Biomonitorización de contaminantes ambientales persistentes y evaluación de efectos subletales en aves silvestres: uso de plumas y biomarcadores de estrés oxidativo

Biomonitoring of persistent environmental pollutants using feathers and assessment of sublethal effects using oxidative stress biomarkers in wildbirds

Tesis Doctoral Europea

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Tesis Doctoral Europea

Biomonitorización de contaminantes ambientales persistentes y evaluación de efectos subletales en aves silvestres: uso de plumas y biomarcadores de estrés oxidativo

Memoria presentada por

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para optar al grado de Doctor con mención de "Doctorado Europeo" por la Universidad de Murcia

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Evaluation of Espín PhD Thesis Summary February 2013-02-27 by C. Sonne

Dear Prof. Dr. Fernández

Below please find my evaluation of Espín's PhD summary (word document and published papers).

In conclusion I find Espín's PhD theses summary and published papers of a quality that allows public defense of the final fully thesis in Spanish.

Yours sincerely,

Christian Sonne

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Evaluation of Espín's PhD Thesis Summary Feb 2013

By

Christian Sonne

GENERAL. Espín has submitted a PhD summary on 57 pp. entitled "*Biomonitoring of persistent environmental pollutants using feathers and assessment of sublethal effects using oxidative stress biomarkers in wild birds*" for pre-review prior to public defense. Together with the summary, four published papers and a review that she co-author has been submitted.

All in all the summary is very well written and easy to understand and overview due to relevant supportive sections re. sampling methods, study design and statistics. Moreover, the sample size given the study species (razorbill, eagle owl and griffon vulture) is reasonable and very relevant literature has been included in the discussion. Solid chemical and biochemical (effect) parameters have been applied.

SPECIFIC. Despite Espín has not solely done the review paper (Chapter I) it gives a nice overview of the use of feathers as biomonitoring OHCs in wild bird species. However, there is novelty from Espín's present PhD thesis as I see and it is unusual to include a review paper like that in a personal thesis. This should, however, not count negative in the evaluation but Espín should may be consider not including it in her PhD thesis as the scientific value of Espín 's PhD thesis is far beyond what is required given the already peer-reviewed published papers.

The Chapter II paper is probably interesting and peer-reviewed and cites relevant literature. However, it is in Spanish and I therefore cannot evaluate its value.

Chapter III is very interesting describing OHC concentrations in internal tissues of the wide-distributed razorbill. Thought this has been done previously it has not been done in this species and it is published in the nice peer-reviewed international journal Chemosphere with is an assurance of the quality.

Chapter IV is tightly associated with paper III and describes the OC-distribution in feather tissue and is already published in journal Ecotoxicology. I can however not evaluate if the relation between OHCs in tissues and feathers has been included in the thesis or in the papers. Please unsure that is undertaken prior to submission.

Chapter V and VI is the last about exposure (Part I). I really like the mercury approach - excellent to combine metals and OHCs in this thesis (just remember to evaluate the OHC vs. Hg relationship if relevant). More over the first is published in the peer-reviewed journal Marine Pollution Bulletin which ensures a certain scientific level. Chapter VI looks into Eagle owl Hg exposure and how it may relate to diet and local pollution. Though it's not published I'm sure it will be.

Chapter VII and VIII are the two papers that describe ROS in relation to element exposure and concentrations. Espín could consider putting together these data when submitting to a scientific journal. It is interesting stuff with some solid scientific correlations that indicate cause-and-effect relationships.

CONCLUSION. I officially support Espín's PhD theses summary of a quality that allows public defense of the final submitted thesis.

External evaluation of the PhD thesis of ESPIN Silvia

Thesis title: **Biomonitoring of persistent environmental pollutants using feathers and assessment of sublethal effects using oxidative stress biomarkers in wildbirds.**

Directors: Antonio Juan García-Fernández and Emma Martínez-López

This thesis includes 8 chapters, 5 of them already published in peer-reviewed journals, and is divided in 2 parts: part 1. Biomonitoring of persistent environmental pollutants in wild birds: feathers as a biomonitoring tool and part 2. Induction of oxidative stress in metal exposed wild birds.

The main objectives of this doctoral thesis, in respect of the three species studied, razorbill, eagle owl and griffon vulture, are very well formulated by the author and comprehensive. The importance of validating the use of feathers for organochlorine pesticides is very clear, timely and justified and supported by an exhaustive literature on the argument. The assessment of oxidative stress related to metals in birds and the research on heavy metals in feathers and tissues of birds are not particularly innovative but the author is very attentive to what was already done in the past and puts together a convincing approach to the three species studied. The sample collection is impressive and statistically credible and the author convincingly integrates the two parts. The number of mercury-contaminated wildlife habitats has increased progressively so the attention of the author to this toxicity is justified. Considerable work has been already carried out on heavy metals in birds; however few studies have been conducted on the effects of these metals on oxidative stress biomarkers in free-living birds exposed to them in nature, so limited data were available.

The rationale describing the need for this work, for selection of the three species considered, for monitoring of biomarkers in addition to direct measurements of heavy metals and OCs in feathers, blood and tissues, and for a focus on OCs in feathers, is well argued. The objectives of the study follow logically from the rationale.

The scope of the work is novel in several ways. It adds to scientific knowledge about the three species considered, about the exposure of this species to environmental contaminants which still pose a threat to wildlife populations, and about the relationships between the values of various biomarkers and the levels of various contaminants in feathers, blood and tissues.

Laboratory analysis, analytical and statistical methods used are up-to-date and quality controlled. The results from the analyses are well described and very clear. The conclusions are sound. The study can be expected to generate a further number of high-quality papers. Overall, the quality and quantity of the scientific output is timely and extremely relevant.

My overall assessment is that the thesis is clearly articulate, well balanced and professionally prepared. The candidate has shown she has fulfilled the criteria needed to be awarded her doctorate.

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External evaluation report for the Doctoral Thesis of Silvia Espin

Biomonitoring of persistent environmental pollutants using feathers and assessment of sublethal effects using oxidative stress biomarkers in wild birds. – Silvia Espin

External referee: Dr. Veerle Jaspers, University of Antwerp, Belgium

General appreciation

Ms. Silvia Espin has proven to have acquired a thorough knowledge about this research field, resulting from the available thesis summary and the published papers. She has systematically developed her research from the analytical protocol for pesticides in feathers, application of this protocol in Razorbill feathers to evaluate them as an alternative tool, a prediction model for Hg levels in Razorbill tissues based on Hg levels in feathers, up to an investigation of the factors that may influence exposure levels in nestling eagle owls. Furthermore, the second part of her thesis aimed to evaluate some of the effects on oxidative stress caused by heavy metal exposure. To my opinion her work is highly relevant for environmental sciences and I believe it is suitable to defend as an European Doctoral Thesis.

Evaluation and relevance of the work

In total the doctoral thesis is a compilation of 8 manuscripts of which 5 have already been published or are in press, four of them in ISI journals, indexed in the Web of Science. Therefore the work presented in this doctoral thesis has been disseminated on an international scale and is evaluated as highly relevant.

I will mainly focus my evaluation on the first part of the thesis (Biomonitoring of persistent environmental pollutants in wild birds: feathers as a biomonitoring tool), as I did not receive the two unpublished papers of Part 2 (Induction of oxidative stress in metal exposed wild birds). However, I would like to raise the question why she focused on OCPs and Hg in Part 1, while the effects of metals were studied in Part 2. The rationale for this choice should be provided, as it is not evident to me.

First, Ms. Silvia Espin started with optimising an analytical method to measure organochlorine pesticides (OCPS) in feathers (Chapter II), then assessed concentrations of OCPs in tissues and feathers of razorbill (Chapter III and IV), developed a predictive model to predict concentrations of Hg in tissues based on concentration of Hg in feathers (Chapter V) and finally she evaluated which factors influence Hg concentration in blood and feathers of nestling eagle owls (Chapter VI). She also contributed as a co-author to a review of feathers as a biomonitoring tool for polyhalogenated compounds (Chapter I).

Chapter I is a very elaborate and comprehensive review on the use of feathers as a biomonitoring tool, which is accepted for publication in one of the top journals in Environmental Sciences. I want to congratulate Ms. Espin for her contribution to this review (she is the second author), which is certainly a big contribution to the research in this area. To my opinion this review should not be placed as the first Chapter of the doctoral thesis, as this work follows naturally from the work she has done previously (Chapter II-VI) and therefore is better placed at the end of Part 1 (as a discussion maybe of part 1). The

important recommendations proposed in this review are as a consequence not applied in the following chapters, since the latter were actually executed previously. Furthermore, if the review is placed at the beginning of the thesis, the thesis starts with a manuscript that Ms. Espin is not the first author of, while on all the other papers she is actually the first author. This may give a biased view on her doctoral work, which is mainly her own (with help from supervisors of course). A doctoral thesis is about the own original contribution to a specific research field and therefore I recommend to start with Chapter II.

Chapter II is about the optimization of the analytical protocol in feathers for OCPs, which I consider the first step towards the completion of her PhD, and therefore should be placed at the beginning. It is a pity that the publication is in Spanish only (although I can read some Spanish, not everyone does) and that the preferred protocol was not mentioned in the English abstract of the paper. This diminished the potential influence of this work on international scale, although it is highly relevant. Therefore, it is good that the preferred method is described in the extended abstract.

Chapter III and IV report for the first time on OCPs in tissues and feathers of Razorbills. High levels were found in comparison to studies on other bird species and these levels are probably related to the razorbill diet and migration. Therefore these studies provide very relevant information both on the use of feathers in this species, but also on the potential influencing factors in OCP exposure in seabirds.

Chapter V and VI look at Hg exposure in Razorbill and Eagle owl, respectively. In Chapter V prediction equations were calculated to model concentrations in tissues (brain and kidney) using concentration of Hg in feather shafts. These equations are therefore a very useful tool to assess internal concentrations using only non-destructive monitoring of feather concentrations. Such equations should also be developed for other pollutants as well. Chapter VI is a very interesting chapter on the potential influencing factors (diet, local contamination, precipitation, and year) on Hg levels in feathers and blood of the eagle owl. I only consider that the correlation coefficient found between the back feathers and the blood is not very strong ($r=0.339$), so I would recommend the authors to check this and if necessary to revise their conclusion on using one unique feather to estimate concentrations in the blood, as this could lead to large margins of error. This may be due to highly variable concentrations in blood, which present a snapshot and it may be better to use feathers to estimate internal tissue concentrations.

Conclusion

Overall the studies performed during the PhD research of Ms. Silvia Espin are original, of high quality and of important relevance to Environmental Sciences. I therefore support and recommend the submission of the thesis and its defense by the candidate Ms. Silvia Espin.

Dr. Veerle Jaspers   Jaspers   19 March 2013

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A mis padres, Juan e Isabel

A mi hermana Isa

A mis abuelos

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ESTRUCTURA DE LA TESIS DOCTORAL

La presente tesis doctoral comienza con una extensa introducción general o revisión bibliográfica que profundiza en la temática de estudio. A continuación se presentan los objetivos generales de la tesis, así como los objetivos específicos de cada capítulo en castellano.

Posteriormente, la tesis se estructura en ocho capítulos, referidos en el texto en números romanos, que corresponden con otros tantos trabajos científicos originales, cinco de ellos ya publicados en revistas científicas. Los capítulos I al V han sido publicados en Environmental Science and Technology (índice de impacto de los últimos 5 años: 5,76), Anales de Veterinaria (Murcia), Chemosphere (índice de impacto de los últimos 5 años: 3,61), Ecotoxicology (índice de impacto de los últimos 5 años: 3,19) y Marine Pollution Bulletin (índice de impacto de los últimos 5 años: 2,93), respectivamente. Los tres últimos capítulos están preparados para ser enviados a revistas científicas internacionales indexadas. Cada uno de estos capítulos se divide en las secciones típicas de un trabajo científico, es decir, en introducción, material y métodos, resultados y discusión, conclusiones y referencias. Todos los capítulos están redactados en el idioma de la revista en la que fueron publicados o se prentede publicar.

Posteriormente se presenta una discusión general, en castellano, sobre lo tratado en los ocho capítulos previos y que conforman el cuerpo científico de la tesis. Esta parte incluye algunas recomendaciones para futuros estudios. Finalmente, se presentan unas conclusiones generales de la tesis en su conjunto en castellano.

INTRODUCCIÓN

1. BIOMONITORIZACIÓN DE CONTAMINANTES AMBIENTALES PERSISTENTES.

A pesar de que algunos compuestos químicos se encuentran en el medio ambiente de forma natural, la actividad humana contribuye a que su presencia aumente en el medio, principalmente a raíz de la revolución industrial. Aunque en los últimos años las emisiones industriales y el uso de determinados productos se han reducido significativamente gracias a mejoras tecnológicas, prohibiciones gubernamentales y un aumento de la conciencia ambiental, estos contaminantes continúan emititiéndose y/o persisten en el medio. Los metales pesados y los compuestos organoclorados destacan entre los contaminantes ambientales por su baja tasa de degradación, su persistencia en el medio y por los efectos que son capaces de producir en los seres vivos.

Desde hace décadas se vienen analizando un gran número de contaminantes ambientales en aire, agua o sedimentos mediante programas de monitorización ambiental, que dan información sobre el grado de contaminación en un lugar determinado. Sin embargo, estos estudios no proporcionan información sobre el grado de exposición a los compuestos tóxicos, la biodisponibilidad de los mismos y los efectos relacionados con dicha exposición en los seres vivos (Beeby, 2001).

Entre los años 40 y principios de los 60 se observó una considerable disminución en las poblaciones de aves, particularmente de aves rapaces, que llegó a relacionarse con el uso de ciertos compuestos clorados como el DDT y su persistencia ambiental (Hotchkiss y Pough, 1946; Newman, 1993; Newton y Wyllie, 1992). Estas observaciones condujeron a un incremento de estudios analíticos sobre muestras biológicas con el fin de mejorar la información sobre la exposición a estos contaminantes. Paralelamente se desarrolló una importante vía de estudios sobre biomarcadores con el fin de ofrecer, además de información sobre la exposición, información útil sobre los posibles efectos de dicha exposición (Moore y Ratcliffe, 1962; Ratclife, 1970). Así, durante las últimas décadas, se han ido desarrollando en distintos países programas de monitorización ambiental, particularmente de biomonitorización, con el objetivo de conocer los niveles de contaminantes ambientales presentes en el medio ambiente, el grado de exposición en los seres vivos y los efectos que pueden causar a largo plazo (García-Fernández y María-Mojica, 2000).

Según el Consejo Nacional de Investigación de los Estados Unidos (NRC, 1991), los objetivos de la monitorización con animales centinela incluyen la recolección de datos para estimar riesgos para la salud humana, identificar contaminantes en la cadena alimentaria, determinar los niveles de contaminación ambiental, e identificar efectos adversos sobre los propios animales. En este sentido, las aves, y más particularmente las aves marinas y las rapaces, han sido ampliamente utilizadas por su posición en la cadena alimentaria, especialmente en el estudio de los contaminantes bioacumulables y biomagnificables a lo largo de la cadena trófica. Así, por ejemplo, la información obtenida de estos estudios de monitorización en aves puede ser utilizada en los procedimientos de evaluación de riesgos regulados por el Reglamento 1907/2006 REACH de la Unión Europea (Duke, 2008). Este Reglamento, que consiste en un sistema integrado de registro, evaluación, autorización y restricción de sustancias y preparados químicos, pretende garantizar un nivel elevado de protección de la salud humana y el medio ambiente. En este sentido, los resultados obtenidos en la monitorización en determinadas aves pueden además incentivar el estudio en otras que pudieran pasar inadvertidas (Duke, 2008).

El uso de marcadores biológicos o biomarcadores es de gran importancia en la evaluación de riesgo tóxico por contaminantes. Un biomarcador puede definirse como la respuesta biológica a una sustancia o sustancias químicas que da una medida de la exposición y, a veces, también del efecto tóxico (Peakall y Walker, 1994). Las características que debería reunir un biomarcador "ideal" son: (i) que sea mensurable en tejidos de fácil obtención o mediante técnica no invasiva y que pueda relacionarse con la exposición y/o grado de daño al organismo; (ii) que se pueda relacionar directamente con el mecanismo de acción de los contaminantes; (iii) que sea mensurable por técnicas muy sensibles que requieran cantidades mínimas de muestra y que sean fáciles de llevar a cabo y rentables económicamente; y (iv) que pueda ser aplicado en diferentes especies (Fossi, 1994). Uno de los obstáculos a este enfoque es la gran variabilidad interespecífica, incluso para el mismo proceso bioquímico. Por tanto, es esencial el conocimiento de los niveles basales de actividad de determinadas enzimas o procesos metabólicos para cada especie. Antes de que pueda ser utilizado en condiciones de campo, un nuevo biomarcador requiere investigación sobre las relaciones dosis-respuesta e influencia de parámetros biológicos (edad, sexo, patrimonio genético, estado reproductivo, etc.) y ambientales (temperatura, salinidad, luz, etc.) sobre los valores de respuesta (Fossi, 1994).

En general, los biomarcadores pueden clasificarse en exposición y efecto. Los biomarcadores de exposición permiten conocer las concentraciones de un determinado compuesto tóxico en el organismo mediante el análisis del mismo o de sus metabolitos en tejidos o fluidos (Gil y Pla, 2001). El conocimiento de la cinética de un compuesto o elemento dentro del organismo nos permite obtener información relevante sobre la intensidad de la exposición. Así pues, la medida directa de los contaminantes en tejidos internos y sangre es el mejor indicador del grado y tipo de exposición a los mismos (García-Fernández *et al.*, 1997; María-Mojica *et al.*, 2000). Sin embargo, razones prácticas, éticas, legales y de conservación abogan por la búsqueda de muestras de obtención poco o nada cruenta para el animal y que sirvan como alternativa a la recolección de tejidos internos. De esta forma, numerosos estudios se han centrado en técnicas no destructivas para la biomonitorización de contaminantes utilizando muestras como la sangre (García-Fernández *et al.*, 1996, 1997; Martínez-López *et al.*, 2005, 2009), los excrementos (Sun *et al.*, 2006), el alimento regurgitado (Newton *et al.*, 1994), los huevos (Malik *et al.*, 2011; Martínez-López *et al.*, 2007), el aceite de la glándula uropigial (Johnston, 1976; Yamashita *et al.*, 2007), el cabello (Covaci *et al.*, 2002; D'Havé *et al.*, 2005) y las plumas (Dauwe *et al.*, 2005; Jaspers *et al.*, 2006; Martínez-López *et al.*, 2004, 2005). En este sentido, las plumas han sido ampliamente utilizadas en estudios de biomonitorización de metales en aves (Furness *et al.*, 1990; Martínez-López *et al.*, 2004, 2005; Pilastro *et al.*, 1993), y más recientemente en la biomonitorización de compuestos orgánicos (Dauwe *et al.*, 2005; Eulaers *et al.*, 2011a; Jaspers *et al.*, 2007a).

Por otro lado, aunque la medición de concentraciones de contaminantes en tejidos de aves es muy útil para conocer el grado de exposición, absorción y acumulación de los compuestos tóxicos, no proporciona información sobre las alteraciones biológicas causadas por la contaminación (Geens *et al.*, 2010). Los efectos tóxicos producidos por los contaminantes en aves han sido ampliamente estudiados y revisados en la literatura (Cortinovis *et al.*, 2008; Frederick y Jayasena, 2010; Kamata *et al.*, 2009; Mateo *et al.*, 2003a; Snoeijs *et al.*, 2004). Por lo tanto, también es necesario buscar biomarcadores de respuesta o efecto para evaluar los cambios sufridos por los organismos, muchos de ellos bioquímicos, como resultado de la exposición a los contaminantes (Gil y Pla, 2001). La sangre es el tejido de elección para estudios no destructivos de biomarcadores, ya que se obtiene fácilmente y permite medir una amplia gama de marcadores biológicos y contaminantes. Sus principales limitaciones se derivan del hecho de que los niveles de residuos pueden deberse a una ingestión

reciente y que algunos biomarcadores fluctúan más que en otros órganos (Fossi, 1994). Se presta especial atención a los parámetros hematológicos (hematocrito, concentración de hemoglobina, etc.) que pueden actuar como sensibles señales de alerta temprana de los posibles efectos tóxicos por contaminantes en la salud y el estado fisiológico (Weeks *et al.*, 1992). Algunas enzimas del metabolismo de aminoácidos como la aspartato transaminasa (AST) y la alanina transaminasa (ALT), o del metabolismo de carbohidratos como la lactato deshidrogenasa (LDH), son indicativas del daño tisular provocado por compuestos tóxicos (Peakall, 1992; Rozman *et al.*, 1974). Por otra parte, el plomo (Pb) influye directamente en la producción del grupo hemo mediante la inhibición de la enzima deshidratasa del ácido δ-aminolevulínico (ALAD). En este sentido, la enzima ALAD se ha utilizado con éxito como biomarcador de contaminación por Pb en aves (Gómez-Ramírez *et al.*, 2011; Martínez-Haro *et al.*, 2011; Martínez-López *et al.*, 2004).

Uno de los mecanismos a los que se atribuye la toxicidad de los metales pesados y los plaguicidas organoclorados es su capacidad de inducir estrés oxidativo, ya sea de forma directa generando especies reactivas de oxígeno (ROS), o de forma indirecta inhibiendo el sistema antioxidante celular (Abdollahi *et al.*, 2004; Ercal *et al.*, 2001). Un aumento en la formación de ROS puede resultar en estrés oxidativo, estado en el cual existe un desequilibrio entre la defensa antioxidante y la producción de especies reactivas de oxígeno, por lo que la defensa es superada por la formación de radicales (Halliwell y Gutteridge, 2007). Un exceso de radicales puede provocar daño oxidativo a lípidos de membrana, ADN y proteínas, y la oxidación de estas biomoléculas puede derivar en una pérdida de función celular y daño a los tejidos (Monaghan *et al.*, 2009). Diversos estudios experimentales han mostrado la capacidad de los metales y plaguicidas organoclorados para inducir estrés oxidativo en diferentes seres vivos (Hassan *et al.*, 1991; Huang *et al.*, 1996; Koner *et al.*, 1998; Sandhir y Gill, 1995; Simon-Giavarotti *et al.*, 2002; Toplan *et al.*, 2003), incluidas las aves (Hoffman *et al.*, 2005; Mateo y Hoffman, 2001; Mateo *et al.*, 2003a; Samanta y Chainy, 1997; Somashekaraiah *et al.*, 1992), por lo que los niveles de moléculas antioxidantes, la actividad de determinadas enzimas antioxidantes, y los productos del daño oxidativo pueden ser interesantes biomarcadores de exposición y efecto a estos contaminantes (Gil y Pla, 2001). Sin embargo, son pocos los estudios que evalúan los efectos de los metales y los plaguicidas en biomarcadores de estrés oxidativo en aves silvestres expuestas a estos contaminantes en condiciones naturales (Barata *et al.*, 2010; Berglund *et al.*, 2007; Koivula *et al.*, 2011; Martínez-Haro *et al.*, 2011).

2. PLUMA COMO HERRAMIENTA DE BIOMONITORIZACIÓN.

Smith *et al.* (2003) recopilaron información sobre los diferentes usos que se dan a las plumas en diferentes campos de la ciencia. Una de las primeras aplicaciones prácticas de las plumas es el estudio de perfiles de elementos y minerales, muy útiles para diferenciar poblaciones de aves (Hanson y Jones, 1968). Además, los análisis de isótopos estables en plumas proporcionan información sobre la alimentación de las aves, su comportamiento migratorio, las estrategias de muda y su origen geográfico (Hobson, 2005). Por otra parte, las plumas son una fuente de ADN para estudios genéticos de filogeografía y estructura de poblaciones (Poulakakis *et al.*, 2008), y ofrecen material para evaluar los niveles de algunos contaminantes (Burger, 1993; Jaspers *et al.*, 2011). Más recientemente, algunos estudios han cuantificado los niveles de corticosterona en plumas para la evaluación del estrés en aves (Bortolotti *et al.*, 2008, 2009).

Los tejidos de queratina, como el cabello, han sido utilizados en estudios de acumulación de contaminantes orgánicos persistentes en mamíferos y seres humanos (Altshul *et al.*, 2004; Covaci *et al.*, 2002; D'Havé *et al.*, 2005; Dauberschmidt y Wennig, 1998). Estos estudios sugirieron que los tejidos de queratina podían ser unidades de biomonitorización interesantes para estos contaminantes persistentes, aunque la acumulación de estos compuestos en el pelo con frecuencia es muy baja. La pluma es el tejido de queratina más importante en las aves, y ha sido utilizada de forma intensiva como método de biomonitorización no destructivo en la contaminación por metales pesados, demostrando que puede usarse como alternativa a los tejidos internos (Burger, 1993; Burgess *et al.*, 2005; Fasola *et al.*, 1998; Garitano-Zavala *et al.*, 2010; Hollamby *et al.*, 2004; Malik y Zeb, 2009; Martínez-López *et al.*, 2005; Zolfaghari *et al.*, 2007). A pesar de ello, todavía son pocos los trabajos que han utilizado la pluma como herramienta no destructiva de biomonitorización de contaminantes orgánicos (Dauwe *et al.*, 2005; Eulaers *et al.*, 2011a; Jaspers *et al.*, 2007a).

Al contrario que el pelo, que crece continuamente, las plumas sólo crecen durante un cierto periodo de tiempo, conectadas al torrente sanguíneo (Jaspers *et al.*, 2004). Los contaminantes pueden llegar a las plumas durante su crecimiento a través de la sangre, produciéndose de esta forma una contaminación interna. Después de la maduración de la pluma, el suministro de sangre se atrofia y se convierten en plumas aisladas del resto del cuerpo (Burger, 1993). Por lo tanto, las plumas pueden contener información sobre las concentraciones circulantes en la sangre durante su desarrollo.

Las plumas tienen una serie de ventajas respecto al resto de tejidos de obtención no cruenta: (i) pueden ser recolectadas independientemente de la época del año, la edad o el sexo, (ii) pueden muestrearse en pequeño número sin causar daño permanente al ave, (iii) pueden obtenerse tanto directamente del animal, como de cadáveres, o recogerse en nidos y suelo en períodos de muda, (iv) están disponibles en museos de historia natural, y (v) son fácilmente recogidas, transportadas y almacenadas a temperatura ambiente. Además de estas ventajas respecto a la toma de muestras, se debe tener en cuenta que hay disponible bastante bibliografía en cuanto a su uso en biomonitorización de contaminantes ambientales, principalmente metales pesados, y que se han encontrado correlaciones significativas entre los niveles de contaminantes en tejidos internos y las concentraciones en plumas. Sin embargo, hay una serie de factores que deben tenerse en cuenta al utilizar la pluma como herramienta de biomonitorización. Algunos factores intraespecíficos como la edad, sexo y condición corporal (Dauwe *et al.*, 2005; Jaspers *et al.*, 2007a, 2011), e interespecíficos como la dieta y el comportamiento migratorio (Behrooz *et al.*, 2009a; Rajaei *et al.*, 2011; Zolfaghari *et al.*, 2007), pueden afectar a las concentraciones de contaminantes en plumas. Además, la interpretación de las concentraciones de contaminantes obtenidas en plumas, así como las correlaciones entre los niveles de contaminantes en tejidos internos y las concentraciones en plumas, pueden ser confusas debido a la contaminación externa en la superficie de las mismas (Dauwe *et al.*, 2003; Jaspers *et al.*, 2004, 2008, 2011; Pilastro *et al.*, 1993). Esta contaminación externa puede producirse tanto por fuentes exógenas, como la deposición atmosférica ya sea en seco o húmedo, como endógenas, es decir, por el acicalamiento de las plumas con aceite de la glándula uropigial (Figura 1).

