiter nisus*) admitted during the non-molting period were sampled. Body condition (BC) was used as an indicator of health status to classify individuals' health as good, poor or cachexia depending on their pectoral muscle score. Mortality was recorded over the non-molting period. Other potential sources of CORTf variation were assessed, such as sex, age and feather type, primary or body covert feathers. While CORTf did not vary with age or sex, significant differences were found between primary and body feathers, highlighting the importance of sampling the same feather type. Our results also revealed that birds in poor BC showed higher CORTf levels than individuals in good condition; however, CORTf levels in cachectic birds did not differ from those in good condition. This finding suggests caution when assuming that only high CORTf levels represent individuals in poor condition, and limits the utility of CORTf for the prediction of BC. The present study also showed that individuals which died following admission had higher CORTf levels than individuals which survived, suggesting the potential utility of CORTf as a metric for the study of subsequent mortality.

2. Introduction

The stress response is defined as the physiological, hormonal and behavioral changes that allow an animal to cope with noxious or unpredictable stimuli (Romero, 2004). The hypothalamic–pituitary–adrenal (HPA) axis is a pivotal element of the stress response and, when activated, triggers a hormonal cascade which results in the release of glucocorticoids (GCs) into the bloodstream. At baseline concentrations, GCs are mediators of essential metabolic processes such as regulation of energy intake, storage and mobilization (Landys et al., 2006; Sapolsky et al., 2000), which play a key role in the promotion of individual daily life processes. During the stress response, release of GCs promotes escape and self-preservation behaviors (Wingfield and Kitaysky, 2002; Wingfield, 2013). However, despite its adaptive value, chronic increases in GCs may negatively affect cognitive ability, growth, immune defense and body condition (BC), with detrimental consequences for an animal's fitness (Romero et al., 2009; Sapolsky et al., 2000; Wingfield and Sapolsky, 2003).

Corticosterone (CORT), the primary stress-induced GC in the avian endocrine system, can provide an index of stress (Bonier et al., 2009; Romero, 2002; Schoech et al., 2009). Blood sampling has been the preferred method to explore the activity of the HPA axis, and several studies have used this matrix to explore CORT variations under different environmental and ecological conditions (e.g. Love et al., 2003; Müller et al., 2007). Yet, this matrix has important limitations. Firstly, this measure only provides information spanning short time periods (Touma and Palme, 2005). Secondly, capture and restraint of animals for sampling can itself induce an increase in CORT secretion into the bloodstream (Romero, 2002; Romero and Reed, 2005). As measuring CORT levels over long time periods is more biologically relevant in ecological studies, feathers have been introduced as a CORT measurement matrix. Feathers accumulate CORT during their growing period via diffusion from the blood quill (Jenni-Eiermann et al., 2015) and its levels are described to be correlated to stressors experienced by the bird during feather growth (Bortolotti et al., 2008). Recent work supports the assumption that there is a correspondence between plasma CORT and CORT integrated in feathers during their growth (Aharon-Rotman et al., 2015; Horak et al., 2013; Jenni-Eiermann et al., 2015). This means that the amount of CORT accumulated in feathers can provide a retrospective long-term integrated record of an individual's HPA axis activity during avian molt (Bortolotti et al., 2008; Fairhurst et al., 2013; Jenni-Eiermann et al., 2015). Additionally, this matrix is easily collected and requires no special storage or transport conditions (Strong et al., 2015).

Despite the strengths of measuring feather CORT (CORTf), there are still significant caveats in the use of feathers. For instance, they have limited usefulness for short-term stressors, for which collecting blood samples (Romero and Fairhurst, 2016) or measuring non-invasively CORT metabolites in droppings (see Möstl et al., 2005) would be more suitable. Additionally, CORT measured in feathers only contains the information of the time the feather grew, making crucial the assessment of the molting period for a proper interpretation of results. At present, there is a need for a deeper knowledge of the rate of deposition of CORT in feathers, as well as the rate of feather growth and its synchrony within a population, as these aspects remain unknown for most species (Romero and Fairhurst, 2016). However, despite the above limitations, feathers constitute a promising tool offering a unique perspective of a past situation that can be very useful in field studies. For instance, in the context of wild bird populations, the measurement of CORTf may provide crucial information about the stages of the annual cycle when these birds are inaccessible for sampling (Kouwenberg et al., 2013).

A growing body of literature has used CORTf as a source of important ecophysiological information (Romero and Fairhurst, 2016). Several studies have reported a relationship between CORTf and different physiological parameters such as parental efficiency (Fairhurst et al., 2012), male ornamentation (Bortolotti et al., 2009), carotenoid expression (Fairhurst et al., 2014), life history events (Bortolotti et al., 2008; Crossin et al., 2013), and environmental and seasonal factors (Fairhurst et al., 2012; Kouwenberg et al., 2016; Legagneux et al., 2013; López-Jiménez et al., 2016) among others. These approaches have used CORTf as a 'stress hormone' indicative of stressful or challenging environments. More recently, new evidence suggests that CORTf can be used as a metric to study the role of carry-over effects (Harms et al., 2015; Pérez et al., 2016; Saino et al., 2012). Harrison et al. (2011) defined 'carry-over effects' as events or processes that occur in one season and influence an individual's performance in a subsequent period. In this context, CORTf may represent a useful biomarker for conservation (Koren et al., 2012). But, despite this growing interest, the use of CORTf as a monitoring tool in wild avian populations is still considered work in progress (Fairhurst et al., 2013; Romero and Fairhurst, 2016).

Studies analyzing CORTf in certain bird species, such as raptors, are rare (Carrete et al., 2013; López-Jiménez et al., 2016; Strong et al., 2015). Raptors are sentinel species of environmental quality because of their position in food webs, their relatively large habitats, and because they are particularly sensitive to ecological changes; thus they are considered an important source of information on ecosystem health (Kovács et al., 2008). At present, several raptor species

around the world are considered endangered as a consequence of anthropogenic disturbances. Wildlife rehabilitation centers represent one of the most widespread conservation means by which injured birds are treated and then released back into the wild (Monadjem et al., 2014). More than the half of the raptors admitted to these centers have traumatic injuries (Molina-López et al., 2011), and despite all efforts of staff, the outcome for nearly 53% of them is euthanasia or natural death (Molina-López et al., 2013). Thus, assessing prognostic indicators is an important goal for rehabilitation centers. Molina-López et al. (2015) suggested basic clinical and hematological parameters as critical for these medical prognostics. In this line, the integrated nature of CORT incorporated in feathers may provide relevant complementary information, giving a retrospective view of the adrenocortical activity of the admitted birds.

The current study attempted to explore CORTf levels in wild Eurasian Sparrowhawks (*Accipiter nisus*) at a rehabilitation center. The main goal of this research was to assess whether CORTf content (deposited during the last molting period in the wild prior to admission to the rehabilitation center) was related to subsequent mortality and health status, reflected by BC. In rehabilitation centers, BC is commonly used as an indicator of individual health status. In fact, it is considered an indicator of sickness and weakness, as well as an indirect means to evaluate endocrine function (Dickens and Romero, 2013).

The hypothesis tested here were:

1. Whether individuals with suboptimal BC showed higher CORTf content than individuals with optimal BC.

2. Whether CORTf concentrations were linked to the mortality rate.

In order to study carry-over effects, it is important to control for other potential sources of variation in the activity of the HPA axis (Busch and Hayward, 2009; Strong et al., 2015). Thus, the present study also focused on the evaluation of possible within- and between-individual differences in CORTf. Therefore, we also predicted that:

3. Different type of feathers would differ in their CORTf concentrations.

4. CORTf levels would differ between juvenile and adult Eurasian Sparrowhawks because juvenile raptors are known to display increased dispersal behaviors in comparison to adults (Newton, 1979).

5. There would be gender-specific influences on CORTf, as the Eurasian Sparrowhawk is a species characterized by a marked female-biased sexual dimorphism (Moss, 1979).

3. Material and Methods

3.1 Source of material

Feathers were sampled from 34 Eurasian Sparrowhawks admitted to Torreferrussa Wildlife Rehabilitation Center (Catalonia, Spain, 3°19'–0°9'E, 42°51'–40°31'N) during the non-molting period, from November to May of 2 consecutive years (2013/2014 and 2014/2015).

Feather samples were taken once the individual had been stabilized after its arrival at the center. Samples were obtained from the interscapular region as this minimized the impact on the bird's fitness in the event of release. Flight feathers were also collected from individuals (*n* = 7) that were euthanized for medical reasons on arrival. All feathers were stored in individually labeled paper envelopes and kept at room temperature until analysis.

3.2 Data collection

Sex, age and BC were recorded at time of admission to the center. Plumage was used to age (n_{adults}=15, $n_{subadults}$ =13, $n_{juvenile}$=3, $n_{unknown}$=3) and sex ($n_{females}$=26, n_{males}=8) individuals. The sex distribution per age class was the following: $n_{female\text{-}adult}$ =12, $n_{female\text{-}subadult}$=10, $n_{female\text{-}juvenile}$=2, $n_{female\text{-}unknown}$=2, $n_{maleadult}$=3, $n_{male\text{-}subadult}$=3, $n_{male\text{-}juvenile}$=1, $n_{male\text{-}unknown}$=1. Of the total 34 Sparrowhawks, 29 (85.3%) were admitted for trauma injuries and five (14.7%) for unknown causes of injury. Within the trauma category, 20.7% of cases were the result of gunshot, 13.8% collisions, and the rest non-determined trauma injuries.

Pectoral muscle volume was used as the measure of BC. The pectoral muscles are the main muscles involved in flight and the largest component of non-fat body mass in birds (Cooper et al., 2015), accounting for approximately 10–25% of total body mass (Hartman, 1961). In birds with low levels of fat stores (threshold adiposity), protein stores may be metabolized (reducing pectoral muscle volume), indicating a dramatic health status of the animal (Jenni et al., 2000). BC was scored by palpating the pectoral muscles to determine the ratio of musculature mass to sternum, following the procedure described by Harrison and Ritchie (1994). Three different classes were distinguished: good BC (B0), poor BC (B1) and cachexia (B2). B0 was characterized by solid rounded pectoral muscles with a slight dip on either side of the sternum; B1 by a prominent sternum, muscle atrophy and noticeable weight loss; and B2 by severe atrophy of

the pectoral muscles. BC was assessed for all individuals by a single veterinary clinician with ample experience in this technique. The outcome for each individual was categorized as 'recovered' (individuals released into the wild), or 'dead' (individuals dying of natural causes or euthanized for medical reasons). Outcomes for both years of the study were determined over the long non-molting period.

3.3 Evaluation of carry-over effects

BC scoring and monitoring of mortality were performed during the non-molting period. As Sparrowhawks were admitted to the center at different times of the non-molting period, the time gap between the molt and the evaluation of carry-over effects was taken into account.

Individuals were classified depending on their month of admittance, to determine whether the time gap could affect interpretation of their CORTf data. October was fixed as the last month of the molting period (Hardy et al., 2006; Gill, 2007). Three groups were then created according to the month of admittance, counting from October: group A (first–second month), group B (third–fourth month) and group C (from the fifth month) (Fig. 1).

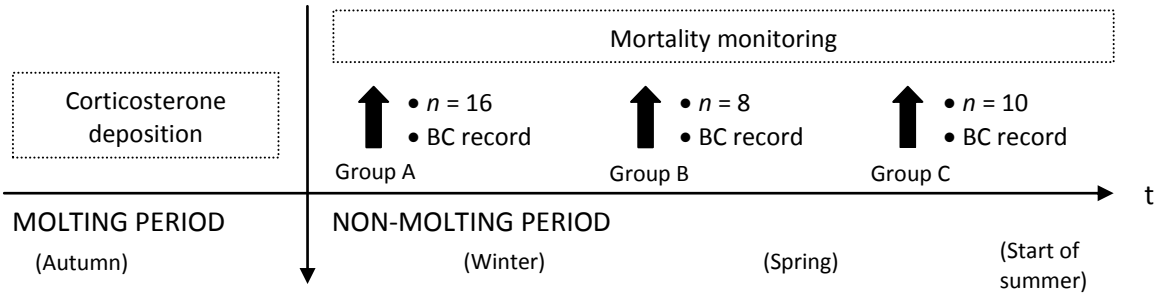

Fig. 1 Time frame of feather sampling and data recording [body condition (BC) and mortality] performed in 34 Eurasian sparrowhawks during the non-molting period. Feather sampling (indicated by an *arrow*) and BC (*BC record*) were carried out at time of individual's admittance to the rehabilitation center, whereas mortality was long-term recorded over the non-molting period. The time gap between molt and data recording was taken into account by grouping individuals into three different groups (*Group A-C*) depending on their month of admittance.

3.4 CORTf extraction procedure

In this study, CORT concentrations were normalized by length and feather mass was controlled to eliminate any potential confounding effects on CORT concentrations (Crossin et al. 2013; Fairhurst et al., 2013; Lattin et al., 2011; Patterson et al., 2015). Four to six interscapular feathers were analyzed per individual to obtain the same range of length (22.6 ± 2.6 cm) and a minimum mass of 30 mg was required. In the case of flight feathers, two primary feathers per individual were used and pooled together (length range, 28 ± 2.3 cm). The two measurements, units pictograms per millimeter and pictograms per milligram, respectively, were highly correlated for the two types of feathers (Pearson correlation test; interscapular feathers, $r=0.80$, $P<0.01$; primary flight feathers, $r=0.98$, $P<0.01$).

In order to extract CORTf, for both types of feather a modified protocol of that described by Bortolotti et al. (2008) was followed. The calamus was removed as it has a different growth rate than the rest of the feather (Bortolotti, 2010). Then, selected feathers of each individual were minced together into pieces of $<3mm^2$. Ten milliliters of methanol (methanol reagent grade 99.9%; Scharlab, Sentmenat, Spain) was added and samples were placed in a vortex (Vortex Mixer S0200-230 V-EU; Labnet International, NJ) for 30 min at room temperature, followed by incubation at 32 °C for 18 h in a shaking water bath (G24 Environmental Incubator Shaker; New Brunswick Scientific, Edison, NJ) for steroid extraction. The methanol was then separated from feather material using vacuum filtration. The resulting supernatant was placed in an oven (Heraeus model T6; Kendro Laboratory Products, Langenselbold, Germany) at 37 °C. Once the methanol was completely evaporated, the dried extracts were reconstituted in 250 µL EIA buffer solution, provided by the EIA assay kit (Corticosterone ELISA kit; Neogen®, Ayr, UK) and frozen at -20 °C until analysis.

3.5 CORT quantification and validation test

CORT analysis was performed using a competitive EIA kit (Corticosterone ELISA kits; Neogen®) and carried out following the manufacturer's instructions. Biochemical assays were performed to validate the immunoassay (Sheriff et al., 2011; Terwissen et al., 2013). We followed the essential criteria for the immunological validation: precision, accuracy, specificity and sensitivity (Sink et al., 2008). Extracts from 25 Eurasian Sparrowhawk were pooled. All samples were measured in two assays with a mean intra-assay coefficient of variation (CV) of 4.41% and an inter-assay CV of 14.22%, indicating good repeatability and precision of the test. The sensitivity was 0.1135 pg mm^{-1} feather. All samples were above the detection limit. Feather

samples showed parallel displacement with the standard curve (standard curve, y = 0.181x - 0.234, R^2=0.98; pooled feather samples, y = 0.237x - 1.382, R^2=0.99). Regarding the dilution test, the efficiency of CORT extraction was 91.28 ± 8.72% with a R^2 of 95%. The average recovery percentage from the spike recovery test was 102.4 ± 17.9%. These results indicated the lack of other components interfering with CORT estimation.

3.6 Statistical analysis

Data were analyzed using R software (R-project, version 3.0.1; R Development Core Team, University of Auckland, New Zealand). All the values are presented as mean ± SD. A P-value<0.05 was established as a criterion for significance. Prior to analyses, a Shapiro–Wilk test was performed to check normality. Because of the non-normality of data, natural logarithm transformations (log_{10}) were applied to achieve a normal distribution and normality was evaluated again. The influence of type of feather on CORTf levels was explored using a two-tailed paired data t-test. A Pearson correlation test was done to evaluate correlation between the two feather types. An ANOVA with type III sum of squares was carried out to detect CORTf differences with respect to sex, age and BC. Year (2013–2014/2014–2015) and time gap between molt and data recording were used as random effects. As the variance of the model was mainly explained by the error (variance=0.03; SD=0.16) rather than by the study year (variance=0.002; SD=0.05) or the time gap (variance=0.002; SD=0.04), the model was refitted by including year and time gap as fixed factors to detect potential influences in the CORTf pattern observed. The ANOVA model was reduced to its simplest form by eliminating any independent variable or interaction that failed to explain significant variations in the dependent variable. This was conducted in a stepwise manner where the least significant terms were sequentially removed (Crawley, 1993). When significant, ANOVA was followed by a post hoc Tukey test to evaluate differences between groups. A generalized linear model was built to test whether sex, age, year, time gap, BC and steroid levels (including their interactions) influenced the probability of mortality in the subsequent non-molting period. The model was reduced to its simplest form, as above. Due to the binary nature of the response variable, mortality (dead=0, alive=1), a binomial error distribution with a logit link function was applied.

4. Results

The content of CORT differed statistically between interscapular and primary flight feathers (paired t-test, t=-5.8012; P<0.01; Fig. 2) with levels ranging from 3.48 ± 1.21 to 10.46 ± 3.73 (pg

CORT mm^{-1} feather) in interscapular and primary flight feathers, respectively. No correlation was found between the two feather types (Pearson correlation test, r=0.29; P<0.05).

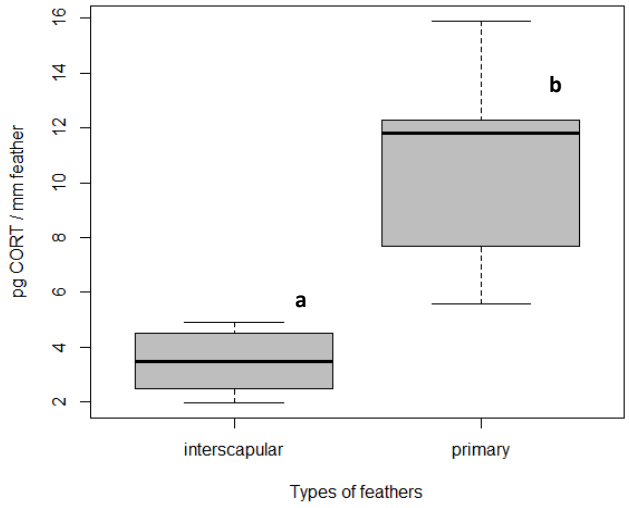

Fig. 2 *Box plot* displaying differences in feather corticosterone (CORTf) concentrations (pg CORT mm^{-1} feather) from interscapular and primary feathers of seven Eurasian sparrowhawk individuals. Two pools, one mixing two flight feathers and the other mixing four to six interscapular feathers were prepared from each individual analyzed.
Different lowercase letter show statistical differences (P<0.01).

CORTf was significantly related to BC ($F_{2.30}$=3.66; P=0.04), whereas there was no link with sex ($F_{1.30}$=3.30; P=0.08), age ($F_{2.30}$=2.39; P=0.12), year ($F_{1.30}$=2.07, P=0.16) or between different time gaps ($F_{2.30}$=1.02, P=0.38). The ANOVA analysis was re-run without the non-significant terms, and the statistical relationship between CORTf concentrations and BC strengthened ($F_{2.33}$=5.77, P<0.01). The following post hoc Tukey multiple comparisons of means revealed, with a 95% family-wise confidence level, that CORTf levels of B1 individuals (5.32 ± 2.12 pg CORT mm^{-1} feather) were significantly higher than those of B0 individuals (3.18 ± 1.01 pg CORT mm^{-1} feather) and B2 individuals (3.16 ± 1.48 pg CORT CORT mm^{-1} feather). Conversely, the values of B0 and B2 individuals did not differ statistically (Fig. 3).

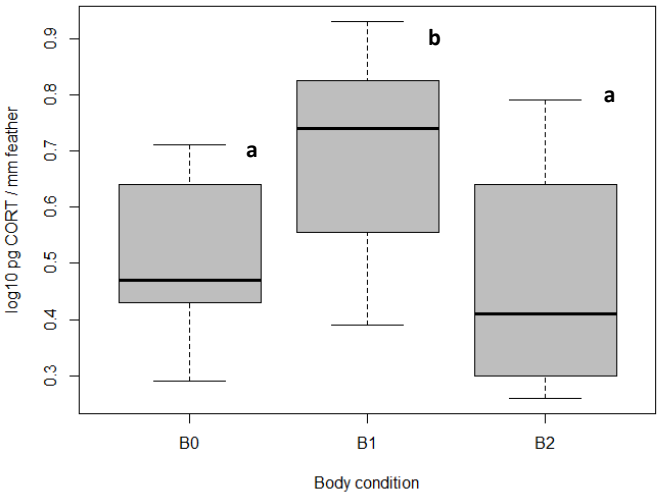

Fig. 3 *Box plot* displaying differences in feather CORT concentrations (pg CORT mm^{-1} feather) between individuals with different BC [good BC *n* = 13 (*BO*); poor BC *n* = 11 (*B1*); cachectic individuals *n* = 10 (*B2*)].
Different lowercase letter show statistical differences (P<0.05).
For abbreviations see Figs. 1 and 2.

Regarding mortality, of the 34 individuals analyzed, 19 (55.88%) died and 15 (44.12%) were alive at the time of analysis. There were non-significant relationships between mortality and sex ($F_{1.30}$=2.99, P=0.08), age ($F_{2.30}$=0.67, P=0.71), year ($F_{1.30}$=2.72, P=0.10), time gap ($F_{2.30}$=0.12, P=0.94), and BC ($F_{2.30}$=1.64, P=0.44); thus, these factors were removed from the model. Only CORTf ($F_{1.33}$=4.21, P=0.04) was positively associated with a high probability of death (Fig. 4a). The estimated survival function derived from this model revealed a positive curvilinear relationship between CORTf and the probability of mortality (Fig. 4b).

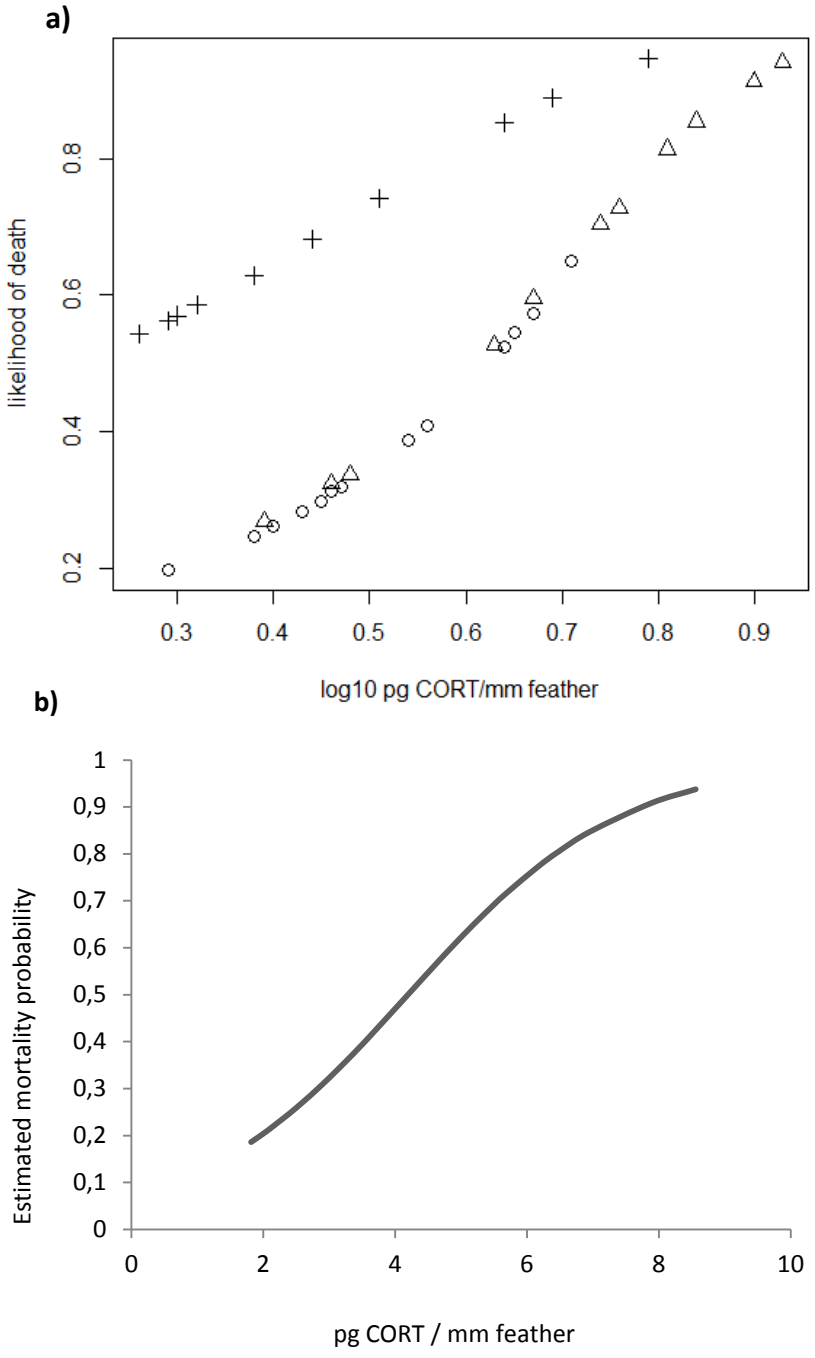

Fig. 4
a) Relationship between feather CORT levels (pg CORT mm^{-1} feather) and likelihood of mortality of individuals sampled during the subsequent non-molting period. Data are from B0 (*n* = 13), B1 (*n* = 11), B2 (*n* = 10) individuals.
b) Estimated mortality for the observed values of feather CORT in Eurasian Sparrowhawk for birds sampled after their annual molting period (November - May of 2 consecutive years, i.e. 2013 - 2015). The survival function was: $y = \exp[-2.60118 + (0.62006 \times Xi)]/1 + \exp[-2.60118 + (0.62006 \times Xi)]$.
For abbreviations see Figs. 2 and 3.

5. Discussion

In the present study we examined the influence of potential sources of variation of CORTf concentrations within and between Eurasian Sparrowhawk individuals. We also studied the relationship between CORT deposited in feathers during the molting period, and the carry-over effects (BC and mortality) recorded in Sparrowhawks in a subsequent period.

5.1 Differences in CORTf content within individuals

Most of the studies analyzing CORTf concentrations have used primary flight (Bortolotti et al., 2008; Bourgeon et al., 2014; Strong et al., 2015; Will et al., 2014) or tail feathers (Harms et al., 2015; Lendvai et al., 2013; López-Jiménez et al., 2016). Few data are available on steroid content in body covert feathers in birds in general. For instance, values exist for Cory's Shearwater (*Calonectris diomedea*) (Fairhurst et al., 2012), Rhinoceros Auklet (*Cerorhinca monocerata*) (Kouwenberg et al., 2016), Swallows (*Tachycineta bicolor*) (Fairhurst et al., 2013), and Red-winged Blackbirds (*Agelaius phoeniceus*) (Kennedy et al., 2013). Only a single study published data on raptors, assessing CORTf in Egyptian Vultures (*Neophron percnopterus*) (Carrete et al., 2013). Our study provides the first published data on CORT from body covert feathers in Eurasian Sparrowhawks and serves to emphasize the need to analyze the same type of feather to avoid within-individual differences.

Concretely, in this study, primary flight feathers displayed significantly higher CORTf levels compared to interscapular feathers. This could be a consequence of different feather growth rates. To date, although very valuable, knowledge of the molting process and its synchrony between individuals is still far from complete for almost all bird species. Work of Hardy et al. (2006) and Gill (2007) indicated that body feathers molt after the breeding season, preceding winter stressors or migration, and are some of the first feathers to molt. Contrary to this, it has been shown that the molt of flight feathers can extend over 2 or more years and individuals can arrest and delay their molt when confronted with high energetic demands (Zuberogoitia et al., 2009; Rohwer et al., 2009). In Spain, both migratory and sedentary populations of Sparrowhawks can be found. Zuberogoitia et al. (2009) described a different timing of molt among them, observing that migratory Sparrowhawks can show delayed molt compared to sedentary ones, and do not even complete their molt in a single year because of the trade-off between molting versus migration. Due to this asymmetrical and incomplete molt between years and populations, variations in ecological and individual conditions at the time of molt of flight feathers may lead to differences in CORTf and misinterpretations in its analysis.

Using body feathers (in our case interscapular) may be advantageous. Their growth is considered synchronous during the first stages of the molting period and different timing of molt between populations has been described for flight and tail feathers, but not for body feathers (Zuberogoitia et al., 2009). Thus, interscapular feathers may reflect steroid hormone levels over a more fixed and defined period of time, even between different Sparrowhawk populations. More importantly, as interscapular feathers have been found to molt before migration, the higher energetic cost demanded by migration (in the case of migratory individuals) would not affect CORTf levels. Another advantage is that their removal is less invasive and does not affect an individual's fitness compared to the removal of flight feathers (Debén et al., 2012).

5.2 Differences in CORTf content between individuals

Attempts to relate differences in steroid concentrations with physiological data did not provide the predicted results. The data presented here revealed that the hormone concentration was statistically indistinguishable between different ages and sexes of birds. In the case of sex, similar results were reported previously in House Sparrows (*Passer domesticus*) (Koren et al., 2012; Romero et al., 2006), in intensively raised broilers (*Gallus gallus domesticus*) (Carbajal et al., 2014) and in some raptor species (Strong et al., 2015). However, other studies reported an age-specific pattern in CORT concentrations (Angelier et al., 2007; Heidinger et al., 2006), in line with the theory that maximum levels of GCs released in response to stress decrease significantly with age. A possible explanation for the lack of consistent age effects may be sample size limitations. As a consequence, CORTf may have diverged most effectively under different stress and demand situations (see the following section ''CORTf content associated with carry-over effects'').

5.3 CORTf content associated with carry-over effects

The importance of events occurring during the molt on subsequent periods has been noted in multiple studies, and feathers have been suggested as a potential tool for the monitoring of carry-over effects across seasons (see Harms et al., 2015). While our study only showed CORTf differences in the case of birds in poor BC compared to optimal and cachectic individuals, other data presented here revealed a relationship between integrated hormonal levels and subsequent mortality recorded at the rehabilitation center. Results should be carefully interpreted due to the time gap between the molt and data recording.

5.3.1. CORTf content associated with BC

A hypothesis of the present study was a substantial difference in CORTf between birds in good condition and birds in bad condition (either classified as poor or cachexia). Although we found a negative relationship between CORTf levels of birds in good condition and those in poor condition, levels in birds presenting cachexia did not differ from those in good condition.

Differences in CORTf concentrations between individuals with optimal and poor BC have been previously described in other studies using different condition metrics (Harms et al., 2010, 2015; Strong et al., 2015). In our study, pectoral muscle mass was used to score BC in agreement with previous research by Strong et al. (2015), who suggested pectoral muscle mass as a resistant BC index over time and found a correlation between it and CORTf. Also, a long-term study of Harms et al. (2015) correlated CORT deposited in feathers during late summer with BC recorded the subsequent winter. Interestingly, a negative relationship was described despite the time gap between CORT deposition into feathers (during molt) and the BC record (scored in a subsequent period) (see section below for more discussion on this issue).

In our research, the utility of CORTf to predict BC is limited by the fact that cachectic individuals displayed significantly lower CORTf concentrations than those in poor condition, and exhibited similar levels to those in optimal condition. Thus, we could not use CORTf to disentangle BC status. Considering that the majority of birds arrived at the rehabilitation center with trauma injuries, the ability of these birds to move and hunt for food would have been compromised, which in turn could have affected each individual's BC. Thus, the time span between the point of injury and arrival at the rehabilitation center could have affected each individual bird's BC more than its CORT levels months before the injury. However, this hypothesis cannot explain the increased CORTf levels noted in birds in poor condition.

Alternatively, despite the general assumption that stress leads to increases in GCs, the fact that the magnitude and direction of change of GC levels in response to stress are still not clear (Bonier et al., 2009; Madliger and Love, 2014) needs to be taken into consideration. Indeed, Dickens and Romero (2013) noted that chronically stressed animals can show higher, unchanged or lower CORT levels. Other authors, such as Mormède et al. (2007) and Schwacke et al. (2014) suggested that low GC levels were a consequence of a decrease in adrenal capacity. Following this line of argument, cachectic Sparrowhawks could have been exposed to high long-term stressful situations that ultimately caused disruption of the endocrine system.

Overall, although the latter hypothesis remains to be tested, findings reported in the present study are of particular interest as they suggest caution in assuming that only high CORT levels indicate low-quality condition. Also, they emphasize the need for future research focused on the stress response of cachectic individuals and those in poor BC.

5.3.2. CORTf content associated with mortality

We observed a significant relationship between CORTf concentration and the probability of death. Individuals with high CORTf concentrations showed the highest probability of mortality. Results published by Koren et al. (2012) and more recently by Harms et al. (2015) indicate that CORTf can be inversely related to survival in House Sparrow and in Eiders, respectively. Our research highlights the importance of events or processes occurring during the molt for subsequent survival, regardless of the time period considered. This latter finding suggests that increases in energetic challenges experienced by individuals in the wild may ultimately have consequences on the survival rate recorded at rehabilitation centers during the subsequent non-molt period.

It is worth noting that cachectic individuals seemed to have a higher probability of death than individuals in good BC and those in poor BC with similar CORTf concentrations (see Fig. 4a). Some of the birds which arrived at the center would have been traumatized because of previous chronic stress and shown high (B1) or relatively low CORTf levels (B2). The worsening status of cachectic individuals probably affected their capacity to overcome an adverse situation, thus they would have had a higher probability of mortality than optimal individuals. On the other hand, the mortality of optimal individuals could have been related to stress experienced during detention at the rehabilitation center, or even to the severity of their injury, rather than to the energetically demanding processes which occurred prior to the molting season. Brown et al. (2005) had already suggested that individuals with intermediate CORTf concentrations may have the best chance of survival, which may support our hypothesis. Further research with an increased sample size is required to improve our understanding of the relationship between CORTf and mortality, focused on disentangling the different responses between BC statuses.

5.4 Future considerations

It is important to note the existence of a variable time gap between the period of feather growth (during which CORT was deposited) and the time of data scoring (when BC and mortality were recorded). Thus, any underlying relationship between CORTf content and BC, or

mortality, could be confounded by this delay. As it was expected that this time gap could affect the relationship between CORTf and the associated carry-over effects, our analyses took into account the interval of time between the molt and admission to the center. However, we found no evidence of any such effect. Despite increasing interest in evaluating carry-over effects, very few studies have reported relationships between CORTf levels and an animal's subsequent fitness (Crossin et al., 2013; Harms et al., 2015; Koren et al., 2012; Strong et al., 2015). To the best of our knowledge, no studies have evaluated the influence of the temporal gap between the molt and recorded fitness parameters. Future work should focus on monitoring this temporal gap with detailed observation of the energetic and stress demands experienced by an individual over the molting period and up to the subsequent non-molting season.

6. Conclusions

The present study emphasizes the need to analyze the same type of feather to avoid within-individual differences, and indicates that interscapular feathers are a suitable type of feather for this. With the aim of a better interpretation of results, further research is needed on feather growth rate and its synchrony between individuals. This study also shows high CORTf levels in Sparrowhawks in poor BC. However, the similar CORTf levels between optimal and cachectic individuals suggests a need for caution when assuming that only high CORTf levels indicate that individuals are in poor condition, and limits the utility of this technique for the prediction of BC. Finally, our research provides evidence that CORT deposited into feathers during the molting period is associated with survival outcomes recorded at admission of Sparrowhawks to the rehabilitation center. Combining information on feathers and individual status recorded at the time of admission may allow the identification of individuals with a lower chance of survival, and thereby help considerably in establishing animal management priorities in wildlife rehabilitation centers.

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CHAPTER II

Corticosterone levels in feathers are predictive of reproductive success in Northern bald ibis, Geronticus eremita

Photo ®: Proyecto Eremita

Corticosterone levels in feathers are predictive of reproductive success in Northern bald ibis*, Geronticus eremita*

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1. Abstract

Measuring corticosterone concentrations in feathers (FCC) has been increasingly used as an integrated index of bird's response to stressors, offering valuable insights into subsequent carry-over effects. However, it is still unclear to what extent corticosterone levels deposited in feathers during molt relate to individual physiology in high-energetic demanding situations. In addition, the stability and repeatability of FCC over the same feather generation and across successive periods are still unresolved issues. We examined these questions by determining FCC in twenty-two captive Northern bald ibis, *Geronticus eremita.* We sampled body feathers on three occasions during two consecutive years (2015-2016) in order to explore within-individual stability of FCC over the same non-molting period and repeatability of FCC among consecutives non-molting periods. Then, we explored whether FCC were (a) associated to individual health status, (b) correlated with the previous reproductive success and (c) had consequences in the future reproductive success. Results showed high stability of FCC among feathers grown during the same molting period, regardless of time of sampling (simultaneous or at different times during the following non-molting period), while FCC were not repeatable between successive non-molting periods. Results also showed that FCC were negatively associated to subsequent reproductive success, while no association was found with previous reproductive success. Overall, our study supports the use of feathers as a stable measure of corticosterone over the same feather replacement generation and emphasizes the potential usefulness of FCC as a biomarker of subsequent reproductive success.

2. Introduction

In response to predictable or unpredictable challenging situations, vertebrates activate the hypothalamic-pituitary-adrenal (HPA) axis, which leads to an increase of glucocorticoid secretion in blood circulation. This stress response enables the animal to redirect its normal activity to an "emergency" state (Wingfield et al., 1998) and prioritize its resources towards self-maintenance (Jenni et al., 2000). In this manner, the probabilities to overcome the adverse situation are increased. However, when the stressor persists or occurs at frequent intervals, the functioning of the HPA axis is altered resulting in abnormal synthesis or deregulation of glucocorticoids (Romero, 2004), which henceforth implies detrimental consequences for the animal's health, survival and reproduction (Romero et al., 2009; Sapolsky et al., 2000; Wingfield and Sapolsky, 2003).

Different methodologies have been developed to quantify the stress response. Blood has been the preferred matrix to measure corticosterone (CORT; the main glucocorticoid in birds) (Love et al., 2003a; Müller et al., 2007), and more recently faecal samples have been used as a less-invasive method (Möstl et al., 2005). However, these matrixes only reflect concentrations of CORT concerning a rather limited period of time (Legagneux et al., 2011; Touma and Palme, 2005). Given the usefulness of a more integrated picture of physiology over time, feathers have been introduced as an alternative matrix (Bortolotti et al., 2008; Romero and Fairhurst, 2016). Feathers are thought to incorporate the amplitude and duration of CORT secretion during the whole period of feather growth (Aharon-Rotman et al., 2015; Bortolotti et al., 2009; Jenni-Eiermann et al., 2015); therefore they provide a relevant measure of the retrospective long-term HPA axis activity during this period of time. An important feature of this matrix is the minimally or non-invasive nature of their collection. In addition, small daily variations in baseline CORT levels have been proven to be insignificant to the global feather CORT concentrations (FCC) (Bortolotti et al., 2008; Fairhurst et al., 2013).

Since the first apparition of FCC in the literature (Bortolotti et al., 2008), a growing number of studies have applied this matrix to examine its relationship with reproductive parameters (Crossin et al., 2013; Kouwenberg et al., 2013), sexual selection (Fairhurst et al., 2014), nutritional stress (Crossin et al., 2013; Will et al., 2014) and environmental factors (Fairhurst et al., 2011; Nordstad et al., 2012). More recently, researchers have explored the use of FCC as a biomarker of carry-over effects relating FCC to subsequent migratory (Harms et al., 2015), survival (Koren et al., 2012; Monclús et al., 2017) and breeding (Harms et al., 2015; Will et al., 2014) performances. However, the reported relationships are complex and it remains unclear

whether FCC is able to reflect and predict individual physiology in front of high-energetic demanding situations (Fairhurst et al., 2017; Harris et al., 2017).

As a relatively new technique, feathers still present some methodological issues that need to be addressed for a proper interpretation of data (Romero and Fairhurst, 2016). For instance, repeatability and stability of CORT in feathers is of special concern. It is vital that FCC are similar among feathers grown during the same molting period and that this measure is stable in feathers over time. Researchers usually analyze CORT from molted feathers, but it results complex to know the exact moment when feathers grew and, consequently, the time gap between feather growth and feather sampling. Given that physical degradation of feather material over time may affect FCC (Romero and Fairhurst, 2016), studying stability of FCC over the same feather generation (referred here as "the same non-molting period") is crucial for data interpretation. Another issue of special concern is the repeatability of FCC across successive periods. This is a relatively poor studied concept and mixed results have been report. For example, great FCC variation was observed between successive years in greater snow geese (*Chen caerulescens atlantica*) and in common eiders (*Somateria millissima*) in relation to climate conditions (Legagneux et al., 2013), while high persistence in FCC was reported in house sparrow (*Passer domesticus*) suggesting that inherent quality of individuals may obscure the effects of extrinsic factors (Aharon-Rotman et al., 2015).

In an effort to address the above-mentioned questions, different objectives were carried out. First, the present study aimed to evaluate within-individual stability of FCC over the same non-molting period. We compared FCC among feathers sampled on the same day and among feathers sampled on different months spanning the same non-molting period. If FCC is indeed a robust and reliable measure, we should find equal hormonal levels among the different feather pools collected. Second, we investigated repeatability of FCC between two consecutive non-molting periods and explored whether FCC relate to health and reproductive success. Specifically, we evaluated whether FCC was a) associated to individual health status, b) correlated to previous reproductive success and c) had consequences in the future reproductive success. We used a captive population of Northern bald ibis *Geronticus eremita* (hereafter called NBI) as the species model. This long-lived species, classified as Critically Endangered (BirdLife International, 2016), has suffered a big decline in numbers over the past decades due to different anthropogenic disturbances (BirdLife International, 2016; Boehm and Bowden, 2010; Serra et al., 2015). So far, several captive breeding and reintroduction programs have been set around Europe (Waldrapp team in Austria, Germany, Italy and

Proyecto Eremita in Southern Spain). However, high euthanasia rate has been described in captive NBI due to chronic ulcerative dermatitis that seemed to be related to feather picking and self-mutilation compulsive disorder (Quevedo, 2009). This study particularly explored FCC and reproductive success in NBI presenting this disease.

3. Material and Methods

3.1 Animals, housing conditions, diet and management

A mixed-sex flock of 22 captive NBI (mean age 18.5 years old; range 4 to 30 years old, 12 males, 10 females) was used in this study. The group was held at Zoobotánico Jerez (Cádiz, Spain) and was part of the *Proyecto Eremita* set in Southern Spain. All animals were born in captivity, kept under the same conditions in an outdoor flight aviary (11m width x 15m length x 8m height), with natural vegetation, water *ad libitum*, subjected to natural photoperiod and temperature, and fed with the same diet (mixture of minced heart of beef, chicken, carrots, pellets for insectivorous birds, multivitamins and carotenes).

Birds of this species are monomorphic and gregarious with a clear social hierarchy (Cramp, 1998). They generally reach sexual maturity around the age of 3 years (Cramp, 1998; Del Hoyo et al., 1992). Breeding activity starts in March (Sorato and Kotrschal, 2006) and eggs are usually laid between March and May, although some flexibility in the timing has been described due to climate conditions (Del Hoyo et al., 1992). Both parents participate in building the nest, incubating the eggs and raising the chicks. Average clutch size is 3 eggs and hatching is asynchronous within intervals from 1 to 3 days (Sorato and Kotrschal, 2006). Nestlings are fully fledged after 42 to 50 days (own observations). Extramarital copulatory behavior is registered during the breeding season and breeding pairs can change in the following year (Cramp, 1998). Birds from the captive NBI group used in this study were free to pair and breed. In 2016, as part of the reintroduction program (*Proyecto Eremita*), eggs were removed from their parents and were artificially incubated and hand rearing by foster parents in order to increase hatching ratio and chick survival.

3.2 Study design

Three samplings were carried out encompassing two consecutive non-molting periods (Fig. 1). Reflecting molt 1, we sampled 11 individuals in October 2014 (Sampling A) and 22 individuals in March 2015 (Sampling B). Reflecting molt 2, we sampled 22 individuals in November 2015 (Sampling C). In this latter case, two pools of feathers from the same anatomical region per

individual were simultaneously collected in 18 NBI. Each sampling procedure achieved different objectives. Samplings A and B were used to evaluate within-individual FCC stability between feathers sampled at different times during the same non-molting period (*n* = 11). Samplings B and C were used to assess within-individual FCC variation between two consecutive non-molting periods and to explore the relationship with health and breeding success (*n* = 22). Finally, Sampling C was used to test within-individual FCC stability between pools of feathers sampled at the same time (*n* = 18).

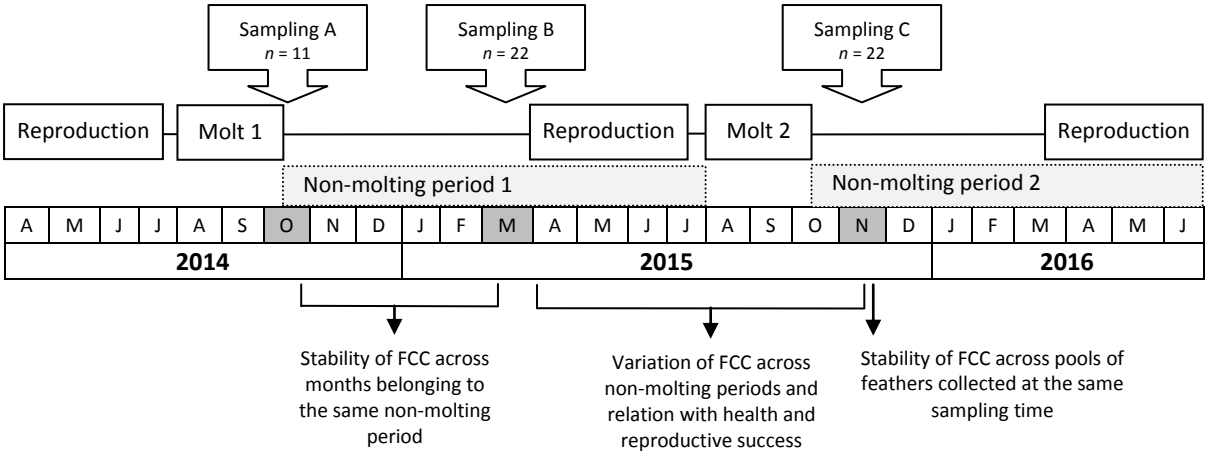

Fig. 1 Time frame of feather sampling and data recording. Northern bald ibis were sampled in three occasions: Sampling A (October 2014); Sampling B (March 2015); and Sampling C (November 2015) encompassing two successive non-molting periods. Reproductive data was recorded during three consecutive years. Specific objectives are described per each sampling procedure in relation to feather corticosterone concentrations (FCC).

3.3 Feather and data collection

A total of 77 samples were analyzed (Supplementary Information Table S1). In all cases, feathers were pulled during routine medical examinations and stored in paper envelopes at room temperature. We sampled interscapular feathers as their collection was judged to have less impact on flight dynamics in birds. These body feathers are replaced each year during the molting period, from August to October (Gill, 2007; Hardy et al., 2006). Medical data and reproductive success were recorded during all the study. Of the 22 NBI, 2 birds (9.1%) presented ulcerative chronic dermatitis and 5 (22.7%) presented chronic disabilities such as flight disabilities (*n* = 2), partial beak amputation (*n* = 2) and microphtalmia (*n* = 1).

3.4 Feather steroid extraction

Length (mm) was the selected unit to homogenize samples, albeit feather mass was controlled in order to eliminate any potential confounding effect on FCC, as previously recommended

(Crossin et al., 2013; Fairhurst et al., 2013; Lattin et al., 2011; Patterson et al., 2015). Three to five interscapular feathers were analyzed per individual in order to obtain the same longitude range (21.9 cm ± 1.5 cm) and a minimum mass of 50 mg was required. In all cases, the two measures (pg mm^{-1} and pg mg^{-1}) were highly correlated (Pearson correlation test; r=0.84, P<0.01).

For FCC extraction, a modified protocol from that described by Bortolotti et al. (2008) was followed with modifications based on Carbajal et al. (2014) and Monclús et al. (2017). Briefly, from each sample, the calamus was removed since it has a different growth rate than the rest of the feather (Bortolotti, 2010). Then, the selected feathers of each individual were minced together with scissors into pieces of < 3 mm^2. Methanol (10 mL; Scharlab, S.L., Sentmenat, Spain) was added and samples were vortex (Vortex Mixer S0200-230 V-EU; Labnet International Inc, NJ, USA) for 30 min at room temperature, and then shaken (G24 Environmental Incubator Shaker; New Brunswick Scientific Co. Inc., Edison, NJ, USA) for 18 h at 32 ºC, for steroid extraction. The methanol was then separated from the feather material using vacuum filtration. The obtained methanol extract was dried in an oven (Kendro Laboratory Products, Langenselbold, Germany) at 37 ºC. Once the methanol was completely evaporated, extract residues were reconstituted in 250 µL buffer solution, provided by the enzyme immunoassay kit (Corticosterone ELISA kit; Neogen® Corporation, Ayr, UK) and shaken for 30 s. Afterwards, samples were immediately stored at -20 ºC until analyses.

3.5 Steroid analysis and validation test

FCC were determined using competitive CORT enzyme immunoassay kits (Neogen® Corporation, Ayr, UK). Biochemical assays were performed to validate the immunoassay following the essential criteria of precision, accuracy, specificity and sensitivity (Mccallister and Smith, 1996; Sink at al., 2008). Extracts from 20 NBI were pooled and used for validation. A mean intra-assay coefficient of variation (CV) of 5.74% and an inter-assay CV of 12.53% were obtained. All samples were above the detection limit (0.556 pg mm^{-1} feather). Pooled samples showed parallel displacement with the standard curve (standard curve: y = 0.092x-0.477, R^2=99%; pool feather samples: y = 0.060x+0.305, R^2=92%). The linearity of dilution showed a R^2 of 98% and a mean percentage error of -11.09 ± 11.29%. The average recovery percentage from spike-and-recovery test was 107.86 ± 13.77%. Cross-reactivity of the CORT antibody is as follows: 38% deoxycorticosterone; 19% 6-hydroxycorticosterone; 5.1% progesterone; 2.7% tetrahydrocorticosterone; 1.5% prednisolone; 1.1% cortisol. Steroids with cross-reactivity <0.9% are not presented.

3.6 Statistical analysis

All analyses were performed using R version 3.2.2 (R-project, R Development Core Team, University of Auckland, New Zealand). For all regression models, the standardized residues were assessed to meet normality and homogeneity of variance. When not, log transformations were applied. In all cases, a P-value<0.05 was established as a criterion for significance.

To test within-individual variations on FCC, we used mixed-effects models with restricted maximum-likelihood method (REML). First, we tested whether the two pools of feathers sampled at the same time from the same individual showed similar FCC (*n* = 18; Sampling C). Second, we tested whether individual FCC varied among months belonging to the same non-molting period (*n* = 22; Sampling A and B). In both cases, we conducted FCC (expressed as pg CORT mm^{-1} feather) as the dependent variable and individual identity as random effect to account for repeated samples on the same individuals. The intra-class correlation coefficient (ICC), expressed as $d^2/(d^2 + \sigma^2)$, was used as a measure of repeatability. It was calculated based on: the variance explained by the model (between-individual variance) (d); and the variance explained by the residual (within-individual variance) (σ) (Nakagawa and Schielzeth, 2010). The ICC ranges from 0 to 1 with values close to 1 meaning that most of the variance is explained by between-individual differences.

We then determined whether FCC were consistent between two consecutive non-molting periods (*n* = 22; Samplings B and C). We used mixed-effects model with REML, normal distribution and identity function. FCC was set as the dependent variable; meanwhile period (Sampling B/Sampling C), sex and age were placed as fixed factors. Individual identity was used as random effect to account for repeated samples. A second mixed-effects model was performed to test for differences in FCC as a function of health and previous reproductive success. Given that the goal was to explore the relationship with physiological parameters, the different periods (Sampling B/Sampling C) as well as individual identity were placed as random effects. Sex and age were placed as fixed factors. For the previous reproductive success, recorded at 2014 and 2015 (Fig. 1), different variables were recorded: 1) breeding success (three levels: successful paired-successful breeding, successful paired-failed breeding, and failed paired-failed breeding); 2) reproductive success (two levels: successful paired-successful breeding, failed paired or failed breeding); 3) fecundity (number of eggs); 4) fledging success (number of young that successfully fledged). We used different models to test each variable separately. Individuals with flight pathologies (*n* = 2) were eliminated, as these birds were not able to arrive at the upper nests. Regarding health, we tested separately in two models: 1)

general health (two levels: healthy vs. unhealthy with the latter including chronic disabilities and skin disease); 2) pathological alterations (three levels: healthy birds, birds with chronic disabilities, birds with skin diseases).

A generalized linear mixed model, with binomial distribution and logit link function, was performed to analyze the effect of FCC on subsequent reproductive success, recorded in 2015 and 2016 (Fig. 1). In the case of 2016, eggs were collected and incubated artificially, thus fecundity and fledging success variables were excluded from the analyses and we only distinguished reproductive success (two levels: successfully paired vs. failed paired). Reproductive success (yes/no) was placed as the dependent variable and the explanatory variables included FCC, age, sex and health (different models for general health and for pathological alterations, see above). Individual identity and periods (Sampling B/Sampling C) were placed as random effects.

In all cases, the initial model contained all the main effects and all the possible interactions. The models were evaluated and ranked using the Akaike's Information Criterion corrected for small sample size (AIC_c) (Burnham and Anderson, 2002). The four most supported models and the null model are reported in AIC_c tables (Table S2, S3 and S4). All models with AIC_c scores within 2 points of the lowest scoring model ($\Delta AIC_c<2$) were considered within the candidate model selection. We used package 'nlme' and 'lme4' (R Core Team 2015).

4. Results

4.1 Within-individual stability of FCC

The two pools of feathers collected in Sampling C showed similar FCC (estimate ± SE=-0.26 ± 0.36, t=-0.72, d.f.= 17, P=0.48; Table 1). The ICC of the model was 0.82 (with d^2=2.29 and σ^2=1.08) showing low within-individual variation. When comparing FCC between Samplings A and B, no statistical difference was found (estimate ± SE=-0.71 ± 0.94, t=-0.75, d.f.=10, P=0.46; Table 1). The ICC of the model was 0.77 (with d^2=2.12 and σ^2=1.16) showing low within-individual variation.

4.2 Variations of FCC across consecutive non-molting periods

4.2.1 Influence of physiological, pathological and reproductive data on FCC

The best-supported model explaining differences in FCC between non-molting periods included sex and period (Table S2). We found that FCC significantly decreased from Sampling B

to Sampling C (estimate ± SE= -1.49 ± 0.62, t=-2.44, d.f.=21, P=0.02; Fig. 2a,b). The mean (± SD) FCC for Sampling B was 8.14 (2.23) pg mm^{-1}, ranging from 4.75 to 13.52 pg mm^{-1}, and for Sampling C was 6.89 (2.46) pg mm^{-1}, ranging from 2.53 to 11.08 pg mm^{-1}. In addition, individuals showed variation in FCC between the two consecutive non-molting periods (Fig. 2a). Sex did not influence FCC (estimate ± SE= 1.10 ± 0.84, t=1.32, d.f.=20, P=0.21; Table 2).

Table 1. Descriptive statistics for feather corticosterone concentrations (FCC; pg CORT mm^{-1} feather) from different pools of interscapular feathers collected at the same sampling time (*n* = 18) and at different sampling time (*n* = 11) in Northern bald ibis.

		FCC (pg CORT mm^{-1} feather)		
		Min	Max	Mean ± SD
Same sampling time	Sampling C - Pool 1	2.61	10.97	7.42 ± 2.05
	Sampling C - Pool 2	3.61	12.36	7.99 ± 2.90
Different sampling times*	Sampling A	6.81	13.11	9.76 ± 1.97
	Sampling B	4.75	13.52	9.01 ± 2.78

* Encompassing the same non-moulting period

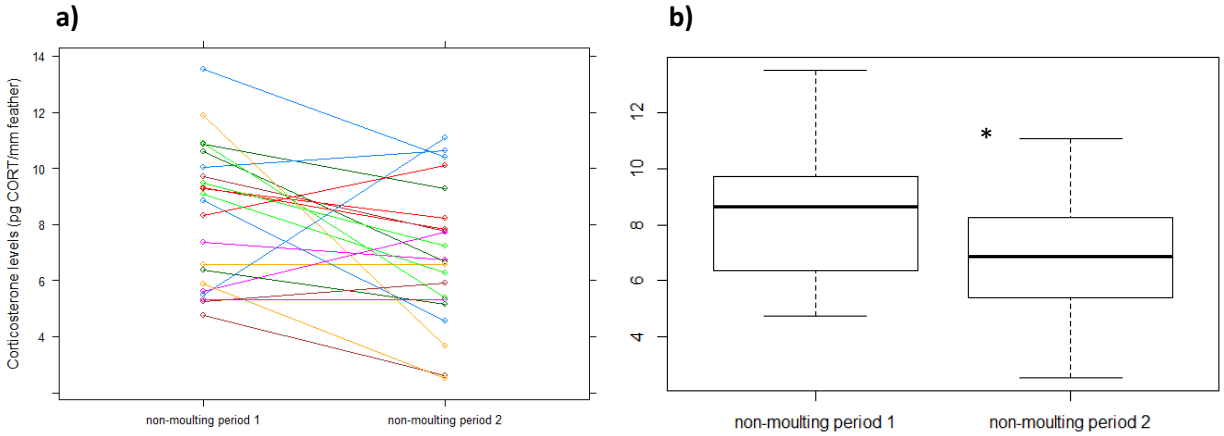

Fig. 2 *Box plot* displaying **a)** individual and **b)** global differences on feather corticosterone concentrations (FCC; pg CORT mm^{-1} feather) between two consecutive non-molting periods in Northern bald ibis (*n* = 22). For information about sampling times see Fig. 1.

Table 2. Concentrations of FCC (pg CORT mm^{-1} feather) of Northern bald ibis raked per sex and sampling time. For information about sampling times see Fig. 1.

			FCC (pg CORT mm^{-1} feather)	
	Sex	n	Mean	SD
Sampling B	Males	12	8.90	1.79
	Females	10	8.06	3.12
Sampling C	Males	12	7.38	2.18
	Females	10	6.35	2.91

The analysis of the effects of previous reproductive performance and health on FCC, including sex and age as control factors, produced no clear best model (Table S3). The best-supported model included sex as fixed factor, but such factor was not significant (in all cases P>0.05). In fact, the null model (only containing the intercept and the random effects) produced ΔAICc < 2 from the best-supported model.

4.3 Relationship with subsequent reproductive success

The most parsimonious model explaining differences on subsequent reproductive success included health, FCC and age (Table S4). The amount of CORT accumulated in feathers grown during the molting period was negatively associated to the following reproductive success (Table 3), with successful birds displaying lower FCC than birds that failed to reproduce (Fig. 3a). Age and health also influenced subsequent reproductive success (Table 3), with unhealthy and older birds being associated to reproductive failure (Fig. 3b, 3c). The second best model with reasonable support (<2 ΔAIC_c; Table S4) included health, FCC, age and sex. In this case, the averaged parameter estimates also showed that FCC, age and health were significantly associated to subsequent reproductive success (all P<0.001) while sex was found non-significant (P=0.33). When health status was classified in three groups (healthy, chronic disabilities and skin disease), candidate models were similar than healthy-unhealthy classification (Table S4). In this case, health was found non-significant (all P>0.05).

Table 3. Model-averaged parameter estimates and associated standard deviation, z- and P-value for the variables contained in the best-supported model explaining success of subsequent reproduction in Northern bald ibis (*n* = 22).

Variable	Estimate ± SE	z-value	P
Intercept	-44.54 ± 9.56 e-04	-434451	<0.001
FCC	8.53 ± 9.68 e-04	8811	<0.001
Age	14.48 ± 1.08 e-03	13788	<0.001
Unhealthy	24.67 ± 9.52 e-04	259206	<0.001
Random effects	Variance	SD	
Period	6.73	2.59	
ID	1.51 e+05	3.87 e+02	

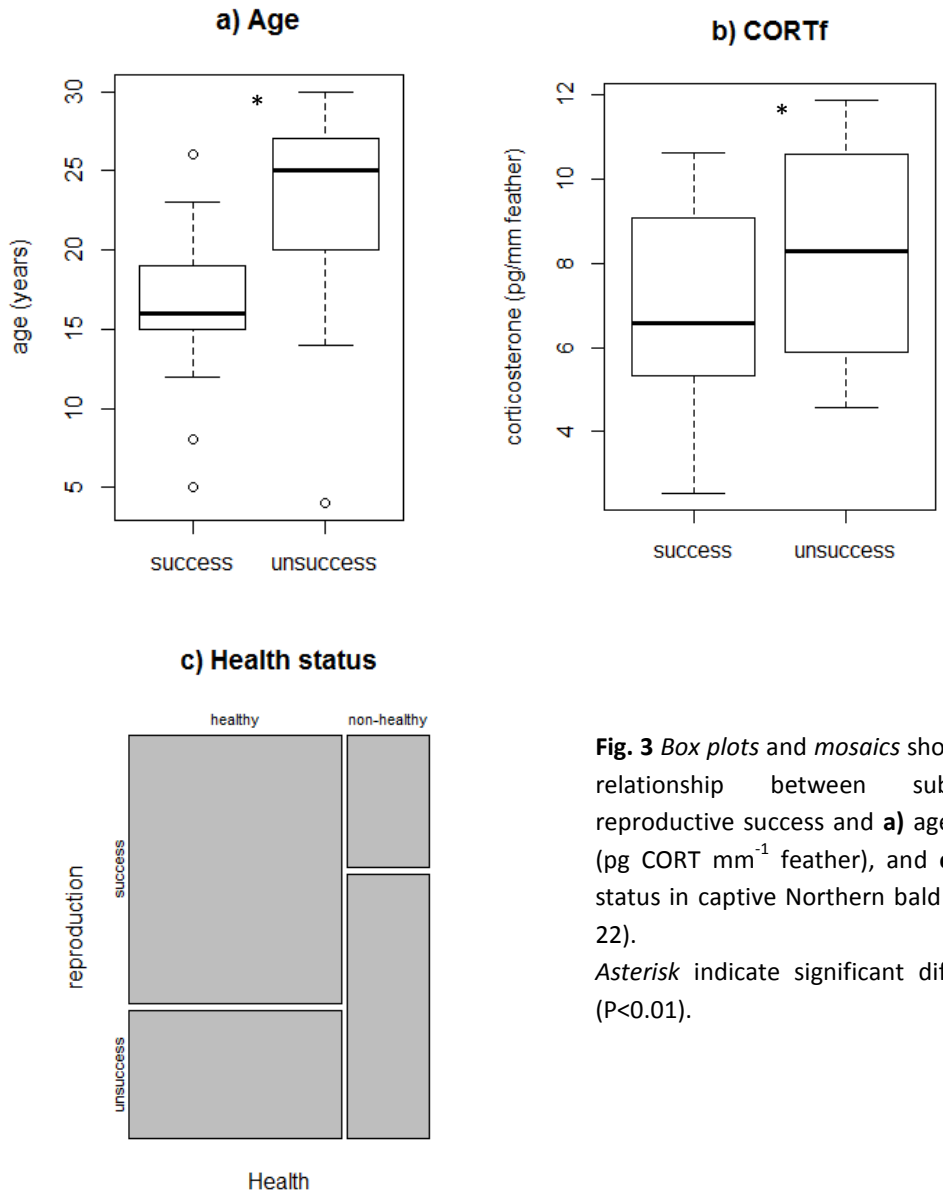

Fig. 3 *Box plots* and *mosaics* showing the relationship between subsequent reproductive success and **a)** age, **b)** FCC (pg CORT mm^{-1} feather), and **c)** health status in captive Northern bald ibis (*n* = 22).
Asterisk indicate significant differences (P<0.01).