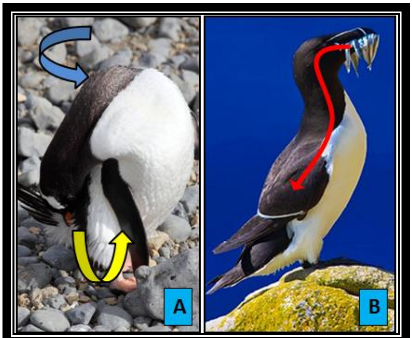

Figura 1. *Posibles rutas de contaminación de plumas: A) Contaminación externa mediante fuente endógena (aceite de la glándula uropigial) y exógena (deposición atmosférica), B) Contaminación interna mediante torrente sanguíneo.*

El tipo de pluma a seleccionar y el patrón de muda de la especie son aspectos de gran importancia a la hora de utilizar esta herramienta de biomonitorización, estrechamente relacionados con la contaminación externa. Las primeras plumas mudadas son más antiguas y tendrán, probablemente, mayor influencia de la contaminación externa. Sin embargo, plumas en desarrollo o mudadas recientemente tendrán poca contaminación externa en su superficie (Jaspers *et al.*, 2004, 2011). Otro factor clave que puede afectar a las correlaciones entre los niveles encontrados en plumas y tejidos internos es el tiempo transcurrido entre el momento en que crecieron las plumas y el momento de la toma de muestras, ya que en este periodo de tiempo las concentraciones de contaminantes presentes en plumas por vía endógena no varían, mientras que los niveles en órganos internos sí pueden verse modificados.

Las plumas pueden considerarse una herramienta de monitorización no invasiva, puesto que pueden tomarse del campo o en nidos sin necesidad de manipular directamente al individuo. Sin embargo, determinados factores como la muda, el momento del muestreo, el tipo de pluma, la contaminación externa y la edad, el sexo y la condición corporal de las aves pueden interferir en los resultados obtenidos y deben tenerse en cuenta. Algunos de estos factores se desconocen cuando las plumas son encontradas en el campo. Por lo tanto, aunque el uso de plumas encontradas puede aportar información interesante, es recomendable poseer la mayor información posible del origen de la pluma, lo que conlleva la manipulación de las aves para la obtención de las mismas. De esta forma, el término “no destructivo” es más apropiado que el témino “no invasivo” al referirnos a la pluma como herramienta de biomonitorización.

Una parte de la presente tesis doctoral se centra en el estudio de plaguicidas organoclorados y mercurio (Hg) en plumas y tejidos internos de aves silvestres. La elección de estos contaminantes se debe a que tanto los compuestos organoclorados como el Hg son contaminantes ubicuos capaces de biomagnificarse a través de la cadena trófica (Braune *et al.*, 2005), lo que provoca que especies de aves situadas en una posición trófica elevada sean más vulnerables a la exposición de estos contaminantes a través de la dieta. En el Capítulo I de la presente tesis se incluye una revisión crítica que profundiza sobre el uso de la pluma como herramienta de biomonitorización de compuestos polihalogenados. En cuanto al uso de las plumas para la monitorización de Hg en aves, numerosos estudios demuestran que son una excelente herramienta para la biomonitorización de este contaminante (Monteiro y Furness, 1995). El Hg es depositado en las plumas durante su formación, uniéndose a la queratina en forma de metilmercurio (MeHg) (Thompson y Furness, 1989a, 1989b),

ya que el Hg tiene una elevada afinidad por los grupos sulfhidrilo (SH) de esta proteína (Burger y Gochfeld, 2001). Se considera que más del 90% de la carga total de Hg en un ave puede ser secuestrada en las plumas durante la muda (Burger, 1993), permaneciendo las concentraciones de Hg relativamente estables (Appelquist *et al.*, 1984). Además, las concentraciones de Hg en tejidos internos se correlacionan con los niveles encontrados en plumas (Thompson *et al.*, 1991). Por otro lado, se ha observado que la distribución del Hg a lo largo de la pluma es relativamente uniforme, ya que no se han encontrado diferencias significativas entre las concentraciones de Hg en barbas internas, barbas externas, cálamo, eje y ápice de la pluma (Figura 2) (Dauwe *et al.*, 2003; Misztal-Szkudlińska *et al.*, 2012).

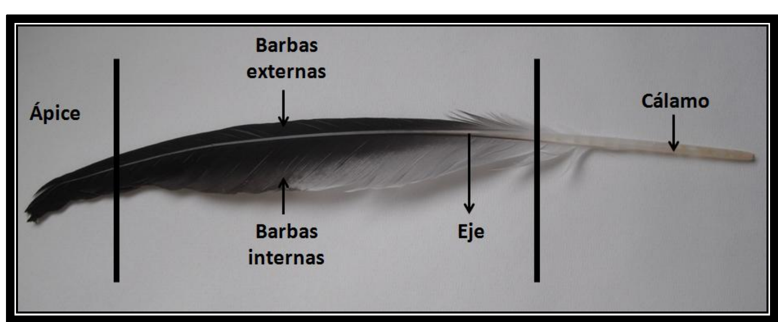

Figura 2. *Partes de una pluma.*

A pesar de que la contaminación externa tiene una influencia importante en las concentraciones encontradas en plumas para la mayor parte de metales (Cardiel *et al.*, 2011; Dauwe *et al.*, 2003; Jaspers *et al.*, 2004; Pilastro *et al.*, 1993), el Hg presente en plumas parece provenir principalmente de su deposición endógena (Jaspers *et al.*, 2004). Como se ha indicado anteriormente, Dauwe *et al.* (2003) no encontraron diferencias significativas entre las concentraciones de Hg en diferentes partes de la pluma (Figura 2), por lo que concluyen que la contaminación externa por Hg es nula o inapreciable, ya que determinadas partes de la pluma como las barbas deberían tener previsiblemente mayor contaminación externa por su mayor superficie. Aunque encontraron niveles de Hg 1,18 veces superiores en barbas que en eje, consideran que podría deberse a una diferente capacidad de acumulación de Hg entre estas dos partes de la pluma, más que a un efecto de contaminación externa (Dauwe *et al.*, 2003). Además, se ha observado que el Hg no se concentra en el aceite de la glándula uropigial, por lo que la contaminación externa de las plumas por esta vía después de su formación es mínima (Frank *et al.*, 1983). Sin embargo, a pesar de que la contaminación externa por metilmercurio es improbable, puede producirse una contaminación externa por mecurio inorgánico (Furness y Camphuysen, 1997). Algunos autores sugieren que el Hg puede quedar adherido en la superficie de la

pluma, ya sea por el aceite de la glándula uropigial o por partículas contaminadas (Condon y Cristol, 2009; Goede y de Bruin, 1984). Por tanto, en la mayoría de estudios se lleva a cabo un lavado de las plumas con agua desionizada y acetona para eliminar la contaminación adherida a la superficie antes de proceder con la técnica analítica (Appelquist *et al.*, 1984; Burger y Gochfeld, 2001; Burger, 1993; Dauwe *et al.*, 2003; Jaspers *et al.*, 2004).

Al igual que ocurre con otros contaminantes, los niveles de Hg en plumas pueden variar según determinados factores como la edad y el sexo. Algunos estudios han descrito mayores concentraciones en machos que en hembras (Lewis *et al.*, 1993; Stewart *et al.*, 1994), lo que se explica por el hecho de que las hembras pueden excretar aproximadamente un 20% del metilmercurio presente en sus tejidos blandos a través de los huevos en desarrollo (Lewis *et al.*, 1993). Por otro lado, se han encontrado mayores niveles de Hg en aves adultas en comparación con los pollos (Burger *et al.*, 2008; Ribeiro *et al.*, 2009). También se han observado diferencias en los niveles de Hg en plumas según la especie estudiada (Monteiro *et al.*, 1995), lo cual puede deberse a diferentes factores como la dieta específica de cada especie y el hábitat en el que residen. Sin embargo, debe tenerse en cuenta que el porcentaje de la carga total de Hg excretado a través de las plumas puede variar según la especie (Wolfe *et al.*, 1998). En este sentido, se ha observado que especies con mayor eficiencia en la desmetilación del Hg, como las aves del orden Procellariiformes, excretan un bajo porcentaje de su carga total de Hg a través de las plumas, aproximadamente un 10% (Kim *et al.*, 1996).

El patrón de muda de las especies tiene una influencia significativa en la concentración de Hg entre diferentes plumas de un mismo individuo (Appelquist *et al.*, 1984). Puesto que el metilmercurio es acumulado en los tejidos blandos desde la última muda del ave, y movilizado durante la siguiente muda (Lewis y Furness, 1991), se ha observado que las primeras plumas en crecer presentan mayores concentraciones de este metal, con concentraciones decrecientes en las sucesivas plumas (Braune y Gaskin, 1987; Braune, 1987; Dauwe *et al.*, 2003; Furness *et al.*, 1986). Además, en aves con patrones de muda secuenciales, los niveles de Hg en las primeras plumas desarrolladas corresponderán a una combinación de los niveles de Hg incorporados en ese momento a través de la dieta junto con el Hg orgánico acumulado entre períodos de muda, mientras que las últimas plumas en crecer al final del período de muda indicarán el consumo actual (Furness, 1993).

Debido al potencial de metilación[1] y a la bioacumulación y biomagnificación del metilmercurio en sistemas acuáticos, la mayor parte de los estudios de biomonitorización de Hg se han centrado en aves piscívoras y ecosistemas acuáticos (Seewagen, 2010). Sin embargo, estas aves no son necesariamente las únicas que sufren los impactos de este contaminante. Aunque menos importante que en los ecosistemas acuáticos (Lindqvist, 1991), la metilación sucede también en ecosistemas terrestres (Cristol *et al.*, 2008; Driscoll *et al.*, 2007; Rimmer *et al.*, 2005, 2010). Algunos estudios han descrito una elevada acumulación de Hg en plumas de rapaces posicionadas en lo alto de cadenas tróficas terrestres (Broo y Odsjö, 1981; Palma *et al.*, 2005). En este sentido, Zolfaghari *et al.* (2007) encontraron diferencias significativas en las concentraciones de Hg en plumas de 18 especies de aves en relación al nivel trófico, observando los mayores niveles de este metal en plumas de rapaces carnívoras no piscívoras.

3. BIOMARCADORES DE ESTRÉS OXIDATIVO CELULAR.

En los organismos aerobios, el oxígeno es esencial para la producción energética, pero, paradójicamente, las reacciones de oxidación-reducción aumentan la producción de especies reactivas de oxígeno (ROS) (Limón-Pacheco y Gonsebatt, 2009). Como indica su propio nombre, las especies reactivas de oxígeno son moléculas altamente reactivas que contienen oxígeno fruto del metabolismo celular normal (Dowling y Simmons, 2009). Estas ROS participan en el control del estado redox celular, pero pueden provocar daños a altas concentraciones, por lo que los seres vivos han desarrollado un mecanismo de defensa antioxidante (Koivula y Eeva, 2010).

Diversos estudios relacionan los efectos tóxicos de los metales y los plaguicidas organoclorados a la inducción de estrés oxidativo, lo cual puede deberse a diferentes mecanismos como la producción de ROS o la alteración del sistema antioxidante celular (Abdollahi *et al.*, 2004; Ercal *et al.*, 2001). Puesto que la presente tesis está enfocada al estudio de metales en relación al estrés oxidativo, nos centraremos en estos contaminantes y su relación con el sistema antioxidante. Las ROS a bajas concentraciones tienen efectos beneficiosos ya que participan en diferentes procesos fisiológicos tales como la defensa contra agentes infecciosos, los sistemas de

[1] Adición de un grupo metilo (-CH_3) a una molécula. En el caso del mercurio, transformación del mercurio inorgánico a metilmercurio, y del metilmercurio a dimetilmercurio, por microorganismos en suelo y agua.

señalización celular, y la inducción de la respuesta mitogénica (Valko *et al.*, 2006). Sin embargo, un aumento en la formación de ROS puede resultar en estrés oxidativo, estado en el cual existe un desequilibrio entre la defensa antioxidante y la producción de especies reactivas de oxígeno, por lo que la defensa es superada por la formación de radicales (Halliwell y Gutteridge, 2007). Un exceso de radicales puede provocar daño oxidativo en lípidos de membrana, ADN y proteínas, con el consecuente daño en funciones celulares y alteraciones tisulares (Hoffman *et al.*, 1998; Valavanidis *et al.*, 2006). Por todo ello, los sistemas antioxidantes juegan un papel importante en la protección del organismo contra el estrés oxidativo inducido por dichos compuestos tóxicos.

Diversos organismos expuestos a contaminantes ambientales han sido utilizados para el estudio de diferentes biomarcadores de estrés oxidativo. Sin embargo, el estudio del estrés oxidativo inducido por metales en poblaciones de aves silvestres sigue siendo escaso (Berglund *et al.*, 2007; Koivula *et al.*, 2011; Martínez-Haro *et al.*, 2011). Puesto que diversos estudios experimentales han demostrado que los metales son capaces de inducir estrés oxidativo en aves (Mateo y Hoffman, 2001; Mateo *et al.*, 2003a), los niveles de moléculas antioxidantes y la actividad de enzimas antioxidantes pueden ser interesantes biomarcadores de exposición y efecto a metales en aves. Varios autores sugieren que es esencial utilizar diferentes biomarcadores puesto que un biomarcador normalmente no es suficiente para describir completamente el mecanismo de estrés (Berglund *et al.*, 2007; Koivula y Eeva, 2010). En este sentido, Halliwell y Gutteridge (1999) concluyeron que no existe un biomarcador universal para estrés oxidativo. Según Costantini y Verhulst (2009), la capacidad antioxidante total o los niveles de un determinado tipo de antioxidante como marcadores de estrés oxidativo sin información adicional sobre daño oxidativo o producción de radicales libres no son suficientes para hacer deducciones sobre estrés oxidativo. Se debe tener en cuenta que altos niveles de ROS no provocan necesariamente estrés oxidativo, ya que pueden ser equilibrados mediante un aumento de las defensas antioxidantes. Además, individuos con niveles de antioxidantes relativamente altos no tienen que estar necesariamente en un mejor estado redox que individuos con menores niveles, ya que dependerá de los niveles de ROS a lo que las defensas tengan que hacer frente (Monaghan *et al.*, 2009). Por tanto, el estrés oxidativo no se puede deducir midiendo sólo un lado de este delicado balance entre la generación de ROS y el sistema antioxidante (Monaghan *et al.*, 2009).

En condiciones estables y constantes, o de homeostasis, tanto los niveles de ROS como de antioxidantes (AO) son bajos, y las defensas son suficientes para equilibrar la producción de ROS, no habiendo estrés oxidativo (Figura 3a) (Monaghan *et al.*, 2009). Se debe tener en cuenta que es improbable que el valor de estrés oxidativo sea exactamente cero, ya que los agentes pro-oxidantes se producen continuamente y por tanto, siempre se genera algo de daño oxidativo (Costantini y Verhulst, 2009). Si se produce un aumento en la producción de ROS, inicialmente se puede exceder la capacidad antioxidante, produciéndose un periodo de estrés oxidativo (Figura 3b). Si el aumento de ROS es pequeño, se puede igualar mediante un aumento en los niveles antioxidantes, previniendo que se produzca más estrés oxidativo (Figura 3c). Si este aumento de ROS es temporal, se volverá a la situación de estabilidad (Figura 3a). Sin embargo, un aumento prolongado de ROS puede provocar un aumento permanente de los niveles base de antioxidantes en el organismo (Figura 3d), siendo así más fácil equilibrar futuros periodos oxidativos (Monaghan *et al.*, 2009).

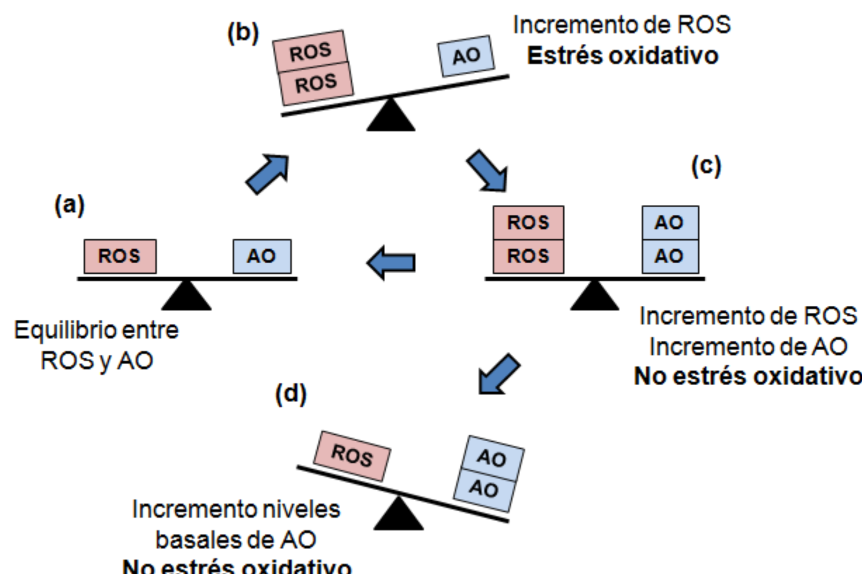

Figura 3. *Relación entre las especies reactivas de oxígeno (ROS), los niveles de antioxidantes (AO) y el estrés oxidativo. Basado en Monaghan et al. (2009).*

También se debe tener en cuenta que, aunque se produzca un aumento de las defensas antioxidantes, esto no implica la prevención del daño oxidativo, ya que la efectividad de este aumento dependerá de la medida en que se anule el aumento de ROS (Monaghan *et al.*, 2009). Por tanto, como sugieren Costantini y Verhulst (2009), un marcador de capacidad antioxidante debe ir asociado con, al menos, un marcador de daño oxidativo cuando se pretende evaluar el estrés oxidativo.

Por último, las diferencias entre especies aviares en cuanto a los niveles basales de parámetros del sistema antioxidante y en cuanto a los efectos que los metales son capaces de producir no están claras (Koivula y Eeva, 2010). Son necesarios más estudios en aves silvestres que nos ayuden a conocer estas diferencias interespecíficas, además de evaluar la posible influencia de otros parámetros como la edad y el sexo sobre el sistema antioxidante (Koivula y Eeva, 2010). Aunque, en general, las hembras tienen una mayor capacidad antioxidante que los machos (Halliwell y Gutteridge, 2007), los trabajos publicados en aves son escasos. En cuanto a la edad, se ha sugerido que el daño oxidativo aumenta con la edad, lo que puede deberse a una mayor producción de ROS y/o a una mayor susceptibilidad a las ROS a mayor edad (Martin y Grotewiel, 2006). También se ha sugerido que aves adultas asignan más recursos a la producción de señales sexuales a expensas de su salud en comparación con aves más jóvenes, por lo que sufren más estrés oxidativo (Cote *et al.*, 2010).

3.1. RADICALES LIBRES.

Como se ha indicado anteriormente, los principales radicales libres son aquellos derivados del oxígeno, conocidos como ROS, aunque existen otros como las especies reactivas de nitrógeno (RNS). Dichos radicales libres son especies que se caracterizan por presentar una estructura con uno o más electrones desapareados, lo que les confiere inestabilidad y una alta reactividad (Valko *et al.*, 2006). Por ello, participan en reacciones de oxidación-reducción donde los radicales tienden a aceptar electrones de otras moléculas reduciéndose, mientras que estas moléculas donadoras quedan oxidadas, convirtiéndose en radicales libres secundarios y creándose una reacción en cadena que puede causar daños biológicos mediante un proceso oxidativo en cascada (Costantini, 2008), particularmente a aquellas moléculas con un alto contenido en ácidos grasos poliinsaturados. Entre las RNS destaca el óxido nítrico ($NO^{\cdot}$), aunque estas especies han sido menos estudiadas y se considera que tienen menor importancia que las ROS en cuanto a la generación de daño (Monaghan *et al.*, 2009). El término ROS se aplica tanto a radicales libres, destacando el anión superóxido ($O_2^{\cdot-}$) y el radical hidroxilo ($OH^{\cdot}$), como a otras moléculas oxidantes como el peróxido de hidrógeno (H_2O_2). Estas ROS se producen continuamente en condiciones normales por medio de diversas vías (Valko *et al.*, 2006). Por ejemplo, la cadena de transporte de electrones mitocondrial puede reducir el O_2 a $O_2^{\cdot-}$ (Rotilio *et al.*, 1995), el citocromo P450 microsomal y su reductasa también producen $O_2^{\cdot-}$ durante la biotransformación

de xenobióticos (Rotilio *et al.*, 1995), y enzimas del citosol como la xantina oxidasa[2] y aldheído oxidasa[3] producen $O_2^{\cdot -}$ y H_2O_2 durante su catálisis (Rotilio *et al.*, 1995; Valko *et al.*, 2006). En eritrocitos, al carecer de mitocondrias, las ROS se producen debido a la elevada presión de oxígeno y el gran contenido en hierro (Fe) del grupo hemo. De esta forma, el $O_2^{\cdot -}$ se puede formar a través de la autoxidación de la oxihemoglobina (Rotilio *et al.*, 1995). El anión superóxido no reacciona directamente con polipéptidos, azúcares o ácidos nucléicos; y su capacidad de peroxidación lipídica es controvertida (Valko *et al.*, 2006). El $O_2^{\cdot -}$ reduce el Fe (III), liberando Fe (II) y oxígeno (Fórmula 1). El Fe (II) liberado puede participar en la reacción de Fenton (Fórmula 2), generando radicales hidroxilo altamente reactivos. La reacción completa que surge de la combinación de estas dos reacciones se denomina reacción de Haber-Weiss (Fórmula 3).

(1) $Me^{(n+1)+} + O_2^{\cdot -} \rightarrow Me^{n+} + O_2$ (Me=normalmente Cu o Fe)

(2) $Me^{n+} + H_2O_2 \rightarrow OH^{\cdot} + HO^{-} + Me^{(n+1)+}$ (Reacción de Fenton)

(3) $O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + HO^{-}$ (Reacción de Haber-Weiss)

Además, el $O_2^{\cdot -}$ puede formar H_2O_2 mediante dismutación enzimática o espontánea. El H_2O_2 también puede formarse directamente mediante catálisis de oxidasas[4] (Rotilio *et al.*, 1995). El H_2O_2 es un oxidante suave con poca capacidad de oxidar lípidos, ADN y proteínas. Sin embargo, al igual que el anión superóxido, pueden generar radicales hidroxilo debido a su participación en las reacciones de Fenton y Haber-Weiss (Fórmulas 2 y 3). El radical $OH^{\cdot}$ es un importante pro-oxidante altamente reactivo y con gran capacidad de provocar daño (Valko *et al.*, 2006), que procede principalmente de la descomposición del H_2O_2 a través de la reacción de Fentón (Fórmula 2), o de la interacción del $O_2^{\cdot -}$ con el H_2O_2 mediante la reacción de Haber-Weiss (Fórmula 3) (Costantini, 2008).

Los productos de peroxidación también pueden tener propiedades pro-oxidantes (Costantini, 2008). Por ejemplo, la toxicidad de los hidroperóxidos (ROOH) es promovida por la presencia de metales como Fe y Cu, que catalizan la escisión del ROOH, generando dos pro-oxidantes altamente reactivos, el radical alcohoxilo (R-O·)

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[2] Cataliza la oxidación de hipoxantina, otras purinas y aldehídos.

[3] Cataliza la oxidación de aldehídos en ácido carboxílico.

[4] Enzimas que catalizan reacciones de oxidación-reducción utilizando oxígeno como aceptor de electrones.

y el alquil peroxilo (R-OO·), favoreciendo el proceso oxidativo en cascada (Costantini, 2008).

Las ROS atacan macromoléculas (lípidos, ácidos nucléicos, proteínas), extrayéndoles un electrón, lo que las oxida y las modifica (Rotilio *et al.*, 1995), generando nuevas ROS y poniendo en marcha una cascada de daños si no son controladas (Monaghan *et al.*, 2009). El daño oxidativo a ácidos grasos poliinsaturados (PUFAs) da lugar a moléculas peroxidadas que se descomponen para formar metabolitos reactivos (Rotilio *et al.*, 1995). De esta forma, la peroxidación lipídica conlleva una compleja reacción en cadena que incluye una serie de reactivos intermedios que pueden provocar daños a proteínas y ADN (Monaghan *et al.*, 2009). Puesto que los lípidos son el mayor componente de las membranas biológicas, la fluidez y permeabilidad de estas estructuras se ven afectadas, además de la función de proteínas de membrana (Rotilio *et al.*, 1995). Entre las consecuencias del daño oxidativo a los ácidos nucléicos destacan la modificación de bases, rotura de anillos de desoxirribosa, rotura de cadenas y aberraciones cromosómicas (Rotilio *et al.*, 1995). En cuanto a las proteínas, pueden producirse modificaciones en la cadena lateral de los aminoácidos con la introducción de grupos carbonilo, o la oxidación de grupos sulfhidrilo. Estas modificaciones provocan una mayor susceptibilidad a proteasas específicas o a la desactivación enzimática (Rotilio *et al.*, 1995). Se ha propuesto que las modificaciones de macromoléculas debidas al estrés oxidativo están implicadas en diferentes procesos fisiológicos y patológicos, incluyendo el envejecimiento, cataratas, artritis, cáncer y enfermedades pulmonares (Rotilio *et al.*, 1995).

3.2. SISTEMA ANTIOXIDANTE.

Como comentábamos anteriormente, los organismos aerobios poseen moléculas antioxidantes, sustancias que tienen la capacidad de inhibir la generación de radicales libres, eliminarlos y reducir la oxidación y el daño provocado por los mismos (Koivula y Eeva, 2010). Los mecanismos de defensa contra el daño oxidativo inducido por los radicales libres operan a tres niveles, prevención, interceptación y reparación (Rotilio *et al.*, 1995). Los principales mecanismos son:

(i) secuestro de metales redox activos pro-oxidantes como Fe y Cu por proteínas específicas (transferrinas[5], metalotioneinas[6], albúmina[7], ceruloplasminas[8]) que

[5] Proteína transportadora específica de hierro en plasma.

[6] Proteínas intracelulares ricas en cisteína que sirven como almacén de cobre y zinc, y secuestran a metales no esenciales.

impide su participación en la reacción de Fenton (Limón-Pacheco y Gonsebatt, 2009; Rotilio *et al.*, 1995);

(ii) secuestro de radicales por proteínas que utilizan O_2, como citocromo oxidasa, ribonucleótido reductasa y oxihemoglobina, siendo el principal mecanismo de prevención de la formación de radicales libres (Rotilio *et al.*, 1995);

(iii) eliminación catalítica de radicales libres y especies reactivas por factores como la catalasa (CAT), superóxido dismutasa (SOD), peroxidasa, y antioxidantes tiol-específicos;

(iv) reducción de radicales libres mediante donadores de electrones, como el glutatión (GSH), vitamina E (α tocopherol), vitamina C (ácido ascórbico), bilirrubina, y ácido úrico (Limón-Pacheco y Gonsebatt, 2009).

(v) la última línea defensiva contra el daño es la eliminación o reparación de las moléculas dañadas (Monaghan *et al.*, 2009). La reparación del ADN es de gran importancia para la función celular, y existen diferentes y complejas vías de reconocimiento y reparación de daños (Kastan y Bartek, 2004). Las proteínas y lípidos dañados también pueden ser reparados, o destruídos y reemplazados (Halliwell y Gutteridge, 2007). Diversas lipasas, proteasas, y enzimas de reparación de ADN participan en esta línea defensiva. Un ejemplo de protección contra daño macromolecular son las proteínas de respuesta al estrés o de choque térmico (Limón-Pacheco y Gonsebatt, 2009).