5. Discussion

5.1 Within-individual stability of FCC

Consistent FCC were observed between the two pools of feathers sampled at the same time from the same individuals. Close agreement in FCC was previously reported in different raptor species between two opposite primary flight feathers (P5) (Strong et al., 2015) and between adjacent secondary flight feathers (S1 and S2) (Lattin et al., 2011) although these types of feathers may have an asynchronous molt (Rohwer et al., 2009). A more recent study (Monclús et al., 2017) showed high variations in FCC between feathers from different body regions, in particular between primary flight and body feathers. One of the major advantages of using

body feathers instead of flight feathers is that they grow in a more defined and fixed period of time (Hardy et al., 2006). Therefore, body feathers are thought to provide more specific and controlled information on the HPA axis activity over time, and may be the most appropriate feather type when different individuals are compared (Monclús et al., 2017). However, the small mass of body feathers, especially in small bird species, may limit their use since FCC are significantly overestimated when feather mass is below a critical threshold resulting in artificially higher amounts of CORT per mg of feather (Berk et al., 2016; Lattin et al., 2011). In this context, pooling body feathers may counteract this effect as it not only allows to increase the sample mass but also standardize the amount of feather material across samples. In addition, pooling feathers of the same type and from the same anatomical region may allow to obtain a more global value of the bird's stress response because differences or defects of single feathers, such as asynchrony in molting time (Romero and Fairhurst, 2016) or poor feather quality (Aharon-Rotman et al., 2015), would be diluted. In line with this, our results confirm that the creation of a pool of interscapular feathers is a good method for assessing HPA axis as it provides consistent results without the disadvantages and potential errors of a single feather measurement.

In addition, the present study showed stability of FCC between feathers grown during the same molting period but collected at two different moments six-months apart. These results suggest that levels of CORT remain fixed in feathers after the end of vascularization (once feather growth has finished) at least for the following six months, thus complementing the very scarce information available in the literature on feather degradation and CORT stability over time (reviewed in Romero and Fairhurst, 2016). Previous studies examined the degradation of CORT among long-time series of feathers collected from museum specimens and suggested no differences in FCC (Bortolotti et al., 2009; Fairhurst et al., 2015). However, the fact that these studies sampled different individuals and used feathers from different years could allow external factors to interfere with the results. By exploring CORT levels between feathers grown at the same time but collected at different times on an individual bird, the present study supports that FCC is a stable metric of long-term CORT levels within individuals over the same feather generation.

5.2 Variations of FCC across consecutive non-molting periods

Levels of FCC varied significantly between the two consecutive non-molting periods analyzed, indicating that birds with high FCC the first year do not necessarily show high levels the following year (Fig. 2a). Although no link was found between high variations of FCC and

individual health status or reproductive failure in the past season, our results provide evidence for fitness-cost associated effects of high FCC in the following reproductive season. Different hypothesis are discussed for the three main findings.

First, increasing FCC were related to subsequent reproductive failure recorded the following season (Fig. 3b). This result is consistent with previous studies in which females of house sparrow (Ouyang et al., 2011) and European starlings (*Sturnus vulgaris*) (Cyr and Romero, 2007) with low plasma CORT levels showed high subsequent reproductive output. Experimentally, it has been demonstrated that elevated CORT levels suppress reproductive behavior (reviewed in Wingfield and Sapolsky, 2003). However, recent work (Pérez et al., 2016) reported the inverse relationship and stated that high CORT levels prepare individuals for reproductive investment. In fact, the direction of the relationship between CORT and successful reproduction may depend on environmental conditions (Cockrem, 2013). While under predictable environment, birds with low CORT responses have greater fitness, birds with high CORT levels breed most successfully under unpredictable or changing environment (Cockrem, 2013). In this line and given that the studied NBI were kept in captivity under stable conditions and available food resources, we hypothesized a negative relationship between FCC and subsequent reproductive performance. As expected, birds with low FCC succeed in their breeding while birds with a long-term increase of FCC failed to breed the next reproductive season. As mentioned above, and in agreement with a previous study (Pérez et al., 2016), we did not find any relationship between FCC and past reproductive performance. These findings suggested fitness-cost associated effects of high CORT levels on subsequent seasons, while indicated no effect of breeding effort on the HPA axis response in confined birds. Thus, birds that fail to breed do not necessarily show an increase of the stress response in the following months. However, high FCC may ultimately have consequences on the subsequent reproductive season.

Second, no association was found between FCC and chronic diseases or disabilities. This could possibly be explained by habituation or acclimation of birds to the adverse situation (Bonier et al., 2009). In our study, unhealthy individuals presented skin diseases or other type of physical disabilities (see section 3.3). It is plausible than under captive conditions and after some time, individuals with physical disabilities were able to adapt to their new circumstances without presenting any HPA alteration. Experimentally, this hypothesis was proven in captive great tits (*Parus major*) (Cockrem and Silverin, 2002), captive American kestrels (*Falco sparverius*) (Love et al., 2003b) and free-living eastern bluebirds (*Sialia sialis*) (Lynn et al., 2010), that under

repeated stress stimulus showed first an increase of CORT levels and progressively a decrease. On the other hand, we found that unhealthy birds showed worse reproductive success the following breeding season (Fig. 3c). Thus, despite having no effect on the HPA axis activity, the presence of chronic disabilities or diseases could affect the reproduction in captive NBI. Given the strong social hierarchy of NBI and that the mating phase is particularly stressful for this species, low-ranking individuals may have more difficulties in pairing and breeding successfully (Sorato and Kotrschal, 2006). In this situation, those birds that arrive at the reproductive season chronically stressed (presenting high FCC) or those with chronic physical disabilities may have more difficulties for breeding. However, this situation may change between consecutive years, at least for birds with high FCC given that their levels may vary yearly.

Lastly, when exploring differences among ages and sex, no statistical FCC variations were found. These findings are in agreement with a previous study of Sorato and Kotrschal (2006) and are probably explained by the sexual monomorphic and symmetrical social structure of NBI. Likewise, previous studies performed on feathers of several other bird species also reported no sex-related differences (Carbajal et al., 2014; Koren et al., 2012; Monclús et al., 2017; Strong et al., 2015). Regarding age, similarities in FCC have been described in raptor species between immature, subadult and adult individuals (Monclús et al., 2017; Strong et al., 2015). Contrary, immature NBI were reported to show higher CORT levels compared to adults, probably reflecting a HPA response to the low-ranking position of these individuals in social hierarchy (Sorato and Kotrschal, 2006). In the present study, the age of the individuals ranged from 4 to 30 years, thus we could not account for differences between immature and adult individuals. However, and similar to health status, we observed that reproductive success decline in old birds (Fig 3a). This negative age-related relationship with reproductive success has already been reported in the literature (Angelier et al., 2007; Blas et al., 2009; López-Jiménez et al., 2016; Sergio et al., 2011) and emphasize the need for controlling age when studying factors affecting reproduction.

6. Conclusions

FCC were stable in body feathers over the same feather generation regardless of time of feather sampling (simultaneous or at different times during the same non-molting period), thus strengthening the potential usefulness of body feathers as a robust metric. In addition, within-individual plasticity in the HPA axis was observed between consecutive years. Birds showing high FCC one year do not necessarily show high levels the following one. Although the present study could not disentangle the cause for high FCC, we provide evidence for the

fitness-cost associated effects of high FCC in a captive population of NBI, being of special interest for management programs of this species. Increases in energetic challenges or long-lasting stressful conditions may ultimately have consequences on the following reproductive season, and this can be predicted by FCC measured in body feathers. In addition, birds with high FCC are thought to not succeed in pairing, but this does not necessarily imply that these birds never succeed again. Age and health status, despite having no effect on the HPA axis activity, may act as potential confounding factors influencing future reproductive success, and should be controlled for a proper interpretation of data. Due to the critically endangered status of NBI and the already great effort performed in developing reintroduction programs, we highly recommend further research elucidating the causes of high FCC.

7. Supplementary Information

Table S1. Relation of sampling procedures, number of individuals and samples of feathers collected.

	Sampling time	*n* individuals	*n* sampling	*n* samples
Sampling A	October 2014	11	1	11
Sampling B	March 2015	22	1	22
Sampling C	November 2015	22	2	44

Table S2. AICc model comparisons examining the effect of sex, age and non-molting period on feather CORT concentrations (FCC; pg CORT mm^{-1} feather). Table includes the five best models, number of parameters in each model (K), the difference between each model and the top model (ΔAIC_c), and models weights (w_i). In bold is shown the best-supported model.

Model description	AIC_c	K	ΔAIC_c	w_i
FCC ~ sex + period	**207.0**	**5**	**0**	**0.741**
FCC ~ null	211.6	3	4.6	0.076
FCC ~ sex + age + period	211.9	6	4.9	0.066
FCC ~ age + sex x period	212.4	7	5.4	0.050
FCC ~ age + period	213.5	5	6.5	0.030

Table S3. AICc model comparisons examining the effect of sex, age, health and previous reproductive performances on FCC (pg CORT mm^{-1} feather). The table includes the four best models, the number of parameters in each model (K), the difference between each model and the top model (ΔAIC_c), and models weights (w_i). In bold are shown the best-supported models ($\Delta AIC_c<2$). Different models were run for the different reproductive and health parameters.

Model description	AIC_c	K	ΔAIC_c	w_i
Fecundity - General health				
FCC~ sex	**213.4**	**5**	**0**	**0.533**
FCC ~ null	**214.0**	**4**	**0.6**	**0.384**
FCC~ age + sex	218.2	6	4.8	0.047
FCC~ age + sex + fecundity + health	220.1	8	6.7	0.019
Fecundity - Pathological alterations				
FCC ~ sex	**213.4**	**5**	**0**	**0.531**
FCC ~ null	**214.0**	**4**	**0.6**	**0.383**
FCC~ age + sex	218.2	6	4.8	0.047
FCC~ age + sex + fecundity + alterations	219.8	9	6.4	0.021
Fledging SU* - General health				
FCC ~ sex	**213.4**	**5**	**0**	**0.520**
FCC ~ null	**214.0**	**4**	**0.6**	**0.375**
FCC~ age + sex	218.2	6	4.8	0.046
FCC ~ age + sex + fledging SU + health	219.2	8	5.8	0.029
Fledging SU* - Pathological alterations				
FCC ~ sex	**213.4**	**5**	**0**	**0.518**
FCC ~ null	**214.0**	**4**	**0.6**	**0.374**
FCC~ age + sex	218.2	6	4.8	0.046
FCC ~ age + sex + fledging SU + alterations	218.9	9	5.5	0.033
Reproduction SU* - General health status				
FCC~ sex	**213.4**	**5**	**0**	**0.444**
FCC ~ null	**214.0**	**4**	**0.6**	**0.320**
FCC ~ age + sex + reproduction SU	216.5	7	3.1	0.093
FCC ~ age + sex + reproduction SU + health	216.7	8	3.3	0.086
Reproduction SU* - Pathological alterations				
FCC ~ sex	**213.4**	**5**	**0**	**0.437**
FCC ~ null	**214.0**	**4**	**0.6**	**0.315**
FCC ~ age + sex + reproduction SU + alterations	216.3	9	2.9	0.100
FCC~ age + sex + reproduction SU	216.5	7	3.1	0.092
Breeding P** - General health				
FCC ~ sex	**213.4**	**5**	**0**	**0.438**
FCC ~null	**214.0**	**4**	**0.6**	**0.316**
FCC~age + sex + breeding P	216.5	8	3.1	0.093
FCC ~age + sex + breeding P + health	216.7	9	3.3	0.085
Breeding P** - Pathological alterations				
FCC~ sex	**213.4**	**5**	**0**	**0.423**
FCC ~ null	**214.0**	**4**	**0.6**	**0.305**
FCC~ age + sex + breeding P + alterations	216.0	10	2.6	0.113
FCC~ age + sex + breeding P	216.5	8	3.1	0.090

* SU= success; **P=performance

Table S4. AICc model comparisons examining the effect of previous FCC (pg CORT mm^{-1} feather), sex, age and health on subsequent reproductive success (R). The table includes the four best models and the null model, the number of parameters in each model (K), the difference between each model and the top model (ΔAIC_c), and models weights (w_i). In bold are shown the best-supported models ($\Delta AIC_c<2$). Different models were run for the different health parameters.

Model description	AIC_c	K	ΔAIC_c	w_i
General health				
R ~ health + FCC + age	**36.3**	**6**	**0**	**0.457**
R ~ health + FCC+ age + sex	**36.5**	**7**	**0.2**	**0.400**
R ~health + age + FCC x sex	38.9	8	2.6	0.124
R ~ FCC x health + FCC : sex + sex + age	44.2	9	7.9	0.009
R ~ null	58.7	3	22.4	0.000
Pathological alterations				
R ~ alterations + FCC+ age	**38.1**	**7**	**0**	**0.511**
R ~ alterations + FCC+ age + sex	**38.7**	**8**	**0.6**	**0.387**
R ~ alterations + age + FCC x sex	41.4	9	3.3	0.098
R ~ alterations + FCC	48.3	6	10.2	0.003
R ~ null	58.7	3	20.6	0.000

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CHAPTER III

Validation of a new protocol extracting corticosterone from feathers of a raptor species

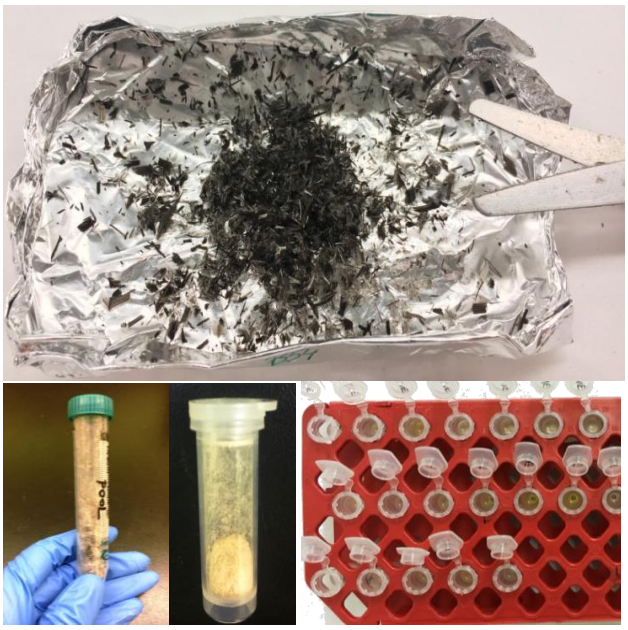

Photo ®: Laura Monclús

Validation of a new protocol extracting corticosterone from feathers of a raptor species

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The abstract of this chapter was presented in a poster format at the 11th International Conference on Behaviour, Physiology and Genetics of Wildlife, Germany 2017, whose abstract is published in Proceedings of the 11th International Conference on Behaviour, Physiology and Genetics of Wildlife. In this thesis, it is presented as a short communication format.

1. Abstract

Measuring corticosterone in feathers is a valuable technique to assess chronic stress in birds that has been widely employed in the literature. However, very few studies have validated the extraction procedure of corticosterone from feathers (CORTf) and the majority has used the classic protocol originally described by Bortolotti et al. (2008). Here, we provided a new optimized protocol for extracting CORTf in a more time-saving and ecological way. We increased the extracting feather surface and used less methanol volume decreasing the ratio methanol:mg feather. We validated biochemically the new protocol using an enzyme immunoassay (EIA) kit and compared the extracted CORTf levels with the ones obtained from the classic protocol. A pool of body feathers (three to five feathers; 37.1 ± 2.2 mm) were sampled from twenty adult red kites (*Milvus milvus*). Each pool of feathers was homogenized and cut with scissors. Then, two new pools of 30 mg were created. The first pool followed the classic protocol and was based on a ratio methanol:mg feathers of 0.33 mL. The second pool followed the new protocol and was based on pulverizing the minced feathers to powder using a ball-mill and decreasing the ratio methanol:mg feather to 0.05 mL. Results showed an acceptable coefficient of variation (CV) (16%) and a positive coefficient of correlation (R^2=0.85, P<0.001) between CORTf levels obtained from both protocols. The intra CV was 5.87%, the recovery percentage of the spike-and-recovery test was 113.42 ± 9.2%, the linearity of dilution showed an R^2= 0.99, and the standard and pool curves showed parallel displacement (R^2=0.93, P=0.02). According to the results, the new protocol successfully extracts corticosterone from feathers proportionately to the classic protocol.

2. Introduction

Measuring corticosterone in feathers is a valuable technique that provides a long-term measure of hormone's integration over time. Corticosterone is the major endpoint of the hormonal cascade mediated by the hypothalamic-pituitary-adrenal (HPA) axis that is initiated in response to stressors (Romero, 2004; Wingfield and Kitaysky, 2002). Bortolotti and colleagues (2008a) were the first authors to report that corticosterone is incorporated in feathers during their growth phase. Given that feathers grow during a certain period of time and then become inert material, feather corticosterone (hereafter, CORTf) is able to provide a historical past record of an individual's HPA axis activity (Bortolotti et al., 2009). However, despite the increasing interest in using CORTf in ecophysiological studies, very few studies have validated the CORTf measurement (Bortolotti et al., 2009, 2008a; Jenni-Eiermann et al., 2015; Lattin et al., 2011). One of the major polemic issues of measuring CORTf concentrations is the extraction efficiency of methanol and the significant effect of feather mass on CORTf concentrations (Romero and Fairhurst, 2016). To date, the majority of studies extracting CORTf have followed the protocol originally described by Bortolotti et al. (2008a, 2008b), some with small modifications (Carbajal et al., 2014; Monclús et al., 2017a). Thus, researches have been extracting corticosterone from feathers using a ratio methanol:mg feathers ranging from 0.25 to 0.7 mL methanol/mg feather (see Berk et al., 2016). However, Berk et al. (2016) recently showed that decreasing the ratio methanol:mg feather did not affect the final CORTf concentrations.

The objective of the present study was to optimize and validate a new protocol for extracting corticosterone from feathers using an enzyme immunoassay (EIA) kit in a more time-saving and ecologic way. We increased the extracting feather area by pulverizing feathers to powder and used less methanol volume in order to decrease the ratio methanol:mg feathers to 0.05 mL (methanol/mg feather), similar to the ratio used for hair cortisol extraction (0.02 mL/mg hair: Davenport et al., 2006). Through the present study, we aimed to biochemically validate the new protocol using a commercial EIA kit (Corticosterone ELISA Kit; Neogen® Corporation) and compared the extracted CORTf levels obtained from the new protocol with the ones obtained from the classic protocol to assess whether CORTf levels were correlated.

3. Material and Methods

3.1 Birds and feather sampling

The species model used in the present study was the red kite (*Milvus milvus*). Feathers from twenty (20) adult red kites that formed part of a population maintained in captivity in a rehabilitation center (Zaragoza, Spain) were used. The sampling of these individuals was done in the framework of another study with captive red kites (Monclús et al., *in preparation*). Six to eleven feathers were plucked from the interscapular region of each bird. After collection, feathers were stored at paper envelopes at room temperature until their analyses.

3.2 Creating feather pools and extracting corticosterone

First, a visual quality examination of all feathers was conducted by the same experienced researcher and feathers with low quality were discarded. As suggested in the literature (Bortolotti et al., 2009, 2008a; Bortolotti, 2010; Jenni-Eiermann et al., 2015), corticosterone concentrations should be normalized by length instead of feather mass, and thus we created a pool of feathers based on the length of feathers (see below). However, as this study was methodological and aimed to compare CORTf concentrations between two extracts obtained from two different protocols, we further use mg to quantify corticosterone instead of mm (see Berk et al., 2016; Lattin et al., 2011).

Three to five feathers were selected and pooled together per each individual to reach similar length among individuals (mean ± SD: 37.1 ± 2.2 cm). After removing the calamus, we cut the feathers of each pool into small pieces < 5 mm^2 with scissors and mixed thoroughly, from which a 30 mg sample was placed in a polypropylene tube for the classic protocol and the rest were minced with a ball-mill (Retsch®, MM2 type, Germany) at 25 Hz for 5 min for obtaining powder. Then, 30 mg of this powder were placed in a new polypropylene tube for the new protocol. For the classic protocol, we added 10 mL of methanol (methanol reagent grade 99.9%; Scharlab, Sentmenat, Spain) to the cut feathers and vortexed (Vortex Mixer S0200-230 V-EU; Labnet International, NJ) for 30 min, followed by overnight incubation at 32 ºC (G24 Environmental Incubator Shaker; New Brunswick Scientific, Edison, NJ) for steroid extraction. Methanol extracts were separated from feather material using vacuum filtration and the resulting supernatant was placed in an oven (Heraeus model T6; Kendro Laboratory Products, Langenselbold, Germany) at 37 ºC until total dryness. For the new protocol, we added 1.5 mL of methanol to the powder and vortexed for 30 min at room temperature, followed by overnight incubation as above. Samples were then centrifuged (Hermle Z300K; Hermle® Labortechnik, Wehingen, Germany) at 6000 rpm at 25 ºC for 15 min. Then, 1 mL of supernatant was transferred to a new aliquot that was placed in an oven at 37 ºC until total dryness. In both cases, dried extracts were reconstituted with 0.25 mL of EIA buffer solution

provided by the EIA commercial assay kit (Corticosterone ELISA kit; Neogen® Corporation Europe, Ayr, UK), shaken for 1 min, and stored at -20 ºC until analysis.

3.3 Corticosterone analysis and validation tests

Corticosterone analysis was performed using a competitive EIA kit (Corticosterone ELISA kit; Neogen® Corporation Europe, Ayr, UK) and carried out following the manufacturer's instructions. All extracts (new protocol and classical protocol) were analyzed in the same EIA kit and were run per duplicate. Extracts used in the new protocol were pooled and were used for the validation tests. We follow the essential criteria of precision, accuracy, specificity and sensitivity for the biochemical validation (Buchanan and Goldsmith, 2004; Sink et al., 2008; Terwissen et al., 2013). The precision within test was calculated using the intra-assay coefficient of variation (CV) from all duplicated samples analyzed. Specificity was given by the parallelism and dilution test. In the parallelism test, two different calibration curves, one created by the EIA kit's standards and the other by adding serial dilution of concentrated feather extract (1:1, 1:2, 1:5, 1:10 and 1:20), were compared. In the dilution test, the pool obtained was serially diluted in EIA buffer provided by the kit. The dilution set ratios were 1:1, 1:2, 1:3 and 1:4. Accuracy was assessed through the spike-and-recovery test, calculated by adding 200, 100 and 50 µl of pool to 50, 100 and 200 µl of pure standard corticosterone solution, respectively. Combinations were repeated with two different pure standard corticosterone solutions (2 and 0.2 ng/mL). Finally, sensitivity was given by the smallest amount of unlabelled hormone that could be distinguished and measured by the EIA. According to the manufacturer, cross-reactivity of the EIA antibody with other steroid hormones is as follows: 38 % deoxycorticosterone; 19 % 6-hydroxycorticosterone; 5.1 % progesterone; 2.7 % tetrahydrocorticosterone; 1.5 % prednisolone; 1.1 % cortisol. Steroids with cross-reactivity below 0.9 % are not presented.

3.4 Statistics

Data were analyzed using R software version 3.2.2 (R-project, R Development Core Team, University of Auckland, New Zealand). A P-value < 0.05 was established as a criterion for significance. Prior to analyses, a Shapiro-Wilk test was used to check normality of data. Parametric Pearson's coefficients were used to calculate correlations between concentrations of CORTf from the two protocols and also between standard and pool lines obtained from the parallelism test. In addition, CV was used to evaluate the % of variation between CORTf concentrations obtained from the two protocols.

4. Results and Discussion

The mean CORTf levels (± SD) was 21.86 (7.26) pg mg^{-1} for the classical protocol and 17.19 (6.39) pg mg^{-1} for the new protocol. The CV found between CORTf levels from the classical and the new protocol was 16.5 %. In addition, a strong and positive correlation was found in CORTf levels between the classical and the new protocols (R^2=0.85; P<0.001) (Fig. 1). These findings indicated a correspondence in levels of the corticosterone extracted from feathers of the same individual between the two protocols used with an acceptable CV.

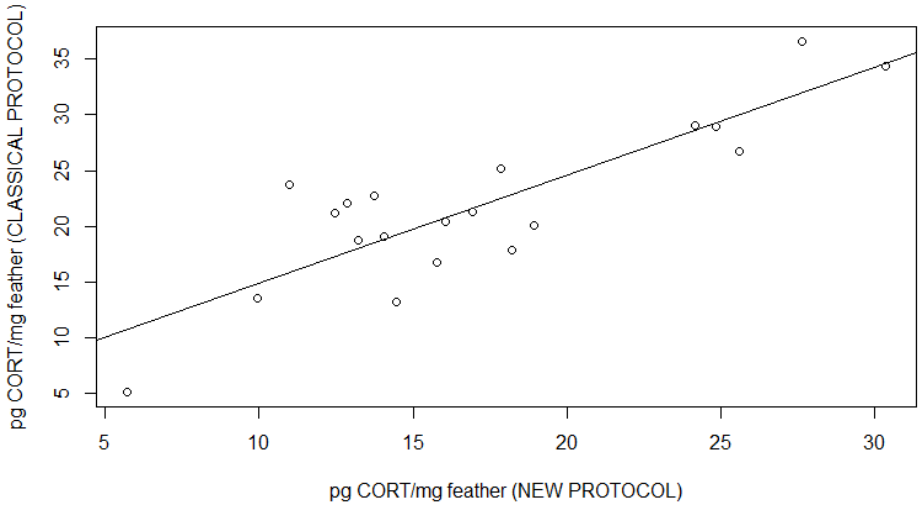

Fig. 1 Correlation between feather corticosterone (CORTf) concentrations obtained from the new and the classical protocol.

Regarding the biochemical validation, the intra-assay CV was 5.87 % revealing an acceptable repeatability within the assay. Fig. 2 illustrates the correlation line acquired in the dilution test, with a R^2 of 0.99, indicating that a correct dose-dependent interaction between corticosterone from feathers extracts and EIA antibodies exists. In addition, the standard and pool curves showed significant parallel displacement (Pearson correlation: R^2=0.93; P=0.02), showing immunological similarities between the standards and the sample hormones. Fig. 3 illustrates the slopes of standard and pool curves. Finally, the average recovery percentage from the spike-and-recovery test was 113.42 ± 9.2%, revealing high efficiency of the method to extract the targeted hormone and indicating that no other components other that corticosterone interfered acutely with the estimation of corticosterone hormone.

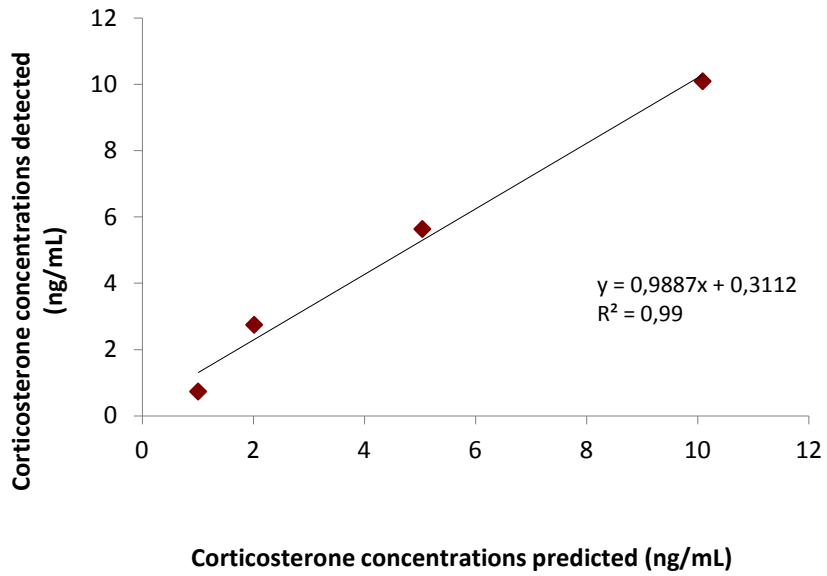

Fig. 2 Linearity of dilution of corticosterone concentrations predicted (standard) and detected (pool) extracted from feathers.

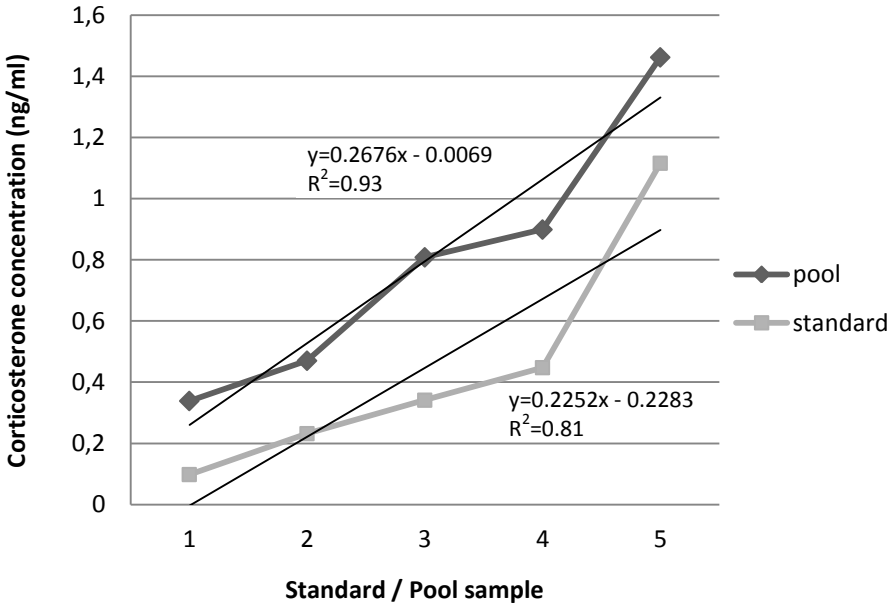

Fig. 3 Parallelism between lines from the standard and sample pool curves.

5. Conclusions

According to the results, the new protocol successfully extracts corticosterone from feathers proportionately to the classic protocol, allowing to reach the same conclusions when corticosterone is extracted from either of both protocols. In addition, results of the validation tests confirm that it is possible to detect corticosterone from feathers using the new protocol with an acceptable repeatability and reliability by using EIA. Overall, the present study

provides an optimized protocol for extracting corticosterone from feathers, facilitating the methodological procedures in a more time-saving and ecological way.

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PART II:

Biomonitoring Envionmental Contamination Through Feathers And Relationship With Adrenal Stress Hormones

CHAPTER IV

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*)

Photo ®: Laura Monclús

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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Accepted with major changes in: Environmental Pollution

1. 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 been investigated yet. 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 vulture chick life, they probably reflect the contaminant burden of the chick from maternal transfer through 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 indeed reflect the concentrations in the egg.

2. 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 important 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 currently 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 choice 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., 2007, 2006). 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 (primaries and secondaries mainly) show an asynchronous moult 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 higher influence of external contamination (Jaspers et al., 2011). Instead, body feathers seem to be advantageous (Eulaers et al., 2014b; Jaspers et al., 2011), as they moult 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.

3. 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). Cinereous vulture is a scavenger raptor that mainly feeds on carcasses, selecting mainly medium-sized carcasses such as rabbit, livestock and big game (Del Moral and De la Puente, 2017). This species breeds in colonies in general of low density, typically building its nest in large trees in forested areas of mountainous zones (Hiraldo, 1977). Clutch size is always one egg, so in successful nests there is always 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 study 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 (own 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 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 simultaneously sampled down and juvenile contour feathers from the nestling. 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 colour 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 and contrast 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 fledged from the nests without any problems.

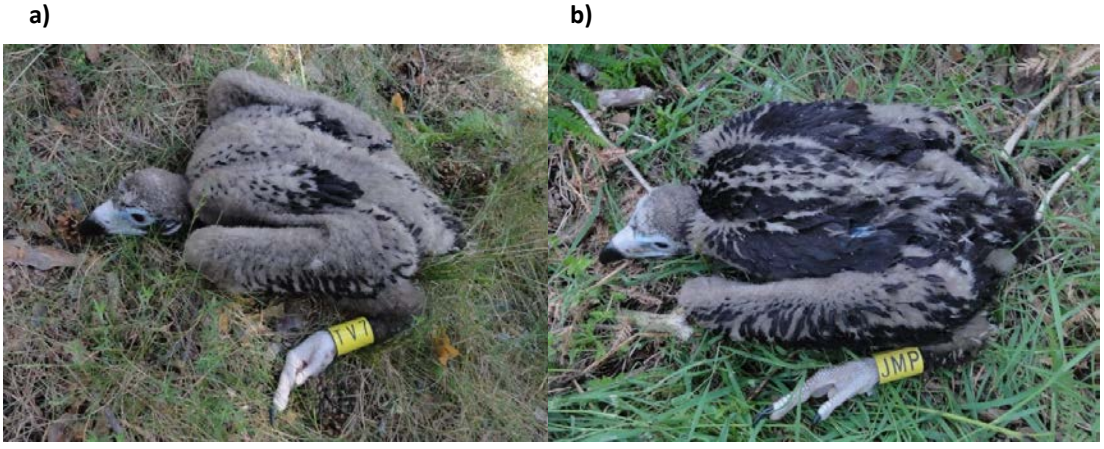

Fig. 1 Nestlings of cinereous vultures at different ages showing different plumage. **a)** Nestlings at approximately 30 d post-hatching covered with the second natal down, which has a grey appearance. Pin feather development mainly of the scapulars, remiges and rectrices is apparent. **b)** Nestlings at approximately 40 d showing black juvenile scapular and wing coverts 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.

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 using a 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 using a 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 10th sample. Recovery of internal standards was on average 76 ± 8%. All compounds were blank-subtracted using the average procedural blanks 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 throughout this study. 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 visualise 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.

4. Results and Discussion

4.1 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 very 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., 2016b). 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 the predominant OCP component in juvenile contour 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 metabolised 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 indicates more probably a long historic exposure rather than 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 ecological factors (Eulaers et al., 2013).

Concentrations of OPEs were more similar between down and juvenile contour feathers than POP compounds, except TCiPP that 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.

4.2 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 that showed no significant difference ($F_{1.15}=0.40$; $P=0.54$) and CB-180 that 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 nearly half 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 ($F_{1.15}=24.18$; $P<0.001$).

Table 1. Concentrations and detection frequency (DF) of PCB, OCP, PBDE and OPE compounds in contour and down feathers (ng g^{-1} dw feather) of nestling cinereous vultures. 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

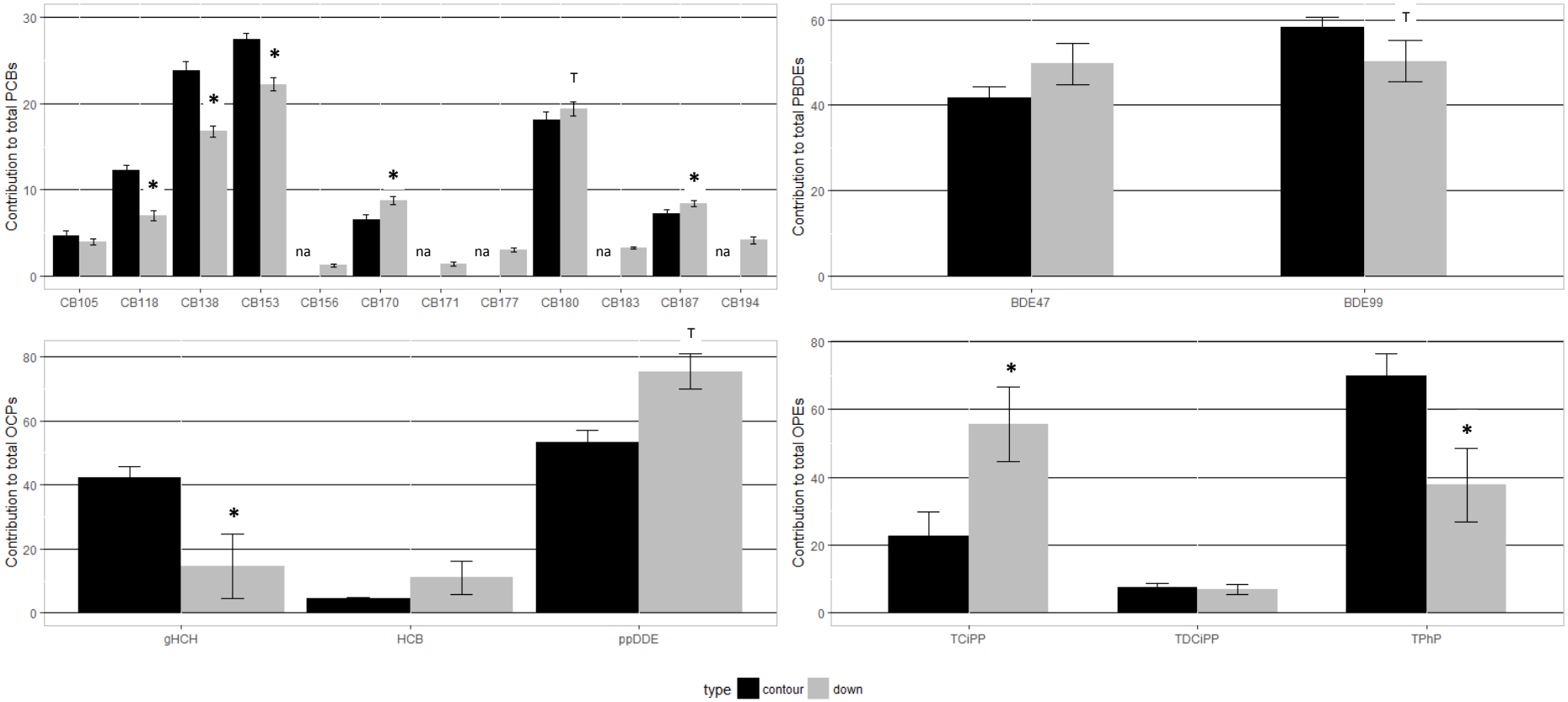

Fig 2 Contribution profile (% mean ± SE) of **a)** PCBs (*n* = 16), **b)** OCPs (*n* = 16), **c)** PBDEs (*n* = 16) and **d)** OPEs (*n* = 7) in contour and down feathers of nestling cinereous vultures. Significant differences ($P<0.05$)* along with tendencies ($0.1>P\leq0.5$)^{T} are shown. Congeners not detected above LOQ in > 38% of all individuals sampled were not statistically analysed ("na").

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 the most persistent compounds. Contrary, juvenile contour feathers showed more presence of the most volatile POPs, such as *γ*-HCH, and the lower chlorinated PCBs. Although PC2 discriminated between several compounds, such as BDE-47, on one hand and BDE-99 on the other, 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 PFR 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 TCEP and 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 and TCEP on one hand and TCiPP on the other, while PC2 discriminated between TCEP 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 and TCEP 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.

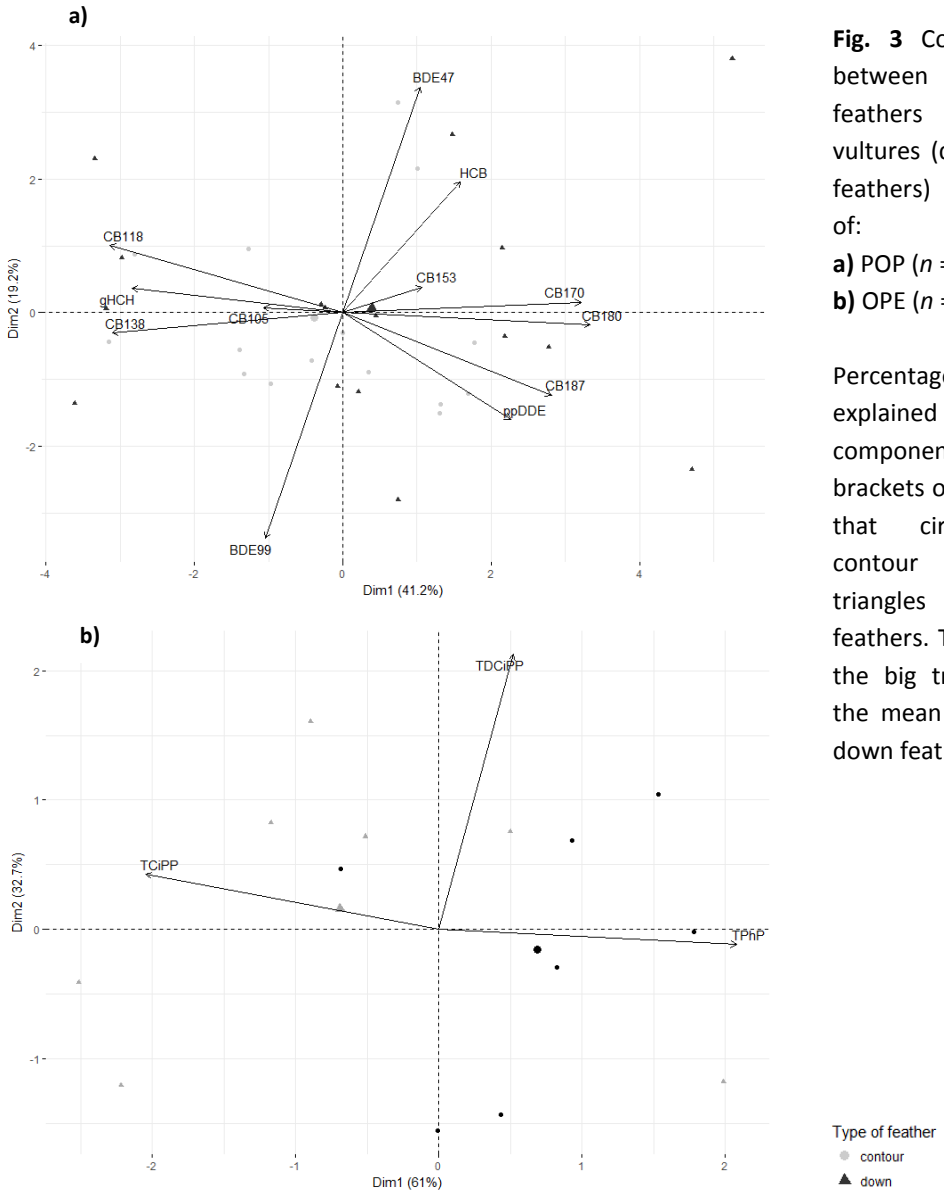

Fig. 3 Correlation biplots between two types of feathers of cinereous vultures (down vs. contour feathers) comparing levels of:
a) POP (*n* = 16)
b) OPE (*n* = 7).

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.

4.3 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 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 reach higher concentrations in juvenile contour feathers, though the latter showing numerically lower concentrations. Contrary, γ-HCH showed a negative, although not significant, correlation (r=-0.43, P=0.09; Table 2), suggesting that higher γ-HCH levels tend to accumulate with advancing age. Finally, none of the OPE compounds showed any significant correlation. It is possible that OPE levels fluctuate highly due to the potential influence of

external contamination (see section "Differences in accumulation between down and contour feathers").

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. 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

4.4 Differences in accumulation between down and contour feathers

Bourgeon et al. (2013) hypothesized that the accumulation of the most persistent pollutants in chicks of a top predator seabird was mainly through maternal transfer via the eggs rather than by food intake, while the least persistent were acquired by dietary transfer. Our results provide support for this hypothesis. We analyzed different feather types that reflect different periods during the nestling development. Since juvenile contour feathers show a longer period of growth than down feathers and only start to grow with advancing nestling age (see Material and Methods section), juvenile contour feathers are exposed to more volatile and less persistent pollutants present in the environment and may thus reflect recent changes in their concentrations (i.e. the increasing levels of *γ*-HCH found in juvenile contour feathers). Alternatively, down feathers grow during the first days of chick life and probably reflect the contaminant burden of the chick by maternal transfer through the egg. High concentrations of high chlorinated PCBs, PBDEs and *p,p'*-DDE were found in down feathers, in concordance with

previous studies describing similar contaminant patterns in eggs (Chen and Hale, 2010; Gómara et al., 2008; Jiménez et al., 2007; Morales et al., 2012).

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

5. 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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CHAPTER V

High levels of corticosterone are related to persistent organic pollutants, but not to organophosphate ester flame retardants, in feathers of nestling cinereous vultures (*Aegypius monachus*)

Photo ®: Javier De la Puente

High levels of corticosterone are related to persistent organic pollutants, but not to organophosphate ester flame retardants, in feathers of nestling cinereous vultures (*Aegypius monachus*)

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1. Abstract

Persistent organic pollutants (POPs) are widely known to disrupt endocrine functions in birds, but their relationship with the response of the hypothalamic-pituitary-adrenal (HPA) axis is still poorly understood. Raising concerns are now focused on the toxic proprieties of emergent organophosphate ester flame retardants (OPEs), however whether OPEs affect the HPA axis response has not been investigated yet. We measured corticosterone concentrations in feathers (CORTf) as a biomarker of long-term HPA axis activity 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 concentration affected 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 reduced duration of the nestling period (P=0.05) and equal fledging success than chicks with lower levels. These findings suggest that POPs, but not OPEs, may increase the HPA axis response of chicks, although levels were not high enough to cause detrimental consequences.

2. Introduction

Apex predators such as birds of prey are widely used as early warning systems for anthropogenic pollution (Furness, 1993; Jaspers et al., 2006). Exposure to persistent organic pollutants (POPs) [i.e. polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs)] has been one of the major concerns for ecosystem and wildlife health due to the global distribution of POPs, 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). Despite the restrictions to their use in many countries, high POP levels still remain in bird samples, particularly in birds of prey (Elliott et al., 2015; Espín et al., 2016), and have been observed to impair bird physiology, neurological functions and reproduction (Chen and Hale, 2010; Eng et al., 2012; Winter et al., 2013). 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. *in preparation*). 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 POPs can act as stressors affecting the endocrine system of birds, for example affecting the thyroid endocrine system (Cesh et al., 2010; Fernie and Marteinson, 2016; Nøst et al., 2012; Rogstad et al., 2017; Van den Steen et al., 2010) or 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 (Table 1), 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 organisms use to deal with stressors (Romero, 2004; Sapolsky, 2000). In the presence of a stressor, the HPA axis responds by releasing glucocorticoids into bloodstream, in birds corticosterone (CORT), with

the aim to restore homeostasis, overcome the stress status and return to initial conditions. This stress response promotes gluconeogenesis (Sapolsky et al., 2000), foraging activity (Landys et al., 2006), escape behavior (Wingfield, 2003) and energy allocation between self-maintenance and non-vital activities (Angelier et al., 2007). However, despite the adaptive value of short-term elevations of CORT, chronically elevated levels have detrimental consequences to cognitive ability, growth, 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). Although the specific mechanisms through which POPs disrupt the HPA axis are still not well known, it has been suggested that POPs may interact with hormone receptors or modulate the hormone synthesis, metabolism, transport and degradation (reviewed in Hampl et al., 2016; Maqbool et al., 2015). Furthermore, POPs may increase the allostatic load of organisms causing important energetic challenges (Bustnes et al., 2001; Nordstad et al., 2012; Monclús et al., *in preparation*). 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 main objective of the present study was focused on investigating the effects of POPs (including PCBs, OCPs and PBDEs) and OPEs on CORT concentrations measured in feathers of nestling cinereous vultures (*Aegypius monachus*). Feathers have become the matrix of preference in biomonitoring environmental pollution in wild 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.

Table 1. CORT changes in birds in the context of POPs exposure.

Authors	POPs	POP matrix	CORT matrix	CORT assessment	Species	Field conditions	Age	Effects
Love et al. 2003	PCBs	Plasma	Plasma	Baseline Stress-induced	American kestrel *Falco sparverius*	Captured	Adults	Reduced stress-induced response and low baseline CORT levels in relation to high PCB exposure.
Verboven et al. 2010	PCBs OCPs PBDEs	Plasma	Plasma	Baseline Stress-induced	Glaucous gull *Larus hyperboreus*	Free-living	Adults	Reduced stress-induced response in males and high baseline CORT levels in relation to high POP exposure.
Bourgeon et al. 2012	OCPs PBDEs	Plasma	Primary wing feather	Long-term	Great skuas *Stercorarius skua*	Free-living	Adults	Negative relationship between CORT and PBDEs, but not with OCPs.
Nordstad et al. 2012	PCBs OCPs	Plasma	Plasma	Baseline	Black-legged kittiwake *Rissa tridactyla*	Free-living	Adults	Baseline CORT only correlated to PCB levels during the pre-breeding period.
Tartu et al. 2014	PCBs OCPs	Plasma	Plasma	Baseline Stress-induced	Black-legged kittiwake *Rissa tridactyla*	Free-living	Adults	Increased stress-induced response to POP levels. No relationship between baseline CORT and POP levels.
Tartu et al. 2015	PCBs OCPs PBDEs	Plasma	Plasma	Baseline Stress-induced	Snow petrel *Pagodroma nivea*	Free-living	Adults	Increased stress-induced response in relation to POP levels. No relationship between baseline CORT and POP levels.
Monclús et al. *in preparation*	PCBs OCPs	Body feathers	Body feathers	Long-term	Red kite *Milvus milvus*	Free-living vs. captive	Juvenile Subadults Adults	Increased CORT in relation to PCBs and DDTs in free-living birds. No relationship in captive birds.

PCBs (polychlorinated biphenyls), OCPs (organochlorine pesticides), PBDEs (polybrominated diphenylethers).
OCPs include DDTs (dichlorodiphenyldichloroethanes).

3. Material and Methods

3.1 Study birds and data collection

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). The cinereous vulture is an endangered species catalogued as Near Threatened by the IUCN (BirdLife International, 2017). This scavenger species is positioned at the top of the food chain and is mainly feeding on medium-size carcasses, rabbit, livestock and big game (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 always one egg (Donázar, 1993). The nests are typically built in large trees in forested mountainous areas (Hiraldo, 1977). The feeding area for the study colony was about 100 Km in the surroundings. Lately, vultures of the study colony were observed to feed on rubbish dump close to Madrid city and remains of plastic bags have been frequently found in their nest (own observations).

Ninety-nine (99) nests of cinereous vulture were monitored and visited during the breeding season of 2016 from a distance in order to avoid disturbances. From mid-February to early-April egg-laying was determined and in late-April to early-June hatching date was recorded. 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). The duration of chick development (in days) was calculated per each individual from hatching to fledging. 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). 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). 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. 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). Descriptive statistics for biological variables are provided in Table S1 of Supplementary Information (SI).

3.2 Contaminant analysis

From the 42 samples collected, 16 were utilized in a previous study (Monclús et al., *submitted EP*) 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., *submitted EP*) and followed those from Eulaers et al. (2014b). 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 (mean feather mass: 0.14 g, range: 0.09 - 0.20 g) and transferred to an analytical glass recipient. Feather samples were then spiked with 100 µl of internal POPs standard (200 pg μl^{-1} CB143; 25 pg μl^{-1} ε-HCL; 25 pg μl^{-1} BDE77) and 25 µl of internal OPEs standard (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 feather analytes were liquid-liquid extracted using hexane:dichloromethane (4:1, v:v). Cleanup of the resulting extracts was performed on Florisil® cartridges (Supelco®) topped with anhydrous Na_2SO_4 (0.5 mg). The cartridges were prewashed with 6 mL of ethyl acetate and 6 mL of hexane and analytes were eluted with 10 mL of hexane:dichloromethane (1:1, v:v) to obtain the first fraction (F1) that was consecutively concentrated to ~200 µL using a gentle flow of nitrogen. OPEs were eluted from the same cartridge with 10 mL of ethyl acetate to obtain the second fraction (F2) that was consecutively concentrated to near dryness using a gentle flow of nitrogen. 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 cleaned F1 extracts were concentrated to near dryness using a gentle flow of nitrogen. 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 iso-tol 9:1, v:v). Fraction F1 was analyzed for POPs and fraction F2 for OPEs. Analysis was done using a gas chromatograph coupled with a mass spectrometer (GC/MS) (see details in Appendix S1).

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 (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).

3.3 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 with a calliper to the nearest 0.1 mm (mean ± SD: 26.5 ± 7.03 cm) and weighted 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). Briefly, after discarding the calamus, feathers were minced with a ball-mill (Retsch®, MM2 type, Germany) for 2 min at 25 Hz. Then, 1.5 mL of methanol (methanol reagent grade 99.9%; Scharlab, Sentmenat, Spain) was added and samples were placed in a vortex (Vortex Mixer S0200-230 V-EU; Labnet International, NJ) for 30 min at room temperature, followed by incubation (G24 Environmental Incubator Shaker; New Brunswick Scientific, Edison, NJ) for 18 h at 32 °C for steroid extraction. Samples were centrifuged (Hermle Z300K; Hermle® Labortechnik, Wehingen, Germany) at 6000 rpm for 20 min at 23 °C and 1 mL of supernatant was transferred to a new aliquot. Samples were then placed in an oven at 37 °C until dryness. Dried extract were reconstituted with 0.25 mL of enzyme immunoassay (EIA) buffer provided by the EIA kit (Neogen® Corporation, Ayr, UK), shaken for 1 min, and immediately stored at -20 °C until analysis. All CORTf values were expressed as a function of feather length (pg mm^{-1}) (Bortolotti et al., 2008) although 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). We provide the biochemical validation for CORTf in Appendix S2.

3.4 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 detection frequencies 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 log-transformed (log10(x+1)) 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). The PCs-POPs explained 41.06 + 18.54% of the total variance and the PCs-all explained 44.20 + 21.10%. Table S2 and S3 provide factor scores for PCs-POPs and PCs-all respectively and Fig. 1 illustrates the PCs. Here, we referred the PC lists as "PC1-POPs", "PC2-POPs", "PC1-all" and "PC2-all". We then explored whether PC1-POPs, PC2-POPs, PC1-all and PC2-all (on separate models) as well as sex and egg-laying date influenced CORTf concentrations. Post-hoc multiple comparisons were made *a posteriori* using Tukey HSD Test. Finally, we tested whether PC1-POPs, PC2-POPs, PC1-all and PC2-all as well as sex, CORTf concentrations and egg-laying date influenced the duration of chick development. In this case, a generalized linear model with Poisson error distribution and log-link function was used. 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 of the PC-all lists (PC1-all and PC2-all). 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 S4 and S5 reporting the best-supported and null models are provided in SI.

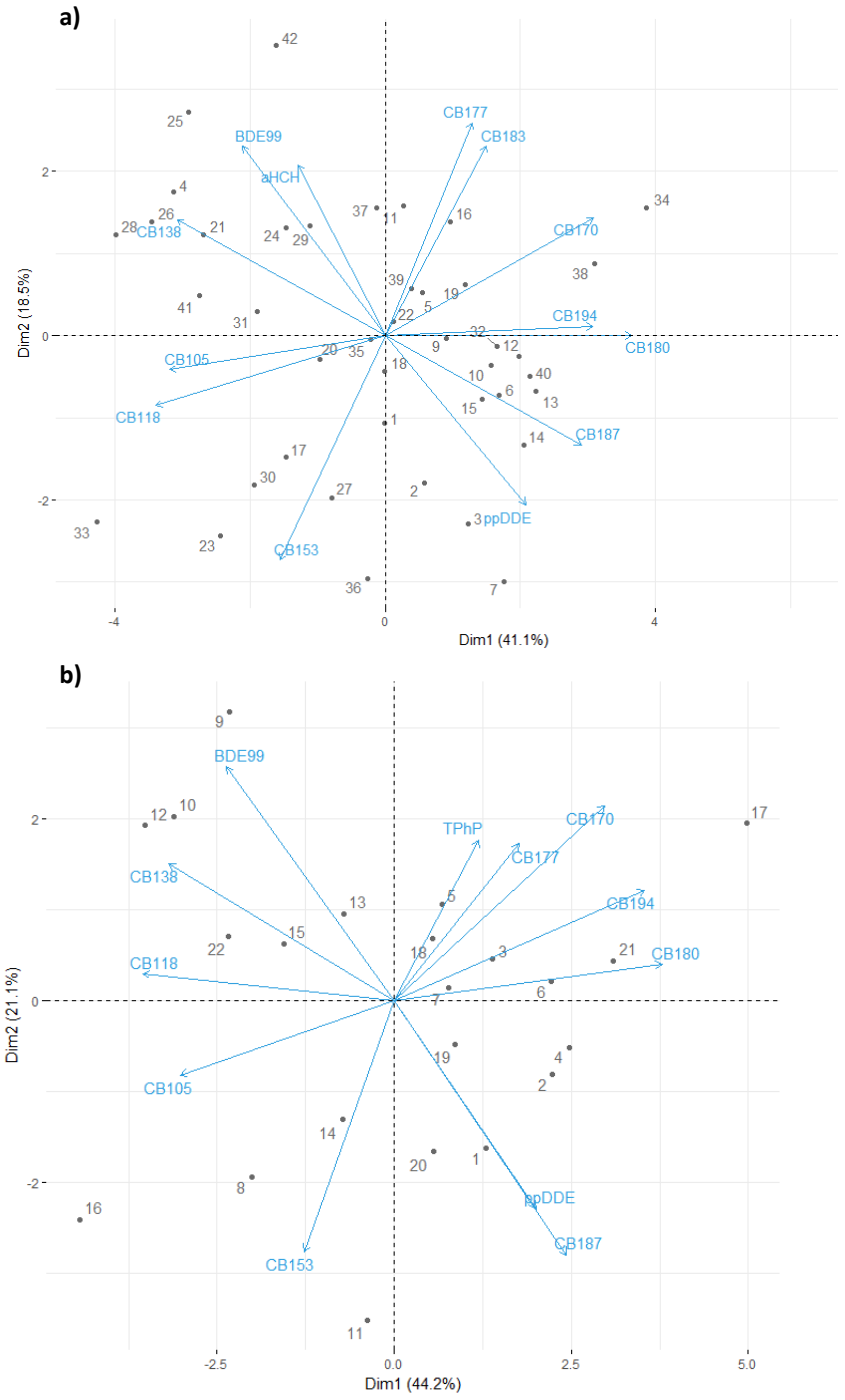

Fig. 1
Principal Component Analysis diagram of:
a) POPs (*n* = 42);
b) POPs and OPEs (*n* = 22) in feathers of nestling cinereous vultures.
Percentage of variation explained by each principal component (PC) is given in brackets on each axis.

4. Results

4.1 Contaminant 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 S6). 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 S4). Concentrations were similar to the concentrations previously reported for the 16 nestlings also included in Monclús et al. (*submitted EP*), except for TPhP and TCiPP that were 1.5 to 2 times lower respectively in the current study (Table S6).

In relation to sex, 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 2). No sex-relation was found for the rest of compounds (all P>0.05).

Table 2. Variations of POP concentrations depending on sex in nestling cinereous vultures (*n* = 42). Only compounds displaying significant differences (P<0.05)* and tendencies (0.1>P≤0.05)^{T} are shown.

CB-153				CB-177			
Variables	Estimate ± SE	t-value	P-value	Variables	Estimate ± SE	t-value	P-value
Intercept	-0.17 ± 0.03	-5.24	<0.001	Intercept	-1.03 ± 0.05	-20.76	<0.001
sexM	-0.08 ± 0.05	-1.86	0.07^{T}	sexM	-0.12 ± 0.07	-1.72	0.09^{T}
HCHs				**γ-HCH**			
Variables	Estimate ± SE	t-value	P-value	Variables	Estimate ± SE	t-value	P-value
Intercept	0.07 ± 0.08	0.87	0.39	Intercept	-0.28 ± 0.15	-1.97	0.05
sexM	-0.24 ± 0.11	-2.12	**0.04***	sexM	-0.37 ± 0.21	-1.79	0.08^{T}

4.2 Relationship between contaminants, CORTf levels and 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 S4). There was a positive and significant relationship between concentrations of CORTf and PC1-POPs (Table 3; Fig. 2a), whereas CORTf was not related to PC2-POPs (Table 3; Fig 2b) nor to PC2-all (Table 3). A positive and significant relationship between CORTf concentrations and egg-laying was found

in all POP models (Table 3), indicating that chicks with later egg-laying date showed higher concentrations of CORT deposited in feathers grown during the third week of life. Specifically, CORTf concentrations of the chicks with latest date of egg-laying (L3; mean ± SE= 3.76 ± 0.48 pg CORT/mm feather) were higher than those chicks with earliest date (L1: mean ± SE= 2.30 ± 0.24 pg CORT/mm feather, Tukey post hoc: P<0.01; L2: mean ± SE= 2.88 ± 0.37 pg CORT/mm feather, Tukey post hoc: P=0.05) (Fig 3a). No significant difference was observed between L1 and L2 groups (Tukey post hoc: P=0.43) (Fig 3a). 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.

Table 3. Model-average parameter estimates and associated standard error (*SE*), *t*-value and *P*-values for the best models explaining CORTf variation in nestling cinereous vulture (*n* = 42 for PC-POPs; *n* = 22 for PC-all). Significant differences (P<0.05)* (P<0.01)** are shown.

Dependent variable	Model	Explanatory variables	Estimate ± SE	t-value	P-value
CORTf	PC1-POPs	Intercept	18.41 ± 6.11	3.01	<0.001
		PC1-POPs	2.35 ± 0.98	2.39	**0.02***
		sexM	-4.17 ± 4.27	-1.00	0.36
		Egg-laying	6.88 ± 2.86	2.41	**0.02***
	PC2-POPs	Intercept	17.67 ± 6.64	2.66	0.01
		PC2-POPs	-0.32 ± 1.49	-0.22	0.83
		sexM	-5.85 ± 5.18	4.69	0.22
		Egg-laying	7.63 ± 3.01	2.49	**0.02***
	PC1-all	Intercept	18.08 ± 9.77	1.85	0.08
		sexM	-7.91 ± 6.42	-1.23	0.24
		Egg-laying	6.58 ± 4.88	1.35	0.20
	PC2-all	Intercept	16.79 ± 9.55	1.76	0.10
		PC2-all	-2.85 ± 2.10	-1.36	0.20
		sexM	-11.87 ± 6.89	-1.72	0.11
		Egg-laying	8.56 ± 4.97	1.72	0.11

4.3 Influence of contaminants and CORTf 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 S5). The duration of chick development was negatively influenced by the date of egg-laying (Table 4). Specifically, 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) and from L3 group (mean ± SE: 106.5 ± 4.6 days; Tukey post hoc: P<0.01) (Fig 3b). No significant difference was observed between L2 and L3 groups (Tukey post hoc: P=0.14) (Fig 3b). Although this relationship was very significant in the POP models, it was no significant in the OPE models

(PC1-all and PC2-all) (Table 4), 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 4). Concentrations of PC2-POPs, PC1-all, PC2-all and CORTf did not influence the duration of chick development (Table 4).

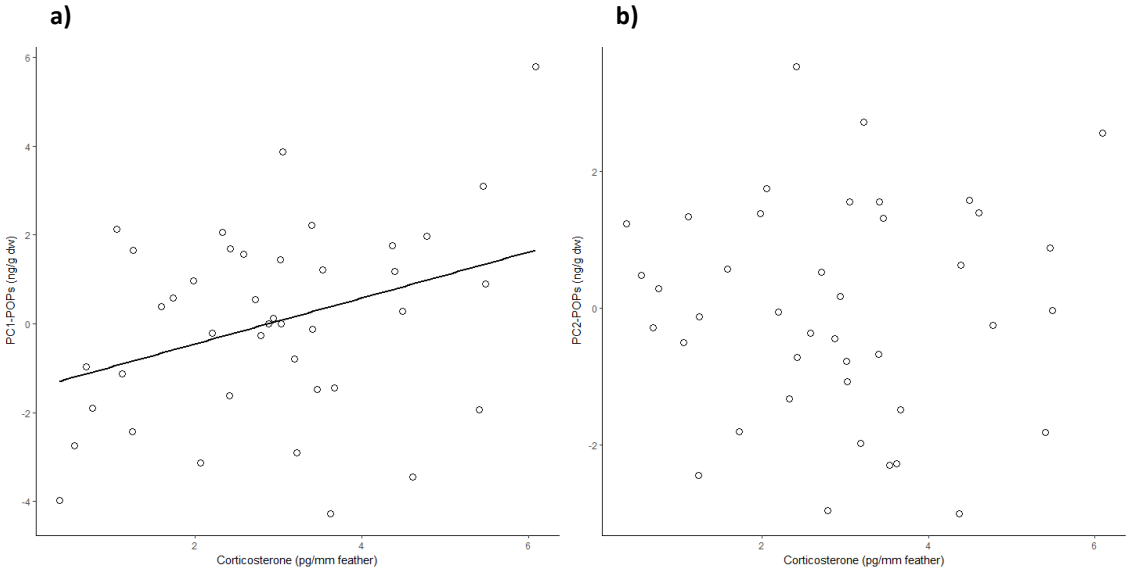

Fig. 2 Relationship between feather CORT concentrations and **a)** PC1-POPs and **b)** PC2-POPs in nestling cinereous vultures (*n* = 42).The line denotes the significant positive relationship and the absence of a line the lack of significance. The empty circles represent each individual.

Table 4. Model-average parameter estimates and standard error (*SE*), *z*- and *P*-value for the best models explaining the duration of development of nestling cinereous vultures (*n* = 42 for PC-POPs, *n* = 22 for PC-all). Tendencies (0.1>P≤0.05)^{T} and significances (P<0.05)* (P<0.01)** (P<0.001)*** are shown.