Una molécula antioxidante al colisionar con un radical libre le cede un electrón, privándole de su capacidad reactiva. De esta forma se produce la oxidación de la molécula antioxidante, transformándose en un radical libre no reactivo que puede regenerarse por la acción de otros antioxidantes (Surai, 2003). Los principales antioxidantes del organismo son el glutatión (GSH), flavonoides, carotenoides, α-tocoferol (vitamina E), vitamina C y ácido úrico. Otro grupo importante de antioxidantes son los enzimáticos, que catalizan reacciones que inactivan y eliminan radicales libres, o participan en el sistema antioxidante de forma indirecta. La glutatión peroxidasa (GPx), glutatión reductasa (GR), glutatión S-transferasa (GST), catalasa (CAT) y superóxido dismutasa (SOD) son algunas enzimas importantes del sistema antioxidante (Halliwell y Gutteridge, 1999; Valavanidis *et al.*, 2006). Los niveles y

[7] Principal proteína plasmática con función de transporte de iones, ácidos grasos, hormonas, bilirrubina, y fármacos entre otros.

[8] Principal proteína transportadora de cobre en plasma.

composición del sistema antioxidante difieren según el tejido y tipo de célula (Costantini, 2008; Limón-Pacheco y Gonsebatt, 2009). Puesto que la mitocondria es uno de los lugares con una mayor producción de radicales libres, está enriquecida con antioxidantes como el GSH y enzimas como SOD y GPx, presentes en ambos lados de sus membranas para minimizar el estrés oxidativo en dicho orgánulo (Valko *et al.*, 2006).

El tripéptido GSH (gamma-glutamil-L-cisteinilglicina) contiene grupos sulfhidrilo (SH) debido al aminoácido cisteína, lo que le proporciona a la molécula su carácter de donador de electrones, y es una de las moléculas no proteicas con SH más abundantes en la mayoría de los organismos (Klaassen *et al.*, 1985). El GSH es sintetizado en dos pasos, en primer lugar la γ-glutamilcisteina sintetasa (γ-GCS) produce la unión γ-peptídica entre el ácido glutámico y la cisteína, y posteriormente la GSH sintetasa agrega la glicina (Limón-Pacheco y Gonsebatt, 2009). Los niveles de GSH se ajustan continuamente debido al balance entre la síntesis de GSH (mediante la enzima GSH sintetasa), el reciclado del disulfuro de glutatión o GSSG (mediante la enzima glutatión reductasa, GR), y su uso por peroxidasas, transferasas, transhidrogenasas y transpeptidasas (Kidd, 1997). El GSH juega un papel importante como agente antioxidante ya que: (i) intercepta directamente radicales libres como el radical hidroxilo; (ii) es sustrato de la reacción enzimática catalizada por la GPx, usándose el grupo sulfhidrilo de la cisteínas como agente reductor; (iii) regula el transporte y metabolismo de xenobióticos mediante la acción de la glutatión-S-transferasa (GST); y (iv) regenera importantes antioxidantes como las vitaminas C y E a sus formas activas (Rotilio *et al.*, 1995; Valko *et al.*, 2006). Además, el GSH mantiene las proteínas con grupos sulfhidrilo en su estado reducido (Kidd, 1997). Ante exposiciones a ROS/RNS o compuestos que pueden generar ROS como el 4-hidroxinonenal (producto de la peroxidación lipídica), aumentan los niveles de GSH mediante modulación de señales celulares que inducen su síntesis (Valko *et al.*, 2006). El GSH también protege a las células frente a la muerte celular programada o apoptosis (Valko *et al.*, 2006), observándose que descensos en los niveles de GSH producen apoptosis celular (Kidd, 1997).

Algunas enzimas ayudan al sistema antioxidante de manera indirecta, como la GST que cataliza la conjugación del GSH con contaminantes como los metales pesados y con peróxidos como los hidroperóxidos lipídicos (Kidd, 1997), o la GR que reduce el glutatión oxidado (GSSG) a su forma reducida (GSH) (Gurer y Ercal, 2000). La glucosa-6-fosfato deshidrogenasa (G6PDH) es una enzima que cataliza la

reducción de NADP+ en NADPH, necesario para que la GR catalice la reacción de transformación del GSSG a GSH (Figura 4).

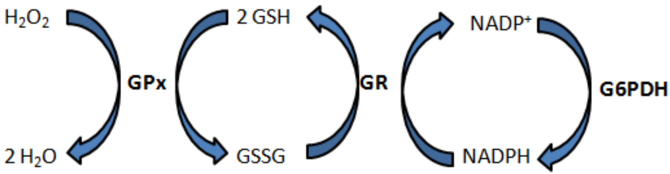

Figura 4. *Función de glutatión peroxidasa (GPx), glutatión reductasa (GR) y glucosa-6-fosfato deshidrogenasa (G6PDH).*

Por otro lado, otras enzimas antioxidantes (GPx, CAT, SOD) participan de forma directa catalizando la ruptura de radicales libres o especies reactivas. La SOD cataliza la eliminación de superóxidos, generando H_2O_2 y oxígeno como productos finales de la dismutación (Fórmula 4). Se han identificado tres isoformas, la isoforma cobre-zinc SOD presente en citoplasma, núcleo y plasma en eucariotas; la isoforma manganeso SOD localizada principalmente en la mitocondria y en procariotas (Limón-Pacheco y Gonsebatt, 2009); y la isoforma hierro SOD presente en procariotas y algunas plantas (Rotilio *et al.*, 1995). La SOD trabaja en sinergia con las enzimas CAT y GPx (Costantini, 2008). La CAT es una enzima que contiene grupos hemo y cataliza la descomposición del peróxido de hidrógeno en agua y oxígeno (Fórmula 5). Se localiza principalmente en orgánulos subcelulares como los peroxisomas. La GPx elimina el H_2O_2 acoplando su reducción con la oxidación del GSH (Fórmula 6). La GPx se encuentra en el citoplasma y en la matriz mitocondrial, lo que la hace más eficaz que la CAT por encontrarse en diferentes compartimentos de la célula. Se trata de una enzima seleniodependiente, aunque existen formas no dependientes de este elemento. La GPx también reduce otros peróxidos, como los hidroperóxidos de ácidos grasos (Fórmula 6) (Limón-Pacheco y Gonsebatt, 2009).

(4) $O_2^{\cdot -} + 2H^+ \xrightarrow{CuZnSOD} H_2O_2 + O_2$

(5) $2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$

(6) $H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$

$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O$

Los micronutrientes dietéticos como β-carotenos, vitamina C y vitamina E también contribuyen al sistema de defensa antioxidante. Las moléculas solubles en agua como la vitamina C son agentes de barrido de radicales muy potentes en la fase acuosa del citoplasma, mientras que las formas liposolubles como β-carotenos y vitamina E actúan como antioxidantes en medios lipídicos. El selenio, cobre, zinc y manganeso también son elementos importantes ya que actúan como cofactores de algunas

enzimas antioxidantes. El selenio es particularmente importante en la protección del medio lipídico contra el daño oxidativo, puesto que es cofactor de la GPx (Limón-Pacheco y Gonsebatt, 2009), además de tener un efecto positivo sobre el GSH, la GR y la SOD (Burk, 2002).

En la figura 5 se presenta un esquema con las diferentes vías por las que el GSH y otros antioxidantes equilibran el estrés oxidativo.

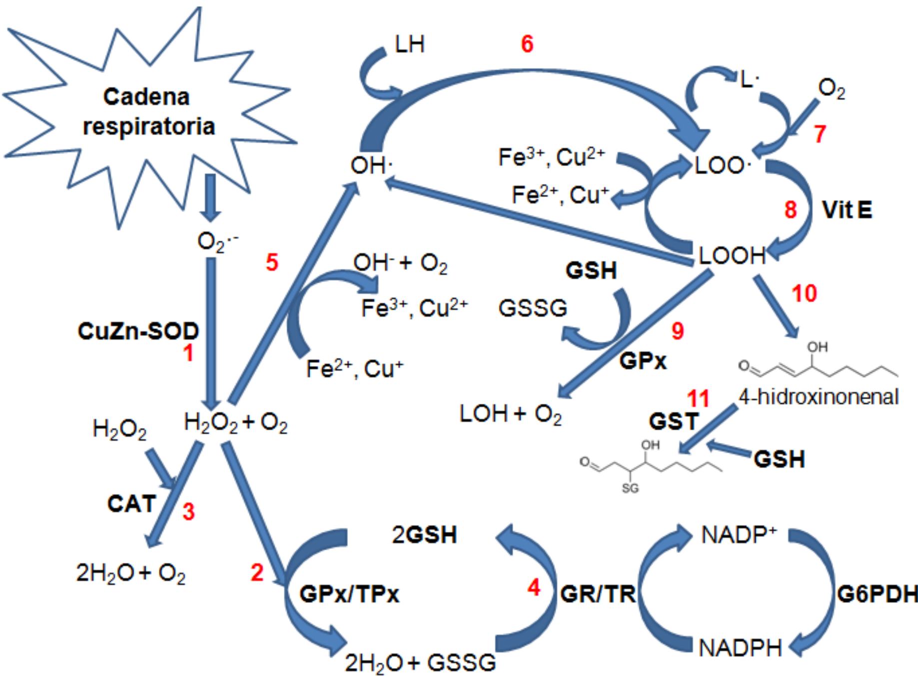

Figura 5. *Funciones del glutatión (GSH) y otros antioxidantes. Modificado de Rotilio et al. (1995) y Valko et al. (2006). Reacción 1. La SOD transforma el* $O2^{·-}$ *en* H_2O_2*. Reacción 2. El* H_2O_2 *es transformado en* H_2O *por la GPx, que utiliza al GSH como donador de electrones. Reacción 3. El* H_2O_2 *también puede transformase en* H_2O *y* O_2 *por la CAT. Reacción 4. El GSSG es reducido a GSH por la GR, que utiliza NADPH como donador de electrones. Reacción 5. Elementos de transición como Fe y Cu transforman el* H_2O_2 *en radicales* $OH^{·}$ *(Reacción de Fenton). Reacción 6. Los radicales* $OH^{·}$ *toman un electrón de los ácidos grasos poliinsaturados (LH) formando un radical lipídico (L·). Reacción 7. El L· puede interactuar con oxígeno molecular para dar un peróxido lipídico (LOO·). Reacción 8. El peróxido lipídico (LOO·) es reducido por la vitamina E formando un hidroperóxido lipídico. Reacción 9. Los hidroperóxidos lipídicos se reducen a alcoholes y* O_2 *por la GPx usando GSH como donador de electrones. Reacción 10. Los hidroperóxidos lipídicos pueden transformarse en aldehídos, como el 4-hidroxinonenal, un fuerte oxidante. Reacción 11. El 4-hidroxinonenal es conjugado con el GSH mediante la GST en un aducto inocuo.*

4. CONTAMINANTES AMBIENTALES PERSISTENTES.

4.1. METALES PESADOS.

4.1.1. CARACTERÍSTICAS DE LOS METALES.

Los metales son compuestos ubicuos que se encuentran de forma natural en el medio ambiente, ya que la mayoría de ellos forman parte de la corteza terrestre. Además, muchos de ellos son esenciales para la vida a determinadas concentraciones. Sin embargo, a pesar de que no son sintetizados por el hombre, las actividades humanas han supuesto un aumento en los niveles de estos contaminantes en el medio.

A continuación se describen las principales características de los 5 metales que serán objeto de estudio en la presente tesis (Tabla 1).

Tabla 1. *Características generales de los metales estudiados.*

Metal	MERCURIO	PLOMO	CADMIO	COBRE	ZINC
Símbolo	Hg	Pb	Cd	Cu	Zn
Número atómico	80	82	48	29	30
Peso atómico	200,59	207,2	112,4	63,546	65,38
Punto de fusión	-38,9ºC	327,4	321	1083	419,5
Punto de ebullición	356,58ºC	1725	765	2595	906
Aspecto y características	Blanco plateado, líquido a temperatura ambiente	Blanco de plata, brillante, maleable y muy dúctil, sólido a temperatura ambiente	Plateado gris metálico, sólido a temperatura ambiente	Metálico rojizo, sólido a temperatura ambiente	Azul pálido grisáceo, sólido a temperatura ambiente
Interés fisiológico	No esencial	No esencial	No esencial	Esencial. Interviene en producción energética. Forma parte de diversas enzimas.	Esencial. Cofactor e integrante de numerosas enzimas.

El plomo (Pb), cadmio (Cd) y mercurio (Hg) son considerados los metales más importantes desde el punto de vista de la contaminación ambiental y toxicológico. Por su parte, el zinc (Zn) y el cobre (Cu) son metales esenciales, aunque la exposición a altas concentraciones que excedan los mecanismos de regulación puede dar lugar a efectos adversos (Eisler, 1993, 1997). Además, el Pb, Cd, Hg y Zn se encuentran dentro de la lista de los 129 contaminantes prioritarios de la Agencia de Protección Ambiental (EPA) de Estados Unidos (US EPA, 2013).

4.1.2. PRESENCIA DE METALES PESADOS EN EL MEDIO AMBIENTE.

A pesar de que metales como el Hg, Pb, Cd, Cu y Zn están presentes de forma natural en la corteza terrestre, gran parte de las emisiones provienen de actividades antrópicas (Gad, 2005a, 2005b, 2005c; Goyer, 1996). Estos metales pueden ser emitidos al medio a través de actividades mineras y operaciones de fundición y refinería de metales; quema de combustibles fósiles; incineración de residuos; y fabricación y uso de insecticidas, herbicidas y fungicidas que los contengan (Gad, 2005a, 2005c, 2005d, 2005e; Goyer, 1996). Particularmente, las emisiones de industrias como la de pulpa y papel y plantas de cloro-álcali también han sido importantes fuentes antrópicas de Hg (Gad, 2005d; Goyer, 1996). En cuanto al Pb, una de las principales fuentes de emisión ha sido el uso de gasolina con Pb como antidetonante (Gad, 2005a), prohibida en España en 1998 (Directiva 98/70/CE, 1998; Real Decreto 785/2001, 2001). Tras la prohibición de uso de la gasolina plomada era esperable una menor exposición de los seres vivos a este metal, sobre todo en zonas próximas a carreteras muy transitadas. Diversos estudios realizados sobre tejidos y sangre de animales de abasto y aves silvestres de la Región de Murcia muestreados, antes y después de la puesta en marcha de la prohibición, demostraron que los niveles tisulares y sanguíneos de Pb habían disminuido de forma significativa en las zonas de alto tráfico (García-Fernández *et al.*, 2003, 2005c). Además, una importante fuente de exposición a Pb en aves es la ingestión de perdigones (Mateo, 2009). En España, el uso de perdigones de Pb está prohibido en humedales incluidos en la lista Ramsar debido al riesgo que suponen para las aves acuáticas (Real Decreto 581/2001, 2001).

En general, en los últimos años la presencia de estos metales en el medio se ha visto reducida significativamente debido a las prohibiciones y restricciones impuestas. En este sentido, junto a las prohibiciones citadas, se han instaurado diferentes medidas de restricción en el uso de metales en determinadas actividades, como el uso de Cd y Hg en la fabricación de pilas (Real Decreto 106/2008, 2008) o el uso de Pb, Hg y Cd en materiales y componentes de los vehículos (Real Decreto 1383/2002, 2003). Los estudios de biomonitorización siguen siendo necesarios para comprobar la efectividad de las medidas impuestas. De esta forma, diversos estudios llevados a cabo en la Región de Murcia (García-Fernández *et al.*, 1995, 1997; Gómez-Ramírez *et al.*, 2011) han demostrado que la exposición a Pb en aves rapaces ha descendido considerablemente en los últimos años (García-Fernández *et al.*, 2008).

4.1.3. CINÉTICA DE LOS METALES EN ORGANISMOS SUPERIORES.

Absorción

La absorción de Hg varía considerablemente dependiendo de la forma del compuesto (Gad, 2005d). Así, a temperatura ambiente, el Hg metálico o elemental se volatiliza a vapores de Hg (Goyer, 1996), siendo la absorción vía inhalatoria de un 75% y la digestiva prácticamente nula (Gad, 2005d). La absorción gastrointestinal de sales inorgánicas de Hg es menor del 15% en ratones y del 7% en humanos, mientras que la absorción del metilmercurio ingerido es del 90-95% (Goyer, 1996).

En cuanto al Pb, las principales vías de absorción son la pulmonar (absorción del 50 al 70%) (Gad, 2005a) y la gastrointestinal (absorción del 10%) (Goyer, 1996), siendo esta última vía muy rápida (Pain y Rattner, 1988).

Respecto al Cd, a pesar de que la absorción vía respiratoria (15-30%, Gad, 2005b; Goyer, 1996) es más eficiente que la digestiva (0,4-2% en mamíferos, Scheuhammer, 1987; y 4-7% en humanos adultos, Gad, 2005b), la mayor parte del Cd acumulado en aves procede de los alimentos que consumen (García-Fernández *et al.*, 1995).

Finalmente, en lo que respecta a los oligoelementos esenciales objeto de esta tesis, hasta el 50% del Cu puede ser absorbido en el tracto gastrointestinal (Eisler, 1997), principalmente en el intestino delgado (Valko *et al.*, 2005), mientras que el 20-30% del Zn ingerido es absorbido por esta misma vía (Goyer, 1996).

Distribución y acumulación

La mayor parte del Hg presente en plasma se encuentra unido a la albúmina u otras proteínas (Valko *et al.*, 2005). El vapor de Hg tiene afinidad por los eritrocitos y el sistema nervioso central debido a su liposolubilidad (Goyer, 1996), mientras que el Hg inorgánico se distribuye y acumula principalmente en los riñones tanto en mamíferos como en aves (Scheuhammer, 1987) unido a metalotioneínas (Piotrowski *et al.*, 1974). En cuanto al MeHg, su carácter liposoluble hace que se acumule principalmente en tejido adiposo y encéfalo (Gad, 2005d), presentando una vida media en aves de entre 2 y 3 meses, mientras que otros compuestos organomercuriales se descomponen rápidamente *in vivo* a Hg inorgánico (vida media de 1-2 semanas) (Scheuhammer, 1987).

Respecto al Pb, una vez absorbido, el 95% es distribuido por el torrente sanguíneo a través de los glóbulos rojos, principalmente unido a la hemoglobina (Gad, 2005a). El Pb en circulación se distribuye a hígado, riñón y otros tejidos blandos como el cerebro, y la concentración va disminuyendo paulatinamente como consecuencia de su acumulación en el tejido óseo que actuará como tercer compartimento (Mautino, 1997). En exposiciones crónicas, aproximadamente el 90% del Pb absorbido se acumula en huesos (García-Fernández *et al.*, 1997), y ante exposiciones agudas este metal se acumula principalmente en hígado y riñón (Longcore *et al.*, 1974; Pattee *et al.*, 1981). La vida media del Pb es de 10 a 30 años en huesos, 40 días en tejidos blandos y de 28 a 36 días en sangre (Mautino, 1997). El Pb acumulado en huesos puede movilizarse años después de que la exposición inicial cese (Gad, 2005a).

En cuanto al Cd, este metal es transportado por la sangre principalmente unido a eritrocitos y a proteínas plasmáticas, particularmente albúmina (Gad, 2005b). El 90% de la carga total de Cd se acumula en hígado y riñón (Scheuhammer, 1987), unido a metalotioneínas (Cherian y Goyer, 1978). Sin embargo, la distribución tisular es dosis-dependiente. De esta forma, exposiciones agudas producen una acumulación mayor a nivel hepático que renal, mientras que la exposición crónica a pequeñas dosis provoca una acumulación en riñón de 2 a 3 veces superior a la hepática (García-Fernández *et al.*, 1995, 1996). El Cd puede alcanzar una vida media de 30 años en el organismo (Gad, 2005b).

En lo referente al Cu, en sangre se une a la albúmina sérica para posteriormente entrar a formar parte integral de una proteína específica de Cu, la ceruloplasmina (Goyer, 1996), que puede contener seis átomos de Cu (Valko *et al.*, 2005). Es acumulado principalmente en hígado y médula ósea (Eisler, 1997). El Cu no es un inductor de metalotioneínas tan efectivo como el Zn o el Cd, sin embargo, se cree que su forma de almacenamiento normal es unido a estas proteínas (Goyer, 1996).

Al contrario que otros elementos traza, el Zn no es acumulado en el organismo formando almacenes permanentes (Valko *et al.*, 2005). Una vez absorbido, el Zn se une principalmente a la albúmina en plasma (Sternlieb, 1988) y es transportado a hígado, páncreas, riñón, bazo y músculo (Cao *et al.*, 2002). El Zn es un potente inductor de metalotioneínas (Gad, 2005e).

Eliminación

La excreción del Hg se produce a través de las heces y la orina (Goyer, 1996), además de las plumas en desarrollo que son una vía de excreción importante en aves (Burger, 1993).

Respecto al Pb, es excretado principalmente a través de las heces (Gad, 2005a), además de transferirse a plumas durante la muda (Honda *et al.*, 1986). Este metal también puede transferirse a los huevos en desarrollo, aunque se ha sugerido que lo hace en baja proporción (Furness, 1993).

En cuanto al Cd, la principal vía de excreción es la orina (Goyer, 1996), aunque también puede eliminarse a través de las plumas (Martínez-López *et al.*, 2005) y durante la formación del huevo en hembras (Burger, 1994).

Finalmente, la mayor parte del Cu acumulado en hígado es excretado por vía biliar (aproximadamente un 80%) (Valko *et al.*, 2005), y el Zn es excretado principalmente a través de las heces (Gad, 2005e).

4.1.4. INTERPRETACIÓN DE CONCENTRACIONES DE METALES PESADOS.

La interpretación de concentraciones de metales se centrará en los tejidos y metales estudiados en la presente memoria.

Mercurio en tejidos internos y plumas

Las concentraciones tisulares de Hg en aves silvestres que habitan áreas alejadas de zonas de contaminación, *a priori*, podrían considerarse como los niveles de fondo, o de base, para el tejido en cuestión. Así, las aves que habitan lugares poco o nada contaminados por Hg presentan niveles de Hg en hígado de 1 a 10 µg/g, encontrándose los mayores niveles en aves piscívoras y aquellas que se alimentan en vertederos (Fimreite, 1974). Por su parte, Scheuhammer (1991) estimó que las concentraciones normales de fondo, o de base, de Hg en plumas de aves rapaces se encontraban en el intervalo de 1 a 5 mg/kg.

Diversos estudios han aportado información que relaciona concentraciones tisulares de Hg con ciertos efectos o alteraciones observadas en los individuos analizados. Así, niveles de Hg de 16 µg/g (peso húmedo) en riñón se relacionaron con alteraciones reproductivas y lesiones cerebrales en Ánade sombrío (*Anas rubripes*) (Finley y Stendell, 1978), mientras que concentraciones mayores de 40 µg/g se

asociaron con muerte por ingestión de metilmercurio en varias especies de aves (Finley *et al.*, 1979). En lo que respecta al hígado, niveles de 1-2 µg/g (peso húmedo) se asociaron a una mayor mortalidad embrionaria y lesiones cerebrales en aves (Zillioux *et al.*, 1993), mientras que en encéfalo, concentraciones de 12-16 µg/g (peso húmedo) fueron detectadas en palomas que presentaban cambios comportamentales (Evans *et al.*, 1982). En cuanto a las plumas, concentraciones dentro del rango de 5 a 40 mg/kg han sido relacionadas con afecciones reproductivas y comportamentales en varias especies de aves (Fimreite, 1979; Heinz, 1979; NAS, 1978; Solonen y Lodenius, 1984; Spann *et al.*, 1972). El amplio rango es debido al diferente patrón de muda de las plumas según la especie (Wolfe *et al.*, 1998).

A continuación se presenta una revisión de las concentraciones de Hg en tejidos internos y plumas en diferentes especies de álcidos (Tabla 2).

Tabla 2. *Concentración de mercurio en tejidos internos y plumas de diferentes especies de álcidos.*

Especie	Concentración media de mercurio (mg/Kg)	Referencia
Peso seco		
Alca común (*Alca torda*)	P: 1,29 (Adultos), P: 1,40 (Pollos)*	(Bond y Diamond, 2009a)
Frailecillo común (*Fratercula arctica*)	P: 1,41 (Adultos), P: 0,99 (Pollos)	
Arao común (*Uria aalge*)	P: 1,65 (Adultos), P: 1,14 (Pollos)	
Arao común (*Uria aalge*)	P: 0,99	(Bond y Diamond, 2009b)
Alca común (*Alca torda*)	P: 1,40	
Frailecillo común (*Fratercula arctica*)	P: 1,81	
Alca común (*Alca torda*)	H: 6,09, R: 3,94, M: 2,67, P: 2,39 (Total)	(Ribeiro *et al.*, 2009)
	H: 5,00, R: 2,99, M: 1,63, P: 0,79 (Juveniles)	
	H: 4,89, R: 3,77, M: 2,14, P: 2,06 (Inmaduros)	
	H: 9,63, R: 4,90, M: 4,54, P: 4,10 (Adultos)	
Frailecillo coletudo (*Fratercula cirrhata*)	H: 2,9**	(Ricca *et al.*, 2008)
Arao común (*Uria aalge*)	H: 4,17	
Arao palomo (*Cepphus columba)*	H: 6,36	
Arao de Brunnich (*Uria lomvia*)	M: 0,38	(Rigét *et al.*, 2007)
Alca común (*Alca torda*)	H: 2,28	(Pérez-López *et al.*, 2006)
Arao común (*Uria aalge*)	H: 1,21	
Frailecillo común (*Fratercula arctica*)	H: 1,77	
Arao de Brunnich (*Uria lomvia*)	H: 0,33-1,61, M: 0,15-0,60	(Savinov *et al.*, 2003)
Arao común (*Uria aalge*)	H: 1,08-1,09, M: 0,33-0,50	
Frailecillo común (*Fratercula arctica*)	H: 1,12-1,37, M: 0,31-0,46	
Arao aliblanco (*Cepphus grylle*)	H: 0,76-1,12, M: 0,27-0,43	
Mérgulo atlántico (*Alle alle*)	H: 0,24-0,51, M: 0,10-0,32	
Alca común (*Alca torda*)	H: 1,71, M: 0,88	
Arao de Brunnich (*Uria lomvia*)	H: 1,11, M: 0,33	(Wenzel y Gabrielsen, 1995)
Arao común (*Uria aalge*)	H: 1,88, M: 0,42	
Arao aliblanco (*Cepphus grylle*)	H: 2,20, M: 0,73	(Nielsen y Dietz, 1989)
Arao de Brunnich (*Uria lomvia*)	H: 2,63, M: 0,73	
Mérgulo atlántico (*Alle alle*)	H: 1,68	(Norheim, 1987)
Peso húmedo		
Mérgulo atlántico (*Alle alle*)	H: 0,26, M: 0,06	(Jæger *et al.*, 2009)
Arao de Brunnich (*Uria lomvia*)	H: 0,37, M: 0,11	
Mérgulo atlántico (*Alle alle*)	H: 0,27, M: 0,08	(Campbell *et al.*, 2005)
Arao aliblanco (*Ceppus grylle*)	H: 1,17, M: 0,34	
Arao de Brunnich (*Uria lomvia*)	H: 1,17, M: 0,33	

*Nota: H: Hígado, R: Riñón, M: Músculo, P: Plumas. Todos los valores corresponden a la media aritmética excepto *= Media marginal y **= Media geométrica.*

En la tabla 3 se presenta una revisión de concentraciones de Hg en plumas de aves del orden Strigiformes.