Dependent variable	Model	Explanatory variables	Estimate ± SE	z-value	P-value
Chick Development (days)	PC1-POPs	Intercept	4.89 ± 0.05	98.78	<0.001
		CORTf	-3.5e-04 ± 1.2e-03	-0.28	0.78
		PC1-POPs	5.5e-03 ± 0.01	0.53	0.59
		sexM	-4.8e-03 ± 0.03	-0.15	0.88
		egg-laying	-0.08± 0.02	-3.53	**<0.001*** **
		PC1-POPs:sex	-0.03 ± 0.01	-1.93	**0.05**^{T}
	PC2-POPs	Intercept	4.89 ± 0.05	100.78	<0.001
		CORTf	-1.10e-03 ± 1.1e-03	-0.95	0.34
		PC2-POPs	0.01 ± 0.01	1.29	0.20
		sexM	0.01 ± 0.03	0.32	0.75
		egg-laying	-0.07 ± 0.02	-3.06	**<0.001*** **
	PC1-all	Intercept	4.09 ± 0.10	42.82	<0.001
		PC1-all	-5.76e0.3 ± 0.01	-0.43	0.67
		sexM	0.02 ± 0.07	0.30	0.76
		egg-laying	-9.35e03 ± 0.05	-0.20	0.84

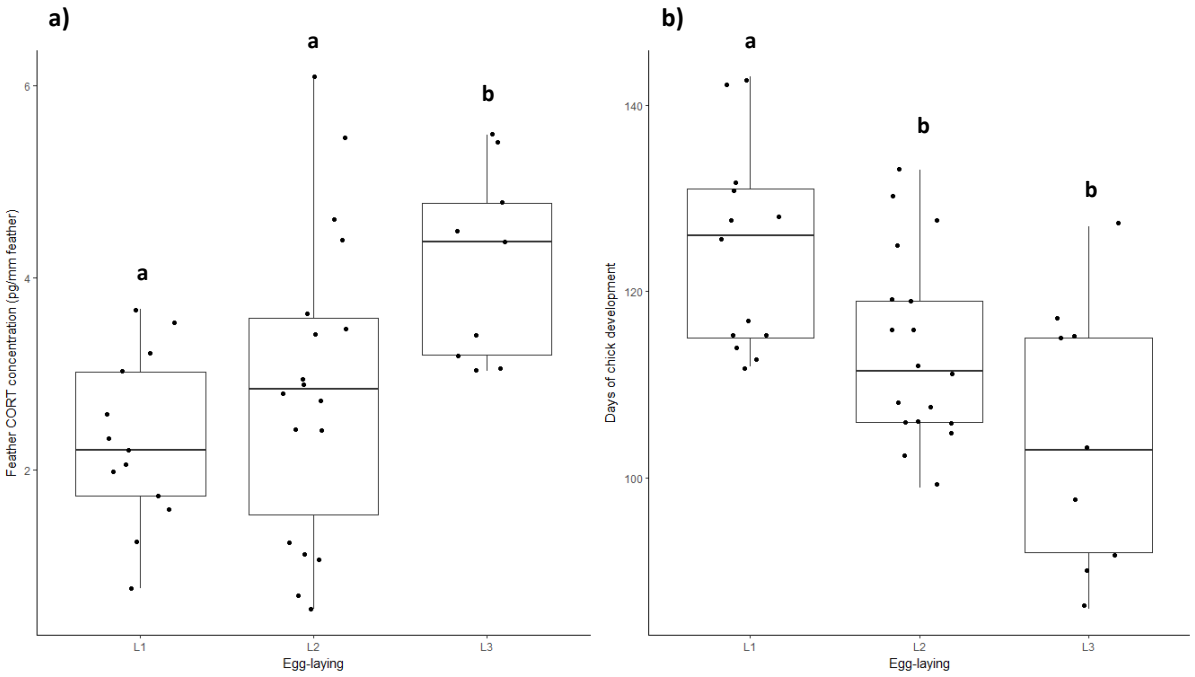

Fig. 3 Relationship between egg-laying (L1, L2, L3) and **a)** levels of feather CORT (pg CORT mm^{-1} feather); **b)** days of chick development in nestling cinereous vultures (*n* = 42). Box plots include the median value (thick line), the interquartile range (top and bottom of the box) and the maximum and minimum values within 1.5 interquartile range (whiskers). *Lower case* indicate significant differences (P<0.05).

5. Discussion

5.1 Contaminant accumulation in nestling cinereous vultures

Few data on contaminants exists in vultures (Gómara et al., 2004; Goutner et al., 2011; Van Drooge et al., 2008) and are particularly scarce when monitoring 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., *submitted EP*). In the latter study, down feathers were reported as a suitable matrix to biomonitor contamination in nestling vultures; further, down feathers were suggested to probably reflect concentrations transferred by the mother to the egg. In the present discussion we compare levels with existing literature and in particular with our preliminary results in nestling cinereous vultures (Monclús et al., *submitted EP*).

The present 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 mainly 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 type analyzed, with different growing 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., *submitted EP*) 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. 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., *submitted EP*). 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., *submitted EP*) 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).

5.2 Relationship between contaminants accumulation, CORTf levels, egg-laying 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 POP concentrations 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 improving energy intake through begging (Kitaysky et al., 2003). On the other hand, contaminants could have acted as environmental stressors. Considering that contaminant concentrations in down feathers probably reflect the contaminant burden transferred by the mother via the egg (Monclús et al., *submitted EP*), 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.

It should be considered that in large raptor species as vultures, new parents may present delayed laying in comparison to older parents (Bernis, 1966) 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 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.

Regarding the relationship with contaminants, 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 OPEs). This is the first investigation exploring whether OPEs affect 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 OPE analysis (n = 22) in comparison to the POP analysis (n = 42). However, the strong and positive effect of the most persistent POP compounds on 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). In our study, the positive relationship between POP 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 influenced CORT concentrations. These two previous studies suggested that high concentrations of 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. *in preparation*) 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" proprieties, 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 affected 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 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 unpublished findings of Monclús et al. (*in preparation*).

6. 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 is correlative, and thus cannot explain the exact impact of contaminants on the CORT regulation, we provide evidence for a strong effect of POPs, especially the most persistent compounds, on the CORT secretion while no effect was found for the OPE compounds. However, although POPs may act as stressors being associated with high CORTf concentrations, they were not observed to impact the growing 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 disrupting consequences. However, because higher contaminant burdens could compromise HPA functioning and 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.

7. Supplementary Information

Appendix S1

Gas chromatography-mass spectrometer (GC/MS) analysis

Analysis of POPs was done using a 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 using a 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 blanks 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 0.05 ng g^{-1} dw for PCBs, HCB and HCHs, 0.1 ng g^{-1} dw for *p,p'*-DDE and *p,p'*-DDD, 0.03 ng g^{-1} dw for PBDEs and 1.0 ng g^{-1} dw for individual OPEs.

Appendix S2

Biochemical validation of feather CORT extraction protocol

Feather CORT concentrations were quantified using a competitive enzyme immunoassay (EIA) kit (Neogen® Corporation, Ayr, UK). Extracts from 40 nestling cinereous vultures were used to biochemically validate the immunoassay, following the essential criteria described by Sheriff et al.[1]. The mean intra-assay coefficient of variation was 3.89% indicating good repeatability and precision of the test. The sensitivity was 0.98 pg mm^{-1} feather CORT and all samples were above the detection limit. Regarding the dilution test, the efficiency for feather CORT extraction was 102.31 ± 18.9% with a R^2=0.99. The average recovery percentage from the spike-and-recovery test was 91.76 ± 13.7%. The standard and pool curves showed parallel displacement (R^2=0.89, P<0.001). These results indicate good accuracy and specificity of the EIA. The cross-reactivity of the CORT antibody is as follows: 38% deoxycorticosterone; 19% 6-

hydroxycorticosterone; 5.1% progesterone; 2.7% tetrahydrocorticosterone; 1.5% prednisolone; 1.1% cortisol. Steroids with cross-reactivity <0.9% are not presented.

[1] Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869–87. doi:10.1007/s00442-011-1943-y

Table S1. Descriptive statistics for biological variables and CORT concentrations in nestling cinereous vultures (*n* = 42).

Variables	Mean	SE	Median	Range (min - max)
Age (d)	56.82	2.22	53.00	35 - 89
Time of development (d)	115.84	1.84	116	86 - 143
Feather CORT (pg mm^{-1} feather)	2.95	1.58	2.91	0.55 - 6.09

Table S2. Extracted principal component (PC) scores for logarithmically transformed concentrations of POPs in down feathers of nestling cinereous vultures (*n* = 42). PC loadings indicate how much each POP compound contributes to PC1 and PC2, respectively.

	PC1-POPs	PC2-POPs
Eigenvalues	5.338	2.410
Proportion of variance	41.058	18.542
Cumulative proportion	41.058	59.600
Loadings		
CB-105	**-0.782**	-0.102
CB-118	**-0.833**	-0.208
CB-138	**-0.756**	0.346
CB-153	**-0.756**	0.346
CB-170	**0.758**	0.351
CB-177	0.315	**0.635**
CB-180	**0.895**	0.001
CB-183	0.368	**0.566**
CB-187	**0.712**	-0.326
CB-194	**0.755**	0.027
p,p'-DDE	**0.511**	-0.505
α-HCH	-0.316	**0.510**
BDE-99	-0.518	**0.565**

Note: HCB, *β*-HCH, *γ*-HCH and BDE-47 were eliminated as these compounds explained very little variation (<0.3 in both PCs).

Table S3. Extracted principal component (PC) scores for logarithmically transformed concentrations of POPs and OPEs in down feathers of nestling cinereous vultures (*n* = 22). PC loadings indicate how much each POP and OPE compound contributes to PC1 and PC2, respectively.

	PC1-all	PC2-all
Eigenvalues	5.205	2.906
Proportion of variance	44.151	21.100
Cumulative proportion	44.151	65.251
Loadings		
CB-105	**-0.736**	-0.201
CB-118	**-0.863**	0.071
CB-138	**-0.776**	0.366
CB-153	-0.263	**-0.589**
CB-170	**0.725**	0.522
CB-177	**0.429**	0.420
CB-180	**0.924**	0.098
CB-187	0.593	**-0.683**
CB-194	**0.862**	0.295
p,p'-DDE	0.488	**-0.559**
BDE-99	-0.577	**0.625**
TPhP	0.292	**0.429**

Note: CB-183, HCB, *α*-HCH, *β*-HCH, *γ*-HCH, BDE-47 and TCiPP were eliminated as these compounds explained very little variation (<0.3 in both PCs).

Table S4. ΔAIC values for regression models explaining variation in feather CORT concentrations. The best-supported models (ΔAIC<2) and the null model are shown. Dependent variables included sex, egg-laying (L1, L2, L3) and contaminants [PC-POP lists (*n* = 42); PC-all lists (*n* = 22)].

Contaminant	Model	K	AIC	ΔAIC	L	w
PC1-POPs	CORT ~ PC1-POPs + sex + egg-laying	5	311.79	0	1	0.54
	CORT ~ PC1-POPs x sex + egg-laying	6	313.79	1.99	0.37	0.27
	CORT ~ Null	2	353.87	42.09	<0.001	<0.001
PC2-POPs	CORT ~ PC2-POPs + sex + egg-laying	5	321.36	0	1	0.59
	CORT ~ PC2-POPs x sex + egg-laying	6	621.36	0.73	0.69	0.40
	CORT ~ Null	2	353.87	32.51	<0.001	<0.001
PC1-all	CORT ~ sex + egg-laying	4	149.28	0	1	0.61
	CORT ~ PC1-all + sex + egg-laying	5	151.24	1.96	0.37	0.23
	CORT ~ Null	2	155.58	6.30	0.04	0.03
PC2-all	CORT ~ PC2-all + sex + egg-laying	5	149.06	0	1	0.36
	CORT ~ sex + egg-laying	4	149.29	0.23	0.89	0.32
	CORT ~ PC2-all x sex + egg-laying	6	149.51	0.45	0.79	0.29
	CORT ~ Null	2	155.58	6.52	0.04	0.01

Table S5. ΔAIC values for regression models explaining variation in time of chick development (days from hatching to fledging). The best supported model and the null model are shown. Dependent variables included feather CORT concentrations (pg CORT mm^{-1} feather), sex, date of egg laying (L1, L2, L3) and contaminants [PC-POPs (*n* = 42); PC-all (*n* = 22)].

Contaminant	Model	K	AIC	ΔAIC	L	w
PC1-POPs	Time of development ~ CORTf + PC1-POPs x sex + egg-laying	6	291.55	0	1	0.43
	Time of development ~ PC1-POPs + sex + egg-laying	4	291.63	0.08	0.96	0.41
	Time of development ~ Null	1	332.11	40.56	<0.001	<0.001
PC2-POPs	Time of development ~ CORTf + PC2-POPs + sex + egg-laying	5	292.98	0	1	0.68
	Time of development ~ CORTf + PC2-POPs x sex + egg-laying	6	294.51	1.54	0.46	0.32
	Time of development ~ Null	1	332.11	39.13	<0.001	<0.001
PC1-all	Time of development ~ PC1-all + sex + egg-laying	4	116.28	0	1	0.37
	Time of development ~ Null	1	116.60	0.32	0.85	0.32
PC2-all	Time of development ~ Null	1	116.60	0	1	0.48
	Time of development ~ CORTf + PC2-all + sex + egg-laying	5	118.13	1.53	0.47	0.22

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CHAPTER VI

Influence of persistent organic pollutants on the endocrine stress response in free-living and captive red kites (*Milvus milvus*)

Photo ®: Carlos Monclús Guardia

Influence of persistent organic pollutants on the endocrine stress response in free-living and captive red kites (*Milvus milvus*)

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1. Abstract

Persistent Organic Pollutants (POPs) have the potential to impair the endocrine regulation of organisms and alter their ability to respond to environmental changes. We studied whether polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) affected the endocrine regulation of free-living and captive red kites (*Milvus milvus*) through studying the dynamics of corticosterone (CORT) and dehydroepiandrosterone (DHEA). We used feathers as a minimally-invasive and integrative matrix. The most abundant compound detected was 4,4'-DDE closely followed by CB-153 and CB-180. Free-living kites showed the highest levels of 4,4'-DDE and CB-180. Age influenced HCB and CB-101 levels, whereas body mass was inversely related to CB-180 and 4,4'-DDT. Interestingly, captive kites showed a ratio DDE/DDT lower than 1 suggesting a relatively recent exposure of DDT, in contrast to free-living kites. Regarding hormonal levels, free-living kites showed higher CORT concentrations than captive kites, reflecting higher allostatic load. In addition, free-living kites showed a positive association between POPs and adrenal hormones, suggesting an increase of CORT as a response of the endocrine system to cope with stressors and a subsequent elevation of DHEA to ameliorate the potential negative effects that high CORT levels could cause to the organism.

2. Introduction

Worldwide contamination of ecosystems has rapidly become one of the main concerns for society today (Bustnes et al., 2008). An important group of contaminants are the persistent organic pollutants (POPs), that although were banned decades ago, are still widespread in the environment (Orton et al., 2013) and can be found in multiple tissues and fluid samples from many species (Espín et al., 2010). One of the major concerns about POPs is the adverse effects that can cause to human (Schettgen et al., 2015) and wildlife health (Letcher et al., 2010; Morales et al., 2012) due to their long environment persistence and their biomagnification and bioaccumulation properties (Henny et al., 2003). In that sense, birds of prey are particularly at risk due to their apex trophic position, long life span and expanded home ranges (Furness, 1993), and have been extensively used as sentinel species to monitor environmental contamination (Espín et al., 2010; Jaspers et al., 2006; Meillère et al., 2016).

The ability of POPs to disrupt the endocrine system has gained increasing attention (Giesy et al., 2003; Hampl et al., 2016; Maqbool et al., 2015) since high POP levels have been associated to physiological, neurological and reproduction impairment (García-Fernández et al., 2008). These contaminants possibly interfere with several steps of endocrine regulation via interacting with hormone receptors, or modulating the hormone synthesis, transport and degradation (Hampl et al., 2016; Maqbool et al., 2015). Although the mechanisms of interaction are still not well known, it has been observed that POPs can disrupt homeostasis and increase dramatically the allostatic load of organisms (Bustnes et al., 2001). Allostatic load is defined as the accumulative physiological costs of neuroendocrine responses that result from repeated or chronic exposure to stressors (McEwen and Wingfield, 2003). In that respect, measuring glucocorticoids can be very useful as these hormones are one of the main mediators of allostasis in vertebrates (McEwen and Wingfield, 2003). In the presence of a stressor, the hypothalamic-pituitary-adrenal (HPA) axis synthesizes and releases glucocorticoid concentrations into blood stream, in birds corticosterone (CORT), with the aim to restore homeostasis and promote behavioral responses to overcome the stress status and return to initial conditions (Wingfield, 2013). However, under chronic and intensive stressors, prolonged elevations of CORT concentrations can have deleterious effects for organisms on a range of fitness-related traits, such as immunosuppression (Padgett and Glaser, 2003), reduced growth (Hull et al., 2007) and low reproduction and survival rates (Cyr and Romero, 2007; Monclús et al., 2017a).

Because the prolonged activation of the HPA axis is not without a cost, there are mechanisms to ameliorate the consequences of an excessive stress response. One of the main mechanisms is the synthesis and secretion of dehydroepiandrosterone (DHEA). This hormone has important protective anti-stress activity due to its anti-glucocorticoid properties, including suppression of glucocorticoid receptors and protection for the neurotoxic effects of high glucocorticoid levels (Hu et al., 2000; Kalimi et al., 1994). The HPA axis is considered to be one of the most important regulatory pathways birds use to deal with changes in the environment (Sapolsky, 2000) and thus an effective functioning is imperative for successful adaptation. However, little is known about the potential effects of POPs on CORT (Nordstad et al., 2012; Verboven et al., 2010), and so far no study has been focused on DHEA.

Earlier studies have used feathers as a non-destructive and minimally-invasive matrix able to measure long-term levels of organic pollutants and steroid hormones. During the period of feather grow, feathers accumulate circulating pollutants and steroid hormones proportionately to blood levels (Bortolotti et al., 2008; Eulaers et al., 2011; Jaspers et al., 2007; Jenni-Eiermann et al., 2015; Van Den Steen et al., 2007). With respect to pollutants, feathers have been reported to provide a valuable assessment of internal body concentrations of POPs (Eulaers et al., 2011; Jaspers et al., 2007, 2006). Notwithstanding, it is important to consider the possible influence of preen oil in the feather contaminant concentrations. Preen oil is an excretion pathway of lipophilic pollutants and can also contribute to the absorption of aerial POPs on the feathers (Espín et al., 2016). The degree of contamination of feathers with preen oil seems to be related to the preening frequency of each species (Espín et al., 2016; García-Fernández et al., 2013) and type of feather, being body feathers the less influenced by this external contamination (Jaspers et al., 2011). Notwithstanding, external contamination by preen oil may be an advantage since contaminants accumulated in preen oil are closely related to blood and internal levels (Eulaers et al., 2011; Jaspers et al., 2011), and thus can enhance the correlation between contaminant concentrations in feathers and in internal tissues (Espín et al., 2016; Eulaers et al., 2011; Jaspers et al., 2008).

The main aim of this study was to investigate whether high concentrations of POPs affect the HPA axis activity through studying the dynamics of CORT and DHEA hormones. This study was the first to analyze feather DHEA (DHEAf) concentrations and explored its relationship with feather CORT (CORTf) concentrations. We chose red kite (*Milvus milvus*) as the model species due to their high position in trophic webs. In addition, high POP levels were previously detected in this endangered species and were suggested to be one of the main concerns for its

conservation (Gómara et al., 2008; Jiménez et al., 2007). In this study, two different types of populations were compared, free-living *vs.* captive kites, in order to study the effects of contaminants in two different living situations (i.e. high energetic requirements in free-living kites *vs*. controlled and available resources in captivity).

3. Material and Methods

3.1 Study species and field procedures

Red kite is a medium-sized raptor endemic from Europe, classified as Near Threatened by the IUCN (BirdLife International, 2017). The distribution range cover most European countries that breeds in whole Europe and winters mainly in Spain (BirdLife International, 2017). Migrants travel from their breeding grounds to Southern areas between August and November and return between February and May (Snow and Perrins, 1998). This species is a generalist predator and usually feed on small to medium-sized mammals and birds, but is also considered a scavenger (BirdLife International, 2017). Between November 2015 and February 2016, 28 captive and 66 free-living red kites were sampled. Captive red kites formed part of a reproductive population maintained in a rehabilitation center (Zaragoza, Spain). Almost all captive kites (28) were born in captivity except 5 individuals that were found in the field and were incorporated to the reproductive program. All captive kites were fed with controlled diet (mice raised in animal facilities). Free-living red kites were captured in a wintering location in Binaced (Huesca, Spain) after their migration from Northern Europe, mainly from Germany, France and Switzerland. Binaced was the chosen area to capture free-living kites as it constitutes one of the main wintering areas in Spain (De la Puente et al., 2009). Free-living kites were trapped with an automatic pull net in a supplementary feeding area for raptors (De la Puente et al., 2009). Immediately after capture, birds were banded with a metal and color ring, and aged by plumage and molt (Forsman, 1999). Blood was collected for sex determination using the PCR protocol developed by Griffiths et al. (1998). Body mass (weight) was accurately measured for each bird (nearest 0.1 g). Juveniles (1st calendar year), subadults (2nd calendar year) and adults (≥3rd calendar year) of both sexes were sampled. We provide age and sex distribution per each type of population in Table S1 in Supplementary Information. Six to eleven body feathers were collected from each individual. We sampled interscapular feathers as their removal is considered to be less invasive than other types of feathers and may not affect the individuals' fitness (Monclús et al., 2017a). These feathers grow during the spring season, mainly during April - August in adults and May - June in juveniles (Forsman, 1999). Feathers were kept in individual paper bags, labeled appropriately and stored at room

temperature until analysis. All free-living kites were released in good state after banding and sampling. Captures and samplings were performed in compliance with the requirements of regional and Spanish authorities.

3.2 Hormonal analysis

All individuals were assayed for hormonal analyses (*n* = 66 free-living; *n* = 28 captive). For each individual, one unwashed feather was measured (length) with a caliper to the nearest 0.1 mm (ranging from 65 to 83 mm) and weighed with a precision scale to the nearest 0.1 mg (ranging from 28 to 38 mg). A method to extract CORT and DHEA from feathers was optimized from that described by Bortolotti et al. (2008) and was properly validated in an earlier study (Monclús et al., 2017b). Briefly, after discarding the calamus, feathers were minced with a ball-mill (Retsch®, MM2 type, Germany) for 5 min at 25 Hz. Then, 1.5 mL of methanol (methanol reagent grade 99.9%; Scharlab, Sentmenat, Spain) was added and samples were placed in a vortex (Vortex Mixer S0200-230 V-EU; Labnet International, NJ) for 30 min at room temperature, followed by incubation (G24 Environmental Incubator Shaker; New Brunswick Scientific, Edison, NJ) for 18 h at 32 °C for steroid extraction. Samples were centrifuged (Hermle Z300K; Hermle® Labortechnik, Wehingen, Germany) at 6000 rpm for 15 min at 25 °C and 1 mL of supernatant was transferred to a new aliquot. Samples were then placed in an oven at 37 °C until dryness. Dried extract were reconstituted with 0.25 mL of buffer provided by the enzyme immunoassay kit (ELISA Neogen® Corporation, Ayr, UK), shaken for 1 min, and immediately stored at -20 °C until analysis. All feather hormone values were expressed as a function of feather length (pg mm^{-1}) (Bortolotti et al., 2008). We provide biochemical validation for CORTf and DHEAf in Appendix S1.

3.3 POPs analysis

Only 56 kites (*n* = 47 free-living; *n* = 9 captive) were assayed for POP analysis as we did not have enough feather mass for both hormone and contaminant analyses for the rest of individuals. In such 56 kites, the feathers remaining after the hormonal analyses were used to analyze POPs (from seven to ten feathers per individual). The captive kites analyzed were all born in captivity. Following Jaspers et al. (2011, 2007), feathers were washed thoroughly with distilled water prior to the analysis of POPs. After the washing procedure, feathers were covered with standard laboratory paper and dried overnight at room temperature. Dried feathers were weighted (from 150 to 250 mg), minced with a ball-mill for 5 min at 25 Hz to obtain powder and transferred to analytical glass recipients. Because low concentrations were

expected, the extraction method had to be refined from that previously described (Morales et al., 2012). Initially, feather samples were spike with 20 ng of surrogate mix solution (described in Appendix S2) and 10 mL of hexane:dichloromethane (1:1; v:v) were added. The samples were then vortexed (1 min) and ultrasonic extracted (10 min) at room temperature. This operation was repeated three times without changing the solvent. Afterwards, the samples were centrifuged at 3000 rpm at 10 ºC for 10 min and the supernatants were transferred to new vials and evaporated to 1 ml using TurboVap® (Caliper®, Massachusetts, USA). The extracts were then purified via Florisil® cartridges (Strata FL-PR 5 g/20 mL, 170 µm, 80 A, Phenomenex® Spain), previously conditioned with 20 mL of hexane:dichloromethane (1:1; v:v). The extracts were eluted with 15 mL of hexane:dichloromethane (1:1; v:v) and the solvents collected were evaporated to almost dryness using a TurboVap®. Finally, the extracts collected were placed in a chromatographic vial with a 100 µL insert and evaporated to 50 µL using ReactiVap® (Pierce®, Rockford, USA).

We analyzed the following compounds: polychlorinated biphenyls (PCBs; CB -28, -52, -101, -138, -153 and -180), dichlorodiphenyltrichloroethane (DDT) and metabolites (4,4'-DDT, 2,4'-DDT, 4,4'-DDE, 2,4'-DDE, 4,4'-DDD and 2,4'-DDD), the four isomers of hexachlorocyclohexane (*α*-, *β*-, *γ*- and *δ*-HCH), hexachlorobenzene (HCB), heptachlor epoxides, *α*- and *β*-endosulphane, trans-, cis- and oxychlordane, metoxychlor, aldrin, isodrin, dieldrin and mirex, hexachlorobutadiene (HCBD) and pentachlorobenzene (PeCB). The concentrations of PCB and OCP were quantified using gas chromatograph (GC, Agilent® 6890, Agilent, CA, USA) coupled with a quadruple mass spectrometer system (MS, Agilent® 5973N, Agilent, CA, USA). The detailed description of GC/MS analysis is given in Appendix S2 and a chromatogram example is provided in Fig S1. For quality assurance, in each batch of 6 samples, a procedural blank was prepared and analyzed. The Limit of Quantification (LOQ) was set at 3 * SD of the procedural blank values. Quality controls (n=5) were performed using feathers from captive red kites. Pooled feathers were spiked with 20 ng of natives (2 ng for PCBs) and 20 ng of surrogates were added to control the extraction and analytical method. Reproducibility and repeatability tests (n=5) were calculated at 0.5 µg/ml of native standards (0.05 µg/ml for PCBs). All calibration curves (range: 0.005-0.5 µg/ml) fitted well with a linear model ($R^2 \geq 0.99$). Details for quality parameters and LOQs are provided in Table S2. Concentrations of analytes were expressed as ng g^{-1} dry weight (dw).

3.4 Statistical analysis

All analyses were carried out using R version 3.2.2 (R-project, R Development Core Team, University of Auckland, New Zealand). Prior to analyses, Shapiro–Wilk tests were performed to check normality. When necessary, data was log-transformed (log(x+1)) to approach a normal distribution. A P-value<0.05 was established as a criterion for significance. We reported average mean ± SE, median and range.

We used linear models (LMs) to test the influence of age (three-level factor: adult, subadult and juvenile), sex, type of population (two-level factor: captive, free-living) and their interactions on body mass. Similarly, LMs were carried out to test the influence of age, sex, body mass, type of population and their interactions on concentrations of CORTf and DHEAf. Pearson's correlation between hormones was also provided for each population. For these analyses, we used data obtained from 94 kites (*n* = 66 free-living; *n* = 28 captive).

Of the total 94 kites, contaminant analysis was only performed in 56 kites (*n* = 47 free-living; *n* = 9 captive). Samples that showed POP concentrations below LOQ, were assigned a value of DF x LOQ with DF (detection frequency) the proportion of measurements with levels above the LOQ (Voorspoels et al., 2002). Different DF was used for the two populations. Compounds with over the 50% of the measurements below the LOQ were excluded from subsequent statistical analysis. We evaluated the effect of age, sex, type of population and body mass on concentrations of each POP compound using LMs. Then, POP compounds were categorized in groups based on similarities in chemical structure and we checked the influence of each group on concentrations of CORTf and DHEAf. Sex, age, type of population and body mass were also included as explanatory variables.

In all cases, all explanatory variables and their interactions were included in the model and were removed when insignificant in a backward stepwise procedure. We used Akaike's Information Criterion (AIC) and Akaike weight (Wi) (likelihood that a given model is the best among all candidate models) to rank models in each set (Burnham and Anderson, 2002). The model with the greatest Wi and lower AIC value indicated the most parsimonious model, that is the model that best fits the data without over-parameterization (Burnham and Anderson, 2002). Models with ΔAIC less than 2 units from the best-supported were also considered (Burnham and Anderson, 2002). To test the strength of the variables, each variable's relative importance (VI) was calculated by summing up the AIC weights of all models which included a certain variable (Burnham and Anderson, 2002). Variables with high VI mean that are included in many models with high AIC weights and thus have strong importance in explaining variation

of the dependent variable. Tables S3, S4, S5 and S6 report the best-supported and the null models.

4. Results

4.1 Biometric parameters

The best-supported model explaining body mass variation was the one including the interaction population*age and sex*age (Table S3). Body mass was statistically higher in females (on average 1055 ± 93.5 g) than in males (911 ± 82.3 g) in all age groups (sex*age, $F_{2.85}$=5.55, P<0.01; sex, $F_{1.85}$=92.14, P<0.001; age, $F_{2.85}$=9.59, P<0.001; Fig. S2). Free-living birds showed higher body mass (on average 996 ± 101.6 g) than captive birds (on average 926 ± 102.7 g), either when type of population was tested alone ($F_{1.87}$=20.42, P<0.001) or through its interaction with age (age*population, $F_{2.85}$=1.28, P<0.001, Fig. S2).

4.2 Dynamics of hormonal levels

Mean hormonal concentrations in feathers of red kites and hormonal concentrations according to population, sex and age are presented in Table 1. The top set candidate models explaining variations in CORTf concentrations included type of population and sex (ΔAIC<2, Table S4). Significant differences were found in CORTf concentrations between the two populations (t=3.11, P<0.01), with free-living kites displaying the highest levels, while no difference was found between sexes (t=1.00, P=0.32) (Table 1). When examining variations in DHEAf concentrations and in CORTf/DHEAf ratio, no clear model was found. In fact, the null model was the best-supported only followed by including sex (ΔAIC=1.85) in the DHEAf model and age (ΔAIC=1.09) in the CORTf/DHEAf ratio model (Table S4), suggesting that these factors explained very little variation. In fact, sex and age did not influence neither DHEAf (sex: t=0.38, P=0.71) nor CORTf/DHEAf ration (age: t>1.2, P>0.05). Fig. 1 illustrates the positive correlation found in free-living birds between concentrations of CORTf and DHEAf (r^2=0.33, P=0.01) and the lack of correlation in captive kites (r^2=0.09, P=0.62).

Table 1 Hormonal concentrations (CORT and DHEA) in feather samples (pg mm^{-1} feather) of red kites according to type of population, age and sex. *Asterisks* define significant differences (P<0.05).