Tabla 3. *Concentración de mercurio en plumas de aves pertenecientes al orden Strigiformes.*

Especie	Concentración media de mercurio (mg/Kg)	Referencia
Búho real (*Bubo bubo*)	0,12 (año 2002) y 0,09 (año 2003) (PCu, Pollos 20-30 días)	(Ortego *et al.*, 2006)
Búho real (*Bubo bubo*)	1,29 (PCu, Aves adultas)	(Lourenço *et al.*, 2011)
Lechuza común (*Tyto alba*)	1,22 (PCu)	
Cárabo común (*Strix aluco*)	0,48 (PCu)	
Mochuelo común (*Athene noctua*)	0,64 (PCu)	
Mochuelo común (*Athene noctua*)	0,12 (P9)-0,36 (P3)[a]	(Dauwe *et al.*, 2003)
Lechuza común (*Tyto alba*)	0,77 (P4)-0,90 (P2)[b]	
Mochuelo común (*Athene noctua*)	0,50 (PS) y 1,10 (PCo)	(Zolfaghari *et al.*, 2007)
Cárabo común (*Strix aluco*)	0,56 (PS) y 0,85 (PCo)	
Búho real (*Bubo bubo*)	0,30 (PS) y 0,71 (PCo)	
Búho real (*Bubo bubo*)	3,20 (IP) y 6,51 (CP). Aves adultas.	(Broo y Odsjö, 1981)
Búho real (*Bubo bubo*)	2,84 (IP) y 1,23 (CP). Aves juveniles, 1-3 meses.	
Búho real (*Bubo bubo*)	4,08 (IP) y 8,00 (CP). Aves adultas.	(Odsjö y Olsson, 1975)
Búho real (*Bubo bubo*)	2,14 (IP) y 5,80 (CP). Aves juveniles, 1-3 meses.	
Búho real (*Bubo bubo*)	2,50 (PCo) y 1,30 (Pin)	(Berg *et al.*, 1966)
Búho real (*Bubo bubo*)	0,70 (CS, Pollos).	(Solonen y Lodenius, 1990)
Cárabo común (*Strix aluco*)	0,50 (CS, Pollos).	
Cárabo uralense (*Strix uralensis*)	0,60 (CS, Pollos).	
Cárabo lapón (*Strix nebulosa*)	0,60 (CS, Pollos).	
Búho chico (*Asio otus*)	0,30 (CS, Pollos).	
Mochuelo boreal (*Aegolius funereus*)	1,50 (CS, Pollos).	
Mochuelo común (*Athene noctua*)	17,00 (AC) y 10,00 (LR)[c]	(Van den Brink *et al.*, 2003)

Nota: [a]P1 es la primaria más interna y primera en mudar y P10 es la pluma más distal y última en mudar; [b]P1 es la pluma más interna y última en mudar y P6 es la primera pluma en mudar; [c]Media geométrica. IP=Población de interior, CP=Población costera, PCo=Plumas de la cola, PS=Plumas secundarias, Pin=Primarias internas, PCu=Plumas corporales, CS=Coverteras secundarias, AC=Área contaminada, LR=Lugar de referencia.

Metales en sangre de aves

La sangre se puede considerar un apropiado indicador de exposición reciente a Pb, Cd y Hg (Evers y Reaman, 1998; García-Fernández *et al.*, 1995; Gómez-Ramírez *et al.*, 2011). La vida media en sangre del Pb es de 28 a 36 días (Mautino, 1997), y de 2,5 meses en el caso del Cd (Ramírez, 2002). El metilmercurio presenta una vida media en sangre de aves entre 2 y 3 meses (Scheuhammer, 1987).

Para poder utilizar la concentración de metales en sangre con el fin de establecer niveles que diferencien entre diferentes tipos de exposición, es necesario conocer las

concentraciones de base consideradas como de “no exposición” o exposición “de base” (García-Fernández, 1994). En este sentido, se han descrito como niveles de fondo aquellas concentraciones encontradas en zonas alejadas de fuentes de contaminación (Franson y Pain, 2011). En la tabla 4 se recogen los valores de metales en sangre encontrados en distintas especies de aves que habitan zonas contaminadas y no contaminadas.

Tabla 4. *Concentraciones de metales en sangre de aves.*

Especie	**Concentración media de metales en sangre (µg/dl)**	**Observaciones**	**Referencia**
Águila perdicera (*Hieraaetus fasciatus*)	Pb= 18,00, Cd= 0,09	ANC	(García-Fernández *et al.*, 1995)
Aguila pescadora (*Pandion haliaetus*)	Pb= 2-4	ANC	(Henny *et al.*, 1991)
Aguila pescadora (*Pandion haliaetus*)	Pb= 0,89; Cd= <0,5; Cu= 29,8; Zn= 315, Hg= 15-55	Pollos	(Langner *et al.*, 2012)
Águila calva (*Haliaeetus leucocephalus*)	Hg= 2-25	Pollos	(Jagoe *et al.*, 2002)
Aguililla calzada (*Aquila pennata*)	Pb= 3,21	ANC	(Martínez-López *et al.*, 2004)
Aguililla calzada (*Aquila pennata*)	Pb= 8,75; Cd= 0,11	ANC	(García-Fernández *et al.*, 1995)
Aguililla calzada (*Aquila pennata*)	Cd= 0,3	ANC. Pollos	(Martínez-López *et al.*, 2005)
Aguilucho pálido (*Circus cyaneus*)	Pb= 6,7 (AC) y 4,2 (ANC)	Pollos	(Henny *et al.*, 1994)
Alimoche (*Neophron percnopterus*)	Pb= 0,56-21,73	Península Ibérica	(Gangoso *et al.*, 2009)
Alimoche (*Neophron percnopterus*)	Pb= 0,51-178	Islas Canarias	
Alimoche (*Neophron percnopterus*)	Pb= 9,33	Durante temporada de caza	
Alimoche (*Neophron percnopterus*)	Pb= 2,88	Fuera de temporada de caza	
Alimoche (*Neophron percnopterus*)	Pb= 14,6; Cd= 0,113; Cu= 0,047; Zn= 361,5		(Donázar *et al.*, 2002)
Autillo californiano (*Megascops kennicottii*)	Pb= 10; Cd= 1,7 (AC)	Pollos	(Henny *et al.*, 1994)
Azor (*Accipiter gentilis*)	Pb= 7,60	ANC	(Martínez-López *et al.*, 2004)
Azor (*Accipiter gentilis*)	Pb= ND; Cd= ND; Cu= 39,9; Zn= 180	ANC	(Stout *et al.*, 2010)
Azor (*Accipiter gentilis*)	Cd= 0,58	ANC. Pollos	(Martínez-López *et al.*, 2005)
Búho americano (*Bubo virginianus*)	Pb= 3,8; Cd= 0,35 (AC)	Pollos	(Henny *et al.*, 1994)
Búho real (*Bubo bubo*)	Pb= 7,6	ANC	(García-Fernández *et al.*, 1997)
Búho real (*Bubo bubo*)	Pb= 8,61 (AC) y 3,7 (ANC)		(Gómez-Ramírez *et al.*, 2011)
Búho real (*Bubo bubo*)	Pb= 8,32; Cd= 0,10	ANC	(García-Fernández *et al.*, 1995)
Búho real (*Bubo bubo*)	Cd= 0,08 (AC) y 0,11 (ANC); Cu= 45,6 (AC) y 32,37 (ANC); Zn= 327,47 (AC) y 351,92 (ANC)	Pollos	(Gómez-Ramírez, 2011)
Buitre leonado (*Gyps fulvus*)	Pb=43,07	AC	(García-Fernández *et al.*, 2005a)
Buitre leonado (*Gyps fulvus*)	Pb= 37,9; Cd= 0,11	ANC	(García-Fernández *et al.*, 1995)

Nota: ANC=Área no contaminada, AC=Área contaminada. ND=No detectado.

Tabla 4. *Concentraciones de metales en sangre de aves (continuación).*

Especie	Concentración media de metales en sangre (µg/dl)	Observaciones	Referencia
Buitre leonado (*Gyps fulvus*)	Pb= 10,4; Cd= <1; Hg= <2	ANC. Juveniles	(Shlosberg *et al.*, 2012)
Buitre leonado (*Gyps fulvus*)	Pb= 14,2; Cd= <1; Hg= 1,2	ANC. Subadultos	
Buitre leonado (*Gyps fulvus*)	Pb= 8,4; Cd= <1; Hg= 1,3	ANC. Adultos	
Busardo colirrojo (*Buteo jamaicensis)*	Pb= ND (AC y ANC); Cd= ND (AC) y 0,9 (ANC)	Pollos	(Henny *et al.*, 1994)
Busardo ratonero (*Buteo buteo*)	Cd= 0,16	ANC. Pollos	(Martínez-López *et al.*, 2005)
Busardo ratonero (*Buteo buteo*)	Pb= 10,8	ANC	(García-Fernández *et al.*, 1997)
Busardo ratonero (*Buteo buteo*)	Pb= 2,74	ANC	(Martínez-López *et al.*, 2004)
Busardo ratonero (*Buteo buteo*)	Pb= 11,80; Cd= 0,09	ANC	(García-Fernández *et al.*, 1995)
Cernícalo americano (*Falco sparverius*)	Pb= 33	Grupo control estudio experimental	(Custer *et al.*, 1984)
Cernícalo americano (*Falco sparverius*)	Pb= 24 (AC) y 8,7 (ANC)	Pollos	(Henny *et al.*, 1994)
Cernícalo americano (*Falco sparverius*)	Pb= 46 (AC) y 25 (ANC)	Adultos	(Henny *et al.*, 1994)
Cernícalo común (*Falco tinnunculus*)	Pb= 10	ANC	(García-Fernández *et al.*, 1997)
Cernícalo común (*Falco tinnunculus*)	Pb= 11,52; Cd= 0,14	ANC	(García-Fernández *et al.*, 1995)
Diversas aves	Pb= <10	ANC	(Hoffman *et al.*, 1981; Pattee y Hennes, 1983)
Diversas aves	Pb= 4,8	ANC	(Scheuhammer, 1989)
Gavilán de Cooper (*Accipiter cooperii*)	Pb= 6,3 (primavera) y 3,2 (otoño)	ANC. Adultos	(McBride *et al.*, 2004)
Gavilán de Cooper (*Accipiter cooperii*)	Pb= 2,8	ANC. Juveniles	(McBride *et al.*, 2004)
Milano negro (*Milvus migrans*)	Pb= 5,4; Cd= 0,68; Cu= 21,1; Zn= 330	AC	(Benito *et al.*, 1999)
Milano negro (*Milvus migrans*)	Pb= 8,4; Cd= 0,18; Cu= 32; Zn= 537	AC	(Blanco *et al.*, 2003)
Milano negro (*Milvus migrans*)	Pb= 4,67; Cd= 0,05; Cu= 36,4; Zn= 482	AC	(Baos *et al.*, 2006a)
Mochuelo común (*Athene noctua*)	Pb= 9,4	ANC	(García-Fernández *et al.*, 1997)
Mochuelo común (*Athene noctua*)	Pb= 7,93; Cd= 0,08	ANC	(García-Fernández *et al.*, 1995)
Quebrantahuesos (*Gypaetus barbatus*)	Pb= 2,33	Pollos	(Hernández y Margalida, 2009)
Quebrantahuesos (*Gypaetus barbatus*)	Pb= 4,56	Juveniles	
Quebrantahuesos (*Gypaetus barbatus*)	Pb= 4,20	Subadultos	
Quebrantahuesos (*Gypaetus barbatus*)	Pb= 5,45	Adultos	

Nota: ANC=Área no contaminada, AC=Área contaminada. ND=No detectado.

La identificación de lesiones características del Pb a altos niveles permite diagnosticar la intoxicación por este metal sin necesidad de establecer umbrales de toxicidad para cada especie (Franson y Pain, 2011). Sin embargo, el tipo habitual de exposición a metales al que se suelen ver sometidas las aves es crónico a bajas dosis (García-Fernández, 1994). Por tanto, el conocimiento de las concentraciones de metales en diferentes especies resulta fundamental cuando se pretende conocer la repercusión de la exposición a concentraciones subletales, donde las lesiones no son

tan evidentes. En un intento de establecer niveles umbral de Pb en sangre, Franson (1996) propuso que niveles de Pb sanguíneos de Falconiformes inferiores a 20 µg/dl debían considerarse como exposición de base; a partir de este nivel y hasta 50 µg/dl nos encontraríamos ante una intoxicación subclínica. Los correspondientes a una intoxicación clínica se encontrarían en el rango de 50-100 µg/dl y, finalmente, los niveles que indicarían una intoxicación grave serían mayores de 100 µg/dl. Estos niveles umbral son solo orientativos, ya que diversos factores pueden interaccionar y causar variaciones en los mismos (Franson y Pain, 2011). Sin embargo, estudios más recientes mostraron que niveles de Pb inferiores a los 15 µg/dl ya se relacionaban con efectos subletales como la inhibición de la enzima ALAD en rapaces y aves acuáticas (Gómez-Ramírez *et al.*, 2011; Martínez-Haro *et al.*, 2011; Martínez-López *et al.*, 2004).

En lo que respecta al Cd sanguíneo, concentraciones inferiores a 0,5 µg/dl se pueden considerar como valores relacionados con exposición de base en humanos. Aún no se ha establecido un umbral de toxicidad para Cd en sangre ni un rango de concentraciones correspondientes a contaminación de base en aves (Martínez-López *et al.*, 2005). Sin embargo, se han observado efectos adversos en faisanes a partir de 1,7 µg/dl (Świergosz y Kowalska, 2000).

En cuanto al Hg, concentraciones sanguíneas de hasta 1 µg/ml en Colimbo grande (*Gavia immer*) no se relacionaron con ningún efecto reproductivo o de comportamiento, razón por la cual se consideró este nivel como el NOAEL[9] en esta especie (Evers *et al.*, 2004). Colimbos con niveles de Hg en sangre entre 1 y 3 µg/ml han mostrado cambios fisiológicos y comportamentales y deficiencias reproductivas (Burgess y Meyer, 2008; Evers *et al.*, 2008). Sin embargo, los efectos a estos niveles no están bien definidos, y se reconoce como LOAEL[10] concentraciones de Hg de 3 µg/ml en sangre para esta especie (Evers *et al.*, 2004). Este LOAEL se asoció con un comportamiento inusual durante la incubación, letargia (Evers *et al.*, 2008), mayores niveles de corticosterona y asimetría en las plumas de vuelo (Evers *et al.*, 2004). Además, se observó una reducción del 41% en el número de volantones con éxito por pareja de Colimbos (Burgess y Meyer, 2008; Evers *et al.*, 2008). Concentraciones por encima de 4 µg/ml se categorizan como de riesgo muy alto, relacionado con cambios comportamentales como menores tiempos de incubación (Evers *et al.*, 2004). En

[9] Siglas en inglés de “*No observed adverse effect level*” o Nivel sin efecto adverso observado.

[10] Siglas en inglés de “*Lowest observed adverse effect level*” o Menor nivel con efecto adverso observado.

cuanto a Colimbos juveniles de 4 a 6 semanas de edad, niveles de Hg en sangre de 0,3 µg/ml o mayores se asocian con menor número de pollos nacidos o que sobreviven a las 8 semanas de vida (Evers y Reaman, 1998).

Con respecto al Cu, a pesar de que es un oligoelemento esencial, puede producir intoxicaciones en aves (Gilbert *et al.*, 1996). Los valores de Cu encontrados en aves sanas ingresadas en centros de recuperación oscilaron entre 13 y 120 µg/dl (García-Fernández *et al.*, 2005b). En cuanto al Zn, la mayoría de los animales pueden tolerar un exceso moderado en la dieta y regular los niveles en su organismo de forma efectiva (Ewan, 1978; Sileo *et al.*, 2003), por lo que altas concentraciones de Zn no son alarmantes desde el punto de vista toxicológico (Goede, 1985). Sin embargo, los mecanismos de homeostasis pueden llegar a fracasar cuando las concentraciones de Zn son extremadamente altas (Sileo *et al.*, 2003). En aves intoxicadas los niveles séricos de Zn descritos pueden oscilar entre 640 y 3200 µg/dl (Carpenter *et al.*, 2004; Sileo *et al.*, 2003; Zdziarski *et al.*, 1994), mientras que una media de 271-313 µg/dl corresponde a aves sanas (García-Fernández *et al.*, 2005b).

4.2. PLAGUICIDAS ORGANOCLORADOS.

4.2.1. CARACTERÍSTICAS DE LOS PLAGUICIDAS ORGANOCLORADOS.

El grupo de los compuestos organoclorados ha sido identificado como uno de los mayores contaminantes medioambientales, tanto de ecosistemas acuáticos como terrestres, como resultado de la actividad humana (Herrera *et al.*, 1996; van Wyk *et al.*, 2001). Además, son considerados por Newton *et al.* (1993) como los compuestos que más daño han causado a la fauna silvestre. Dentro de este grupo se encuentran los plaguicidas organoclorados (OC) y los bifenilos policlorados (PCBs).

Los organoclorados comprenden un grupo de compuestos orgánicos de síntesis derivados de hidrocarburos complejos, en los que un hidrógeno es sustituido por cloro. Las principales propiedades físico-químicas de los organoclorados son su gran estabilidad química, y por tanto persistencia y resistencia a la biodegradación, y su solubilidad en disolventes orgánicos y lípidos (Álvarez y Cruz, 1989). Los OC pueden ser divididos en cinco grupos (Smith, 1991): DDT y análogos, hexaclorociclohexano (HCH), ciclodienos y compuestos similares, toxafeno y compuestos relacionados, y estructuras tipo mirex y clordecona.

A continuación se describen las principales características de los 16 plaguicidas objeto del presente estudio, englobados en los tres primeros grupos (Tabla 5).

Tabla 5. *Características generales de los plaguicidas organoclorados.*

Compuesto	Estructura	Usos	Características	Observaciones	Prohibición	DL_{50}	Log Kb
DDT (Diclorodifenil tricloroetano)	Cl, Cl, Cl, Cl, Cl	Amplia gama de plagas agrícolas y vectores de enfermedades	Elevada persistencia. Forma cristales incoloros. Resistente a la destrucción por la luz y la oxidación. Bioacumulación significativa.	Mezcla de isómeros p,p´-DDT (más abundante y activo)	Orden 4 Febrero 1994 En 2008 cesa la producción de Dicofol (producto con DDT)	225 mg/Kg (O/R)	6,11
DDD (Diclorodifenil dicloroetano)	Cl, Cl, Cl, Cl	Insecticida		Metabolito del DDT	Orden 4 Febrero 1994	3400 mg/Kg (O/C) 1200 mg/Kg (D/C)	
DDE (Diclorodifenil dicloroetileno)	Cl, Cl, Cl, Cl	No presenta acción insecticida	Elevada persistencia	Resulta de la degradación del DDT en sistemas biológicos			
HCH (Hexacloro ciclohexano)	Cl, Cl, Cl, Cl, Cl, Cl Lindano (γ-HCH)	Insecticida, agente contra ecoparásitos en productos veterinarios y farmacéuticos, tratamiento suelos y semillas (lindano)	El lindano es un polvo cristalino entre blanco y amarillo. Se acumula moderadamente.	Mezcla de isómeros: α (60-70%), β (6-8%, casi inactivo), δ (2-5%), γ-HCH (12-15%, mayor actividad insecticida)	Orden 4 Febrero 1994 (HCH) Decisión 2000/801/CE (Lindano)	1752,8 mg/Kg (O/R) >8000 mg/Kg (D/R) 1362,5mg/Kg (O/C) 1786,3mg/Kg (D/C)	α= 2,39 β = 2,68 δ = 1,45 γ = 1,72
Aldrín	Cl, Cl, Cl, Cl, Cl, Cl	Insecticida para suelo, algodón, plagas del césped, gusanos blancos y gusanos de la raíz del maíz	Color nulo-marrón oscuro. Forma líquida o sólida. Bioacumulación significativa.	Se metaboliza rápidamente a su epóxido dieldrín	Orden 4 Febrero 1994	39-60 mg/Kg (O/R) 98 mg/Kg (D/R)	1,80-4,01
Dieldrín	Cl, Cl, Cl, Cl, Cl, Cl, O	Protección madera contra el ataque de insectos y termitas, e industria para combatir plagas textiles	Forma copos de color tostado. Estable en presencia de luz, humedad, álcalis y ácidos moderados. Bioacumulable.	Epóxido del aldrín	Orden 4 Febrero 1994	46 mg/Kg (O/R) 60-90 mg/Kg (D/R)	3,31

DL_{50}=Dosis Letal 50, O=Oral, D=Dérmica, R=Ratas, C=Conejos. Kb=Coeficiente de bioacumulación.

Tabla 5. *Características generales de los plaguicidas organoclorados (continuación).*

Compuesto	Estructura	Usos	Características	Observaciones	Prohibición	DL_{50}	Log Kb
Endrín	Cl Cl Cl Cl Cl Cl O	Control de insectos, roedores y pájaros	Sólido cristalino incoloro-color tostado. Bioacumulación considerable.	Epóxido del isodrín. Transformación a aldehído por luz y calor	Orden 4 Febrero 1994	7,5-17,8 mg/Kg (O/R) 15 mg/Kg (D/R)	4,07
Heptacloro	Cl Cl Cl Cl Cl Cl Cl	Insecticida y plagas del maíz	Polvo blanco. Estable en presencia de luz, aire, humedad y calor moderado. Fuerte tendencia a la bioacumulación.	Se convierte en epóxido, de mayor toxicidad	Orden 4 Febrero 1994	100-163 mg/Kg (O/R) 195-250 mg/Kg (D/R)	3,83
Endosulfán	O S O O Cl Cl Cl Cl Cl Cl	Control de insectos en alimentos, cultivos no alimentarios y protector de madera	Sólido cristalino marrón. Estable a la luz solar e inestable en medios alcalinos. Hidrolizado lentamente por agua y ácidos. No se considera bioacumulable.	Mezcla de isómeros: I (70%) y II (30%). Se transforma a sulfato (toxicidad similar)	Decisión de la Comisión 2005/864/CE	18-43 mg/Kg (O/R) 78-130 mg/Kg (D/R)	2,75

DL_{50}=Dosis Letal 50, O=Oral, D=Dérmica, R=Ratas, C=Conejos. Kb=Coeficiente de bioacumulación.

4.2.2. PRESENCIA DE PLAGUICIDAS ORGANOCLORADOS EN EL MEDIO AMBIENTE.

La mayor fuente de contaminación del medio ambiente por plaguicidas es el depósito que resulta de la aplicación de estos productos químicos para controlar plagas agrícolas en cultivos, masas forestales, suelos o aguas estancadas o para luchar contra plagas que causan problemas de salud al ser humano (García, 2002).

Como consecuencia, estos compuestos pueden estar presentes tanto en el suelo como en la atmósfera e hidrosfera. En el caso de la atmósfera, además de la pérdida directa por tratamientos aéreos, pueden proceder de volatilización durante la aplicación o a partir de superficies tratadas, efluentes industriales e incluso las tormentas de polvo (Albajes, 1992). La presencia de fitosanitarios en el agua suele tener mayor repercusión ya que se difunden rápidamente, pueden circular hasta lugares más lejanos, y sus residuos alcanzan las cadenas tróficas cercanas al hombre de manera más directa. Los plaguicidas que son transportados a ambientes acuáticos proceden principalmente de la escorrentía y lixiviación de áreas de cultivo, efluentes procedentes de plantas industriales químicas que vierten sus productos y, como se ha citado anteriormente, el transporte atmosférico también ha sido demostrado (García, 2002).

Aunque los plaguicidas han sido diseñados para ofrecer una alta especificidad de acción, su uso genera efectos indeseados como la persistencia ambiental de residuos tóxicos, generación de organismos resistentes y la contaminación de recursos hídricos con degradación de la flora y fauna (Rubio, 2004).

La persistencia de los organoclorados favorece la incorporación a las cadenas tróficas, la acumulación en los tejidos grasos humanos y animales, y la biomagnificación. Estos compuestos son ingeridos por los herbívoros o penetran directamente a través de la piel de organismos acuáticos como los peces y diversos invertebrados. El plaguicida se concentra aún más al pasar de los herbívoros a los carnívoros alcanzando elevadas concentraciones en los tejidos de los animales que ocupan los eslabones más altos de la cadena alimentaria (Wayland *et al.*, 1991).

La presencia y la persistencia de estos residuos en el suelo, agua, aire, plantas y animales hace aumentar el interés y la preocupación por el estudio de su acumulación a lo largo del tiempo y los efectos que conlleva la exposición a dichos compuestos (Keith, 1996).

4.2.3. CINÉTICA DE LOS PLAGUICIDAS ORGANOCLORADOS EN ORGANISMOS SUPERIORES.

Absorción

Las principales vías de entrada de estos compuestos en las aves son la oral, debido al consumo de presas, y la pulmonar por contaminación atmosférica. Una vez ingeridos, la absorción intestinal de estos compuestos está influenciada por los constituyentes de la dieta, como la fibra y la grasa, y también por la ingesta total de comida (Heath y Vandekar, 1964).

Distribución, acumulación y metabolismo

Al tratarse de sustancias apolares son más difíciles de eliminar, por lo que tienen tendencia a acumularse, especialmente en tejido adiposo. Así, el DDT y su metabolito DDE son acumulados en el tejido adiposo en alta proporción; los isómeros del hexaclorociclohexano son almacenados en diferente grado debido a las diferencias de metabolización existente entre los isómeros; y el dieldrín es almacenado en alta proporción mientras que el endrín lo hace en menor grado. Además de almacenarse en el tejido adiposo y en el hígado, pueden estar localizados y causar toxicidad en cerebro, riñón y glándulas adrenales (Smith, 2004).

El organismo intenta convertir estas sustancias en otras menos lipofílicas y más polares para favorecer su eliminación a través de reacciones de biotransformación como las reacciones de oxidación. Pueden ser metabolizados por el sistema microsomal citocromo P-450 a derivados hidroxílicos, por deshidrocloración como ocurre en el caso del lindano, o por conversión a epóxidos más estables como es el caso de la formación del dieldrín desde el aldrín (Smith, 2004). Otras rutas de metabolización también implican la conjugación con glutatión o la formación de glucurónidos. El mayor número de reacciones de biotransformación se produce en el hígado, sin embargo, en algunos organismos también son importantes las reacciones que tienen lugar en pulmón, intestino, piel y riñón (María-Mojica, 1998).

Eliminación

Normalmente, los plaguicidas padres son eliminados por la bilis o por las heces, mientras que los metabolitos se eliminan por la orina si presentan una alta polaridad, como por ejemplo los conjugados glucurónicos. Esto podría implicar la reabsorción en el tracto intestinal y el transporte a hígado y riñón donde se producen las transformaciones (Smith, 2004).

Otra vía de eliminación de interés en mamíferos es la láctea (Miranda Filho *et al.*, 2009), debido al alto contenido lipídico (3-5%) en leche y el elevado fluido sanguíneo que llega al tejido mamario (Jensen, 1983). También en mamíferos, la eliminación de DDT, DDE, dieldrín, HCH y HCB por el pelo ha sido estudiada, relacionando su presencia en la grasa de la piel y del pelo (Covaci *et al.*, 2008; Matthews *et al.*, 1976).

Las aves, durante el periodo de puesta, eliminan compuestos organoclorados con la yema del huevo debido a la elevada afinidad de éstos por los lípidos que forman parte del mismo (Ross *et al.*, 2008; Van den Steen *et al.*, 2009). Algunos estudios han analizado compuestos organoclorados en el aceite de la glándula uropigial (Jaspers *et al.*, 2008), considerándolo otro método de eliminación de insecticidas lipofílicos en aves (Hall *et al.*, 1971). Como se ha explicado previamente, las plumas también pueden considerarse un método de eliminación de estos compuestos en aves.

4.2.4. INTERPRETRACIÓN DE CONCENTRACIONES DE PLAGUICIDAS ORGANOCLORADOS.

Diversos trabajos han asociado las concentraciones de plaguicidas organoclorados presentes en encéfalo con efectos tóxicos en aves. En este sentido, el encéfalo parece ser el tejido más útil para diagnosticar muerte por insecticidas (Stickel *et al.*, 1969). Sin embargo, la información en cuanto a la relación entre los niveles de organoclorados en plumas y sus efectos en aves es muy escasa.