Hormonal concentrations					
			CORT	DHEA	RATIO
Population	Free-living	Mean ± SE	3.30 ± 0.22*	0.42 ± 0.06	29.04 ± 10.68
	(*n* = 66)	Median (range)	2.90 (1.33-13.47)	0.33 (<0.01-3.38)	10.39 (2.01->100)
	Captive	Mean ± SE	2.40 ± 0.16*	0.33 ± 0.08	39.16 ± 16.42
	(*n* = 28)	Median (range)	2.26 (0.96-4.27)	0.17 (<0.01-1.80)	11.70 (2.26->100)
Sex	Males	Mean ± SE	3.17 ± 0.27	0.45 ± 0.08	38.69 ± 12.70
	(*n* = 52)	Median (range)	2.68 (0.96-13.47)	0.33 (<0.01-3.38)	9.92 (2.78->100)
	Females	Mean ± SE	2.81 ± 0.16	0.32 ± 0.05	24.68 ± 12.39
	(*n* = 42)	Median (range)	2.70 (1.19-4.27)	0.23 (<0.01-1.80)	11.58 (5.77->100)
Age	Adult	Mean ± SE	2.86 ± 0.36	0.37 ± 0.09	46.08 ± 21.84
	(*n* = 26)	Median (range)	2.97 (1.19-5.72)	0.17 (<0.01-1.80)	10.83 (2.27->100)
	Subadult	Mean ± SE	3.04 ± 0.52	0.44 ± 0.09	20.52 ± 19.71
	(*n* = 14)	Median (range)	2.65 (1.33-8.04)	0.38 (0.03-0.92)	9.29 (4.24->100)
	Juvenile	Mean ± SE	3.07 ± 0.25	0.39 ± 0.07	21.50 ± 8.67
	(*n* = 54)	Median (range)	2.64 (0.96-13.47)	0.28 (<0.01-3.38)	9.73 (2.09->100)

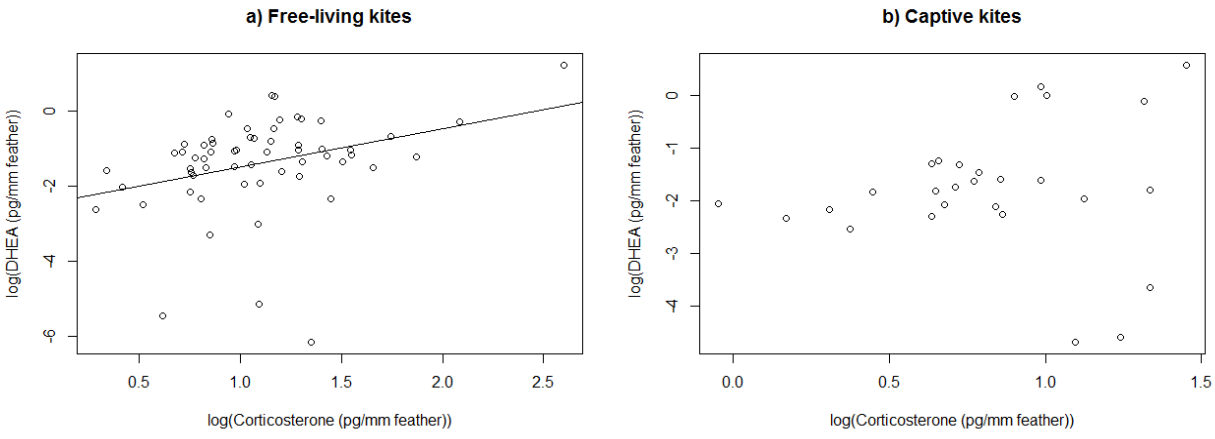

Fig. 1 Linear fit of the relationship between feather DHEA concentrations and feather CORT concentrations (pg mm^{-1} feather) in red kites for **a)** free-living population (*n* = 66) and **b)** captive population (*n* = 28). The correlation was significant for the free-living birds (y = 0.49x-0.81; R^2=0.30, P<0.001) but was not significant for the captives (R^2=0.09, P=0.62).

4.3 Dynamics of contaminant levels

Out of the 30 POPs analyzed in feathers of red kites, we could identify 21 compounds (PCBs: -28, -52, -101, -138, -153 and -180; OCPs: 4,4'-DDT, 2,4'-DDT, 4,4'-DDE, 2,4'-DDE, 4,4'-DDD, 2,4'-DDD, HCB, HCBD and PeCB). Only five PCBs (CB -52, -101, -138, -153, -180) and five OCPs (4,4'-DDT, 4,4'-DDE, HCB, HCBD and PeCB) were detected in at least 50% of the population (Table 2) and these were included in further statistical analyses. CB-101 and CB-180 were detected below the 50% in captive kites (Table 2) but were also included in statistics as these compounds were highly detected in free-living kites. The most abundant compounds in free-living kites were 4,4'-DDE closely followed by CB-153 and CB-180 and 4,4'-DDT was the predominant in captive kites followed by CB-153 and HCB (Table 2; Fig. S3).

Table 2. Concentrations (ng g^{-1} dw) and detection frequencies (DF) are shown for POP compounds in feathers of red kites. Significant differences ($P<0.05$)* and tendencies ($0.05 \leq P > 0.1$)^{T} between populations are shown.

	DF %	Mean ± SE ng g^{-1} dw	Median ng g^{-1} dw	Min - max ng g^{-1} dw
Free-living individuals (*n* = 47)				
∑PCBs		9.13 ± 2.63	5.13	0.56 - 122.44
CB 52	88	0.81 ± 0.20	0.37	0.01 - 7.06
CB 101	53	0.85 ± 0.31	0.21	0.03 - 13.83
CB 138	100	1.94 ± 0.59	0.83	0.28 - 27.36
CB 153	98	3.10 ± 0.63	2.01	0.10 - 30.09
CB 180	80	2.43 ± 1.08^{T}	0.68	0.04 - 49.26
∑DDTs		8.33 ± 2.06	4.40	1.23 - 165.83
4,4'DDT	81	2.19 ± 0.92	0.68	0.25 - 41.29
4,4'DDE	91	6.10 ± 1.56*	1.91	0.16 - 44.19
DDE/DDT ratio		5.04 ± 1.03	1.92	0.36 - 33.43
HCB	100	2.07± 0.14	1.77	0.81 - 6.03
PeCB	100	0.80 ± 0.09	0.64	0.08 - 4.19
HCBD	98	0.25 ± 0.02	0.21	0.03 - 0.84
Captive individuals (*n* = 9)				
∑PCBs		6.89 ± 2.12	6.96	0.39 - 18.99
CB 52	88	1.30 ± 0.57	0.44	0.01 - 2.12
CB 101	34	0.60 ± 0.31	0.03	0.03 - 2.17
CB 138	100	1.39 ± 0.45	0.83	0.12 - 4.24
CB 153	89	2.15 ± 0.47	1.77	0.10 - 5.09
CB 180	45	1.45 ± 0.79^{T}	0.03	0.04 - 7.09
∑DDTs		4.09 ± 1.88	6.95	1.23 - 31.25
4,4'DDT	56	2.38 ± 1.30	0.30	0.29 - 9.62
4,4'DDE	78	1.71 ± 0.66*	1.02	0.16 - 6.03
DDE/DDT ratio		1.73 ± 0.71	0.70	0.31 - 6.64
HCB	89	2.03 ± 0.45	1.47	0.04 - 4.36
PeCB	100	0.87 ± 0.14	0.80	0.41 - 1.45
HCBD	100	0.23 ± 0.03	0.21	0.13 - 0.42

For ∑PCBs, CB-52, CB-138, CB-153, HCBD and PeCB the null model was the best-supported (Table S5). The model including age was the best-supported for CB-101 and HCB (Table S5), albeit the null model was only 0.07 and 0.47 AIC points less for CB-101 and HCB respectively. Following the best-supported model we found that juvenile individuals showed a tendency of increase of CB-101 levels (estimate ± SE=1.07 ± 0.56, t=1.91, P=0.06) and a significant increase of HCB levels (estimate ± SE=0.36 ± 0.17, t=2.08, P=0.04) compared to adults (estimate ± SE= -2.49 ± 0.45 and estimate ± SE=0.35 ± 0.14, respectively). The model including type of population and body mass was the best-supported for CB-180 levels (Table S5). We found that free-living individuals showed a tendency to increase CB-180 levels compared to captive individuals (estimate ± SE=1.18 ± 0.62, t=1.90, P=0.06) and an inverse relationship between body mass and levels of CB-180 (estimate ± SE=-0.005 ± 0.002, t=-2.36, P=0.02) (in both cases intercept=3.09 ± 2.03). Finally, for ∑DDTs and 4,4'-DDE the best-supported model was the one including type of population (Table S5). While no variation was found between populations when the sum of DDTs was tested (t=1.63, P=0.11), a significant increase of 4,4'-DDE levels was found in free-living kites in comparison to captive kites (estimate ± SE=1.11 ± 0.49 vs. -0.25 ± 0.44, t=2.29, P=0.03). For 4,4'-DDT, the best-supported model included body mass (Table S5). We found an inverse relationship between body mass and levels of 4,4'-DDT (estimate ± SE=-0.003 ± 0.002 vs. 3.15 ± 1.21, t=-2.72, P<0.01).

4.4 Relationship between hormonal and contaminant concentrations

The best-supported model examining hormonal variations included population, ∑PCBs and ∑DDTs (Table S6). Type of population was included in all the best models and was a strong predictor of CORTf and DHEAf variations presenting high VI (Table 3). Both ∑PCBs and ∑DDTs showed high VI explaining variations of CORTf (Table 3), whereas only ∑PCBs explained DHEAf variation with high VI (Table 3). The estimates showed higher concentrations of CORTf and DHEAf in free-living kites compared to captives (Table 4). A positive relationship was also found between ∑PCBs and CORTf and DHEAf concentrations, while ∑DDTs was only associated to increasing concentrations of CORTf (Table 4). Positive and significant correlations were also found in free-living kites (Table 5).

Table 3. Variable Importance (VI) of different parameters explaining **a)** feather CORT concentrations and **b)** feather DHEA concentrations in red kites. The values were obtained from the model selection procedure. Different models were run for each of the persistent organic pollutant (POP): ∑PCBs, ∑DDTs, HCB, HCBD, PeCB. Variables with a high VI (≥0.80) are shown in bold.

a) feather CORT concentrations						
Variable	POP	Population	Sex	Age	Weight	Weight x sex
∑PCBs	**0.99**	**1.00**	0.45	0.60	0.37	0.34
∑DDTs	**0.80**	**0.99**	0.18	0.33	0.12	0.09
HCB	0.07	**0.99**	0.37	0.15	0.07	0.01
HCBD	0.03	**0.99**	0.07	0.17	0.46	0.01
PeCB	0.03	**0.99**	0.07	0.17	0.46	0.01
b) feather DHEA concentrations						
Variable	POP	Population	Sex	Age	Weight	Weight x sex
∑PCBs	**0.93**	**0.99**	0.53	0.22	0.07	0.02
∑DDTs	0.55	**0.95**	0.27	0.12	0.04	0.01
HCB	0.33	**1.00**	0.76	0.47	0.26	0.01
HCBD	0.09	**0.94**	0.53	0.23	0.03	<0.01
PeCB	0.08	**0.95**	0.53	0.22	0.03	<0.01

Table 4. Model-averaged parameter estimates and associated standard error (*SE)*, *t*- and *P*- values for the best models explaining feather CORT and feather DHEA concentrations in red kites. ∑PCBs and ∑DDTs were included as focal POPs explaining feather CORT variation in separate models, and only ∑PCBs was included as focal POPs explaining feather DHEA due to their high VI. Significant differences (P<0.05)* (P<0.01)** (P<0.001)** and tendencies (0.05≥P<0.1)^{T} are shown in bold.

Model	Dependent	Independent	Estimate ± SE	t-value	P-Value
∑PCBs	CORTf	Intercept	0.53 ± 0.13	4.12	**<0.001*****
		Population: free-living	0.36 ± 0.11	3.07	**<0.001*****
		Log(∑PCBs)	0.16 ± 0.05	3.23	**0.001*****
	DHEAf	Intercept	-3.34 ± 0.47	-7.15	**<0.001*****
		Population: free-living	1.02 ± 0.43	2.39	**0.02***
		Log(∑PCBs)	0.61 ± 0.18	3.48	**<0.001*****
∑DDTs	CORTf	Intercept	0.72 ± 0.12	6.22	**<0.001*****
		Population: free-living	0.34 ± 0.13	2.72	**<0.01****
		Log(∑DDTs)	0.07 ± 0.04	1.85	**0.06**^{T}

Table 5. Correlations for hormonal concentrations and contaminants in feathers of red kites. Significant differences (P<0.05)* (P<0.01)** are shown in bold.

Parameters	Captive individuals			Free-living individuals		
	n	r^2	P	*n*	r^2	P
CORTf-∑PCBs	9	-0.41	0.27	47	0.36	**0.01***
CORTf-∑DDTs	9	-0.09	0.80	47	0.29	**0.04***
CORTf-HCB	9	-0.21	0.59	47	<0.01	0.96
CORTf-HCBD	9	-0.09	0.80	47	-0.15	0.29
CORTf-PeCB	9	-0.14	0.72	47	-0.07	0.64
DHEAf-∑PCBs	9	0.45	0.23	47	0.43	**<0.01****
DHEAf-∑DDTs	9	0.15	0.70	47	0.21	0.15
DHEAf-HCB	9	0.05	0.89	47	0.15	0.29
DHEAf-HCBD	9	0.08	0.84	47	-0.09	0.54
DHEAf-PeCB	9	0.15	0.71	47	0.06	0.68

5. Discussion

The present study is the first to report PCB and OCP concentrations in feathers of red kites comparing free-living *vs*. captive individuals. Moreover, the effect of these compounds on the HPA endocrine profile was tested. Firstly, a different endocrine profile was found between free-living and captive individuals, suggesting a different allostatic load between these two types of populations. Secondly, free-living individuals showed a slightly increase of contaminant levels compared to captive individuals. Finally, a relationship between PCBs and CORTf and DHEAf as well as between DDTs and CORTf were found in free-living individuals, suggesting that these compounds may affect the HPA axis response. Implications of each of these results are discussed below.

5.1 Dynamics of hormonal levels

Free-living kites showed higher concentrations of CORTf compared to captive kites and similar concentrations of DHEAf and similar CORTf/DHEAf ratio. The elevation of CORTf concentrations in free-living kites could have different explanations. First, the wild habitat with potential poor food availability of free-living kites could lead to high energetic requirements affecting CORTf levels in contrast to the husbandry conditions with easy food acquisition of captive kites (Kitaysky et al., 2010; Will et al., 2014). Second, red kites can have a wide period of molt, which can range from April to October (Forsman, 1999), and may overlap with the end of the breeding period and the early autumn migration. In such case, high CORTf levels could

respond to the high energy demands required during reproduction (Pérez et al., 2016) or to the physiological preparation process of birds prior migration (Pérez-Tris et al., 2014). Thus, elevations of CORTf levels would not necessarily reflect chronic stress (Dickens and Romero, 2013), but could also indicate a mechanism to cope successfully with high energetic demanding situations. Finally, it could also be possible that the stress-response were activated to handle a wide range of environmental conditions, such as for example environmental pollution (Meillère et al., 2016; Nordstad et al., 2012).

This study measured DHEA in feathers for the first time in the literature. As mentioned in the introduction, DHEA is a hormone that has the ability to rescue the negative effects caused by chronic elevations of glucocorticoids (Hu et al., 2000; Kalimi et al., 1994). In humans, DHEA has been increasingly studied and measured in minimally-invasive matrixes (Gallagher et al., 2006; Wolkowitz et al., 2001). In such studies, it has been observed that under acute stress stimulus, DHEA increases as cortisol (the main glucocorticoid in humans) rises. Alternatively, under conditions of chronic stress, levels of DHEA remain unchanged or progressively diminish while levels of cortisol increase or do not change, resulting in an elevated cortisol/DHEA ratio (Wolkowitz et al., 2001). Hence, under a scenario of allostasis overload, when the demands exceed the resources available to meet the costs (McEwen and Wingfield, 2003), one could expect to find high levels of cortisol (or CORT in birds) and low DHEA levels, resulting in an elevated CORT/DHEA ratio. In our study, we found that neither DHEAf nor the CORTf/DHEAf ratio differed between populations and, more importantly, we found a positive correlation between CORTf and DHEAf in free-living kites. These findings suggest a different allostatic load between free-living and captive kites, and an adaptive response of the endocrine system in free-living kites that would increase their secretion of DHEA in order to ameliorate the potential damaging effects of high CORT levels. In addition, free-living kites showed better body condition than captives (Fig S2), which would rule out a poor health status of these individuals (Dickens and Romero, 2013).

5.2 Dynamics of contaminant levels

Among analyzed compounds, 4,4'-DDE was the most predominant POP followed by CB-153 and CB-180. Information on concentrations of POP compounds in feathers of red kites is lacking, and therefore meaningful comparisons are difficult to make. However, similar contribution profiles of contaminants can be found in feathers of other raptor species, with *ortho* PCB, especially -153, -138 and -180, and 4,4'-DDE as the most predominant contaminants (Abbasi et al., 2017; Eulaers et al., 2011; Jaspers et al., 2007). Here, we reported

concentrations of HCB and DDT similar to levels previously reported in feathers of black kites (*Milvus migrans*) from Iran (Abbasi et al., 2017), white-tailed eagles (*Haliaeetus albicilla*) from Norway (Eulaers et al., 2011) and common buzzards (*Buteo buteo*) from Belgium (Jaspers et al., 2007, 2006). However, PCB levels were 3 to 18 times lower than those reported in other studies (Eulaers et al., 2011; Jaspers et al., 2011, 2007), probably because we only included 5 congeners in the ∑PCBs sum in comparison to the minimum 11 congeners included in other studies. It is also important to consider that the different feather type analyzed (body feather *vs.* tail or primary feather) can lead to contaminant variations due to the different feather growth rate and consequently to the different spatial and temporal contaminant exposure (Jaspers et al., 2011).

Despite a ban on the use of DDT in Europe in the 70s, this prohibition was not effective in Spain until the 90s (Orden de 4 de Febrero). This fact could explain the different DDE/DDT ratios observed between free-living and captive kites. The metabolite 4,4'-DDE represented more than 70% of the total ∑DDT in free-living kites (Fig S3), indicating either a historic exposure of DDT and the capacity of organisms to metabolize it to DDE, or the degradation of DDT itself to DDE in the environment (Espín et al., 2010; Jiménez et al., 2007). In contrast, captive kites showed a DDE/DDT ratio generally lower than 1 (Table 2), suggesting recent exposure to parent DDT. This unexpected finding indicate the presence of non-degraded DDT in the environment despite its prohibition, and was in agreements with previous studies performed in Spain that have already reported this concerning elevation of DDT (Espín et al., 2010; Gómara et al., 2008). More recently, some studies have suggested that traces of DDT could be attributed to the synthesis of dicofol, a pesticide widely used in Spain (García-Fernández et al., 2008; Martínez-López et al., 2009). However, we were not able to detect dicofol in our samples. In addition, we found that levels of CB-180 and 4,4'-DDE varied between the two types of population, with free-living kites displaying the highest concentrations (although CB-180 only showed a tendency). In the literature, some factors such as industrialized gradient from urban - rural geographical areas, trophic positions, dietary habits and migratory status have been attributed to causing differences in contaminant concentrations between types of populations (Espín et al., 2010; Jaspers et al., 2007; Ortiz-Santaliestra et al., 2015). In a previous study performed on European eel (*Anguilla anguilla*) (Blanchet-Letrouvé et al., 2014), CB-180 appeared to be the only congener able to discriminate amongst sampling sites. In our study, free-living kites were sampled in Spain after their migration from Northern European countries where they molted their body feathers (Forsman, 1999). Given that kites may have migrated from different geographical areas (Germany, France

or Switzerland), they were exposed to different contaminant concentrations during their molting period and, thus, the sampled feathers reflected the contaminant load from those areas. Unfortunately, in this study we could not account for geographical information and thus we could not disentangle POP differences within free-living kites. In fact, this could be the explanation for the high deviance found in the contaminant concentrations in this type of population (Table 2; Fig. S3).

Other factors such as age, sex and body condition have also been reported to influence POP concentrations. Since red kites are very long-lived and thus exposed to contaminants over many years, one could expected to find higher POP concentrations in adult individuals (Jaspers et al., 2013). While we did not find any age-relationship in the most persistent POP compounds, juvenile individuals showed elevated levels of CB-101 and HCB. These compounds are less persistent in the environment and HCB, in particular, can show inter-annual variations (Eulaers et al., 2013) as well as a more rapid clearance rate compared to other POPs (Clark et al., 1987). In fact, levels of these compounds have been related to recent exposure or external contamination of feathers from air-borne sources, more than to biomagnification or bioaccumulation (Dauwe et al., 2005). Due to juveniles and adults molt their feathers in different areas and different time of year, they can be exposed to different contaminant exposure. Regarding sex, we did not find any relationship with POP concentrations even though we expected diminished POP levels in females due to maternal deposition of POPs into eggs (Bustnes et al., 2008b). This could be due to the time gap existing between egg hatching and molt of feathers during which concentrations of POP in females could increase again. Other authors (Espín et al., 2010; Ólafsdóttir et al., 2005) reached the same conclusion after sampling razorbills (*Alca torda*) and black guillemots (*Cepphus grylle*) during the non-breeding season. Finally, we found an inverse relationship between concentrations of 4,4'-DDT and CB-180 and body mass. Body mass has been reported to be an important factor affecting contaminant levels (Bustnes et al., 2008) and birds with decreasing fat deposits have usually been associated to elevated concentrations of POP in blood (Bustnes et al., 2010). These compounds have higher affinity for fatty tissues and, thus, under poor physiological status of the individual leading to lipid mobilization, these compounds are more released into the blood and consequently more levels are accumulated in feathers. However, these results should be interpreted cautiously given the time gap between contaminant deposition in feathers (spring) and body mass record-taking (winter) and the possible interference of preen oil (Eulaers et al., 2014).

5.3 Effects of contaminants in hormone concentrations

Concentrations of PCB and DDT were positively associated to CORTf and DHEAf concentrations in free-living kites. Interestingly, while levels of PCB were positively correlated to levels of both hormones, levels of DDT were only correlated to high levels of CORTf. These findings suggested that although PCBs and DDTs influenced both hormones, PCBs affected the HPA axis activity more severely than DDTs.

The positive association between POP and CORTf concentrations in free-living kites reflected the endocrine response of birds facing environmental stressors by the activation of the HPA axis. In agreement with our results but using a more acute CORT matrix, Nordstad et al. (2012) found a positive relationship between PCB and baseline CORT levels during the pre-breeding season in kittiwakes (*Rissa tridactyla*). Similarly, Verboven et al. (2010) observed that high levels of OCP and other contaminants, such as flame retardants, were positively associated to high baseline CORT levels in glaucous gulls (*Larus hyperboreus*). In that study, males also showed a reduced stress-induced response, suggesting a compromise ability to adapt to a changing environment. In the present study, we also reported increasing DHEAf levels along with POPs and a positive correlation between CORTf and DHEAf. These findings suggested an adaptive response by the HPA axis that increased the secretion of DHEA in order to ameliorate the potential negative effects that high CORT levels could cause to the organism. Therefore, we suggested that although POPs influenced the HPA axis, birds were able to show compensatory mechanisms.

Captive kites, in contrast, did not show any correlation, between CORTf and DHEAf nor between these hormones and contaminants. The higher contaminant concentrations observed in free-living kites compared to captive kites would explain part of this different endocrine condition. High POP levels could cause an increase of the allostatic load of free-living birds and in turn an increase of the glucocorticoid secretion (Sapolsky, 2000). However, it is important to consider that the contaminant variation was only observed in two compounds (4,4'-DDE and CB-180) and such variation was not as large as could it be expected. For instance, Jaspers et al. (2009) reported a 10-to-30-fold contaminant variation between urban and rural magpies (*Pica pica*), while in our study we only observed a 2- to-3-fold. On the other hand, and as stated before, free-living kites could show high allostatic load due to the possible overlapping of breeding and migration seasons with the molting period (Forsman, 1999), incrementing in that way their energy requirements. In such situation, POPs, acting as environmental stressors,

could affect in a more intensive way the HPA axis activity of free-living birds because of their already high allostatic load rather than their high amount of contaminants *per se*.

6. Conclusions

In conclusion, this study is a step forward in the use of feather measurements in bird ecotoxicology research. By implementing the measurement of a new steroid hormone in feathers, DHEAf, this study explores the adaptive response of an organism in managing chronically stressful situations. High POP concentrations could act as endocrine disruptors and affect the functioning of the HPA axis, explaining therefore the high concentrations of CORTf and DHEAf reported in free-living kites. Captive kites, in contrast, showed low POP and CORTf levels. However, the different endocrine profiles of these two types of population could also be explained by the influence of other physiological factors such as migration and reproduction. These periods could overlap with the molting season and therefore contribute to increase the allostatic load of free-living kites. In this line, POP levels could affect the HPA axis activity of free-living birds in a more intensive way than captive birds, due to their already higher allostatic load. Further research focused on the mechanism of interaction with the HPA axis is needed to elucidate the effects of contaminants in context with other physiological factors and to assess the threshold of contaminant burden when allostatic load could turn into allostatic overload.

7. Supplementary Information

Appendix S1

Biochemical validation of the new optimized feather hormone-extraction protocol

Two competitive enzyme immunoassay (EIA) kits were performed to analyze CORT (Neogen® Corporation, Ayr, UK) and DHEA (Arbor Assays®, Mi, USA). Extracts from 20 red kites were used to biochemically validate the immunoassays, following the essential criteria described by Sheriff et al.[1]. The mean intra-assay coefficient of variation (CV) was 5.9% for CORT and 6.3% for DHEA. The inter-assay CV was 6.9% and 9.1% for CORT and DHEA respectively. The sensitivity was 0.53 pg mm^{-1} feather for CORT and 0.007 pg mm^{-1} feather for DHEA. All samples were above the detection limits. Regarding the dilution test, the efficiency for CORT extraction was 105.1±26.3% with a R^2=0.99 and 102.7±2.9% with a R^2=0.99 for DHEA extraction. The average recovery percentage from the spike-and-recovery test was 113.42±9.2% for CORT and

117.84±15.9% for DHEA. The standard and pool curves showed parallel displacement for CORT (R^2=0.93, p=0.02) and for DHEA (R^2=0.96, p<0.01). Overall, these results indicated good repeatability, precision and accuracy for both immunoassays.

[1] Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869–87. doi:10.1007/s00442-011-1943-y

Appendix S2

Chemicals and Internal Standards

The surrogate mix solution was created using $^{13}C_6$ Hexachlorobenzene (100 µg/mL in acetone), PCB-209 (10 µg/mL in cyclohexane) and PCB-65 (98% pureness). Both $^{13}C_6$ Hexachlorobenzene and PCB-209 were acquired from Dr. Ehrenstorfer (Augsburg, Germany), and PCB-65 was acquired from Accustandard (New Haven, USA). The natives used were the following: DDTs and metabolites (4,4'-DDT, 2,4'-DDT, 4,4'-DDE, 2,4'-DDE, 4,4'-DDD and 2,4'-DDD), the four isomers of hexachlorocyclohexane (*α*-, *β*-, *γ*- and *δ*-HCH), hexachlorobenzene (HCB), heptachlor epoxides, *α*- and *β*- endosulphane, trans-, cis- and oxychlordane, metoxychlor, aldrin, isodrin, dieldrin and mirex, at a concentration of 10 µg/mL in toluene, and the PCB congeners (CB -28, -52, -101, -138, -153 and -180) at 1 µg/mL in toluene, were obtained from Pesticide-mix 1 from Accustandar (New Haven, USA). The hexachlorobutadiene (HCBD) (5000 µg/mL in methanol) and pentachlorobenzene (PeCB) (98% pureness) were obtained from Sigma Adrich (St Louis MO, USA). Organic solvents, dichloromethane, n-hexane and isoctane reagents were purchased from Merck (Darmstadt, Germany).

Condition of GC/MS and analysis

For the analysis of PCBs and OCPs, an Agilent® 6890 gas chromatograph (GC, Agilent, CA, USA) coupled with a quadruple mass spectrometer system (MS, Agilent® 5973N, Agilent, CA, USA) was used. The GC was equipped with a 30 m long x 0.25 m ID x 0.25 µm film thickness HP-5 MS column coated with (5%-Phenyl)-methylpolysiloxane (Phenomenex® column, CA, USA) and the MS was operated in electron ionization mode (EI, 70 eV). The temperature program was set at 70 ºC, kept for 1 min, then increased to 175 ºC at 6 ºC/min, kept for 4 min, raised to 235 ºC at 3 ºC/min, and finally raised to 315 ºC at 7 ºC/min and kept at 315 ºC for 10 min. The total runtime was of 55.5 min. The splitless mode was used for injection of 2 µL (inlet temperature 225 ºC) with 7 min solvent delay. Helium was used as a carrier gas at 75.9 kPa with a 1.2

ml/min flow. Temperatures for the injector, transfer line and ion source were 300, 280 and 250 ºC, respectively. Acquisition was performed in time scheduled Multiple Reaction Monitoring (MRM) with two transitions per compound (transitions are shown in Table S1). The software used in this study was Agilent® Massunter Quantitative Analysis B.3.01/Build 3.1.170.0 (Agilent, Santa Clara, CA, USA).

Table S1. Summary of age and sex distribution per type of population of red kites sampled for hormonal and contaminant analysis.

	Hormonal analysis			Contaminant analysis		
	N total	N males	N females	N total	N males	N females
Free-living kites	66	36	30	47	25	22
Adults	11	4	7	10	4	6
Subadults	11	7	4	7	5	2
Juveniles	44	25	19	30	16	14
Captive kites	28	16	11	9	6	3
Adults	15	9	6	7	5	2
Subadults	3	2	1	1	0	1
Juveniles	10	5	5	1	1	0

Table S2. Summary of the transitions and quality parameters for each chemical compound of POPs in feathers of red kites (*n* = 56).

Compound	Recovery ± SD (%)	Repeata-bility (%)	Reproduci-bility (%)	LOQ (ng/g)	Quantifier transition	CE (eV)	Qualifier transition	CE (eV)
PCBs								
CB28	117 ± 5	3.4	1.3	0.20	258→186	30	258→188	30
CB52	116 ± 9	2.6	0.4	0.02	292→222	30	292→220	30
CB101	116 ± 11	1.3	8.6	0.06	326→256	50	326→219	50
CB138	97 ± 12	1.4	8.1	0.11	360→290	30	360→288	30
CB153	102 ± 12	0.9	6.0	0.20	360→290	30	360→288	30
CB180	112 ± 12	1.4	4.7	0.07	394→359	20	394→324	20
DDTs								
4,4'-DDT	122 ± 5	6.8	10.0	0.59	235→199	20	235→165	20
2,4'-DDT	99 ± 8	2.2	2.6	0.38	235→165	40	237→167	40
4,4'-DDD	99 ± 6	6.1	10.2	0.37	235→165	40	237→167	40
2,4'-DDD	110 ± 9	1.0	6.7	0.48	235→200	20	235→165	20
4,4'-DDE	110 ± 8	1.7	1.4	0.33	318→248	20	318→246	20
2,4'-DDE	100 ± 7	2.1	1.9	0.32	318→246	20	318→248	20
OCPs								
HCBD	77 ± 6	2.0	3.5	0.07	260→225	20	260→190	30
PeCB	90 ± 5	2.8	0.5	0.31	250→215	30	250→180	30
α-HCH	106 ± 8	1.8	3.6	0.41	219→183	20	219→147	20
β-HCH	116 ± 11	1.2	14.8	1.22	219→183	20	219→147	20
γ-HCH	120 ± 11	1.2	5.0	0.59	219→183	20	219→147	20
δ-HCH	124 ± 15	2.8	0.7	0.96	219→183	20	219→147	20
HCB	119 ± 4	2.6	2.4	0.09	284→249	20	284→214	20

Aldrin	116 ± 11	3.7	2.1	0.65	263→193	40	263→191	40
Dieldrin	111 ± 9	1.0	1.8	4.19	241→206	30	263→193	30
Endrin	>150	1.1	4.5	1.47	263→228	20	263→193	20
Isodrin	116 ± 9	3.1	2.8	4.07	263→228	30	263→193	30
Heptachlor	>150	3.2	2.8	0.08	272→237	20	272→235	20
Heptachlor epoxides	134 ± 10	4.5	5.5	4.92	353→191	45	353→193	45
Oxychlordane	106 ± 7	4.7	15.8	1.10	185→121	20	185→85	20
α-Endosulfane	101 ± 9	4.7	4.1	2.79	241→206	40	241→170	40
β-Endosulfane	100 ± 8	0.8	2.1	2.52	241→206	15	241→170	15
α-Chlordane	109 ± 8	2.5	2.4	1.51	375→339	30	375→266	20
γ-Chlordane	100 ± 9	1.0	1.8	0.37	375→266	20	373→266	20
Metoxychlor	113 ± 7	9.4	12.1	0.48	227→169	30	227→141	30
Mirex	75 ± 10	1.0	6.0	0.66	332→262	40	332→260	40

Table S3. ΔAIC values for regression models explaining variation in body mass. The best model and the null model are shown. Dependent variables include type of population (free-living, captive); sex; and age (adult, subadult, juvenil).