En un estudio experimental, pollos de Pelícano blanco (*Pelecanus erythrorhynchos*) fueron tratados a través de la dieta con 144 y 72 ppm de PCBs o DDTs (DDT+DDD+DDE), respectivamente, durante 10 semanas (Greichus *et al.*, 1975). El hígado acumuló los mayores niveles de estos compuestos (PCBs 290 ppm, DDTs 135 ppm, peso húmedo), seguido del encéfalo (PCBs 110 ppm, DDTs 35 ppm) y las plumas (PCBs 120 ppm, DDTs 48 ppm). Estos autores sugirieron que las plumas son preferibles a otros tejidos como indicadoras de las concentraciones de DDTs y PCBs en encéfalo, basándose en la similitud de concentraciones encontradas entre encéfalo y plumas. Greichus *et al.* (1975) no observaron signos de intoxicación severa, aunque encontraron alteraciones en los niveles de vitamina A en hígado y de potasio, calcio y proteínas en suero. Por tanto, el umbral de efectos subletales probablemente se encuentre por debajo de estas concentraciones de DDTs y PCBs en plumas (48 y 120 ppm, respectivamente). Sin embargo, debe tenerse en cuenta que en este estudio no se indicaba un lavado de la pluma, por lo que es probable que existiera contaminación externa por el acicalamiento con aceite de la glándula uropigial.

Hay pocos trabajos de biomonitorización que proporcionen información de las concentraciones de organoclorados en plumas y en tejidos internos, y prácticamente no se ha abordado la interpretación de las concentraciones en plumas con relación a los posibles efectos adversos (Behrooz *et al., 2*009a,b). Por tanto, resulta evidente que son necesarios más estudios experimentales y de campo para aproximarnos al conocimiento de los valores umbral en plumas indicativos de no efecto en las diferentes especies.

En la tabla 6 se presenta una revisión de las concentraciones de plaguicidas organoclorados de interés en la presente tesis doctoral en plumas de diferentes especies de aves. Sin embargo, en el capítulo I se presenta una revisión más completa de compuestos polihalogenados en plumas.

Tabla 6. *Concentraciones de plaguicidas organoclorados en plumas de aves.*

Especie	**Concentración media/mediana (ng/g) de plaguicidas organoclorados en plumas**	**Referencia**
Pingüino emperador (*Aptenodytes forsteri*)	∑HCH = 0,103-0,112, ∑DDT = 0,031-0,036	(Sen Gupta *et al.*, 1996)
Chorlitejo mongol (*Charadrius mongolus*)	∑HCH = 4,4, ∑DDT = 34	(Tanabe *et al.*, 1998)
Chorlitejo mongol (*Charadrius mongolus*)	∑HCH = 17, ∑DDT = 26	
Carbonero común (*Parus major*)	∑DDT = 6,13	(Dauwe *et al.*, 2005)
Busardo ratonero (*Buteo buteo*)	∑DDT = 4,9	(Jaspers *et al.*, 2006)
Garza real (*Ardea cinerea*)	∑HCH = 3,5, ∑DDT = 21	(Jaspers *et al.*, 2007a)
Polla de agua (*Gallinula chloropus*)	∑HCH = 2,4, ∑DDT = 12	
Gaviota argéntea (*Larus argentatus*)	∑HCH = 1,1, ∑DDT = 12	
Lechuza común (*Tyto alba*)	∑HCH = 5,3, ∑DDT = 48	
Búho chico (*Asio otus*)	∑HCH = 2,5, ∑DDT = 110	
Busardo ratonero (*Buteo buteo*)	∑HCH = 1,9, ∑DDT = 9,4	
Gavilán (*Accipiter nisus*)	∑HCH = 4,6, ∑DDT = 230	
Cernícalo vulgar (*Falco tinnunculus*)	∑HCH = 1,2, ∑DDT = 110	
Busardo ratonero (*Buteo buteo*)	∑DDT = 8,18 (VE), 12,5 (VI), 2,71 (EE), 5,23 (EI)	(Jaspers *et al.*, 2007b)
Urraca (*Pica pica*)	∑DDT = 3,07-27,2	(Jaspers *et al.*, 2008)
Águila moteada (*Aquila clanga*)	∑HCH = 21, ∑DDT = 26	(Behrooz *et al.*, 2009a)
Gavilán (*Accipiter nisus*)	∑HCH = 33, ∑DDT = 81	
Cernícalo vulgar (*Falco tinnunculus*)	∑HCH = 48, ∑DDT = 20	
Esmerejón (*Falco columbarius*)	∑HCH = 82, ∑DDT = 24	
Alcotán europeo (*Falco subbuteo*)	∑HCH = 23, ∑DDT = 11	
Halcón peregrino (*Falco peregrinus*)	∑HCH = 60, ∑DDT = 13	
Mochuelo europeo (*Athene noctua*)	∑HCH = 46, ∑DDT = 73	
Autillo europeo (*Otus scops*)	∑HCH = 212, ∑DDT = 295	
Búho campestre (*Asio flammeus*)	∑HCH = 28, ∑DDT = 62	
Perdiz chucar(*Alectoris chukar*)	∑HCH = 18, ∑DDT = 3	
Francolín común (*Francolinus francolinus*)	∑HCH = 22, ∑DDT = 2	
Tórtula turca (*Streptopelia decaocto*)	∑HCH = 65, ∑DDT = 15	
Abubilla (*Upupa epops*)	∑HCH = 18, ∑DDT = 15	
Cuervo común (*Corvus corax*)	∑HCH = 16, ∑DDT = 4	
Urraca (*Pica pica*)	∑HCH = 76, ∑DDT = 11	
Carraca europea (*Coracias garrulus*)	∑HCH = 18, ∑DDT = 12	
Avutarda hubara (*Chlamydotis undulata*)	∑HCH = 32, ∑DDT = 15	
Cuco común (*Cuculus canorus*)	∑HCH = 18, ∑DDT = 14	
Urraca (*Pica pica*)	∑DDT = 3,07 (área urbana) y 34,2 (área rural)	(Jaspers *et al.*, 2009)

Nota: ND = No detectado, LOQ = Límite de cuantificación. VE=Vaina externa, VI=Vaina interna, EE=Eje externo, EI=Eje interno. DDT = diclorodifeniltricloroetano, DDE = diclorodifenildicloroetileno, DDD = diclorodifenildicloroetano, HCH = hexachlorociclohexano.

Tabla 6. *Concentraciones de plaguicidas organoclorados en plumas de aves (continuación).*

Especie	Concentración media/mediana (ng/g) de plaguicidas organoclorados en plumas	Referencia
Matín pescador Pío (*Ceryle rudis*)	∑HCH = 25, ∑DDT = 96	(Behrooz *et al.*, 2009b)
Alción de esmirna (*Halcyon smyrnensis*)	∑HCH = 40, ∑DDT = 19	
Martinete común (*Nycticorax nycticorax*)	∑HCH = 46, ∑DDT = 24	
Avetorillo común (*Ixobrychus mintus*)	∑HCH = 49, ∑DDT = 37	
Focha común (*Fulica atra*)	∑HCH = 26, ∑DDT = 5	
Garcilla bueyera (*Bubulcus ibibs*)	∑HCH = 24, ∑DDT = 9	
Avetoro común (*Botaurus stellaris*)	∑HCH = 39, ∑DDT = 7	
Calamón (*Prophyrio porphyrio*)	∑HCH = 32, ∑DDT = 22	
Gaviota picofina (*Larus genei*)	∑HCH = 55, ∑DDT = 11	
Pelícano común (*Pelecanus onocrotalus*)	∑HCH = 32, ∑DDT = 15	
Cerceta común (*Anas crecca*)	∑HCH = 15, ∑DDT = 20	
Ánade real (*Anas platyrhynchos*)	∑HCH = 12, ∑DDT = 10	
Silbón europeo (*Anas penelope*)	∑HCH = 31, ∑DDT = 11	
Cuchara común (*Anas clypeata*)	∑HCH = 50, ∑DDT = 39	
Porrón pardo (*Aythya nyroca*)	∑HCH = 5, ∑DDT = 85	
Azor común (*Accipiter gentilis*)	β-HCH = 0,30, DDE = 43,9	(Eulaers *et al.*, 2011a)
Pigargo europeo (*Haliaeetus albicilla*)	β-HCH = 0,19, DDE = 8,30	
Águila real (*Aquila chrysaetos*)	β-HCH = 0,28, DDE = 33,3	
Pigargo europeo (*Haliaeetus albicilla*)	Rectriz izquierda: p,p'-DDE=27, ∑HCH=1,7	(Jaspers *et al.*, 2011)
	Rectriz derecha: p,p'-DDE=34, ∑HCH=2,6	
	Plumas corporales: p,p'-DDE=250, ∑HCH=12	
	Segunda primaria: p,p'-DDE=19, ∑HCH=2,8	
	Quinta primaria: p,p'-DDE=14, ∑HCH=1,1	
	Octava primaria: p,p'-DDE=12, ∑HCH=1,1	
Ánade real (*Anas platyrhynchos*)	p,p'-DDT= 1,4, o,p'-DDT= 7, p,p'-DDD= 1,3, p,p'-DDE= 14,3, o,p'-DDE= 0,2, α-HCH= ND, β-HCH= 15, γ-HCH= 6	(Rajaei *et al.*, 2011)
Cerceta común (*Anas crecca*)	p,p'-DDT= 1, o,p'-DDT= 40,5, p,p'-DDD= 3,2, p,p'-DDE= 50,5, o,p'-DDE= 2, α-HCH= 1, β-HCH= 25, γ-HCH= 21,5	
Ánade rabudo (*Anas acuta*)	p,p'-DDT= 0,5, o,p'-DDT= 40, p,p'-DDD= 2,4, p,p'-DDE= 17, o,p'-DDE= 0,04, α-HCH= 1, β-HCH= 5, γ-HCH= 12	
Gaviota cana (*Larus canus)*	p,p'-DDT= 1,8, o,p'-DDT= 7,9, p,p'-DDD= 0,7, p,p'-DDE= 28, o,p'-DDE= 0,4, α-HCH= 6, β-HCH= 21, γ-HCH= 7,5	
Gaviota enana (*Larus minutus*)	p,p'-DDT= 5,8, o,p'-DDT= 50,2, p,p'-DDD= 12,3, p,p'-DDE= 96, o,p'-DDE= 12, α-HCH= <LOQ, β-HCH= 17, γ-HCH= 4	
Gaviota reidora (*Larus ridibundus*)	p,p'-DDT= 6,7, o,p'-DDT= 10,5, p,p'-DDD= 2, p,p'-DDE= 99, o,p'-DDE= 0,5, α-HCH= 4,70, β-HCH= 9, γ-HCH= 11	
Zampullín chico (*Tachybaptus ruficollis*)	p,p'-DDT= 15, o,p'-DDT= 1, p,p'-DDD= 5, p,p'-DDE= 13, o,p'-DDE= <LOQ, α-HCH= <LOQ, β-HCH= 2,5, γ-HCH= 4,5	
Zampullín cuellinegro (*Podiceps nigricollis*)	p,p'-DDT= 1,3, o,p'-DDT= 10,3, p,p'-DDD= 3,4, p,p'-DDE= 69, o,p'-DDE= 0,3, α-HCH= 8,4, β-HCH= 9, γ-HCH= 26	
Somormujo lavanco (*Podiceps cristatus*)	p,p'-DDT= 1, o,p'-DDT= 1, p,p'-DDD= 1, p,p'-DDE= 89, o,p'-DDE= 0,2, α-HCH= <LOQ, β-HCH= 4, γ-HCH=7	
Cormorán grande (*Phalacrocorax carbo*)	p,p'-DDT= 2,3, o,p'-DDT= 16, p,p'-DDD= 4, p,p'-DDE= 21, o,p'-DDE= 0,5, α-HCH= 1, β-HCH= 13, γ-HCH= 7	
Pigargo europeo (*Haliaeetus albicilla*)	DDE = 6,59	(Eulaers *et al.*, 2011b)

Nota: ND = No detectado, LOQ = Límite de cuantificación. VE=Vaina externa, VI=Vaina interna, EE=Eje externo, EI=Eje interno. DDT = diclorodifeniltricloroetano, DDE = diclorodifenildicloroetileno, DDD = diclorodifenildicloroetano, HCH = hexachlorociclohexano.

Por otro lado, existen diversos estudios que relacionan los niveles de estos contaminantes en tejidos internos con efectos adversos en aves. En especies de aves de la familia Ardeidae, niveles de DDE en hígado de 124 µg/g (peso húmedo) se relacionaron con roturas de la cáscara de huevo (Pratt, 1972), mientras que niveles de 570 µg/g se relacionaron con la muerte de las aves (Call *et al.*, 1976). En tejido encefálico, concentraciones de DDTs superiores a 20 µg/g o concentraciones de dieldrín mayores a 4 µg/g se asociaron con mortalidad en aves (Stickel *et al.*, 1969, 1970). Se ha comprobado que la exposición a DDE es la principal causa de disminución del grosor de la cáscara de huevo, relacionándose valores de 1 a 1,5 µg/g (peso húmedo) de DDE en huevos con una pérdida entre 5 y 10% de grosor, y valores de 59 µg/g con una disminución del 44% (Blus *et al.*, 1974, 1979; Martínez-López *et al.,* 2007; Risebrough, 1972).

Otros trabajos han demostrado la capacidad de estos compuestos de provocar alteraciones comportamentales. Tórtolas domésticas (*Streptopelia risoria*) expuestas a 10 ppm de DDE en la dieta durante 63 días que presentaban residuos en encéfalo de 2,9 µg/g, mostraron alteraciones del comportamiento reproductivo (Haegele y Hudson, 1977). Igualmente, Ánades reales (*Anas platyrhynchos*) que recibían diferentes dosis de dieldrín en su dieta (4, 10 y 30 ppm) presentaron alteraciones del comportamiento que fueron relacionadas con un descenso en los niveles de aminas biógenas como la serotonina, la dopamina y la noradrenalina. Los niveles hepáticos y encefálicos que se detectaron en los individuos expuestos a 4 ppm en dieta fueron 2,3 µg/g y 0,12 µg/g, respectivamente (Sharma *et al.*, 1976).

A continuación se presenta una recopilación de concentraciones de plaguicidas organoclorados en tejidos internos de álcidos (Tabla 7) obtenidos en diferentes estudios que son de utilidad para la interpretación de resultados en la presente tesis.

Tabla 7. *Concentraciones de plaguicidas organoclorados en tejidos internos de álcidos.*

	Concentración OC (ng/g peso húmedo) en tejidos de álcidos			
Compuesto	**Mérgulo atlántico *(Alle alle)***	**Arao de Brunnich *(Uria lomvia)***	**Arao aliblanco *(Cepphus grylle)***	**Referencias**
α-HCH	H: 0,54, G: 42,9	H: 0,30, G: 11,4	H: 0,3, G: 44,2	(Buckman *et al.*, 2004)
β-HCH	H: 2,8, G: 93,9	H: 2,2, G: 35,4	H: 5,4, G: 111	
Lindano (γ-HCH)	H: 0,17, G: 12,2	H: 0,19, G: 3,8	H: 0,05, G: 5,7	
∑HCH	H: 3,5, G: 149	H: 2,6, G: 50,6	H: 5,7, G: 161	
p,p'-DDE	H: 8,9, G: 348	H: 31,8, G: 621	H: 51,7, G: 533	
p,p'-DDD	H: ND, G: ND	H: 0,49, G: 10,1	H: 1,4, G: 38,1	
p,p'-DDT	H: 0,50, G: 16,0	H: 1,1, G: 26,4	H: 1,5, G: 31,0	
∑DDT	H: 9,4, G: 364	H: 33,4, G: 658	H: 54,5, G: 602	
Dieldrín	H: 4,4, G: 175	H: 4,1, G: 76,4	H: 7,9, G: 135	
α-HCH*	H: ND	H: 0,17	H: 0,27	(Borgå *et al.*, 2007)
β-HCH*	H: 1,67	H: 0,86	H: 1,52	
Lindano (γ-HCH)*	H: ND	H: ND	H: ND	
p,p'-DDE*	H: 32,14	H: 30,60	H: 32,22	
p,p'-DDD*	H: ND	H: ND	H: 0,62	
p,p'-DDT*	H: ND	H: ND	H: ND	
∑HCH*	NA	H:1,13	H:1,58	(Borgå *et al.*, 2001)
∑DDT*	NA	H: 26,93	H: 31,46	

*Nota: H=Hígado, G=Grasa, ND=No detectado, NA=No analizado. *Los valores han sido calculados según los datos proporcionados por Borga et al. 2001 y 2007 de concentración de organoclorados (ng/g peso lipídico) y porcentaje de lípidos de la muestra.*

4.3. FACTORES QUE AFECTAN A LA PRESENCIA DE CONTAMINANTES AMBIENTALES PERSISTENTES EN AVES.

La presencia de los metales pesados y plaguicidas organoclorados en los distintos tejidos de aves está determinada por distintos factores como son el sexo, la edad y la dieta. Los niveles más altos de plaguicidas organoclorados (Donaldson *et al.*, 1999; Tanabe *et al.*, 1998; Wienburg y Shore, 2004) y de Hg, Cd, Cu y Zn (Barjaktarovic *et al.*, 2002; Debacker *et al.*, 2001; Gochfeld y Burger, 1987; Nielsen y Dietz, 1989; Savinov *et al.*, 2000) en machos, puede deberse a que las hembras pueden transferir parte de estos compuestos a los huevos (Bustnes *et al.*, 2008; Lewis *et al.*, 1993; Moss *et al.*, 2009). Sin embargo, se ha observado que los niveles de Pb en hembras son mayores que en machos, lo que puede deberse a que durante la formación de la cáscara aumenta la demanda de calico, lo que induce una mayor absorción intestinal de Pb por la existencia de un mecanismo de absorción común para ambos elementos (Finley y Dieter, 1978; Pattee, 1984).

Respecto a la edad, aves adultas muestran mayores concentraciones de organoclorados (Borgå *et al.*, 2001; Donaldson *et al.*, 1997; Vorkamp *et al.*, 2004), Hg (Dietz *et al.*, 1990; Ribeiro *et al.*, 2009), Cd, y Pb (Gochfeld *et al.*, 1996), lo que puede indicar una mayor acumulación durante un periodo más largo de tiempo.

En cuanto a la dieta, los máximos niveles de organoclorados se detectan en aves piscívoras, seguidas de insectívoras, omnívoras y herbívoras (Kunisue *et al.*, 2002). Además, en aves rapaces se ha demostrado que cuanto mayor es el porcentaje de aves en la dieta, mayores son los niveles de organoclorados (Van Drooge *et al.*, 2008). Las concentraciones de Hg en plumas también son menores en aves herbívoras, seguido de las aves predadoras de invertebrados y finalmente las aves que se alimentan de vertebrados (Zolfaghari *et al.*, 2007). Además, cuanto mejor es la condición corporal o mayor es el contenido en tejido adiposo, una mayor proporción de compuestos liposolubles como los organoclorados se acumulan en esos tejidos y no están disponibles para causar toxicidad (Van Wezef *et al.*, 1995). La condición corporal también es un factor que se debe tener en cuenta en el caso de los metales (Kalisińska *et al.*, 2010; Kojadinovic *et al.*, 2007; Richards *et al.*, 1987).

El hábitat es un factor extrínseco de gran importancia, ya que aves que habitan áreas próximas a zonas mineras, fundiciones o refinerías, áreas urbanas e industriales, zonas de caza con perdigones de Pb, o zonas agrícolas, tienen mayor probabilidad de estar expuestas a estos contaminantes y sufrir sus efectos (Gangoso *et al.*, 2009; García-Fernández *et al.*, 1995; Gómez-Ramírez *et al.*, 2011; Jaspers *et al.*, 2009; Martínez-López *et al.*, 2009).

Otros factores de interés son las propiedades físico-químicas de los compuestos que determinarán su biodisponibilidad, la ruta y el tiempo de exposición; y las condiciones ambientales como temperatura y humedad (Dikshith, 1991; Kendall y Scanlon, 1984; Prokop *et al.*, 2003).

4.4. EFECTOS DE LOS CONTAMINANTES AMBIENTALES PERSISTENTES EN AVES.

Los compuestos organoclorados y los metales pesados tienen consecuencias no sólo letales, sino también subletales sobre distintos parámetros como el sistema inmunitario, la función reproductiva, el desarrollo nervioso e incluso sobre la aparición de tumores. Este tipo de efectos se asocian generalmente a exposiciones ambientales crónicas a bajas dosis.

4.4.1. Alteraciones sobre el éxito reproductivo en las aves.

Éxito de la puesta

Entre los efectos más frecuentemente observados destacan la disminución de la puesta de huevos por nido, el número de huevos eclosionados y el número de pollos que sobreviven tras varias semanas de vida. La primera evidencia de que el DDT está involucrado en el declive de poblaciones de aves se inició a partir de observaciones de nidos de Halcón peregrino (*Falco peregrinus*), donde se encontró un número de huevos rotos tan alto que causó una reducción considerable en el número de crías producidas por cada pareja de adultos por año. Además, se observó una reducción significativa del grosor medio de la cáscara comparando huevos puestos en 1947, previo al uso del DDT, con huevos puestos en 1952 (Newman, 1993). Otras especies con resultados semejantes fueron el Águila calva (*Haliaeetus leucocephalus*) y el Águila pescadora (*Pandion haliaetus*) en los Estados Unidos; y el Águila real (*Aquila chrysaetos*), el Esmerejón (*Falco columbarius*), el Cernícalo vulgar (*Falco tinnunculus*) y el Cormorán (*Phalacrocorax*) en Gran Bretaña (Ratcliffe, 1970). Ciertos autores han estudiado los niveles de organoclorados y el grosor de la cáscara de huevo y su relación con el éxito reproductivo (Cortinovis *et al.*, 2008; Helberg *et al.*, 2005; Kamata *et al.*, 2009; Martínez-López *et al.*, 2007; Mason *et al.*, 1997). Ratclife (1970) realizó un experimento con Halcón peregrino, proporcionándoles una dosis de DDT similar a la que podrían haber ingerido estas aves en el alimento durante los años 60, resultando en una reducción en el grosor de la cáscara entre un 8 y un 17% respecto al grupo control. Un estudio más reciente en Búho real mostró una disminución del grosor de la cáscara de huevo del 17% cuando los niveles de DDE fueron 8 µg/g (peso húmedo) (Gómez-Ramírez *et al.*, 2012); mientras que en un estudio realizado por Martínez-López *et al.* (2007), una concentración de DDE de 1,5 µg/g (peso húmedo) en huevos se asoció con una disminución del 10% en el grosor de la cáscara de Aguililla calzada (*Hieraaetus pennatus*).

En cuanto a los efectos del Cd y Pb sobre el tamaño de la puesta y el grosor de la cáscara de huevo, los resultados son contradictorios. Mientras que algunos estudios experimentales describen disminución de la puesta y del grosor de cáscara en Gallo doméstico (*Gallus gallus*), ánades, y codornices (Edens y Garlich, 1983; White y Finley, 1978); otros trabajos no han observado estos efectos en rapaces en cautividad (Pattee, 1984) ni en otras aves de vida libre (Dauwe *et al.*, 2004; Furness, 1996; Henny *et al.*, 1991; Scheuhammer, 1987). La exposición por inyección e inmersión de

huevos de Ánade real a Cd y Pb aumenta la mortalidad embrionaria y reduce el éxito de eclosión del huevo, siendo mayores los efectos en el caso del Cd (Kertész y Fáncsi, 2003). Además, las sales de Cd depositadas en el blastodermo de embriones de pollos causan alteraciones teratogénicas (Schowing, 1984). Sin embargo, estos efectos no deberían tener relevancia para los estudios en aves silvestres puesto que las aves no parecen transferir Cd al huevo, o lo hacen en pequeñas concentraciones (Furness, 1996).

A pesar de que el Hg no produce disminución en el grosor de la cáscara de huevo (Heinz, 1974; Hill y Shaffner, 1976; Spann *et al.*, 1972), se ha observado que la exposición a metilmercurio provoca la reducción de la puesta en Ánade real (Heinz, 1979). Además, se ha observado un aumento de la puesta de huevos sin cáscara y una reducción del peso medio del huevo en adultos de Faisán común (*Phasianus colchicus*) tratados con metilmercurio en época reproductiva (Fimreite, 1971). La exposición a metilmercurio también puede producir disminución de eclosiones debido a mortalidad embrionaria y aumento del número de huevos sin fertilizar (Borg *et al.*, 1969; Frederick y Jayasena, 2010). Se ha demostrado que el metilmercurio es teratogénico en aves, provocando malformaciones (Heinz y Hoffman, 2003; Hoffman y Moore, 1979). Una breve inmersión de huevos de Ánade real en soluciones de metilmercurio es capaz de producir aberraciones esqueléticas en los embriones (Hoffman y Moore, 1979). Además, la exposición a Hg inorgánico provoca disminución de la fertilidad de huevos en Codorniz japonesa (*Coturnix coturnix japonica*) (Hill y Shaffner, 1976).

El Pb, Cd y Hg parecen influir en el crecimiento de las aves expuestas. En estudios experimentales se ha observado una alteración de la actividad de enzimas del metabolismo cerebral relacionada con un retraso en el crecimiento en Cernícalos americanos (*Falco sparverius*) expuestos a Pb (Hoffman *et al.*, 1985). La exposición a Cd en Garceta azul (*Egretta caerulea*) provoca un menor crecimiento óseo (Spahn y Sherry, 1999). Pollos expuestos a Hg inorgánico también mostraron retraso en el crecimiento (Grissom y Thaxton, 1985; Parkhurst y Thaxton, 1973).

Además de los efectos en la cáscara de huevo, ya descritos por numerosos autores, la inyección de o,p'-DDT (1-100 µg/g de huevo) en yema de huevos de Codorniz japonesa antes de la incubación provocó una reducción de tamaño del oviducto izquierdo dosis-dependiente, y un desarrollo anormal del derecho en hembras, mientras que se observó asimetría en los testículos de los machos tratados

(Kamata *et al.*, 2009). En cuanto a los metales, la exposición a Pb produce diferentes alteraciones testiculares en Tórtola doméstica, tales como disminución del peso testicular y degeneración de túbulos seminíferos (Kendall y Scanlon, 1981; Veit *et al.*, 1983). También se ha observado reducción del peso gonadal en Codorniz japonesa ante la exposición a Hg inorgánico (Hill y Soares, 1984). La exposición a Cd también es capaz de provocar atrofia testicular en aves (Furness, 1996; Scheuhammer, 1987), además de reducir la motilidad espermática, provocar necrosis de los conductos eferentes, dañar las células de Sertoli, disminuir la concentración sérica de testosterona, y provocar abortos y teratogénesis en los fetos (Satoh *et al.*, 2002). Todo parece indicar que la exposición elevada y continua a estos contaminantes puede poner en riesgo las poblaciones de aves por una menor fertilidad causada por la disfunción del tracto reproductivo.

Disrupción endocrina

Algunos compuestos organoclorados han sido identificados como estrogénicos débiles o precursores de estrógenos por hidroxilación hepática (Eroschenko y Palmiter, 1980; Kupfer y Bulger, 1987), por lo que pueden actuar disminuyendo o estimulando la producción de determinadas hormonas o alterando las rutas metabólicas de las mismas. La administración de DDT, aldrín, dieldrín y lindano produce efectos estrogénicos en los animales de experimentación (Fry y Toone, 1981; Raizada *et al.*, 1980; Tiemann, 2008). También ha sido identificado como estrogénico el endosulfán (Eroschenko y Palmiter, 1980; Fry y Toone, 1981; Fry, 1995; Varayoud *et al.*, 2008). La contaminación por DDT en California se ha relacionado con feminización en gaviotas machos; y la inyección de DDT en huevos fértiles de gaviotas provoca el desarrollo de tejido ovárico y oviductos en embriones machos (Fry y Toone, 1981; Fry, 1995; Fry *et al.*, 1987; Hoyer, 2001). Inyecciones de o,p'-DDT en huevos de gaviotas también produjeron anormalidades, tanto en los embriones machos como hembras (Fry, 1995). Cortinovis *et al.* (2008) determinaron que la concentración media de testosterona y 17β-estradiol en huevos de Somormujo lavanco (*Podiceps cristatus*) disminuía conforme aumentaba la concentración de p,p'-DDE.