Independent variable	Explanatory variables	K	AIC	ΔAIC	L	w
Body mass	Population x age + sex : age	10	1073.39	0	1	0.97
	Null	2	1158.18	84.79	<0.01	<0.01

Table S4. ΔAIC values for regression models explaining variation in feather CORT concentration (CORTf), feather DHEA concentration (DHEAf) and feather CORT/DHEA ratio (RATIO). The best models (ΔAIC<2) and the null model are shown. Dependent variables include type of population (free-living, captive); sex; age (adult, subadult, juvenile); and body mass. Separate models were run per each hormone and for the ratio.

Independent variables	Explanatory variables	K	AIC	ΔAIC	L	w
CORTf	Population	3	89.86	0	1	0.52
	Population + sex	4	90.83	0.97	0.62	0.32
	Null	2	97.03	7.17	0.03	0.01
DHEAf	Null	2	314.24	0	1	0.53
	Sex	3	316.09	1.85	0.39	0.21
RATIO	Null	2	283.98	0	1	0.52
	Age	3	285.07	1.09	0.58	0.30

Table S5. ΔAIC values for regression models explaining variation in dry weight concentrations of 10 contaminants in feathers of red kites. Only the best models (ΔAIC<2) are shown. Dependent variables include type of population (free-living, captive); sex; age (adult, subadult, juvenil); and weight. Separate models were run per each contaminant group and per each compound.

Dependent variables	Explanatory variables	K	AIC	ΔAIC	L	w
∑PCBs	Null	2	150.87	0	1	0.53
	Population	3	152.37	1.51	0.47	0.25
CB52	Null	2	206.47	0	1	0.42
	Sex	3	207.44	0.96	0.62	0.26
	Population + sex	4	208.42	1.95	0.38	0.16
CB101	Age	3	233.67	0	1	0.33
	Null	2	233.74	0.07	0.96	0.32
	Age + sex	5	234.48	0.81	0.67	0.22
CB138	Null	2	148.94	0	1	0.43
	Weight	3	149.63	0.69	0.71	0.30
	Population + weight	4	150.80	1.86	0.39	0.17
CB153	Null	2	127.96	0	1	0.39
	Population + weight	4	128.76	0.81	0.67	0.26
	Weight	3	128.80	0.84	0.66	0.25
CB180	Population + weight	4	222.29	0	1	0.43
	Population + weight + age	6	223.72	1.42	0.49	0.21
	Weight	3	223.98	1.69	0.42	0.19
∑DDTs	Population	3	187.02	0	1	0.30
	Population + weight	4	187.27	0.26	0.88	0.26
	Null	2	187.72	0.71	0.70	0.21
4,4'DDT	Weight	3	164.27	0	1	0.49
	Population + weight	4	165.30	1.03	0.60	0.29
4,4'DDE	Population	3	195.03	0	1	0.46
	Population + weight	4	196.41	1.38	0.50	0.23
HCB	Age	4	102.09	0	1	0.35
	Null	2	102.56	0.47	0.80	0.28
	Population + weight + age	6	103.79	1.70	0.43	0.15
HCBD	Null	2	98.23	0	1	0.53
	Weight	3	98.87	0.64	0.72	0.38
PeCB	Null	2	101.63	0	1	0.43
	Population + age	5	103.38	1.75	0.42	0.18

Table S6. ΔAIC values for regression models explaining variation in feather CORT concentration (CORTf) and feather DHEA concentration (DHEAf). The best models (ΔAIC<2) and the null model are shown. Dependent variables include type of population (free-living, captive); sex; age (adult, subadult, juvenil); body mass (BM); and contaminant compounds ∑PCBs, ∑DDTs and HCB, HCBD and PeCB.

Compound	Dependent variables	Explanatory variables	K	AIC	ΔAIC	L	w
∑PCBs	a) CORTf	Population + log(∑PCBs)	4	36.72	0	1	0.40
		Population + sex + age + weight:sex + log(∑PCBs)	9	38.21	1.49	0.48	0.19
		Population + age + log(∑PCBs)	6	38.63	1.91	0.38	0.15
		Null	2	52.37	15.65	<0.001	<0.001
	b) DHEAf	Population + log(∑PCBs)	4	182.06	0	1	0.40
		Population + sex + log(∑PCBs)	5	182.57	0.51	0.78	0.31
		Null	2	195.66	13.59	<0.001	<0.001
∑DDTs	a) CORTf	Population + log(∑DDTs)	4	43.55	0	1	0.45
		Population	3	45.06	1.51	0.47	0.21
		Null	2	52.37	8.82	0.01	<0.001
	b) DHEAf	Population	3	191.60	0	1	0.40
		Population + log(∑DDTs)	4	192.34	0.73	0.69	0.28
		Population + age + log(∑DDTs)	6	193.59	1.99	0.37	0.15
		Null	2	195.66	4.05	0.13	0.05
HCB, HCBD, PeCB*	a) CORTf	Population	3	45.06	0	1	0.53
		Population + BM	4	46.28	1.21	0.55	0.29
		Null	2	52.37	7.31	0.03	0.01
	b) DHEAf	Population	3	191.60	0	1	0.41
		Population + sex	4	192.22	0.62	0.73	0.30
		Null	2	195.66	4.05	0.13	0.05

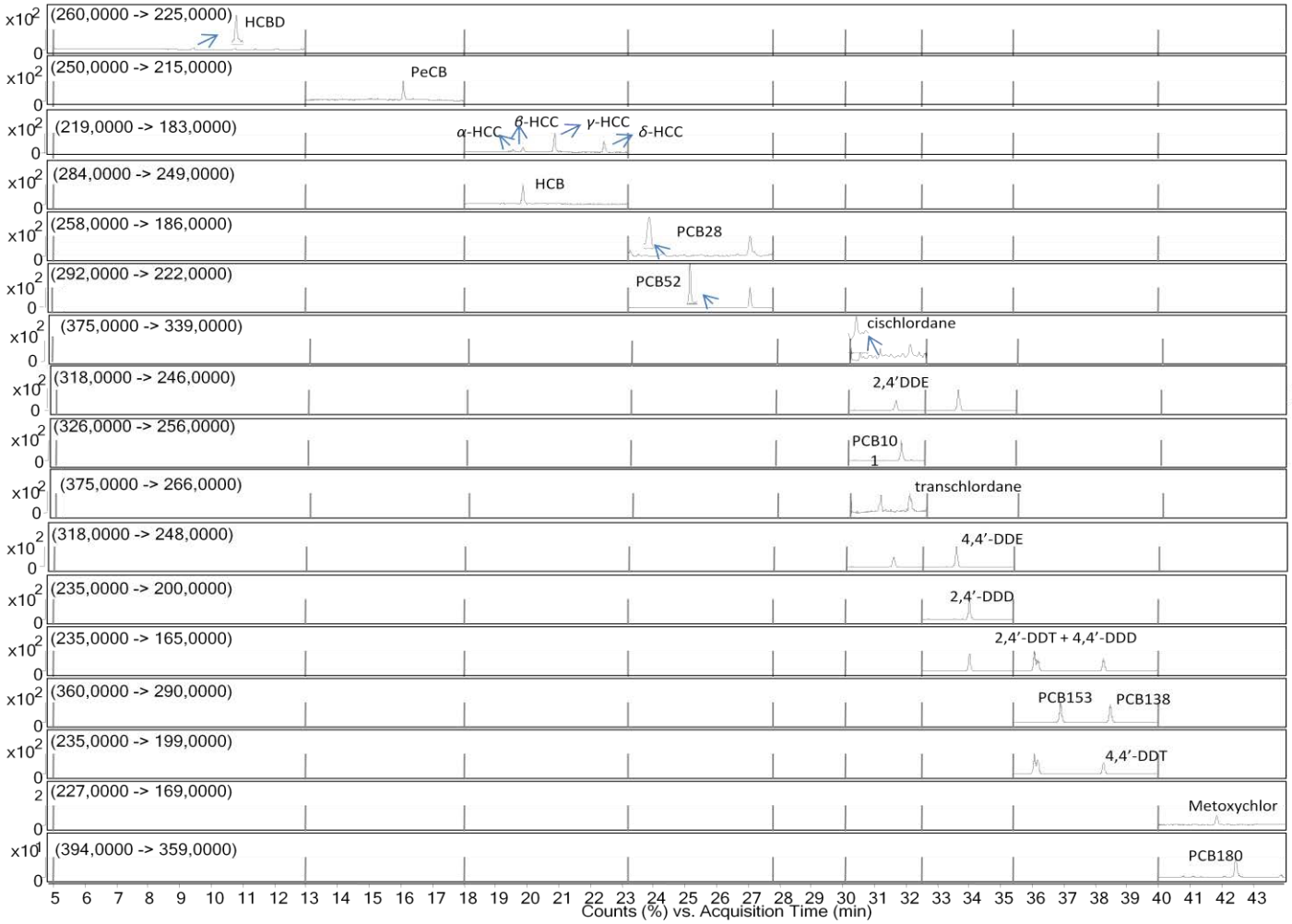

Fig. S1. GC/MS chromatogram of one feather sample as an example. Each contaminant compound detected in the sample is illustrated. Transitions are specified.

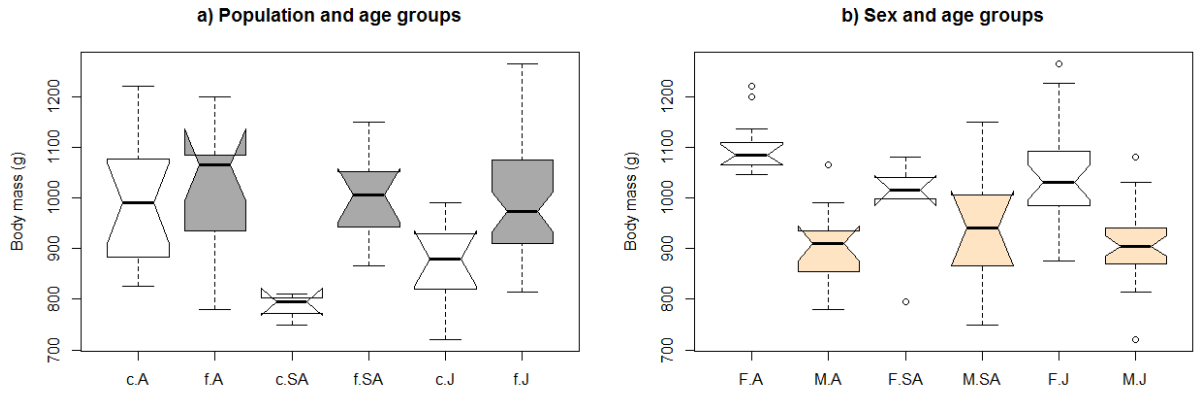

Fig. S2. *Notch box plots* displaying the relationship between body mass and **a)** age: adult (A), subadult (SA) and juvenile (J) depending on the type of population: captive (c) and free-living (f) and **b)** sex: females (F) and males (M), depending on the age groups. Shown are median value (thick line) along interquartile range (top and bottom of box), and maximum and minimum values within 1.5 interquartile range (whiskers).

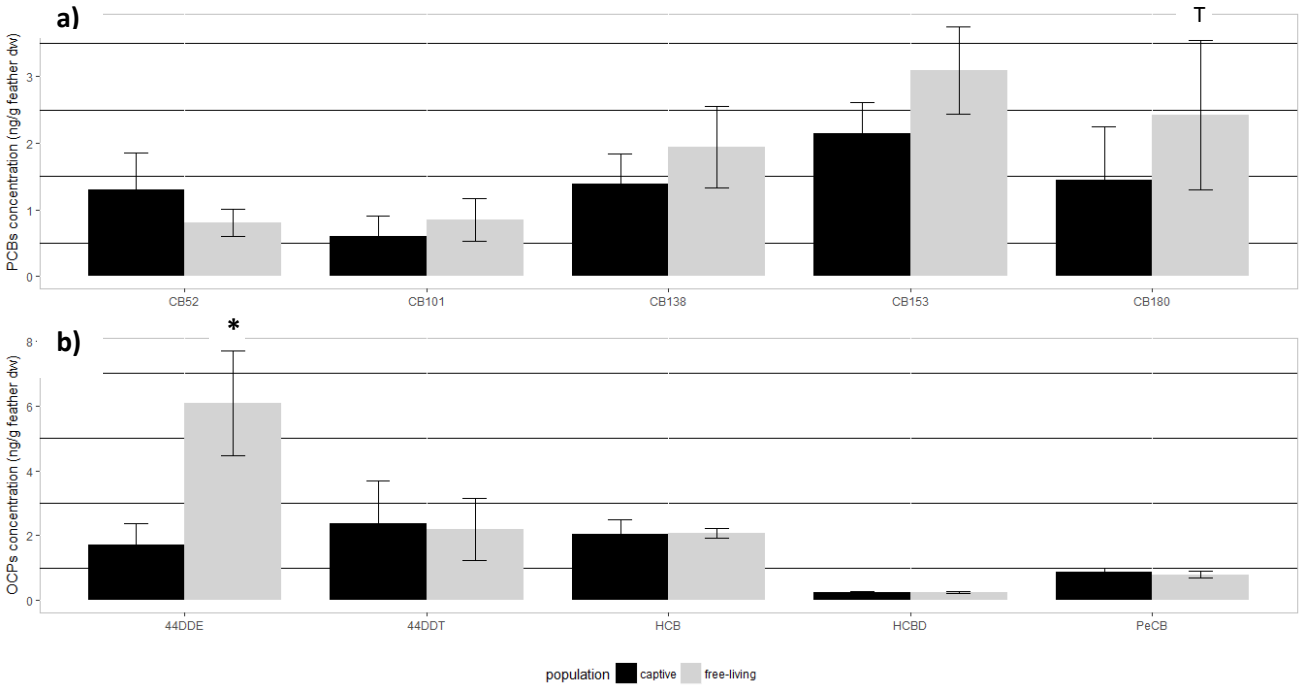

Fig. S3. Concentrations of **a)** PCBs and **b)** OCPs in feathers of red kites from two different types of population: free-living (*n* = 44) and captive (*n* = 9). Data are shown as means ± SE. Asterisk (*) defines significant differences (P<0.05) and ^{T} defines a tendency (0.1>P≤0.05).

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GENERAL DISCUSSION

The main purpose of this doctoral thesis was to evaluate and optimize the use of feathers to assess long-term levels of corticosterone and biomonitor environmental pollutants exploring their effects on birds. To achieve this objective, different studies were carried out using raptor species classified as Least Concern (Eurasian sparrowhawk: Chapter I) and Near Threatened (red kites and cinereous vultures: Chapter IV, V and VI) and a pelecaniform species classified as Critically Endangered (Northern bald ibis: Chapter II).

The following discussion is a compilation of the most important results of this doctoral thesis and is presented in line with the specific objectives formulated. Each of the five specific objectives defined is addressed in a separate section below. Author point of view, including suggestions for future studies, is provided at the end.

1. Suitability of different types of feathers to measure corticosterone and monitor environmental pollutants.

The first objective of the present thesis was to evaluate and compare different types of feathers to assess CORT and environmental pollutants as a means of long-term adrenal activity (Chapter I) and biomonitor contaminant exposure (Chapter IV).

Feathers have been widely used in the literature to assess long-term concentrations of CORT and as a biomonitoring tool of environmental pollutants (i.e. Abbasi et al., 2016; Harms et al., 2010; Legagneux et al., 2013; López et al., 2016; Pollack et al., 2017; Strong et al., 2015). At the start of this thesis, almost all the literature assessing CORT in feathers had used flight (primary and secondary) and rectrices feathers. Instead, body feathers were rarely used and only one study had reported CORT levels on body feathers using a raptor species (Egyptian vultures *Neophron percnopterus*; Carrete et al., 2013). The use of body feathers may be a very valuable alternative to the use of large feathers, especially when sampling endangered or protected bird species, due to their removal is less invasive, do not generally result in bleeding and do not affect individual's flight (Espín et al., 2014). Ease and minimally or non-invasive sampling properties provided by body feathers are also interesting advantages for biomonitoring studies. Therefore, the first step of this thesis aimed to determine the suitability of body feathers. To this end, CORT concentrations were compared between flight and body feathers in Eurasian sparrowhawks, reporting significant differences (Chapter I). Flight feathers displayed higher levels of CORT (~ 3 times higher) than body feathers, indicating that levels from both types of feathers are not directly comparable. In addition, no correlation was found

between the two types of feathers, indicating that flight and body feathers accumulate different concentrations of CORT. It is important to consider that feathers accumulate CORT only during their period of growth, which may vary on duration and frequency over the year depending on the type of feather. Therefore, it is important, on one hand, to identify the growth period of the feather sampled, and on the other, consider the biological context (i.e. if the feather growth overlap with reproduction or migration) to establish the relationship with biological and ecological parameters. Unfortunately, the specific molting process and its synchrony between individuals is unknown for almost all bird species and all feather types, being very difficult to monitor in wild bird species (Romero and Fairhurst, 2016). For example, flight feathers are known to take up to two or three years to complete their growth and can even arrest their molting process in front of high energetic demanding situations (i.e. reproduction, migration or poor habitat quality) (Forsman, 1999; Rohwer et al., 2009; Zuberogoitia et al., 2009). In fact, these feathers are described to molt serially from several foci and several "waves" (Forsman, 1999). This means that the same flight feathers from two different birds are not necessarily grown at the same time. Therefore, using flight feathers to compare CORT concentrations among individuals can lead to misinterpretations as these feathers may reflect different spatial and temporal status within and between bird populations. Alternatively, body feathers are described to grow in a more synchronous way during the first stages of the molting period, after the breeding season and preceding winter stressors and migration (Forsman, 1999; Gill, 2007; Hardy et al., 2006). Body feathers may thus reflect CORT concentrations over a more fixed and defined period of time. In support to this, we observed that body feathers displayed lower variability in CORT levels in comparison to flight feathers (Fig. 2 Chapter I), therefore we suggested body feathers as a suitable type of feather to sample in order to obtain more specific information in time and minimizing confounding factors.

Regarding contaminants, body feathers have widely been used to monitor environmental pollutants (i.e. Abbasi et al., 2016a; Eulaers et al., 2011b; Jaspers et al., 2011), being reported to provide more specific information than large feathers (Jaspers et al., 2011). In addition, analytical feasibility and biological relevance were also demonstrated (Eulaers et al., 2013, 2011a, 2011b). Therefore, in this thesis we used body feathers to biomonitor environmental contaminants (Chapter IV, V and VI). Additionally, we present new findings that expand the application of body feathers, especially in nestlings (Chapter IV).

Feathers from nestlings are the best indicators of local contamination status and, because feather sampling can be done close to feather growth, they reduce temporal displacement and provide more accurate information (Eulaers et al., 2011a). Chapter IV was set out to test, for the first time in the literature, the suitability of nestling down feathers to monitor concentrations of POPs and OPEs in comparison to contour body feathers (feathers with a vane, which had been used in all previous studies). These two types of feathers present different grow time during chick development, being down feathers the first plumage to grow and contour body feathers the subsequent one, constituting the juvenile plumage and lasting up to subadult molt (Bernis, 1966; Forsman, 1999). Therefore, they provide information of consecutive nestling periods. Higher detectability and concentrations of targeted POP and OPE compounds were found in down feathers in comparison to contour body feathers. Following Bourgeon et al. (2013), we hypothesized that down feathers reflect the contaminant burden of the chick transferred by maternal ovoposition rather than by food intake. If confirmed, down feathers could be particularly interesting because they may provide information not only of natal conditions, i.e. chick development, growth and subsequent fledging success, but also of maternal conditions, i.e. egg-laying, hatching success and nest attendance. In addition, down feathers can be easily collected at nests once chicks have naturally molted to juvenile plumage, adding suitability of down feathers as a non-invasive biomonitoring technique.

Selecting the most proper type of feather for each study will depend on the endpoint of the study and the species of choice. However, body feathers represent a suitable type of feather to avoid within-individual interferences (i.e. avoiding overlap with reproduction and migration), being more easy and ethical to collect. In addition, due to the sampling of body feathers can be done more close to their growth, they can provide more accurate information, especially when using nestling down feathers.

2. Consistency, stability and repeatability of corticosterone levels in body feathers and optimization of corticosterone extraction methodology.

The second objective of this thesis was to determine individual consistent, stability and repeatability of feather CORT concentrations in body feathers over time (Chapter II). In addition, a new and optimized protocol for extracting CORT from feathers was validated (Chapter III).

One of the main reasons why body feathers are rarely used in the literature is the small sample size of this feather type. Different authors have reported overestimations of CORT levels when

feather mass is below 20 mg (Berk et al., 2016; Lattin et al., 2011). To avoid this artifact and to accomplish with the minimum mass requirements, pools of body feathers were used in almost all the studies of the present thesis (Chapter I, II, III, V). By pooling body feathers, the sample size is increased and the amount of feather material across samples can be standardized. Once the suitability of body feathers were assured (Chapter I; Section 1), it was necessary to evaluate the robustness of body feathers (Chapter II). To this purpose, three key factors were identified: (1) Consistency: Sampling feathers from the same anatomical location, which overlap in growth time, should be informative of the same circulating CORT levels and thus should provide similar feather CORT levels. (2) Stability: CORT is usually analyzed from molted feathers but it results complex to know the exact moment when feather grew, and consequently the time gap between feather growth and feather sampling. To be a robust matrix, feathers collected at any time before the following molt period should exhibit similar CORT concentrations, hence CORT levels should be stable in feathers at least over the same feather generation. (3) Repeatability: It is important to know whether CORT levels are repeatable across consecutive years and if levels reflect inherent quality of individuals (Aharon-Rotman et al., 2015) or change due to extrinsic factors (Legagneux et al., 2013).

Findings from Chapter II showed that different pools of body feathers (feathers collected from the interscapular region grown at the same molting period) displayed similar CORT levels, indicating that body feathers are a robust and consistent matrix. Furthermore, similar CORT levels were found between two pools of body feathers collected at different times before the following molt (i.e. the first pool was collected right after the molt and the second six months after). These findings indicated that CORT levels remain fixed in body feathers after the end of vascularization, at least for the following six-months, completing the very scarce available information in the literature on feather degradation and CORT stability over time (reviewed in Romero and Fairhurst, 2016). It is worth mentioning that we explored feather CORT stability using a pool of body feathers instead of a single feather, and thus single feather variations could be diluted by the global value obtained by the pool. This implies that defects in quality or slight between-feather asynchronies within individuals may not be problematic. Finally, we found dissimilate CORT levels between years, indicating that birds with high CORT levels one year do not necessarily show high levels the following one. Therefore, feather CORT could probably be rather influenced by environmental factors encountered during molt (i.e. climate) than by intrinsic measures of quality (i.e. body condition), as previously suggested in the literature (Harms et al., 2015; Legagneux et al., 2013).

The second part of this objective aimed to optimize the extracting methodology. The protocol applied in the first two studies of this thesis (Chapter I and II) was based on the methanol-based extraction technique originally described by Bortolotti et al. (2008a, 2008b) in feathers of red-legged partridges (*Alectoris rufa*) and followed the modifications applied by Carbajal et al. (2014) in feathers of farm broilers (*Gallus gallus domesticus*). Chapter I and II used a ratio methanol:mg feathers of 0.33 (mL methanol / mg feather). So far, the volume of methanol used for extracting CORT have ranged from 0.25 to 0.7 mL in almost all the studies published (reviewed in Berk et al., 2016). However, the ratio methanol:mg feathers has recently been reported to not affect the final feather CORT concentrations (Berk et al., 2016). Following this latter finding, Chapter III aimed to optimize the extraction protocol in order to facilitate the methodological procedures in a more time-saving and ecologic way. By increasing the surface of the feather and decreasing the volume of methanol used, we explored the efficiency of a ratio methanol:feather of 0.05 mL, similar to the ratio used in hair (0.02 mL methanol / mg hair; Davenport et al., 2006). To investigate the feasibility of this new protocol, the same pools of feathers were split and were simultaneously analyzed per duplicate using the new optimized protocol and the classic protocol. A strong and positive correlation was found between the two protocols (Chapter III), indicating a correspondence in levels and confirming the feasibility of the new protocol. Once the new optimized protocol was validated (see below), it was used for conducting the studies of Chapters V and VI.

The assay validation step was a crucial point for the developing of this thesis. As stated in the literature (Buchanan and Goldsmith, 2004; Touma and Palme, 2005), validation tests cannot be generalized or extrapolated from one technique to another or among species or matrixes. This means that one validated technique used in one lab cannot serve as a validation for another lab. A careful validation is thus mandatory for new matrixes and species analyzed for the first time in each lab in particular (Touma and Palme, 2005), although this step is often forgotten in the literature (Buchanan and Goldsmith, 2004). Differences in the extracting protocols, methodologies, and enzyme immunoassay (EIA) commercial kits (which may have different cross-reactivities and affinity to antibody binding), can explain differences in the steroid analyses among labs (Tate and Ward, 2004).

In the present thesis, all studies used the same commercial EIA kits (Neogen® Corporation, Ayr, UK) to quantify feather CORT concentrations, including the validation tests (Chapter I, II, III, V, VI). The only exception was the use of another commercial EIA kit (Arbor Assays®, Mi, USA) for the detection of DHEA (Chapter VI). Both commercial immunoassays were selected because

their broad assay range of hormonal detection (ranging from 0.05 to 5 ng for CORT and from 0.096 to 60 ng for DHEA per mL of feather extract) and the positive previous experience of our lab with these EIA kits (Carbajal et al., 2014; Tallo-Parra et al., 2015). As previously supplied for hair (Tallo-Parra et al., 2015), a complete battery of validation tests was done for validating CORT analyses in body feathers of different bird species. The validation tests presented in this thesis for feather CORT and DHEA include the evaluation of precision, accuracy, specificity and sensitivity, additionally to the information provided by the EIA manufacturer, for the following species: Eurasian sparrowhawk (Chapter I); Northern bald ibis (Chapter II); red kites (Chapter III, VI); and cinereous vultures (Chapter V). Overall, the precision tests showed an intra-assay coefficient of variation around 5% and an inter-assay around 10%, considerably below the 15% considered as acceptable precision (Crowther and Walker, 2001; Food and Drug Administration, 2015). The accuracy of our tests was among 95 to 99% and the specificity tests showed a mean recovery percentage of 106 ± 14.08%. The dilution curves were parallel to the standard curve and sensitivity was in all cases much lower than the hormone concentrations from feather extracts. Results were similar to those previously described for feather CORT (Carbajal et al., 2014; Jenni-eiermann et al., 2015; Kouwenberg et al., 2013), although this thesis presented a more extended battery of validation tests in comparison to those studies. In addition, this was the first time that the extraction of DHEA from feathers was biochemically validated in the literature. This hormone is a steroid hormone, and thus have similar chemical characteristics to CORT; being highly soluble in lower alcohols such as methanol (the most solvent alcohol for steroid extraction; see Pöstch and Moeller, 1996). Overall, validation tests performed in this thesis confirmed that feather CORT and feather DHEA extracted from feathers through the presented protocols (classic and optimized), and analyzed through the commercial Neogen® and Arbor® kits, are reliable and can be used for research (Crowther and Walker, 2001; Food and Drug Administration, 2015).

3. Feather corticosterone as a biomarker of bird fitness.

The third objective of this thesis was to evaluate the usefulness of feathers as a biomarker of bird survival (Chapter I) and reproduction (Chapter II).

Levels of feather CORT were analyzed in Chapters I, II, V and VI of this thesis. Despite we used two different protocols for the extraction of feather CORT (Chapter I and II used the classic protocol while Chapter V and VI used the optimized protocol), the fact that CORT levels from both protocols were significantly and positively correlated (Chapter III) enable us to compare CORT concentrations among species and studies.

Maximum levels of feather CORT (mean ± SE) were found in Northern bald ibis (7.52 ± 0.51 pg CORT mm^{-1} feather; Chapter II), being two times higher than in Eurasian sparrowhawk (3.94 ± 0.30 pg CORT mm^{-1} feather; Chapter I), red kites (3.30 ± 0.22 pg CORT mm^{-1} feather; Chapter VI) and cinereous vultures (2.95 ± 0.24 pg CORT mm^{-1} feather; Chapter V). Differences between Northern bald ibis and raptors could be attributed to the different taxonomy: Northern bald ibis belongs to the Pelecaniforme order (BirdLife International, 2017a) while raptors are from the Accipitriforme order (BirdLife International, 2017b, 2017c, 2016). Moreover, it should be considered that ibis were captive birds while almost all raptors were free-living (except captive red kites in Chapter VI). However, this does not seem to be the cause since high CORT levels would be rather expected in free-living birds than in captivity because of evident requirements for survival of these individuals (Chapter VI). Interestingly, similar CORT levels were found between raptor species (at least between free-living birds) despite their different age groups. CORT levels in nestlings (cinereous vultures) were similar to adults (either sparrowhawks or red kites) contrary to previous studies that had reported differences between chicks and adults; i.e. in American kestrels (*Falco sparverius*; Love et al., 2003). Indeed, variation in the adrenocortical response to stress have been reported in nestlings due to chick development (see "The Developmental Hypothesis": Blas et al., 2009). Notwithstanding, we are comparing adults and nestlings from different raptor species, and thus we cannot rule out differences between nestling and adults within the same species. In addition, the type of feather sampled in nestling cinereous vulture (down feathers; Chapter V) was different from that used in adult sparrowhawks and kites (contour body feathers; Chapter I, VI). As explained in section 1, down feathers have different characteristics compared to contour body feathers, showing different contaminant accumulation patterns (Chapter IV). Considering that they reflect different nestling periods, down feathers could probably differ in CORT concentrations as well. Unfortunately, due to sampling limitations, we were not able to analyze CORT in that type of feathers and thus we could not account for such information. Further research is thus recommended.

An assessment of CORT in feathers of wild Eurasian sparrowhawks (Chapter I) and captive Northern bald ibis (Chapter II) allowed us to study the relationship between the HPA axis activity and health status, reproduction and survival. Carry-over effects, i.e. events occurring in one season affecting individual's performance in a subsequent period, have been noted in multiple studies (Bêty et al., 2003; Legagneux et al., 2012; Saino et al., 2012). Due to CORT can mediate or indicate the expression of such carry-over effects, feather CORT has been suggested as a potential tool to monitor carry-over effects across seasons (Harms et al., 2015;

Pérez et al., 2016). In support to this, we reported that high CORT levels could predict mortality in wild Eurasian sparrowhawk (Chapter I) and reproductive failure in captive Northern bald ibis (Chapter II).

One of the key points of Chapter I is that we sampled sparrowhawks arrived at a rehabilitation center after some traumatic injury. We could then monitored the health status of these birds during their stay at the rehabilitation center and related its body condition and survival rate to the past HPA axis activity when these birds were in the wild. Feathers were completely grown when birds arrived at the center; therefore they provided an indication of the stress response or energetic demands experienced by the bird during the molting period, some months earlier. It was reported a negative relationship between high levels of feather CORT and survival rate recorded at the rehabilitation center. This negative relationship was in agreement with previous reports of Koren et al. (2012) in wild House sparrow (*Passer domesticus*) and Harms et al. (2015) in wild common eiders (*Somateria mollissima borealis*). Although these earlier studies provided very valuable information, they did not consider the interval of time between the feather growth and the record of fitness parameters (referred to as "time gap"). We argued that this time gap could be an important confounding factor influencing the capacity of feather CORT to predict subsequent mortality. However, we did not find any evidence for such effect. Birds admitted six months after the molting period showed the same significant relationship between feather CORT and mortality than birds admitted only one month later.

Besides time gap, we also explored the influence of body condition (Chapter I). Earlier studies described a negative relationship between high levels of CORT in feathers and subsequent poor body condition (Harms et al., 2015, 2010; Strong et al., 2015). In our study, we found a substantial difference in feather CORT concentrations between birds in good condition and those in poor condition. However, those birds presenting worse body condition, classified as cachectic individuals, showed similar levels of feather CORT than individuals in good condition. On one hand, it could be possible that chronically stressed birds presented poor body condition the next season. However, the fact that cachectic individuals showed similar levels than birds in good condition evidences that there are limiting factors when using feather CORT to predict body condition and suggest caution when assuming that only high CORT levels represent individuals in poor condition.