Diversos estudios han demostrado que la exposición a organoclorados en Gaviota hiperbórea (*Larus hyperboreus*), Urogallo (*Tympanuchus phasianellus*) y Paloma (*Columba livia*) provoca cambios comportamentales durante el cortejo, en la construcción y protección del nido, y en el cuidado de los pollos, aumentado el riesgo de ser depredados (Bustnes *et al.*, 2005; Colborn, 2002; McEwen y Brown, 1966).

Quinn *et al.* (2008) observaron que una sola exposición a p,p'-DDE en embriones de Codorniz japonesa acelera el desarrollo sexual en hembras y reduce los comportamientos reproductivos masculinos. Entre los efectos observados en Ibis blancos (*Eudocimus albus*) expuestos a metilmercurio destacan cambios en la conducta de emparejamiento, con comportamientos homosexuales entre machos (Frederick y Jayasena, 2010).

4.4.2. Alteración del sistema nervioso.

Tanto los plaguicidas organoclorados como los metales son contaminantes con capacidad de producir efectos neurotóxicos que provocan afecciones en el sistema motor, sensorial, cognitivo o autónomo (Evangelista de Duffard y Duffard, 1996; Keifer y Firestone, 2007; Walker, 2003; Wennberg, 1994).

Los plaguicidas organoclorados actúan alterando la membrana de las células nerviosas causando cambios en la cinética del Na^+ y K^+, pudiendo estar también implicados en el transporte del calcio o en la actividad de la enzima Ca^{2+}ATPasa (Smith, 1991; Woolley *et al.*, 1985). El DDT y sus metabolitos suprimen la diferenciación de las células neuronales e inducen la apoptosis *in vitro* (Shinomiya y Shinomiya, 2003). Por otro lado, se ha relacionado la exposición ambiental a DDT con la aparición de diversas alteraciones neuroanatómicas durante el desarrollo del sistema nervioso en individuos jóvenes de Zorzal robín (*Turdus migratorius*), incluyendo cerebros más pequeños, y reducción del tamaño neuronal y del volumen del núcleo intercolicular, estructura crítica para el comportamiento sexual (Iwaniuk *et al.*, 2006).

Respecto a los metales, estos pueden interactuar con proteínas involucradas en la transducción de señales, como canales y bombas de Ca^{2+} (Nicotera y Rossi, 1993). En cuanto al Pb, el sistema nervioso es uno de los principales sistemas afectados por este metal (Redig y Arent, 2008). Esta afectación, en casos de intoxicación aguda, se caracteriza por debilidad muscular, reducción de reflejos, parálisis del buche, esófago, proventrículo y molleja, letargia, parálisis de extremidades y cuello, convulsiones y ceguera (Lumeij, 1985; Mateo *et al.*, 2003a). Se trata de síntomas relacionados con desmielinización del sistema nervioso central y periférico (Hunter y Haigh, 1978; Hunter y Wobeser, 1980; Platt *et al.*, 1999). La exposición a Pb provoca anomalías en la locomoción, equilibrio, termorregulación, aprendizaje y reconocimiento de individuos en Gaviotas argénteas (*Larus argentatus*) (Burger y Gochfeld, 2005).

El Hg es capaz de provocar degeneración de la médula espinal y lesiones cerebrales en aves (Fimreite, 1971; Finley y Stendell, 1978; Hill y Soares, 1984; Pass *et al.*, 1975). Entre los signos de intoxicación aguda por metilmercurio se describen temblores, reducción de la ingesta de alimentos y la consecuente pérdida de peso, debilidad progresiva en extremidades, dificultad para volar, andar y mantenerse erguido, ataxia, parálisis y/o convulsiones (Pass *et al.*, 1975; Scheuhammer, 1987). Palomas tratadas con metilmercurio o Hg inorgánico mostraron reducción de aprendizaje y alteraciones comportamentales, produciéndose cambios permanentes en la postura y en la coordinación motora (Evans *et al.*, 1982; Leander *et al.*, 1977). También se ha observado que crías de patos cuyos padres han sido tratados con metilmercurio acuden menos a las llamadas de sus progenitores (Heinz, 1975, 1979). Además, el canto de aves que habitan áreas contaminadas por Hg presenta una menor diversidad de notas y es emitido a frecuencias tonales más bajas que los cantos en los sitios de referencia (Hallinger *et al.*, 2010). Las alteraciones del comportamiento pueden conllevar una mayor probabilidad de depredación, muerte por inanición o el padecimiento de enfermedades infecciosas que favorezcan la muerte por otras causas (Burger y Gochfeld, 1997; Heinz *et al.*, 1983).

El Cd también es capaz de provocar alteraciones en el comportamiento, como ha sido evidenciado en ánades juveniles expuestas a este metal, que mostraron anomalías en el comportamiento de huida (Heinz *et al.*, 1983).

4.4.3. Alteraciones sobre el sistema inmune.

Algunos contaminantes ambientales pueden llegar a ser inmunotóxicos, incrementando, por tanto, la susceptibilidad al padecimiento de enfermedades infectocontagiosos (Grasman *et al.*, 1996; Vos y Luster, 1989). En este sentido, se ha encontrado una relación positiva entre la presencia de nemátodos y las concentraciones hepáticas de varios organoclorados en Gaviota hiperbórea (Sagerup *et al.*, 2000). Grasman *et al.* (2000) y Bustnes *et al.* (2004) observaron que un aumento en la concentración de organoclorados provocó un aumento en los niveles de linfocitos en Gaviotas argénteas y Gaviotas hiperbóreas, respectivamente. Estudios realizados por Grasman *et al.* (1996) sobre Gaviota argéntea y Pagaza piquirroja (*Hydroprogne caspia)* demostraron la asociación entre la supresión de las células T y la exposición a compuestos organoclorados, siendo los PCBs los más fuertemente asociados con la inmunosupresión.

También se ha observado que determinados metales, entre los que se incluyen el Pb, el Hg y el Cd, pueden causar efectos adversos sobre el sistema inmune en aves, alterando tanto la inmunidad humoral como la celular (Bridger y Thaxton, 1983; Exon, 1984; Fair y Myers, 2002; Hawley *et al.*, 2009; Kenow *et al.*, 2007; Kumar *et al.*, 1999; Redig *et al.*, 1991; Rocke y Samuel, 1991; Snoeijs *et al.*, 2004; Spalding *et al.*, 2000; Trust *et al.*, 1990; Vodela *et al.*, 1997). En general, los metales pesados se consideran inmunosupresores. Aunque se sugiere que esta alteración puede estar relacionada con efectos citotóxicos directos sobre las células del sistema inmune, carcinogénesis, deficiencias nutricionales o respuesta al estrés, es necesario seguir investigando acerca de los mecanismos implicados (Exon, 1984; Grasman y Scanlon, 1995; Hoffman *et al.*, 2009).

4.4.4. Carcinogenicidad y genotoxicidad.

Valerón *et al.* (2009) observaron una asociación entre la exposición a combinaciones de plaguicidas organoclorados y la inducción de procesos de transformación en células mamarias humanas. El dieldrín provocó un aumento en el volumen y número de tumores en glándulas mamarias de ratones (Cameron y Foster, 2009). Por su parte, Wong y Matsumura (2007) concluyeron que el β-HCH actúa como promotor del cáncer de mama. Los DDTs, HCHs, heptacloro y endosulfán están clasificados como posibles carcinógenos en humanos (Grupo 2B) (IARC, 1987a, 1991, 2001; U.S. EPA, 2006). Sin embargo, el aldrín, dieldrín y endrín no son clasificables respecto a su carcinogenicidad en humanos, por lo que están catalogados en el grupo 3 (IARC, 1987b).

Tanto el Pb como el Cd han mostrado suficiente capacidad de inducir carcinogénesis en animales de laboratorio (Goyer, 1996). El Cd está clasificado como carcinógeno en humanos (Grupo 1) y el Pb inorgánico como probable carcinógeno en humanos (Grupo 2A) por la Agencia Internacional para la Investigación del Cáncer (IARC) (IARC, 1997, 2006). Aunque la carcinogenicidad de estos metales no está totalmente demostrada en aves, sí que se ha observado genotoxicidad en pollos de Milano negro (*Milvus migrans*) expuestos a Cd (Baos *et al.*, 2006b). Estos mismos autores encontraron diferencias interespecíficas, siendo el Milano negro más sensible que la Cigüeña blanca (*Ciconia ciconia*). Se ha demostrado que la inoculación directa de Zn en testículos de aves y roedores induce la aparición de tumores testiculares (Goyer, 1996; NAS, 1979), aunque este metal no es carcinogénico por cualquier otra vía (Eisler, 1993).

El metilmercurio induce la aparición de tumores en riñón de ratones, y existen suficientes evidencias de su carcinogenicidad en animales de experimentación. Está catalogado como posible carcinógeno en humanos (Grupo 2B). Aunque algunos estudios muestran que el Hg inorgánico causa un aumento de diferentes tipos de tumores en ratas y ratones, no hay evidencias suficientes de su carcinogenicidad en animales de experimentación. El Hg inorgánico y el Hg metálico no pueden ser clasificados respecto a su carcinogenicidad en humanos, por lo que actualmente están clasificados dentro del grupo 3 (IARC, 1997).

4.4.5. Alteraciones tisulares.

El Hg es capaz de producir lesiones renales en Estornino pinto (*Sturnus vulgaris*) (Nicholson y Osborn, 1984). La exposición a Cd en ratas puede inducir una nefropatía caracterizada por necrosis de los túbulos proximales, proteinuria, glucosuria, aumento de los niveles de Cd en orina y disminución en riñones, con la presencia de metalotioneínas en plasma (Goyer *et al.*, 1984). Hay evidencias de que el mecanismo es similar en aves, aunque es improbable que estas lesiones tengan lugar en individuos de vida libre si la exposición se produce a bajas dosis (Scheuhammer, 1987).

El Pb también afecta al riñón, principalmente en forma de nefrosis y aparición de cuerpos de inclusión intranucleares en aves intoxicadas por Pb (Beyer *et al.*, 1988; Locke *et al.*, 1966; Pattee *et al.*, 1981; Romero *et al.*, 2007). El Pb produce también necrosis miocárdica y necrosis fibrinoide arterial (Pattee *et al.*, 1981). La intoxicación aguda por Cu en aves se manifiesta principalmente por la erosión de la molleja y el proventrículo (Henderson y Winterfield, 1975; Jensen y Maurice, 1978; Poupoulis y Jensen, 1976). En el caso de la intoxicación por Zn en aves, la pancreatitis es la lesión característica (Beyer *et al.*, 2005; Sileo *et al.*, 2003).

4.4.6. Alteraciones bioquímicas.

Los compuestos organoclorados ejercen efectos tóxicos a nivel hepático y renal, lo cual se ve reflejado en las alteraciones de determinados parámetros bioquímicos sanguíneos. Así, un aumento en los niveles de enzimas como creatin quinasa (CK), aspartato aminotransferasa (AST) y lactato deshidrogenasa (LDH) se ha evidenciado en plasma de aves a las que se les administró organoclorados vía oral (Dieter, 1974, 1975).

El metilmercurio afecta a diversos parámetros bioquímicos, reduciendo las concentraciones de ácido úrico, albúmina, fósforo inorgánico y proteínas totales en plasma; aumentando la actividad de la enzima AST; e inhibiendo la actividad de la LDH en Garzas blancas (*Ardea alba*) (Hoffman *et al.*, 2005). Tanto el Cd como el Pb interfieren en la actividad de varias enzimas, a menudo por competencia con otros elementos esenciales como el Zn o el calcio (Eisler, 1985; Godwin, 2001). Algunas de las alteraciones de parámetros bioquímicos por exposición a Pb y Cd son la disminución de la actividad fosfatasa alcalina (Kertész y Hlubik, 2002; Mateo *et al.*, 2003a, 2003b; Rozman *et al.*, 1974) y el aumento de la actividad alanina aminotrasferasa (Cain *et al.*, 1983; Hoffman *et al.*, 1981; Rozman *et al.*, 1974). Por otro lado, la exposición a Pb se ha relacionado con un aumento de la glucosa, la creatinina, el colesterol y la CK en suero/plasma (Hoffman *et al.*, 1981, 1985; Mateo *et al.*, 2003a). Además, el Pb es un potente inhibidor de enzimas que intervienen en la síntesis del grupo hemo del eritrocito (ALAD, ferroquelatasa, coproporfirinógeno oxidasa, hemo sintetasa). Destaca el efecto del Pb en la ALAD porque su inhibición por Pb es mayor que en el resto de enzimas y es, además, el primer cambio bioquímico cuantificable tras la exposición a Pb (Tola *et al.*, 1973). Una inhibición del 45-59% de la actividad de esta enzima es suficiente para provocar anemia en pollos de Cernícalos americanos expuestos a Pb (Henny *et al.*, 1994; Hoffman *et al.*, 1985). Diversos estudios han encontrado correlaciones significativas entre los niveles de Pb y la actividad enzimática de la ALAD en aves silvestres (Gómez-Ramírez *et al.*, 2011; Martínez-Haro *et al.*, 2011; Martínez-López *et al.*, 2004).

4.4.7. Alteraciones relacionadas con el estrés oxidativo.

Además de los procesos endógenos, diversos procesos exógenos como la exposición a metales, compuestos clorados y radiación, pueden generar directamente o inducir indirectamente la producción de ROS en las células (Valko *et al.*, 2006). En la presente tesis nos hemos centrado en el estudio de metales pesados en relación al estrés oxidativo, por lo que a continuación se describirán los efectos de estos contaminantes en el sistema antioxidante.

Como se ha comentado anteriormente, algunos estudios sugieren que uno de los mecanismos que influyen en la toxicidad de los metales es la inducción de ROS (Ercal *et al.*, 2001). En este sentido, los metales redox activos como el Fe y el Cu catalizan la reacción de Fenton, generando así radicales libres. Sin embargo, los metales redox inactivos como el Pb, Cd y Hg inducen estrés oxidativo indirectamente afectando a los

niveles de antioxidantes enzimáticos y no enzimáticos ricos en grupos sulfhidrilo. Aunque este parece ser el principal mecanismo del estrés oxidativo generado por los metales redox inactivos, estos también pueden generar ROS indirectamente afectando a la mitocondria (Koivula y Eeva, 2010).

Pb, Hg y Cd son metales con afinidad para compartir electrones, por lo que tienden a formar enlaces covalentes, principalmente con el GSH y grupos sulfhidrilo de las proteínas (Bondy, 1996; Quig, 1998). Precisamente esta interacción de los metales en el metabolismo del GSH es esencial en el efecto tóxico de estos contaminantes (Hultberg *et al.*, 2001). El proceso de conjugación metal-GSH ayuda a excretar metales tóxicos en la bilis, sin embargo, reduce la capacidad antioxidante (Quig, 1998). Cuando el GSH es agotado por estos metales, se comienza a sintetizar nuevo GSH a través del ciclo γ-glutamil (Ercal *et al.*, 2001), pero si el agotamiento del GSH continúa debido a una exposición crónica a metales, este tripéptido no es suministrado eficientemente (Hultberg *et al.*, 2001; Quig, 1998; Stohs y Bagchi, 1995). Un único átomo de Cd o Hg puede unirse hasta a dos moléculas de GSH provocando su excreción (Zalups y Lash, 1996). Varias enzimas del sistema de defensa antioxidante pueden proteger de este desequilibrio. Sin embargo, muchas de estas enzimas pueden ser inactivadas por la unión de metales a su centro activo si contienen grupos SH (Quig, 1998). Además, estos metales alteran la estructura y función de proteínas como la bomba sodio-potasio debido a su unión directa a grupos SH libres (Quig, 1998).

Una de las respuestas de adaptación de los organismos ante la reacción de estos metales con los grupos SH es aumentar los niveles basales de GSH, y la actividad de GPx y GR (Quig, 1998). Otra de las respuestas adaptativas y de protección ante estos metales es la inducción de la síntesis de metalotioneínas (Quig, 1998). Estas proteínas intracelulares sirven como almacén de Cu y Zn, y secuestran a los metales no esenciales (Quig, 1998). Las metalotioneínas son ricas en cisteína, y tienen mayor afinidad por el Hg y el Cd que por el Zn (Hamer, 1986). Por tanto, la unión del Hg y Cd a estas proteínas produce la liberación del Zn, lo que induce la síntesis de metalotioneínas (Hamer, 1986).

A continuación se describirán los posibles mecanismos por los que los metales inducen el estrés oxidativo:

Plomo

Aunque se sabe que el Pb no puede iniciar la peroxidación lipídica de forma directa, diversos estudios muestran que es capaz de alterar la composición, estructura y función de las membranas celulares (Ercal *et al.*, 2001; Gurer y Ercal, 2000). De esta forma, se ha demostrado un aumento en las concentraciones de malondialdehído (MDA), compuesto resultante de la oxidación de ácidos grasos, tras la exposición *in vitro* de ácidos grasos poliinsaturados a Pb. Además, los niveles de MDA generados aumentaban con el número de dobles enlaces de los ácidos grasos, sugiriendo que se trata de un proceso de peroxidación (Yiin y Lin, 1995), ya que los ácidos grasos con ninguno, uno o dos dobles enlaces son más resistentes al ataque oxidativo que los ácidos grasos poliinsaturados que tienen más de dos dobles enlaces (Gurer y Ercal, 2000). Por otro lado, se ha observado una alteración provocada por este metal en la composición de los fosfolípidos de membrana en eritrocitos, ya que el Pb^{2+} se une fuertemente a la fosfatidilcolina, provocando un descenso en sus niveles (Gurer y Ercal, 2000). Estos datos sugieren que la modificación de la composición de lípidos de membrana puede alterar la integridad de la misma, aumentando su susceptibilidad a la peroxidación lipídica (Gurer y Ercal, 2000). Además, el Pb altera la actividad de enzimas de membrana y la composición de proteínas de membrana en eritrocitos (Fukumoto *et al.*, 1983; Hasan *et al.*, 1971; Raghavan, 1981). Con todo ello, aun no está claro si el estrés oxidativo es la causa o la consecuencia de los efectos tóxicos del Pb, pero probablemente la exposición a Pb aumenta la vulnerabilidad de las membranas al ataque de ROS alterando su integridad, permeabilidad y función mediante el deterioro de sus componentes (Gurer y Ercal, 2000).

El estudio de los efectos del Pb en la membrana de eritrocitos en particular ha sido más intenso debido a que los glóbulos rojos tienen una gran afinidad por este metal, conteniendo la mayor parte del Pb presente en el flujo sanguíneo, y son más vulnerables al daño oxidativo que otras células (deSilva, 1981; Leggett, 1993). En este sentido, se ha demostrado que el Pb es capaz de aumentar la susceptibilidad osmótica y mecánica de los eritrocitos (Waldron, 1966), además de disminuir la deformabilidad y acortar su vida útil (Hernberg *et al.*, 1967; Levander *et al.*, 1977). Algunos metales como el Pb^{2+}, Hg^{2+} y Cu^{2+} son potentes agentes hemolíticos, sugiriéndose que la peroxidación lipídica inducida por estos metales es el mecanismo por el que se produce la hemólisis (Ercal *et al.*, 2001). Teniendo en cuenta que el Pb no puede iniciar la peroxidación lipídica mediante una acción directa, se han estudiado posibles mecanismos indirectos del inicio de la peroxidación de lípidos de membrana (Ribarov y

Bochev, 1982; Ribarov *et al.*, 1981). La interacción de metales pesados con la oxihemoglobina (oxyHb) es una importante fuente de radicales superóxido en los eritrocitos. En este sentido, se ha observado que el Pb^{2+} aumenta la autooxidación de la hemoglobina, y esta actividad se ve inhibida por las enzimas SOD y CAT, sugiriendo que $O_2\cdot^-$ y H_2O_2 están involucrados en este proceso. Por tanto, se cree que el Pb puede generar ROS en su interacción con la hemoglobina, provocando daño peroxidativo en membranas de eritrocitos (Ribarov y Bochev, 1982; Ribarov *et al.*, 1981).

Los efectos que el Pb puede provocar sobre el sistema hematológico son bien conocidos. La anemia es un síntoma de la intoxicación por Pb, ya que este metal inhibe la síntesis del grupo hemo de la hemoglobina, y modifica la morfología y supervivencia de los eritrocitos (Gurer y Ercal, 2000). Este metal es capaz de inhibir la actividad de las enzimas ácido δ-aminolevulínico deshidratasa (ALAD) y ferroquelatasa, ambas involucradas en la síntesis del grupo hemo. El Pb se une a los grupos SH de la enzima ALAD provocando su inactivación (Farant y Wigfield, 1982). Por tanto, la condensación de dos moléculas de ácido δ-aminolevulínico (ALA) para formar porfobilinógeno (catalizado por la ALAD) y la incorporación de Fe a la protoporfirina (catalizada por la ferroquelatasa) son bloqueados provocando una inhibición de la formación del grupo hemo. Esta reducción de grupo hemo estimula a la ALA-sintetasa por retroalimentación y, como consecuencia, los niveles de ALA aumentan y se acumulan en sangre (Figura 6) (Gurer y Ercal, 2000). Tanto el ALA como el acoplado ALA/oxihemoglobina, tienen alto potencial para autooxidarse, provocando la formación de ROS (Monteiro *et al.*, 1986, 1989). Además, la inhibición de la ferroquelatasa causa liberación de iones de Fe que quedarán disponibles para participar en la reacción de Fenton y de Haber-Weiss (Valko *et al.*, 2005).

El Pb también interfiere en el sistema de defensa antioxidante de las células, alterando la actividad de enzimas antioxidantes como SOD, CAT y GPx, y modificando los niveles de moléculas antioxidantes como el GSH (Gurer y Ercal, 2000). Como se ha dicho anteriormente, el Pb presenta una elevada afinidad por los grupos SH, por lo que son capaces de inhibir enzimas y reducir la cantidad de moléculas antioxidantes que presentan este grupo funcional (Flora *et al.*, 2008). Diversos estudios han observado un descenso de los niveles de GSH ante exposiciones a Pb en ratas (Korsrud y Meldrum, 1988) y aves (Mateo *et al.*, 2003a; Somashekaraiah *et al.*, 1992). Sin embargo, estudios experimentales en aves también han observado un aumento en los niveles de GSH tras la exposición a Pb (Hoffman *et al.*, 2000a; Mateo y Hoffman,

2001; Mateo *et al.*, 2003a), lo que puede deberse a una inducción de la enzima γ-glutamil cisteína sintetasa que participa en la síntesis del GSH (Griffith, 1999), como propone Mateo *et al.* (2003a). Mateo *et al.* (2003a) también sugieren que una inhibición de la actividad de la enzima GPx puede ser en parte la causa del aumento en los niveles de GSH, debido al menor uso del GSH por parte de la GPx. Sin embargo, el aumento de GSH en aves expuestas que presentan una actividad normal de enzimas antioxidantes sugiere que existen otros mecanismos implicados (Mateo y Hoffman, 2001).

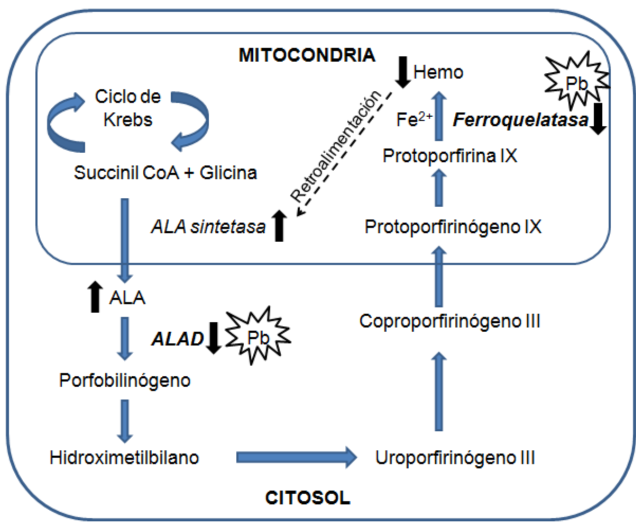

Figura 6. *Efectos del Pb en la síntesis del grupo Hemo. ALA: ácido delta-aminolevulínico, ALAD: ácido delta-aminolevulínico deshidratasa. Basado en Gurer y Ercal (2000).*

La enzima G6PDH también contiene grupos SH, y tanto estudios *in vitro* (Lachant *et al.*, 1984; Vallee y Ulmer, 1972), como estudios experimentales con Ánade real y Barnacla canadiense (*Branta canadensis*) (Hoffman *et al.*, 2000b; Mateo *et al.*, 2003a) han observado una inhibición de su actividad por la exposición a Pb. Sin embargo, otros trabajos han encontrado aumentos en la actividad de esta enzima ante la exposición a este metal (Gelman *et al.*, 1978; Gürer *et al.*, 1998). Estos resultados contradictorios parecen deberse a la concentración y duración de la exposición, así como a la magnitud del estrés oxidativo en la célula (Gelman *et al.*, 1978). La enzima GR, encargada de reducir el disulfuro de glutatión (GSSG) a GSH manteniendo el sistema de defensa antioxidante indirectamente, presenta un enlace disulfuro en su centro activo (Fahey y Sundquist, 1991). El Pb interfiere con este enlace e inhibe la actividad enzimática en ratas (Sandhir y Gill, 1995; Sandhir *et al.*, 1994) y embriones de aves (Somashekaraiah *et al.*, 1992), lo que evita la reducción del GSSG haciendo a la célula más susceptible al daño oxidativo (Gurer y Ercal, 2000).

Por otra parte, la GPx, CAT y SOD son metaloenzimas que ejercen su actividad antioxidante eliminando el $O_2\cdot^-$ y el H_2O_2, y necesitan elementos esenciales para su funcionamiento (Gurer y Ercal, 2000). El selenio es cofactor de la GPx, y el Pb es un antagonista del selenio, por lo que la actividad de esta enzima puede verse inhibida ante la exposición a este metal (Schrauzer, 1987), como se ha observado en diferentes estudios experimentales en aves (Mateo *et al.*, 2003a; Somashekaraiah *et al.*, 1992). Por otro lado, la CAT presenta el grupo hemo como grupo prostético, y el Pb reduce la absorción de Fe, metal pesente en este grupo, y como se ha explicado previamente, inhibe la síntesis del grupo hemo. El descenso de la actividad de CAT se ha atribuido a la interferencia del Pb en ambos procesos (Sandhir y Gill, 1995; Sandhir *et al.*, 1994). Por último, la SOD necesita Cu y Zn para ejercer su actividad, y el Pb reemplaza a ambos metales, por lo que es capaz de inhibir su actividad (Gelman *et al.*, 1978; Mylroie *et al.*, 1986). En resumen, los efectos inhibitorios del Pb sobre las enzimas antioxidantes debilitan las defensas de las células y las hacen más susceptibles al ataque oxidativo (Gurer y Ercal, 2000).

Finalmente, diferentes estudios experimentales demuestran que el Pb es capaz de provocar una aumento de la peroxidación lipídica en aves (Mateo y Hoffman, 2001; Mateo *et al.*, 2003a; Somashekaraiah *et al.*, 1992). Como sugieren Mateo y Hoffman (2001), diversos mecanismos podrían ser responsables de la peroxidación lipídica inducida por el Pb. Entre ellos destaca la generación de ROS, especies capaces de atacar a las membranas, por la acumulación de ALA como consecuencia de la inhibición de la ALAD o por la autooxidación de la oxihemoglobina. Además, el Pb puede provocar efectos en el sistema antioxidante mediante la unión directa de Pb o de subproductos de la peroxidación lipídica al GSH, lo que puede afectar al estado redox celular, y mediante la inhibición de enzimas que previenen la peroxidación de lípidos, como la GPx, SOD, CAT, GR, GST y G6PDH (Mateo y Hoffman, 2001).