With regards to reproduction, Chapter II reported a negative relationship between high CORT levels and reproductive failure recorded the subsequent reproductive season. One of the most interesting results of this study was that ibis showing high levels of feather CORT one year do

not necessarily showed high CORT levels the following year. Hence, those individuals with high CORT levels that consequently fail to breed one year do not necessary fail to breed the following one. In this study, the analysis of feather CORT was conducted in ibis maintained in captivity, providing with a more controlled and homogeneous population, and reducing the influence of possible confounding factors, such as body condition, food availability or high energetic requirements. We suggested that feather CORT variations among years probably reflected extrinsic factors (i.e. climate conditions; Legagneux et al., 2013) rather than individual quality, although in this study we could not identify the cause of high CORT levels. Another interesting finding was that similar CORT levels were found between healthy and unhealthy individuals (i.e. ibis showing chronic disabilities such as skin diseases or flight alterations). Due to birds were maintained in captivity, with available food resources, the unhealthy individuals probably habituated to their new circumstances without presenting any HPA axis alteration (see Lynn et al., 2010). Notwithstanding, these birds showed worse reproductive success than healthy birds. Similarly, reproductive success declined in old birds. These two findings showed that not only high CORT levels can impair reproduction, but also other biological parameters such as age and health, thereby caution is required in the analysis of data. This is why in our study, age and health status were included as covariates in the statistical model. Results showed that age and health status, although showing worse reproductive success, were not associated to high CORT levels.

Determining feather CORT may be very valuable in rehabilitation centers and also in *in situ* reproduction programs to identify individuals with lower chance of survival and reproduction, respectively. However confounding factors (i.e. body condition, health status, age) should be taken into account in monitoring studies for a proper interpretation of data.

4. Usefulness of feathers to biomonitor environmental contamination and in especial relation with adrenal stress hormones.

The fourth objective of this thesis was to evaluate the usefulness of feathers to biomonitor environmental pollution and study the relationship with adrenal stress hormones (Chapter V and VI).

To address this objective, we used wild birds of prey as model species due to they have overwhelming been used as sentinel species to biomonitor environmental pollution (Espín et al., 2016; Gómez-Ramírez et al., 2014; Helander et al., 2008). An assessment of feather contaminants in cinereous vultures (Chapter IV, V) and red kites (Chapter VI) allowed us to

report levels of persistent organic pollutants (Chapter IV, V, VI) and flame retardants (Chapter IV, V) and to study subsequent relationships between these pollutants and the endocrine stress response (Chapters V, VI). To facilitate the comprehension of results, I address the discussion of this objective in two different sections: 4.1 biomonitoring environmental contamination through feathers and 4.2 relationship with feather CORT concentrations.

4.1. Biomonitoring environmental contamination through feathers

In the literature, different factors have been attributed to influence contaminant accumulation patterns, such as for example the geographical origin of the individuals (i.e. urban *vs.* rural birds; Jaspers et al., 2009; Meillère et al., 2016), the trophic position (i.e. bird-eating raptors *vs*. raptors that feed on mammals; Elliott et al., 2009; Mañosa et al., 2003), feeding habits (i.e. specialist *vs*. opportunistic species; van Drooge et al., 2008), migratory habits (i.e. migratory *vs.* resident birds; Espín et al., 2010) and biological parameters (i.e. age, sex, body condition; Bustnes et al., 2008; Jaspers et al., 2013), among others. With this in mind, the present thesis explored pollutants in different raptor species with different migratory habits and biological parameters. Particularly, Chapter V was conducted using nestling cinereous vultures from Spain. Chapter VI was conducted using juvenile, subadult and adult red kites, and two different types of populations were compared: free-living kites migrating from Northern Europe and wintering in Spain *vs.* captive kites maintained at a rehabilitation center in Spain.

In general, most of the available literature has used non-keratinous matrixes such as blood, eggs, muscle, liver and kidney to biomonitor environmental pollution (Couderc et al., 2015; Espín et al., 2010; Gómara et al., 2008; Jiménez et al., 2007; Morales et al., 2012; Nordstad et al., 2012; Van Drooge et al., 2008). Although increasing studies have used feathers to biomonitor chronic exposure to pollutants (Abbasi et al., 2016a; Eulaers et al., 2013, 2011b; Jaspers et al., 2011), information of POPs in feathers is still lacking for many species, making it very difficult to compare between populations and relate contamination in the bird to a particular source. At the start of this thesis, no published study existed on feathers of red kites, and only one study had reported POP levels in two Asian vulture species, Indian vultures (*Gyps indicus*) and white-rumped vultures (*Gyps bangalensis*) (Abbasi et al., 2016a). Even more scarce was the information reporting OPE levels in feathers with only one study conducted on white-tailed eagles (*Haliaeetus albicilla*) (Eulaers et al., 2014a). Comparisons were then made with published reports performed in other raptor species and also in other matrixes.

The observed high detectability and concentrations of high-chlorinated PCB congeners (CB-138, -153 and -180) and *p,p'*-DDE in both species (Chapters V, VI) was in agreement with previous literature in feathers (Abbasi et al., 2017; Eulaers et al., 2013, 2011b; Jaspers et al., 2007) and also in other matrixes such as eggs (Morales et al., 2012) and blood (Gómara et al., 2004; Sletten et al., 2016). In free-living red kites (Chapter VI), *p,p'*-DDE was the predominant congener followed by CB- 153 > CB-180 > *p,p'*-DDT > CB-138. Alternatively, in captive red kites *p,p'*-DDT exhibited the highest concentrations among targeted compounds (followed by CB-153 > *p,p'*-DDE > CB-180 > CB-138) and the ratio DDE/DDT was lower than 1 in almost all individuals. This was a rather surprising result. The use of DDT was banned in Spain in the 90s (Orden de 4 de Febrero) and thus the ratio DDE/DDT was expected to be higher than 1 indicating a more percentage of *p,p'*-DDE to the total DDTs. The metabolite DDE indicates a historic exposure of DDT and the capacity of organisms to metabolize it to DDE, or the degradation of DDT itself to DDE in the environment (Espín et al., 2012, 2010; Jiménez et al., 2007). This difference between free-living and captive kites would be due to their different procedure; free-living kites molt their feathers in Northern Europe while captive kites molt their feathers in Spain. Interestingly, high levels of DDT had already been reported in different Spanish bird samples: in eggs of red kites (Gómara et al., 2008; Jiménez et al., 2007) and also in internal tissues of other bird species [i.e. razorbills *Alca torda* (Espín et al., 2010) and raptor species (Luzardo et al., 2014)]. These earlier results, added to our findings, are particularly concerning. Two different explanations may be: (i) the extreme environmental persistence of DDT and the proximity to the African continent (where DDT is still in use against malaria) and (ii) a likely illegal use of this pesticide in Spain, despite of its ban. In addition to this, it has recently been suggested that DDT can be an intermediate product in the synthesis of dicofol (García-Fernández et al., 2008; Martínez-López et al., 2009), a pesticide still in use in Spain. However, dicofol was not detected neither in red kites (Chapter VI) nor in cinereous vulture nestlings (Chapter V).

Unlike captive kites, *p,p'*-DDT was not a predominant compound in nestling cinereous vultures (Chapter V), indicating a total different *scenario* to that illustrated in captive kites (Chapter VI). In nestling cinereous vultures, *p,p'*-DDE was the chief contributor followed by *γ*-HCH and BDE-99. This profile was in agreement with earlier reports in vultures (Goutner et al., 2011) denoting the importance of vultures in agricultural lands and rubbish dumps. The dissimilate *p,p'*-DDT levels between species could be explained by: different ages (adults *vs.* nestlings), different spatial exposure and food source (captivity with controlled food supply *vs*. free-living conditions) or different type of feather analyzed (contour feathers *vs.* down feathers).

Notwithstanding, and given the negative effects of DDT impacting not only on raptor populations but also on human health, a more extended exploration of the DDE/DDT profile is highly recommended in Spain. In addition, the high predominance of lindane (*γ*-HCH) in cinereous vultures was also a surprising result. The use of lindane was definitely banned in 2001 in Spain (Decision, 2000/801/EC) and, since then, levels of this pesticide have generally diminished (Martínez-López et al., 2009). However, high levels are still detected in some raptor species (Goutner et al., 2011; Van Drooge et al., 2008) and authors have denounced possible illegal sprayings in Spain. If this is the case, free-living birds inhabiting Spain lands (nestling cinereous vultures; Chapter V) would have been more exposed to lindane than those birds maintained in captivity (captive red kites; Chapter VI) or those migrating from Northern Europe (free-living red kites; Chapter VI). Lindane has a shorter environmental half-life in comparison to the other OCPs (Blus and Henny, 1985) and is relatively rapidly metabolized and excreted in organisms (Moisey et al., 2001). Thus, high concentrations of lindane denote recent exposure. Finally, due to sample constraints, PBDEs were only analyzed in cinereous vultures (Chapter V) and thus we could not account for differences in red kites (Chapter VI).

In terms of concentrations, maximum levels of PCBs, *p,p'*-DDE and HCB were found in red kites (Chapter VI) in comparison to nestling cinereous vultures (Chapter V). The highest difference was recorded for PCB compounds with a mean (±SE) in free-living kites of 9.13 (2.63) ng g^{-1} dw, 6.89 (2.12) ng g^{-1} dw in captive kites and 3.03 (0.24) ng g^{-1} dw in cinereous vultures. The compound *p,p'*-DDE, although maximum amounts were recorded in free-living kites (6.10 ± 1.56 ng g^{-1} dw), was higher in nestling cinereous vultures (3.28 ± 0.28 ng g^{-1} dw) than in captive kites (1.71 ± 0.66 ng g^{-1} dw). Finally, HCB was 10 times higher in red kites (free-living: 2.07 ± 0.14 ng g^{-1} dw; captive: 2.03 ± 0.45 ng g^{-1} dw) than in nestling cinereous vultures (0.10 ± 0.01 ng g^{-1} dw). Besides the above-mentioned differences, PCB levels detected in this thesis were far below those previously reported. For instance, levels in nestling cinereous vultures (Chapter V) were 4 to 12 times lower to those reported in nestlings of different raptor species in Norway (Eulaers et al., 2014a, 2013); and levels in red kites (Chapter VI) were 3 to 18 times lower to those reported in Flemish raptors (Jaspers et al., 2006, 2007). Alternatively, PCB levels reported in Asian raptor species (Abbasi et al., 2017, 2016a, 2016b) were similar to our findings. In general, Spain has been reported as a country with intermediate (Gómez-Ramírez et al., 2012) or low (Luzardo et al., 2014) PCB levels in comparison to other European countries. Alternatively, levels of HCB, *p,p'*-DDE, and BDE-99 were more similar to levels previously reported in the literature (see Abbasi et al., 2017; Eulaers et al., 2014, 2013; Jaspers et al., 2011).

The present thesis (Chapter IV and V) is a step forward in the study of OPEs in birds. Rising concerns about potential adverse effects of OPEs on wildlife and human health have lead to several studies exploring their concentrations in different biota samples (Barón et al., 2014; Guigueno and Fernie, 2017; Kucharska et al., 2015; Sundkvist et al., 2010) and investigating their bioaccumulation and biomagnification properties (Brandsma et al., 2015; Fernie et al., 2015; Greaves and Letcher, 2014). However, only one study has reported levels of OPEs in feathers (Eulaers et al., 2014a). In the present thesis, the four OPE targeted compounds (TCEP, TCiPP, TPhP and TDCiPP) were detected in down feathers (Chapter IV) but only two (TPhP and TCiPP) could be quantified in more than 50% of the population, with TPhP reaching the highest concentrations (Chapter V). In comparison to nestling white tailed-eagles (Eulaers et al., 2014a), nestling cinereous vultures showed 2 times lower TCiPP levels and similar TPhP levels (Chapter V). This earlier study in nestling white tailed-eagles showed higher concentrations of TCiPP than TPhP, in contrast to our findings. As explained in section 1, our analysis of OPEs was conducted in down feathers, in contrast to the contour body feathers used by Eulaers et al. (2014a). We suggested that down feathers may reflect the initial contaminant concentrations of the chick, probably transferred by the mother through egg ovoposition (Chapter IV). Following that hypothesis, we expected higher concentrations of TCiPP than TPhP as indeed found in our preliminary study (Chapter IV) and because TCiPP, in comparison to TPhP, has higher affinity for the egg yolk and thus it is transferred *in ovo* (Greaves and Letcher, 2014). Interestingly, when OPEs were detected in contour body feathers, TCiPP was the predominant compound (Chapter IV). It is possible that the different feather type analyzed influences the levels. However, it is worth mentioning that the washing protocol validated for POP compounds (Jaspers et al., 2011, 2008) is not validated for OPEs, and thus levels of OPEs may vary highly due to external contamination (see section 5).

4.2. Relationship with feather corticosterone concentrations

Once we had investigated the accumulation patterns of pollutants in feathers, we explored the relationship between levels of contaminants and CORT (Chapter V, VI) and, for the first time in the literature, DHEA (Chapter VI). A strong and positive relationship was found between contaminants and CORT levels in nestling vultures (Chapter V) and free-living kites (Chapter VI), but not in captive kites (Chapter VI). Levels of contaminants were also positively associated to DHEA (Chapter VI). These findings may first suggest that contaminants affect the HPA axis activity of free-living birds while do not affect birds maintained in captivity with controlled diet and husbandry conditions. Different factors should nonetheless be taken into account.

First, different compounds were analyzed in the two studies. Chapter VI was mainly focused on the analysis of PCBs and DDTs, while Chapter V also included the analysis of the emergent pollutants PBDEs and OPEs. Different statistical methods were also used. In the literature, one of the main constraints in analyzing the effects of contaminants has been the high correlations detected among compounds (see for example Table 18 in Chapter VI). Consequently, some authors have decided to evaluate only the effects of the most persistent (PCB-153, *p,p'*-DDE, BDE-47) and less persistent (HCB, PCB-28) compounds (Eulaers et al., 2013), while others have conducted a sum of the compounds and analyze the effects of POPs in general (Tartu et al., 2015). Notwithstanding, the compounds taken into the POP sum may vary among studies, making comparisons very difficult. In addition, the different chemical properties between groups of contaminants can make them to behave differently. Therefore, we decided to approach the study of the POPs effects in two different manners: (i) by analyzing each group of contaminants using separate AIC-based selection and different models (Chapter VI) (see also Nordstad et al., 2012) and (ii) by reducing the dimensionality of data using Principal Component Analysis (PCA) and ranking the influence of each compound creating PC-lists (Chapter V) (see also Bourgeon et al., 2013; Verboven et al., 2010). In Chapter VI, we observed that either PCBs and DDTs affected CORT concentrations while HCB, HCBD and PeCB had no effect. In Chapter V, we found that the most persistent compounds (i.e. high-chlorinated PCBs and *p,p'*-DDE) were positively associated to high levels of CORT, while the less persistent pollutants (low-chlorinated PCB and HCB) and BDE-99 were not. In addition, in Chapter V, OPE compounds were also observed to not affect the HPA axis activity. These differences among compounds are suggestive of different noxious effects depending on the contaminant class, as previously suggested (Cesh et al., 2010; Nordstad et al., 2012). Both studies of the present thesis indicate an effect of the high-chlorinated PCBs and DDTs on the HPA axis, while no effect was found for the rest of compounds. The positive relationship POPs-CORT has been previously reported in black-legged kittiwakes (*Rissa tridactyla*) (Nordstad et al., 2012) and glaucous gulls (*Larus hyperboreus*) (Verboven et al., 2010), in which increasing POP concentrations were related to high baseline CORT concentrations. However, no previous literature had explored this relationship using a long-term matrix such as feathers. This thesis allowed us to study the influence of environmental pollutants on the HPA axis activity in a long-term perspective and demonstrated that the most persistent compounds affected the HPA axis activity.

Second, despite the positive relationship found between POPs and CORT in free-living birds (either in free-living migratory kites or in nestling cinereous vultures), it was not significant in

captive kites. In addition, a different endocrine profile was found between free-living and captive kites (Chapter VI). One of the key points of the study presented in Chapter VI was the analysis of DHEA in feathers for the first time in the literature. DHEA is a hormone that has the ability to rescue the negative effects caused by chronic elevations of glucocorticoids (Hu et al., 2000; Kalimi et al., 1994). In humans, this hormone has increasingly been studied for its "anti-glucocorticoids" properties (Gallagher and Young, 2002; Gallagher et al., 2006). It has been observed that under acute stress stimulus, DHEA increases as glucocorticoids rise. Alternatively, under chronic stress, levels of DHEA remain unchanged or progressively diminish while levels of glucocorticoids increase or do not change, resulting in elevated ratios glucocorticoids/DHEA (Wolkowitz et al., 2001). In our study, free-living kites showed significant higher levels of CORT than captive kites but displayed similar DHEA levels and ratio CORT/DHEA. On one hand, the CORT elevation in free-living kites could respond to the higher energetic requirements meet in the wild (i.e. food shortages, reproduction or migration constraints due to molt overlapping with these periods), thereby reflecting a higher allostatic load in comparison to captive kites. In addition, the contaminant burden was in general higher in free-living kites than in captive kites and POPs were positively correlated to CORT and DHEA levels only in free-living kites (Chapter VI). No differences were found between different ages. Overall, these findings indicated that POPs could have acted as endocrine disruptors affecting the functioning of the HPA axis; explaining therefore the high concentrations of CORT reported in free-living kites and, in turn, the subsequent elevation of DHEA. This latter hormone would be suggestive of an adaptive response of the HPA axis which may secrete DHEA in order to ameliorate the potential negative effects that high CORT levels could cause to the organism. However, despite detectability and accumulation levels seem to be higher in free-living than in captive kites, the contaminant variation was only statistically significant for two compounds (PCB-180 and *p,p'*-DDE), and such variation was not as large as it could be expected (Table 2 Chapter VI). Previously, a study of Jaspers et al. (2009) reported a 10-to-30 fold contaminant variation between urban and rural magpies (*Pica pica*), while in our study we only observed 2-to-3 fold. Under this scenario, we suggested that POPs could affect in a more intensive way the HPA axis activity of free-living birds because of their higher allostatic load rather than their higher amount of contaminants *per se*.

Finally, it is worth considering that Chapter V explored the relationship between contaminants and CORT in nestlings. Sampling at the nestling stage may offer several advantages such as mitigating possible age-confounding factors, providing a small-scale geographical accuracy and reducing the influence of biological and ecological parameters, i.e. reproduction, dispersion,

migration and foraging activities (Elliott et al., 2009; Eulaers et al., 2013, 2011a). In addition, nestling feathers can be sampled close to the period of feather growth, thus reducing the time gap between feather growth and feather sampling and reflecting accurate internal state of contamination (Eulaers et al., 2011a). By using down feathers, Chapter V studied the effects of contaminants on the HPA axis activity and their impact on the development of nestlings. The suitability of down feathers for biomonitoring contamination was tested in Chapter IV and is deeper discussed in Section 1, hence it is not discussed here again. In Chapter V, we found a positive relationship between POP and CORT levels; those chicks with high POP burden also showed high CORT levels. However, POPs were not found to negatively impact the growing of the chicks. Contrary, chicks with high levels of CORT and also males with high levels of the most persistent POP compounds showed rapid development and equal fledging success than chicks with lower contaminant burden or lower CORT concentrations. These findings highlighted the plasticity of CORT in the framework of benefits towards deleterious effects and suggested that contaminant levels were not high enough to cause detrimental consequences.

In general, and as mentioned above (Section 4.1), contaminant levels detected in feathers of red kites (Chapter VI) and cinereous vultures (Chapter V) were intermediate (or in some cases low) in comparison to levels previously reported in the literature. We suspect that contaminant levels were not high enough to cause direct detrimental consequences; for instance, free-living kites showed good body condition and were able to migrate from Northern Europe (Chapter VI), and nestling cinereous vultures showed a rapid growth rate and fledged successfully (Chapter V). Notwithstanding, a positive and significant relationship was indeed found between POPs (high chlorinated PCBs and *p,p'*-DDE in both studies) and CORT levels, indicating an impact of these contaminants on the HPA axis activity in these birds. Although no direct effect could be reported in our studies, contaminants can have farther-reaching consequences than the ones studied in this thesis (for instance we did not monitor reproductive success or survival). In addition, pollution stress is additive to the existing natural stress (or other types of anthropogenic stress) and the combination of different stressors may be more detrimental than single stressors acting alone. Consequently, associations between contaminants and fitness components, or CORT in this case, may vary depending on environmental conditions and preexisting stressors (Bustnes et al., 2008). In this thesis, we could observe how higher, although slightly, contaminant accumulation in free-living birds in comparison to captive birds made the difference between influencing or not the HPA axis activity. In addition, despite biological and geographical differences, the two free-living bird

populations analyzed showed a positive association between contaminants and CORT, highlighting the impact of contaminants on the HPA axis activity.

5. Confounding factors in relation to corticosterone and contaminant feather measurement.

Factors such as molt period, feather collection time, type of feather, external contamination of the feather's surface, as well as biological factors (i.e. age, sex, diet, body condition, health status, genetics, migratory movements and life-history stages) can influence CORT levels (Madliger et al., 2015) and also contaminant accumulation (Smith et al., 2007). In this thesis, we controlled for some confounding factors and assessed their influence on CORT levels and pollutant accumulation. Some confounding factors have already been discussed in previous sections for easy comprehension of results, thus I will only mention them here and not further discussed.

Throughout the present discussion, and also when conducting this thesis, one of the key factors for interpreting data and establishing relationships with biological factors, have been the molt period and the time gap between feather growth and feather sampling (studied in Chapter I). One of the major constraints when working with free-living birds is that the molting period is often unknown and feathers are fully grown when collected; thus, information of the location and the timing of the feather growth have to be inferred or assumed. Choice of the feather type would depend on the species, the molting pattern and the endpoint of the study, and it should be homogenized within studies and also across studies when meta-analyses are performed. Results of this thesis, and other studies of the literature, highlight body feathers as a suitable, feasible and valuable matrix to biomonitor not only stress response but also environmental contamination, while minimizing confounding factors i.e. feather size, molt and age of the feather (more discussed in Sections 1-4).

Sex and age are two of the most important biological confounding factors that should be controlled for the analysis of CORT and contaminants. In the present thesis, we included these factors as covariates in the statistical models of all studies (Chapter I, II, V, VI). Similar CORT levels were found between sex and age groups in all the species analyzed. These findings are in agreement with earlier studies reporting no sex-related (Carbajal et al., 2014; Fairhurst et al., 2017; Koren et al., 2012; Strong et al., 2015) nor age-related (Strong et al., 2015) differences in CORT measured in feathers. However, other studies performed in a more acute matrix (i.e. blood) reported large CORT variations depending on the age of the individual (Angelier et al.,

2007; Heidinger et al., 2006; Sorato and Kotrschal, 2006). These latter studies evaluated differences in the stress-induced CORT response (acute response) and did no evaluate chronic levels of CORT, as we did by using feathers. Due to small variations in baseline CORT levels have been proven to be insignificant to the global feather CORT value (Fairhurst et al., 2013), it is possible that CORT differences between age groups are not strong enough to be reflected in feathers. In addition, it can also be possible that feather CORT concentrations diverge most effectively under different stress and demand situations rather than between ages and sexes (Chapter I). In addition, body condition (Chapter I) and health status (Chapter II) have been identified as important confounding factors. These factors are further discussed in Section 3.

In regards to contaminants, we did not find any age-related variation in POP levels in red kites, except for PCB-101 and HCB that were higher in juvenile kites (Chapter VI). These compounds are less-persistent than the other POP compounds and may be rather influenced by external contamination than by age differences; for example, see great inter-annual variations reported in HCB levels in feathers of different raptor species (Eulaers et al., 2013) and also feather washing constraints (discussed below). The lack of age-effect in the rest of compounds was a surprising result as we had expected great variations between juveniles and adults due to their different life-time accumulation, metabolism capacity and dispersion movements (Borga et al., 2005; Jaspers et al., 2007, 2006). It is possible that the different geographical origin between free-living kites, which was unknown, caused the high deviance found in the contaminant levels between these kites and, at the same time, masked the age effect. Notwithstanding, we neither find any age-relation in captive kites, which support the lack of difference between juveniles, subadults and adults. Regarding sex, we also expected to find differences in the contaminant patterns due to the influence of maternal transfer of contaminants into eggs (Bustnes et al., 2008). However, we did not find any variation. This could be due to the time gap existing between the egg laying and the molt of feathers during which concentrations of POPs in females could increase again. Other studies have reached similar results and conclusions when using feathers (Espín et al., 2010; Ólafsdóttir et al., 2005), suggesting that feathers could be an useful tool to biomonitor contaminant levels during the non-breeding period in order to avoid sex-relate differences during the breeding season. Finally, we also evaluated the influence of body condition on contaminant patterns. Decreasing body mass has been associated to elevated concentrations of POPs in blood (Bustnes et al., 2010, 2008). In support to this, we reported an inverse relationship between concentrations of 4,4'-DDT and PCB-180 and body mass (Chapter VI). An important confounding factor that should be taken into account is the influence of preen oil and the washing constraints of feathers (see below).

Other biological confounding factors, such as migratory and feeding habitats, are discussed along the Sections 3 and 4.

When analyzing contaminants in feathers, it is important to consider that external contamination on the surface of the feather (either caused by preen oil or atmospheric sources) can influence contaminant concentrations (Espín et al., 2016). Jaspers et al. (2011) validated a washing protocol to eliminate atmospheric particles, but this protocol seemed to not remove preen oil (or not remove it at all). The degree of contamination by preen oil seems to be related to the preening frequency of each species (Espín et al., 2016; García-Fernández et al., 2013) and differ between types of feathers, being body feather one of the feathers less influenced by this external contamination (Jaspers et al., 2011). In addition, contamination by preen oil may be an advantage since contaminants accumulated in preen oil has been closely related to blood and internal levels (Eulaers et al., 2011b), and thus can enhance the correlation between contaminant concentrations in feathers and internal tissues (Espín et al., 2016; Eulaers et al., 2011b; Jaspers et al., 2008). This is the reason why we used the washing protocol described by Jaspers et al. (2011) (Chapter IV, V, VI). Notwithstanding, the influence of preen oil or atmospheric deposition onto the preen oil is still of great concern for the evaluation of POPs. In addition, the efficiency of this washing protocol has not been investigated yet for the analysis of OPEs, therefore variations in OPE concentrations may be greatly influenced by external contamination (Chapter IV, V) and caution should be taken in the interpretation of data. Further research on this issue is therefore crucial.

Finally, the unit by which CORT and contaminants are measured in feathers can also influence the levels. Bortolotti (2010) suggested that CORT is incorporated in feathers proportionately to growth rate and not feather mass, indicating that the correct unit to express the amount of CORT in feathers is length (mm) and not mass (mg). So far, almost all the studies have expressed feather CORT concentrations in length (e.g. Fairhurst et al., 2017; Jenni-Eiermann et al., 2015; Strong et al., 2015), contrasting with the toxicological studies that have mostly expressed the amount of contaminants in mass (e.g. Abbasi et al., 2017, 2016a; Eulaers et al., 2011a; Jaspers et al., 2006, 2011, 2007). Following the theory of Bortolotti (2010), García-Fernández et al. (2013) suggested that contaminant concentrations in feathers should preferably be expressed as a function of deposition rate in addition to a mass-based unit, instead of simply using mass. These authors suggested a mathematical former based on the feather growth rate data, which can be calculated when growth bars are visible using ptilochronology. However, this information is often unavailable and cannot be easily applied in

most of the species. Therefore, almost all toxicological studies have reported contaminant concentrations in mg. In the present thesis, in order to facilitate comparability among studies, the amount of CORT in feathers is expressed per mm (Chapter I, II, V and VI) and the amount of contaminants per mg (Chapter IV, V and VI). However, mm and mg were controlled in most of the studies performed and strong and positive correlations were found between both units for the analysis of CORT (Chapter I, II, V and VI: $0.80 < r > 0.84$, $P < 0.001$) and contaminants (Chapter VI: $0.79 < r > 0.94$, $P<0.001$).

6. Author points of view: Future studies

Feathers is a matrix with an exceptional potential to be used in field and lab studies when non-destructive sampling is required and long-term perspective of stress and contaminant exposure is pursued. Three factors are crucial to conduct an adequate analysis: good experimental design selecting the appropriate type of feather in accordance with the endpoint of the study; lab analysis following validated protocols; and data interpretation taking into account potential physiological confounding factors. All the advantages that have been numbered in this thesis for the use of body feathers make them suitable when working with wild birds or when non-invasive methods are required. However, there are still many gaps of knowledge that should be studied in order to better understand and interpret data.

Below I list some remaining questions that have resulted from the present thesis and that should be addressed in future studies:

- Stability in feathers:
  - Experimentally test the effect of extreme environmental conditions (e.g. intense UV radiation, high temperatures) on the stability of CORT in feathers.
  - Evaluate the stability of pollutants in feathers over time (at least during the same feather generation).
- Preen oil and washing constraints:
  - Deeper study the influence of preening in the contaminant levels in feathers. Is preen oil an important confounding factor?
  - Validation of the washing procedure to analyze OPEs in feathers. How OPEs are deposited into feathers?
- Time gap:
  - Monitor the time gap between the last molt period and time of sampling in biomonitoring carry-over effects of chronic stress and high contamination.

- Hormones:
  - Experimentally test the relationship between CORT and DHEA.
  - Study the stress-induced CORT response in birds presenting cachectic condition or chronic disabilities or pathologies. Do these birds respond to stressors similar to healthy birds?
- Contaminants:
  - Study whether contaminant levels in nestling down feathers indeed reflect the concentrations in eggs.
  - Test the usefulness of nestling down feathers to biomonitor deleterious effects of contaminants on reproductive success (i.e. date of egg-laying; clutch size, nest-site attentiveness; hatching success; fledging success).
- Relationship hormones - contaminants:
  - Study the mechanism of action of pollutants impairing the HPA axis activity.
  - Monitor nestlings over their first year of life: Do high concentrations of CORT and POPs influence dispersion and survival rate in juveniles?
  - Establish the threshold value of contaminants in feathers causing negative effects.

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CONCLUSIONS

The present doctoral thesis aimed to evaluate and optimize the use of feathers to assess long-term levels of corticosterone and biomonitor environmental pollutants exploring their effects on birds. Under this aim, the studies conducted in this thesis allowed us to reach the following conclusions:

Specific objective 1:

To test the suitability of different types of feathers to measure corticosterone and monitor environmental pollutants.

CONCLUSION 1.1: Feather corticosterone concentrations differ depending on the type of feather, being body feathers more suitable than flight feathers.

CONCLUSION 1.2: Nestling down feathers can be useful for contaminant monitoring (POPs and OPEs).

Specific objective 2:

To determine individual consistency, stability and repeatability in feather corticosterone levels over time and validate a new protocol for the extraction of feather corticosterone.

CONCLUSION 2.1: Concentrations of corticosterone in body feathers are consistent and stable on an individual level over the same feather generation.

CONCLUSION 2.2: Feather corticosterone concentrations differ from year to year indicating individual flexibility.

CONCLUSION 2.3: A new and optimized method for extracting and quantifying feather corticosterone concentrations has been validated.

Specific objective 3:

To examine the potential uses of feather corticosterone as a biomarker of bird fitness (survival and reproduction).

CONCLUSION 3: High feather corticosterone concentrations appear to be predictive of high mortality rate and reproductive failure in the studied species, demonstrating the potential of this tool to be used in bird management programs.

Specific objective 4:

To evaluate the usefulness of feathers to biomonitor environmental pollutants and investigate potential relationships with adrenal stress hormones.

CONCLUSION 4.1: Unexpected high levels of DDT and lindane have been detected in feathers of captive red kites and nestling cinereous vultures, respectively.

CONCLUSION 4.2: In free-living but not in captive red kites, levels of PCBs and DDTs are shown to enhance adrenal stress response, increasing both corticosterone and dehidroepiandrosterone hormone concentrations.

CONCLUSION 4.3: In nestling cinereous vultures, high levels of PCBs and DDE, but not PBDEs nor OPEs, are related to high concentrations of corticosterone but do not affect growing rate or fledging success.

Specific objective 5:

To examine potential confounding factors in relation to corticosterone and contaminant measurements in feathers.

CONCLUSION 5.1: Sex and age do not influence feather corticosterone nor contaminant concentrations in the studied species.

CONCLUSION 5.2: Body condition and health status can influence the capacity of feather corticosterone to be a specific biomarker of bird fitness.