En la figura 7 se presenta un esquema con los posibles mecanismos implicados en el estrés oxidativo producido por la exposición a Pb.

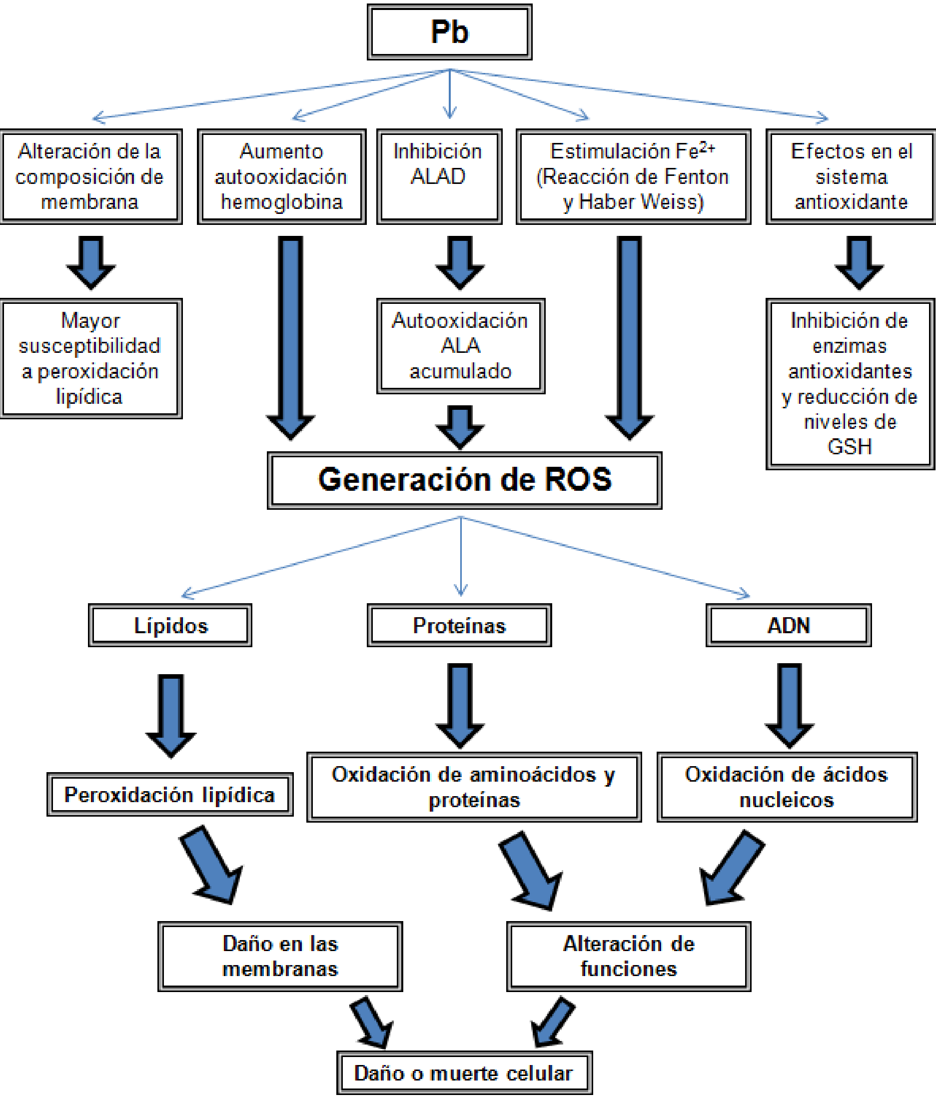

***Figura 7**. Posibles mecanismos de estrés oxidativo inducido por el plomo. Basado en Gurer y Ercal (2000).*

Mercurio

Diferentes estudio *in vivo* e *in vitro* sugieren que la exposición a Hg inorgánico y orgánico induce estrés oxidativo (Valko *et al.*, 2005). Tanto el Hg^{2+} como el metilmercurio pueden unirse covalentemente al GSH y a la cisteína de las proteínas debido a su afinidad por los grupos SH (Figura 8). De hecho, uno de los principales efectos intracelulares del Hg es la inducción y unión a metalotioneínas, debido a que son ricas en cisteína (Valko *et al.*, 2005). De esta forma, los niveles de GSH se ven reducidos en presencia de este metal (Ercal *et al.*, 2001), pudiendo provocar daño oxidativo por la acumulación de ROS que normalmente son eliminadas por el GSH (Sarafian, 1999). Diversos estudios en aves han encontrado correlaciones negativas

entre los niveles de Hg y la concentración de GSH (Henny *et al.*, 2002; Hoffman *et al.*, 1998, 2009).

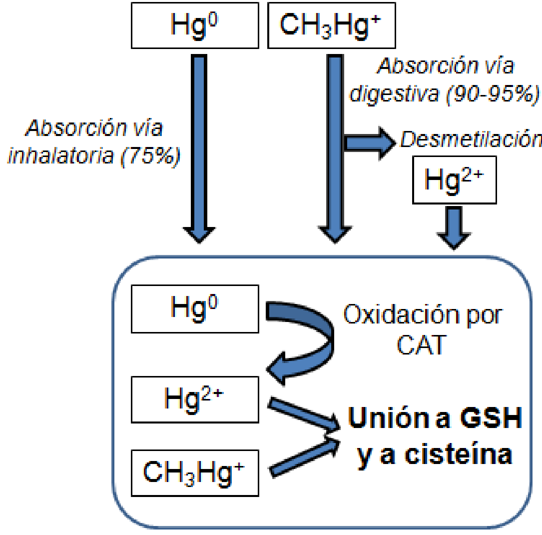

Figura 8. *Absorción de vapor de mercurio elemental y mercurio orgánico. Basado en Quig (1998).*

Otro de los mecanismos implicados en el estrés oxidativo inducido por el Hg es la inhibición de la fosforilación oxidativa mitocondrial. El Hg inorgánico aumenta la producción de H_2O_2 alterando la eficiencia de la fosforilación oxidativa y del transporte de electrones (Lund *et al.*, 1991). Por su parte, el MeHg acelera la tasa de transferencia de electrones en la cadena de transporte de electrones, generando $O_2\cdot^-$ y H_2O_2 (Verity *et al.*, 1975). De esta forma, diferentes estudios han observado que ante la exposición a Hg se produce disminución en los niveles de GSH, un aumento en la formación de H_2O_2 y peroxidación lipídica (Lund *et al.*, 1991, 1993).

Por otro lado, tanto el Hg orgánico como inorgánico alteran la homeostasis del calcio (Ercal *et al.*, 2001). Se cree que el MeHg aumenta el calcio intracelular acelerando el flujo de calcio desde el medio extracelular y movilizando las reservas de calcio intracelulares. En cuanto al Hg^{2+}, aumenta el calcio intracelular tan solo mediante el aumento del flujo desde el medio extracelular (Tan *et al.*, 1993). Los niveles elevados de calcio citoplasmático activan enzimas hidrolíticas como las fosfolipasas[11], aumentando la generación de ácido araquidónico, que influye en la

[11] Enzimas que hidrolizan los enlaces éster presentes en los fosfolípidos. Según el enlace éster que escinden se clasifican en A1, A2, B, C o D.

lipooxigenasa[12] y ciclooxigenasa[13] y resulta en la producción de $O_2\cdot^-$ (Ercal *et al.*, 2001). Por tanto la activación de la fosfolipasa A2 está relacionada con el aumento de ROS (Bazan, 1989; Keyser y Alger, 1990). Otra consecuencia del aumento de calcio es la conversión de la xantina dehidrogenasa a xantina oxidasa, que cataliza reacciones con $O_2\cdot^-$ y H_2O_2 como subproductos (Ercal *et al.*, 2001). También se ha sugerido que el Hg^{2+} desplaza al Fe y el Cu de sus lugares de unión acelarando la formación de ROS a través de la reacción de Fenton (Huang *et al.*, 1996; Sarafian, 1999).

Diversos estudios han observado que el Hg induce la peroxidación lipídica en ratas (Fukino *et al.*, 1984; Huang *et al.*, 1996) y aves expuestas (Henny *et al.*, 2002; Hoffman *et al.*, 2005, 2011). En este sentido, el selenio tiene un efecto protector ya sea por la unión directa al Hg, o como cofactor de la GPx (Huang *et al.*, 1996).

Por último, además de la unión directa del Hg al GSH, este metal es capaz de inhibir la actividad de dos enzimas clave en el metabolismo del GSH, la GSH sintetasa y la GR (Zalups y Lash, 1996), además de otras enzimas antioxidantes como SOD, CAT y GPx (Gstraunthaler *et al.*, 1983). Sin embargo, también se ha demostrado que el Hg es capaz de estimular la actividad de la cobre-zinc SOD, xantina oxidasa, CAT, GR y GPx, y aumentar los niveles de GSH (Ariza *et al.*, 1998; Hussain *et al.*, 1999; Woods y Ellis, 1995). Esta activación del sistema antioxidante se interpreta como un efecto protector de las células ante el estrés oxidativo inducido por el Hg. En este sentido, Hoffman *et al.* (2005) observaron un aumento en la actividad de GST, GR y G6PDH en Garzas blancas expuestas a metilmercurio, lo que interpretan como un mecanismo compensatorio, aunque sugieren que este mecanismo puede depender de la especie, ya que no se ha observado en otras especies como ánades o cormoranes (Henny *et al.*, 2002). También se ha sugerido que los cambios en los niveles de GSH dependen del momento de la exposición y la concentración de Hg (Ji *et al.*, 2006), observándose un aumento en la concentración de GSH cuando se trata de una exposición a bajas dosis de este metal (Henny *et al.*, 2002).

[12] Enzima que interviene en la síntesis de los leucotrienos a partir del ácido araquidónico.

[13] Enzima que interviene en la síntesis de prostaglandinas a partir del ácido araquidónico.

Cadmio

El Cd no es capaz de generar radicales libres de forma directa (Valko *et al.*, 2005). Sin embargo, puede inducir estrés oxidativo mediante la generación indirecta de radicales como el $O_2\cdot^-$ e OH^- (Galán *et al.*, 2001). También ha sido demostrada la generación de H_2O_2 que puede suponer una fuente significativa de radicales a través de la reacción de Fenton (Watanabe *et al.*, 2003). Price y Joshi (1983) propusieron un mecanismo que explica la capacidad del Cd para producir radicales libres indirectamente. El mecanismo consiste en que el Cd puede reemplazar al Cu y Fe en varias proteínas citoplasmáticas y de membrana como la ferritina[14], aumentando los niveles de Cu y Fe libres capaces de participar en el estrés oxidativo mediante la reacción de Fenton (Casalino *et al.*, 1997).

La mayoría de estudios han observado un aumento en los niveles de GSH tras la exposición a Cd (Rana y Verma, 1996; Shaikh *et al.*, 1999), lo que se asocia con un mecanismo de protección (Stohs y Bagchi, 1995). Sin embargo, en algunos casos se ha observado una inhibición (Karmakar *et al.*, 1998), probablemente debida a una producción indirecta de ROS que excede la capacidad de regenerar el GSH (Stohs y Bagchi, 1995). En presencia de Cd a bajas concentraciones se atenúa la eliminación de H_2O_2, lo que sugiere que el Cd participa en la inhibición de los sistemas de eliminación del H_2O_2 (CAT, GSH/GR) (Filipic y Hei, 2004). De esta forma, se ha observado que el Cd es capaz de inhibir la actividad enzimática de GR, CAT y SOD (Hussain *et al.*, 1987; Koizumi y Li, 1992), aunque también se ha descrito la inducción de la actividad de algunas enzimas. En eritrocitos de Búho real y Busardo ratonero (*Buteo buteo*) expuestos *in vitro* a Cd y Pb, se produjo un aumento de la actividad peroxidasa, GST y CAT (Hernández-García, 2010). Ognjanović *et al.* (2003) también observaron un aumento en la actividad de SOD, CAT, GPx, GR y GST, y en los niveles de glutatión reducido, vitamina C, vitamina E y peróxidos lipídicos tras la expoxición a Cd. Su capacidad de producir peroxidación de lípidos (Manca *et al.*, 1991; Yiin *et al.*, 2001) puede deberse a la formación indirecta de radicales libres y a la alteración de los niveles de GSH y metalotioneínas, que permite a los radicales atacar a los lípidos de membrana (Ercal *et al.*, 2001).

Se ha demostrado que la vitamina E reduce la formación de TBARS debida al Cd (Beytut *et al.*, 2003), el selenio tiene un efecto protector sobre la inhibición de la GR

[14] Principal proteína de almacén de hierro.

(Ulusu *et al.*, 2003), y que la deficiencia de Zn aumenta la susceptibilidad a la formación de radicales libres inducidos por este metal (Oteiza *et al.*, 1999).

Cobre

El Cu es esencial a determinadas concentraciones y su deficiencia aumenta la susceptibilidad de las células al daño oxidativo, ya que disminuye la capacidad de las células para producir SOD (Valko *et al.*, 2005). Sin embargo, cuando el Cu se encuentra en exceso respecto a las necesidades celulares, es capaz de mediar la producción de radicales libres y la oxidación directa de lípidos, proteínas y ADN (Valko *et al.*, 2005). Tanto el ion cúprico (Cu (II)) como el ion cuproso (Cu(I)) pueden intervenir en reacciones de oxidación y reducción. El ion cúprico en presencia de agentes reductores como el ácido ascórbico o el GSH, puede reducirse a ion cuproso, capaz de catalizar la formación de radicales hidroxilo mediante la descomposición del peróxido de hidrógeno en la reacción de Fenton (Fórmula 7) (Lloyd *et al.*, 1997).

(7) $Cu(I) + H_2O_2 \rightarrow Cu(II) + \rightarrow OH^{\cdot} + HO^{-}$

Cuando se considera al Cu como un metal que participa en la reacción de Fenton, debe tenerse en cuenta que el Cu libre intracelular se limita a menos de un ion de Cu libre por célula, por lo que la disponibilidad de Cu libre está muy restringida (Rae *et al.*, 1999).

Estudios *in vitro* demuestran que el Cu es capaz de oxidar lipoproteínas de baja densidad (LDL) y de alta densidad (HDL) debido a la formación del radical hidroxilo y la consecuente peroxidación de lípidos que puede producirse por este radical (Burkitt, 2001; Raveh *et al.*, 2000). No solo los iones de Cu libres están envueltos en la oxidación de LDL, sino también las ceruloplasminas, ya que si los radicales las atacan, pueden liberar iones de Cu disponibles para la oxidación de estas lipoproteínas (Mukhopadhyay y Fox, 1998). Por otro lado, se ha confirmado en estudios experimentales que el Cu es capaz de inducir roturas de ADN y oxidación de bases mediante las ROS (Brezova *et al.*, 2003).

Zinc

El Zn es un metal redox inerte, es decir, no participa en la reacciones de oxidación-reducción (Valko *et al.*, 2005). La exposición crónica a Zn en un organismo produce un aumento en la síntesis de metalotioneínas, mientras que un descenso de los niveles de Zn generalmente provoca una mayor susceptibilidad al estrés oxidativo

(Valko *et al.*, 2005). Las deficiencias de Zn se han asociado con mayor daño oxidativo por oxidación de lípidos, proteínas y ADN (Kraus *et al.*, 1997; Oteiza *et al.*, 2000). Aunque muchos cationes inducen el estrés oxidativo, en Zn actúa como un estabilizador de membrana y previene la formación de ROS (Stohs y Bagchi, 1995).

Las funciones del Zn como antioxidante pueden dividirse en dos mecanismos. El primero de ellos es la protección de los grupos sulfhidrilo de las proteínas y enzimas contra el ataque de los radicales libres u oxidación (Valko *et al.*, 2005). Gibbs *et al.* (1985) sugirieron tres posibilidades estructurales que podrían explicar la estabilización de los grupos SH: (i) la unión directa del Zn a estos grupos SH, (ii) la unión del Zn a un punto de unión cerca de los grupos sulfhidrilo, y (iii) la unión del Zn a otro punto de la proteína que resulta en un cambio en su conformación. Cualquiera de estos modelos resultaría en una menor reactividad de estos grupos SH (Valko *et al.*, 2005).

El segundo mecanismo antioxidante del Zn es un efecto antagonista ante metales redox activos como el Fe y el Cu. En el proceso de oxidación de proteínas, las modificaciones oxidativas se producen predominantemente alrededor del lugar de unión del metal redox (Valko *et al.*, 2005). De esta forma, el Zn puede desplazar estos metales de su lugar de unión, siendo estos eliminados de la célula y reduciendo la posibilidad de que el metal redox activo participe en la formación de radicales hidroxilo mediante la reacción de Fenton (Valko *et al.*, 2005).

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OBJETIVOS

El objetivo general de la presente tesis doctoral es la evaluación de la exposición a plaguicidas organoclorados y metales pesados, así como de los efectos subletales inducidos por la exposición a metales en el sistema antioxidante, en diferentes especies de aves silvestres.

De esta forma, la tesis se ha dividido en dos partes. La primera parte se centra en la biomonitorización de contaminantes ambientales persistentes con especial atención en el uso de la pluma como herramienta de biomonitorización. Para ello, en primer lugar se realiza una revisión sobre el uso de la pluma como herramienta de biomonitorización de compuestos polihalogenados (Capítulo I) y se desarrolla una técnica analítica para el análisis de plaguicidas organoclorados en plumas, evaluando también la influencia de la contaminación externa (Capítulo II). Posteriormente, se realiza un estudio de plaguicidas organoclorados en tejidos internos y en plumas de Alca común (*Alca torda*) (Capítulos III y IV) y, finalmente, se estudian los niveles de Hg en tejidos internos y plumas en dos especies de aves, el Alca común como especie marina (Capítulo V) y el Búho real (*Bubo bubo*) como especie terrestre (Capítulo VI). De esta forma, además de ampliar la bibliografía disponible en aves marinas, se pretende evaluar la exposición a Hg y el uso de la pluma para la biomonitorización de este metal en una especie de ave estrictamente terrestre, ya que los trabajos a este respecto en aves no asociadas a ambientes acuáticos o marinos son escasos.

En la segunda parte de la tesis, se evalúan los posibles efectos subletales que la exposición a metales pesados puede provocar en el sistema antioxidante de dos especies de aves silvestres, el Búho real (Capítulo VII) y el Buitre leonado (*Gyps fulvus*) (Capítulo VIII). De esta forma, debido a los pocos estudios disponibles en cuanto a niveles de parámetros del sistema antioxidante y efectos que los metales son capaces de producir en biomarcadores de estrés oxidativo en aves silvestres, se pretende proporcionar información en este sentido en dos nuevas especies de aves terrestres. Además, se pretende proporcionar nuevos datos que ayuden a establecer niveles de no efecto en aves silvestres.

A continuación se describen los objetivos específicos de cada capítulo:

Parte 1.Biomonitorizacizacion de contaminantes ambientales persistentes en aves silvestres: pluma cómo herramienta de monitorización.

- *Capítulo I. Feathers as a biomonitoring tool of polyhalogenated compounds: a review.*

Realizar una revisión crítica sobre el uso de la pluma como herramienta de biomonitorización de compuestos polihalogenados. El objetivo de esta revisión es tanto proporcionar información sobre los niveles de compuestos orgánicos en plumas de diferentes especies, como evaluar los factores que influyen en las concentraciones de estos contaminantes.

- *Capítulo II. Desarrollo de un método analítico para la extracción de plaguicidas organoclorados en plumas.*

Desarrollar un método analítico para la extracción de 16 compuestos organoclorados en plumas y evaluar la interferencia por contaminación externa en los niveles encontrados en plumas así como la distribución de dichos compuestos entre partes de la pluma (barbas y ejes).

- *Capítulo III. Assessment of organochlorine pesticide exposure in a wintering population of Razorbills (Alca torda) from the southwestern Mediterranean.*

Evaluar la exposición a plaguicidas organoclorados y el patrón de distribución de estos compuestos en tejidos internos de una población de Alca común procedente del Mediterráneo, teniendo en cuenta la edad, género y condición corporal de los individuos.

- *Capítulo IV. Razorbill (Alca torda) feathers as an alternative tool for evaluating exposure to organochlorine pesticides.*

Evaluar la utilidad de la pluma como herramienta de biomonitorización de plaguicidas organoclorados en Alca común y estudiar la influencia de factores como la edad y el sexo de las aves en las concentraciones de estos contaminantes en plumas.

- *Capítulo V. Razorbills (Alca torda) as bioindicators of mercury pollution in the southwestern Mediterranean.*

Evaluar la exposición a mercurio en Alca común, así como la influencia de la edad y el sexo de los individuos en las concentraciones observadas, y desarrollar ecuaciones de predicción para estimar las concentraciones de Hg en órganos diana de sus efectos (encéfalo y riñón) usando las concentraciones de Hg en plumas.

- *Capítulo VI. Factors influencing mercury concentrations in nestling Eagle Owls (Bubo bubo).*

Evaluar la exposición a mercurio en pollos de Búho real procedentes del sureste de la península Ibérica y estudiar la influencia de la dieta, el área de estudio y las condiciones climáticas en las concentraciones de este metal.

Parte 2. Inducción de estrés oxidativo en aves silvestres expuestas a metales pesados.

- *Capítulo VII. Effects of heavy metals exposure on oxidative stress biomarkers in Eurasian Eagle owl (Bubo bubo).*

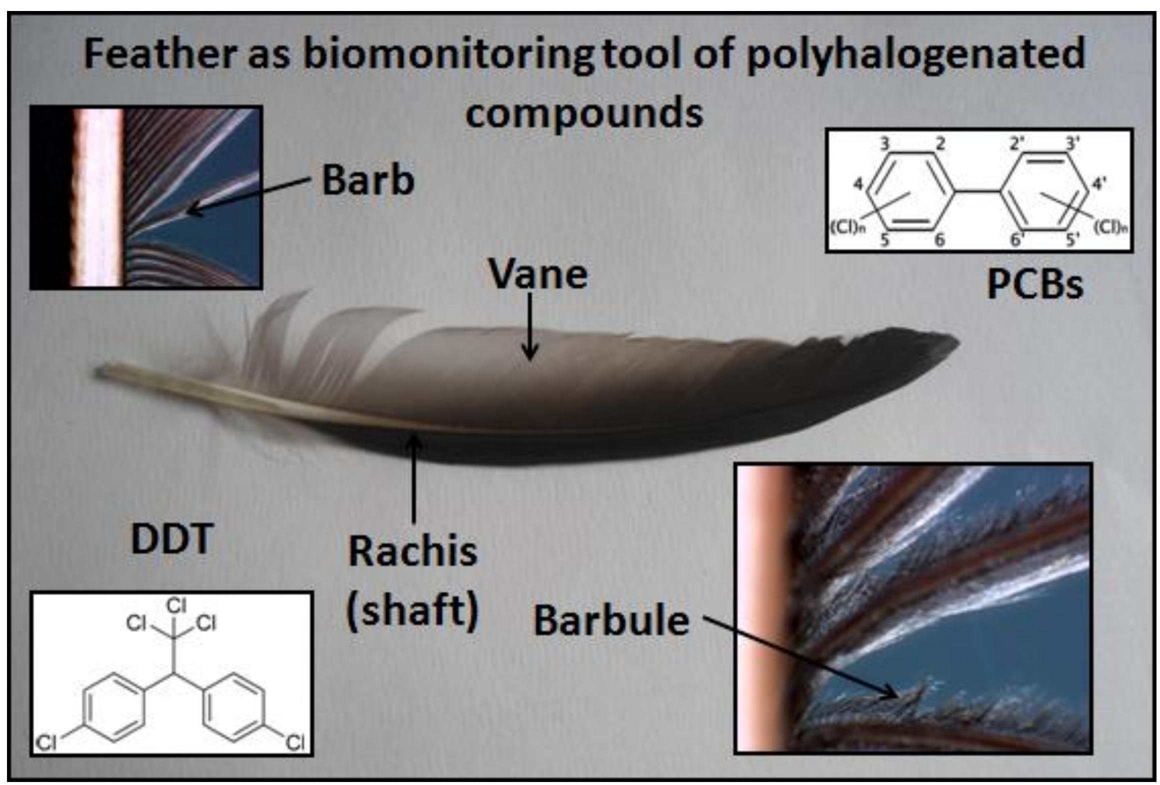
Evaluar la exposición a metales (Pb, Cd, Hg, Cu y Zn) en sangre de Búho real de la Región de Murcia y estudiar los efectos de la exposición a dichos metales en biomarcadores de estrés oxidativo, incluyendo el contenido en glutatión total (tGSH), la actividad de enzimas antioxidantes (GPx, SOD, CAT y GST), y la peroxidación lipídica (TBARS).

- *Capítulo VIII. Effects of heavy metals exposure on oxidative stress biomarkers in Griffon Vulture (Gyps fulvus).*

Evaluar la exposición a metales (Pb, Cd, Hg, Cu y Zn) en sangre de Buitre leonado procedente de dos áreas de la Comunidad Valenciana y estudiar los efectos de la exposición a dichos metales en biomarcadores de estrés oxidativo, incluyendo el contenido en glutatión total (tGSH), la actividad de enzimas antioxidantes (GPx, SOD, CAT y GST), y la peroxidación lipídica (TBARS).

CHAPTER I

Feathers as a biomonitoring tool of polyhalogenated compounds: a review.



Antonio J. García-Fernández, Silvia Espín, Emma Martínez-López. Feathers as a biomonitoring tool of polyhalogenated compounds: a review. Environmental Science and Technology (in press).

Abstract

Feathers have many advantages that make them an excellent nondestructive tool for monitoring polyhalogenated compounds (PHCs). This paper proposes a review on the PHCs in feathers and factors influencing the pollutant load. Special attention has given to external contamination and the main analytical methods used to detect these compounds in feathers. Some authors have found strong and significant correlations between the concentrations of PHCs in feathers and internal tissues, providing positive expectations for their future use in the field of ecotoxicology. However, changes in diet, time elapsed between the previous molt period and sampling, sample size, and/or external contamination have been suggested as possible causes to explain the lack of correlations reported in some studies. Further studies with newly grown feathers and blood samples would be required in order to clarify this issue. Although atmospheric deposition has been reported as cause of external contamination, preening oil seems to be the most relevant factor contributing to this process. Unfortunately, washing techniques tested to date are not able to effectively remove the surface contamination from barbs and shafts, and therefore, it is necessary to develop methods able to discriminate between internal and external contamination. Finally, in this review, deposition rate is proposed as a measurement unit, as this allows comparisons between different parts of the same feather, as well as between different feathers.

1. Introduction

Polyhalogenated compounds (PHCs) are global contaminants which are widely distributed within ecosystems and include organochlorine compounds such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCs); brominated flame retardants such as polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyls (PBBs); and perfluorinated compounds (PFCs) such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). Many of these have been synthesized for industrial use (e.g., PCBs, PBDEs) or as agrochemical products (e.g., dichlorodiphenyltrichloroethane or DDT, lindane, chlordane) (Jones and De Voogt, 1999). Living organisms are exposed to these environmental pollutants worldwide and many are not readily degraded becoming highly persistent as a consequence.

Moreover, PCBs, OCs and PBDEs produce chronic effects on biota and are considered hormone disruptors and immunosuppressive agents, as well as causing adverse effects on the nervous and reproductive systems (Fernie *et al.*, 2005; Fry, 1995; Furness *et al.*, 1993; McArthur *et al.*, 1983). Although most of these compounds have already been banned in developed countries, they are still frequently found in the tissues and fluid samples of several species (Espín *et al.*, 2010b (see Chapter III); Martínez-López *et al.*, 2009; Piqué *et al.*, 2006; van Drooge *et al.*, 2008).

Due to the sensitivity of birds to environmental changes and their position in the food chain, they can accumulate high levels of contaminants and are therefore useful for the biomonitoring of environmental pollution (Furness, 1993). Measuring pollutants directly using internal tissue samples from birds is the best known indicator of the degree of exposure to accumulative compounds (García-Fernández *et al.*, 1997). Yet recently, the need to look for alternative samples to internal tissues has arisen due to practical, ethical and conservationist reasons.

Feathers offer many advantages as a useful research material, and may even be considered the ultimate nondestructive monitoring tool (Bortolotti, 2010). Table 8 shows the strengths and weaknesses of using feathers as a biomonitoring tool for polyhalogenated compounds.

Smith *et al.* (2003) collected data on the different uses given to feathers in a range of scientific fields, emphasizing the importance of the information that this material is able to provide. One of the earliest practical applications of feathers was the study of elemental or mineral profiles, which can be useful for differentiating between bird

populations (Hanson and Jones, 1968). Furthermore, the analyses of stable isotopes can provide information on the feeding ecology, migration behavior, molting strategies, and geographic origins of bird species (Hobson, 2005). Feathers are also a source of DNA for the genetic study of phylogeographical and population structures (Poulakakis *et al.*, 2008), as well as offering a ready source of material for assessing the levels of certain contaminants (Espín *et al.*, 2012 (see Chapter IV); Jaspers *et al.*, 2011). More recently, studies have managed to quantify corticosterone in feathers in order to evaluate how birds cope with stress (Bortolotti *et al.*, 2008, 2009a, 2009b).

The use of feathers in toxicology has an early beginning. However, the vast majority of feather-based studies have been concerned with metal contamination (Burger, 1993; Burgess *et al.*, 2005; Fasola *et al.*, 1998; Hollamby *et al.*, 2004; Martínez-López *et al.*, 2005; Zolfaghari *et al.*, 2009), demonstrating that feathers can be used as an alternative to internal tissues. The latest findings show excellent future prospects for organic-pollutant biomonitoring in avian populations (Espín *et al.*, 2012 (see Chapter IV); Jaspers *et al.*, 2006, 2009).

Table 8. *Strengths and weaknesses of using feathers as a biomonitoring tool for polyhalogenated compounds.*

	Strengths and weaknesses of using feathers as a biomonitoring tool for polyhalogenated compounds	
	Strengths	**Weaknesses**
Sampling and Methodology	Collected in small numbers without causing permanent damage to the bird	Need to control the molting pattern of the species
	Collected regardless of the season, age or gender	Lack of certified reference standards
	Transported and stored at room temperature	Availability of the ideal feather
	Obtained from carcasses or picked up following the molting season	
	Available in natural history museums	
Exposure	Direct relationship with environmental pollution	Difficulties differentiating between internal and external contamination
	Several works available	Concentrations in feathers are only correlated with levels of PHCs in some tissues
	Strong and significant correlations between concentrations of PHCs in feathers and internal tissues	Correlations influenced by the time between when feathers were grown and when internal tissues were collected
	Small fractions of the feather may be of interest, i.e. studying the incidence of acute exposures to certain pollutants	Interpreting feather concentrations is complex due to the molt strategy and the influence of preening activity on feathers, which in turn depends on the age of the feather
	The whole feather is able for divulging information about exposure over a wide period of time	
Effects	Correlations between feathers and target tissues of toxic effects	Little data available on the relationship between PHCs in feathers and their effects
	In the future, less invasive samples valid for estimating adverse effects	

Keratinized tissues have been used to measure persistent organic pollutants in mammals (Altshul *et al.*, 2004; Covaci *et al.*, 2002; D'Havé *et al.*, 2005; Dauberschmidt and Wennig, 1998). These studies suggested that keratin could be used for biomonitoring persistent pollutants and feathers are the main keratin-containing tissue in birds. Feathers are connected to the bloodstream only for a certain period of time during their initial development and molt period (Burger, 1993). Organic pollutants can reach feathers during their growth period via the blood, thus producing internal contamination. When they mature, vascular connections undergo atrophy and compound concentrations remain stable (Burger and Gochfeld, 2000). Therefore, feathers can provide information on concentrations in the blood circulation at the time of their growth. No data are available in the literature on the specific chemical structure and binding capacities of organic compounds to feather tissue. However, once incorporated, contaminants seem to be permanently retained, as it is possible to detect nonpersistent pollutants (e.g., low chlorinated PCBs or α, δ and, γ-hexachlorocyclohexane) in feathers over long periods of time. Accordingly, feathers from museum collections have been used to detect OC and PCB concentrations as indicators of past exposure (Behrooz *et al.*, 2009a).

In the first instance, feathers may be seen as a successful noninvasive tool since feathers lost in the field or in nests can be collected without having to directly handle individual birds. However, factors such as the molt period, collection time, type of feather, external contamination on the feather's surface, as well as age, gender or the nutritional status of birds could interfere with the results and should be considered (Espín *et al.*, 2012 (see Chapter IV); Jaspers *et al.*, 2007b, 2011). Some of these factors are unknown when feathers are found in the field. Therefore, although the use of lost feathers is feasible, greater control of the feather and/or the bird is desirable, thus requiring the handling of individual birds and in turn making the term *nondestructive* more appropriate than that of *noninvasive*.

2. Growth rate of feathers

Pollutants can only reach feathers via the blood during their growth periods (Burger, 1993). It is therefore necessary to understand how they grow. The growth rate of a feather depends on the species and may vary in different parts of an individual (Bortolotti, 2010) (Table 9). Ptilochronology could be useful in this sense. Ptilochronology studies the alternating pale and dark bands on feathers, which are indicative of daily growth (Grubb, 1989). Using this technique, the deposition rate of

pollutants in feathers could be calculated. Table 9 shows feather growth rates for different bird species.

Table 9. *Feather growth rates for different bird species.*

Species	Order[1]	Type of feather[2]	Growth rate[3]	Reference
Acrocephalus arundinaceus	PA	P	3.4	(Rohwer *et al.*, 2009)
Acrocephalus schoenobaenus	PA	P	2.6	(Rohwer *et al.*, 2009)
Agelaius phoeniceus	PA	P	3.8-4.4	(Linz and Linz, 1987)
Anas platyrhychos	A	P	4.5	(Rohwer *et al.*, 2009)
Anser anser	A	P	5.5	(Prevost, 1983)
Anser caerulescens	A	P	7.7-8.0	(Prevost, 1983)
Anser rossii	A	P	7.9	(Prevost, 1983)
Branta bernicla	A	P	3.8-7.8	(Prevost, 1983)
Branta canadensis interior	A	P	6.8-8.7	(Prevost, 1983)
Branta leucopsis	A	P	7.0-7.6	(Prevost, 1983)
Bucephala islandica	A	P	4.1	(van de Wetering and Cooke, 2000)
Bugeranus carunculatus	GR	P	9.0-13.0	(Prevost, 1983)
Buteo buteo	AC	P	4.6	(Ontiveros, D., 1995)
Buteo buteo	AC	S	4.1	(Ontiveros, D., 1995)
Buteo buteo	AC	R	4.0	(Ontiveros, D., 1995)
Carduelis chloris	PA	P	2.1-2.6	(Newton, 1967)
Carduelis chloris	PA	S	2.4	(Newton, 1967)
Carduelis chloris	PA	R	2.0	(Newton, 1967)
Carduelis flammea	PA	P	2.6-3.2	(Prevost, 1983)
Carpodacus mexicanus	PA	P	2.2-3.7	(Prevost, 1983)
Corvus frugilegus	PA	P	2.7-4.9	(Prevost, 1983)
Corvus monedula	PA	P	2.8-4.3	(Prevost, 1983)
Coscoroba coscoroba	A	P	5.0	(Prevost, 1983)
Coturnix coturnix	G	P	4.3-4.7	(Prevost, 1983)
Cygnus cygnus	A	P	9.0	(Prevost, 1983)
Cygnus olor	A	P	5.5-8.3	(Prevost, 1983)
Falco tinnunculus	F	P	3.8-4.6	(Prevost, 1983)
Glyphorynchus spirurus	PA	R	1.9	(Stratford and Stouffer, 2001)
Grus grus	GR	P	9.0	(Prevost, 1983)
Grus japonensis	GR	P	11.0	(Prevost, 1983)
Grus leucogeranus	GR	P	9.0	(Prevost, 1983)
Grus vipio	GR	P	9.0	(Prevost, 1983)
Gymnogyps californianus	AC	P	4.41	(Finkelstein *et al.*, 2010)
Gypaetus barbatus	AC	P	6.6	(Prevost, 1983)
Gyps africanus	AC	P	4.4	(Prevost, 1983)
Halcyon leucocephala	C	P	3.6	(Prevost, 1983)
Hemiprocne mystacea	AP	P	2.9	(Rohwer and Wang, 2010)
Hemiprocne mystacea	AP	R	2.3	(Rohwer and Wang, 2010)

Table 9. *Feather growth rates for different bird species (continued).*

Species	Order[1]	Type of feather[2]	Growth rate[3]	Reference
Larus hyperboreus	CH	P	6.0-10.0	(Prevost, 1983)
Larus marinus	CH	P	7.0-12.0	(Prevost, 1983)
Luscinia luscinia	PA	P	4.2	(Prevost, 1983)
Luscinia svecica	PA	P	3.2	(Rohwer *et al.*, 2009)
Meleagris gallopavo	G	P	7.5	(Prevost, 1983)
Milvus migrans	AC	P	4.6	(Ontiveros, D., 1995)
Milvus migrans	AC	S	4.3	(Ontiveros, D., 1995)
Milvus migrans	AC	R	4.6	(Ontiveros, D., 1995)
Motacilla alba	PA	P	4.5	(Prevost, 1983)
Oceanodroma homochroa	PR	P	1.4-2.0	(Ainley *et al.*, 1976)
Oceanodroma homochroa	PR	R	1.8-2.2	(Ainley *et al.*, 1976)
Oenanthe oenanthe	PA	P	3.0-4.5	(Prevost, 1983)
Pandion haliaetus	AC	P	5.7	(Prevost, 1983)
Pandion haliaetus	AC	S	3.1	(Prevost, 1983)
Pandion haliaetus	AC	R	3.2	(Prevost, 1983)
Passer domesticus	PA	P	2.6-2.7	(Prevost, 1983)
Phasianus colchicus	G	P	5.0-7.2	(Prevost, 1983)
Phylloscopus trochilus	PA	P	3.1	(Rohwer *et al.*, 2009)
Pica pica	PA	P	1.8-3.4	(Prevost, 1983)
Pipra pipra	PA	R	1.6	(Stratford and Stouffer, 2001)
Pyrrhula pyrrhula	PA	P	2.1-2.7	(Newton, 1967)
Pyrrhula pyrrhula	PA	S	2.3-2.5	(Newton, 1967)
Pyrrhula pyrrhula	PA	R	2.3	(Newton, 1967)
Serinus canarius	PA		2	(Wolf *et al.*, 2003)
Sterna hirundo	CH	P	5.1	(Ricklefs, 1979)
Streptopelia roseogrisea	CO	P	5-6	(Riddle, 1908)
Streptopelia roseogrisea	CO	R	5-6	(Riddle, 1908)
Sturnus vulgaris	PA	P	4-4.5	(Dawson, 2003)

[1]A=Anseriformes, AC=Accipitriformes, AP=Apodiformes, F=Falconiformes, C=Coraciiformes, CO=Columbiformes, CH=Charadriiformes, G=Galliformes, GR=Gruiformes, PA=Passeriformes, PR=Procellariiformes. [2]P=Primaries, S=Secondaries, R=Rectrices. [3]mm feather/day.

These alternative bands or growth bars were first described by Riddle (1908) who called them “fundamental bars” (Figure 9A). Growth bars are often difficult to recognize depending on the species, as they are only visible under certain lighting angles and are seen as alternately dark and pale crossbands running over wing and tail feathers, being more rarely visible on other contour feathers (Erritzoe, 2006). The dark bands are formed during the day and the pale ones overnight (Erritzoe, 2006). In diurnal birds the dark band is due to a higher metabolism in the daytime when more melanin is produced (Michener and Michener, 1938; Wood, 1950). Therefore, each pair of bands together constitutes a 24 hour period of feather growth (Michener and Michener, 1938).

The difference in the width of the bands is probably due to variation in the food intake during the period of feather growth (Grubb, 1989, 1991; Møller, 1996). Growth bars are often confused with fault bars (Figure 9B). Riddle (1908) described fault bars as 1 mm wide (or less) translucent cross stripes where a disturbance has taken place during the growth of the feather. Unlike fault bars, it seems that growth bars do not harm the stability of the feather (Erritzoe, 2006).

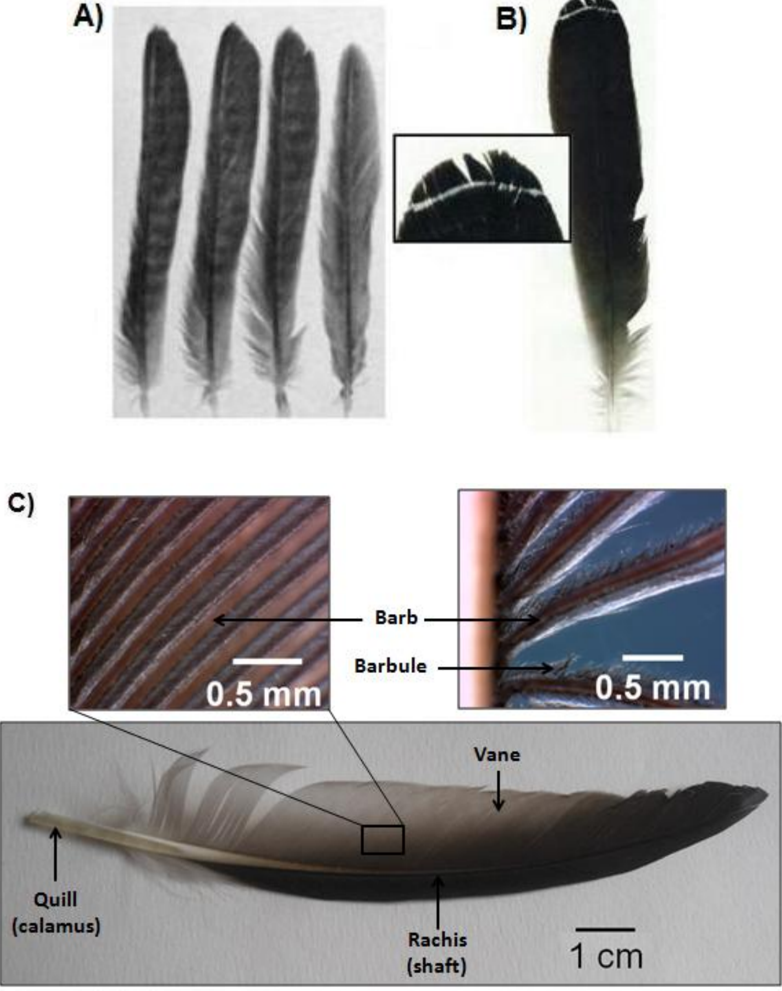


***Figure 9**. A) Tail feathers with clearly visible growth bars. Olive-backed Pipit (Anthus hodgsoni) (Photo: Wolf Dieter Busching). B) A distinctive fault bar from a Jay's (Garrulous glandarius) tail feather (Photo: Wolf Dieter Busching). C) Structure of a primary feather from a Razorbill (Alca torda).*

The growth rate for much of the length of the feather is relatively uniform (Bortolotti, 1984a,b). Strictly speaking, the longitudinal part of the feather that grows on a given day shows the pollutant levels circulating in the blood that day. Therefore, pollutant concentrations detected in a given section of the distal part of a feather may differ with

those detected in a proximal section of the same feather. In some cases, the analysis of a small fraction of the feather may be of interest, that is, when studying the incidence of acute exposures to certain pollutants. However, the whole feather is able to offer information regarding exposure over wider periods of time. Furthermore, it should be noted that not all individuals of a population molt their feathers synchronously (Remisiewicz *et al.*, 2009), so the results in feathers of different individuals are probably not related to exactly the same time period.

3. Deposition rate of pollutants in feathers

Recently, Bortolotti (2010) has shown that different factors could affect the chemical analysis of feathers and that perhaps the expression of results as “contaminant mass per feather mass” is not the most appropriate unit of measure. In this review we highlight two of these factors and propose a different way of expressing the results.

The first factor is the influence of feather mass. Bortolotti (2010) suggests that the variation in mass among feathers affects the interpretation of their contaminant concentrations. A given amount of contaminant deposited in a heavier feather will result in a diluted concentration. In this regard, a range of studies have shown that the vane typically has a higher pollutant concentration than the shaft (Figure 9C details feather structure), consistent with the dilution artifact (Bortolotti, 2010). The second factor is the influence of feather growth rate. Slower growing feathers are likely to have a higher contaminant load than faster growing feathers. In addition to these factors, we must take into account the species studied and their molting process.

Therefore, the key factor could be the use of deposition rate as unit of measurement, which allows comparisons to be made between different parts of the same feather as well as between different feathers. To express the results in terms of deposition rate (load of pollutant that enters a feather daily), we propose the following equation (Eq.1):

Deposition rate (ng contaminant /day) = (Eq.1)
mass contaminant (ng) / pairs of growth bars measured (day)

As explained above, we can use ptilochronology to calculate this, since one pair of growth bars (dark and light) represents a 24-h period of growth. However, in certain bird species the growth bars are not visible, making this calculation impossible. In this case, a second option including growth rate data could be of interest (Eq.2):

Deposition rate (ng contaminant /day) = (Eq.2)
mass contaminant (ng) / feather length (mm) * growth rate (mm feather/ day)

The latter option has two disadvantages. First, one must know the growth rate of the different feathers of each species (see Table 9). This information is unavailable, or scarcely so, for most species and therefore ecological studies are required in order to provide this data. On the other hand, it should be noted that not all individuals in a given species have the same feather growth rate, and it is possible to find different growth rates associated with different nutritional resources (Wolf *et al.*, 2003). Taking all this into account, we suggest the use of equation 1 whenever possible, as complementary data for future studies.

The results described in previous studies could be transformed to the proposed unit, as it would be interesting to provide results taking into account the mass of contaminant per the mass of the feather, as well as per the growth rate of the feather, since it expresses the mean daily input of pollutants in feathers. In this sense, in one of our previously published papers (Espín *et al.*, 2012 (see Chapter IV)) in which organochlorine pesticides were analyzed in the feathers of Razorbill (*Alca torda*), the primary feather growth rate was presumed to be 3.3 mm/day. Since we could not visualize the growth bars, we calculated the growth rate using the time a whole feather takes to grow (six weeks according to Wickett (1999)) and the length of the feather sampled. In order to transform these units we multiplied the results (in pollutant mass per feather mass) by the proportional weight of whole feather used (in our case 0.2 g), thus obtaining the contaminant mass, to which the aforementioned equation was later applied. Therefore, a mean ∑Organochlorine pesticide concentration of 870.48 ng/g found in primary feathers by Espín *et al.* (2012) should be equivalent to 5.3 ng organochlorine pesticides/day when expressed in deposition rate units.

4. *Feathers and organic compounds*

Several PHCs have been analyzed in feathers, including PCBs, OC, PBDEs and PFCs in both terrestrial and aquatic species. Furthermore, studies which have analyzed these contaminants in feathers have covered species from very few locations such as Belgium, southwest Iran, and southeast Spain (Table 10). The scientific literature available on this subject is still scarce and quite recent. Table 10 presents a historical review, in chronological order, which shows the species studied, the aim of the study, as well as sampling area and year. Table 11 shows PHC concentrations according to species.

Table 10. *Review of the historical use of feathers as samples for PHC analysis.*

Historical use of feathers as a tool for polyhalogenated compound analysis			
Species	**Aim**	**Sampling area and year**	**Reference**
Pheasants (*Phasianus colchicus*)	Accumulation and effects	U.S. (1971)	(Hall *et al.*, 1971)
Double-crested Cormorant (*Phalacrocorax auritus*), Mallard Duck (*Anas platyrhynchos*), White Leghorn (*Gallo gallo*)	Distribution pattern	1974	(Greichus and Greichus, 1974)
Double-crested Cormorant (*Phalacrocorax auritus*), White Pelicans (*Pelecanus erythrorhynchos*)	Effects in parasite fauna of birds.	U.S. (1974)	(Greichus *et al.*, 1974)
Japanese Quail (*Coturnix coturnix japonica*)	Experimental kinetic study	U.S. (1974)	(Ivie *et al.*, 1974)
White Pelican (*Pelecanus erythrorhynchos*)	Distribution and effects	U.S. (1972)	(Greichus *et al.*, 1975)
Poultry	Report on levels in feathers	Slovenia (1992)	(Zupancic-Kralj *et al.*, 1992)
Emperor Penguin (*Aptenodytes forsteri*)	Report on levels in feathers	Antarctica (1987-1988)	(Sen Gupta *et al.*, 1996)
Long-billed Mongolian Plover (*Charadrius mongolus*; sub sp. *C. atrifrons*), Short-billed Mongolian Plover (*Charadrius mongolus*; sub sp. *C. schaeferi*)	Accumulation and potential hazards	India (1995)	(Tanabe *et al.*, 1998)
Burrowing Owls (*Athene cunicularia hypugea*)	Report on levels in feathers	U.S.	(Gervais *et al.*, 2000)
Great Tit (*Parus major*)	Report on levels. Correlations with internal tissues	Belgium (2000, 2002)	(Dauwe *et al.*, 2005)
Common Buzzards (*Buteo buteo*)	Report on levels. Correlations with internal tissues	Belgium (2003-2004)	(Jaspers *et al.*, 2006)
European Starlings (*Sturnus vulgaris*)	Usefulness as a non-destructive biomonitor	2007	(Van Den Steen *et al.*, 2007)
3 aquatic species and 5 terrestrial species	Report on levels. Correlations with internal tissues	Belgium (2003-2004)	(Jaspers *et al.*, 2007b)
Common Buzzards (*Buteo buteo*)	External contamination	Belgium (2003-2004)	(Jaspers *et al.*, 2007a)
Common Magpie (*Pica pica*)	External contamination	Belgium (2006)	(Jaspers *et al.*, 2008)
Grey Heron (*Ardea cinerea*), Herring Gull (*Larus argentatus*), Eurasian Sparrowhawk (*Accipiter nisus*), Eurasian Magpie (*Pica pica*), Eurasian Collared Dove (*Streptopelia decaocta*)	Report on levels	Belgium	(Meyer *et al.*, 2009)
18 species from Accipiteridae, Falconidae, Strigidae, Phasianidae, Columbidae, Upupidae, Corvidae, Coraciidae, Otididae and Cuculidae families	Report on levels in feathers	Iran (1991-1996)	(Behrooz *et al.*, 2009a)
15 species from Alcedinidae, Ardeidae, Rallidae, Laridae, Plecanidae and Anserinae families	Report on levels in feathers	Iran (1994-1996)	(Behrooz *et al.*, 2009b)
Common Magpies (*Pica pica*)	Report on levels in feathers	Urban area and Rural area from Belgium (2006)	(Jaspers *et al.*, 2009)
Clapper Rails (*Rallus longirostris*)	Report on levels in feathers	U.S. (1999-2000, 2006-2007)	(Summers *et al.*, 2010)
Mallard Duck (*Anas platyrhynchos*)	Analytical method of extraction	Spain (2009)	(Espín *et al.*, 2010a) Chapter II
Northern Goshawks (*Accipiter gentilis*), White-tailed Eagles (*Haliaeetus albicilla*), Golden Eagles (*Aquila chrysaetos*)	Report on levels. Correlations with internal tissues	Norway (2008)	(Eulaers *et al.*, 2011a)
Cattle Egret (*Bubulcus ibis*) and Little Egret (*Egretta garzetta*)	Report on levels and potential effects	Pakistan (2009)	(Malik *et al.*, 2011)
Razorbill (*Alca torda*)	Report on levels and potential effects	Spain (2007)	(Espín *et al.*, 2012) Chapter IV
White-tailed Eagles (*Haliaeetus albicilla*)	Influencing factors on levels	Greenland (1997-2009)	(Jaspers *et al.*, 2011)
10 species from Phalacrocoracidae, Podicipedidae, Laridae, and Anatidae families	Report on levels. Correlations with internal tissues	Iran (2008)	(Rajaei *et al.*, 2011)
White-tailed Eagles (*Haliaeetus albicilla*)	Report on levels. Correlations with internal tissues	Norway (2009)	(Eulaers *et al.*, 2011b)
Barn Owl (*Tyto alba*)	Correlations with internal tissues. External contamination	Belgium (2008-2009)	(Jaspers *et al.*, 2013)

Early studies used feathers to determine the concentration of PHCs in several bird species treated with certain doses of these pollutants (Greichus and Greichus, 1974;

Greichus *et al.*, 1975; Hall *et al.*, 1971; Ivie *et al.*, 1974). In 2005, Dauwe *et al.* (2005) conducted a pilot study on the Great Tit (*Parus major*) in order to evaluate the usefulness of feathers as a biomonitoring tool for organic pollutants by analyzing fat tissue in order to check whether feathers reflected levels in internal tissues. Jaspers *et al.* (2006) conducted the first study on levels of organic compounds in the feathers of a predatory bird, the Common Buzzard (*Buteo buteo*). Following this, Van Den Steen *et al.* (2007) performed an experimental evaluation of the usefulness of feathers as a nondestructive biomonitor of PCBs in European Starlings (*Sturnus vulgaris*). Later still, several studies have evaluated the influence of external contamination on organic contaminant levels found in the feathers of several bird species (Behrooz *et al.*, 2009b; Rajaei *et al.*, 2011; Summers *et al.*, 2010; Van Den Steen *et al.*, 2007) as well as the correlations between levels in feathers and internal tissues (Espín *et al.*, 2010b, 2012 (see Chapters III and IV); Jaspers *et al.*, 2007b, 2011; Rajaei *et al.*, 2011). Some studies have even reported on the concentrations of organic pollutants in feathers from museum collections (Behrooz *et al.*, 2009a,b).

Table 11. *Levels of polyhalogenated compounds in feathers of different species as per literature.*

Polyhalogenated compounds in feathers of different species			
Species	**n**	**Mean/median concentrations (ng/g)**	**Reference**
Emperor Penguin (*Aptenodytes forsteri*)	3	∑HCH = 0.103-0.112, ∑DDT = 0.031-0.036, ∑PCB = 0.106-0.114	(Sen Gupta *et al.*, 1996)
Long-billed Mongolian Plover (*Charadrius mongolus*; sub sp. *C. atrifrons*)	1	∑HCH = 4.4, ∑DDT = 34, PCBs = <20, ∑CHL = <0.2, HCB = 0.3	(Tanabe *et al.*, 1998)
Short-billed Mongolian Plover (*Charadrius mongolus*; sub sp. *C. schaeferi*)	1	∑HCH = 17, ∑DDT = 26, PCBs = <20, ∑CHL = <0.2, HCB = 0.3	
Great Tit (*Parus major*)	9-30	∑DDT = 6.13, ∑PCB = 49.7, ∑PBDE, PBB, HCB, OxC and TN = ND	(Dauwe *et al.*, 2005)
Common Buzzard (*Buteo buteo*)	43	∑DDT = 4.9, ∑PCB = 26, ∑PBDE = 1.4	(Jaspers *et al.*, 2006)
Grey Heron (*Ardea cinerea*)	9	∑HCH = 3.5, ∑DDT = 21, ∑CHL = 0.32, HCB = 5.0, ∑PCB = 91, ∑PBDE = 7.7	(Jaspers *et al.*, 2007b)
Common Moorhen (*Gallinula chloropus*)	11	∑HCH = 2.4, ∑DDT = 12, ∑CHL = ND, HCB = ND, ∑PCB = 25, ∑PBDE = ND	
Herring Gull (*Larus argentatus*)	12	∑HCH = 1.1, ∑DDT = 12, ∑CHL = 0.46, HCB = ND, ∑PCB = 220, ∑PBDE = 11	
Barn Owl (*Tyto alba*)	9	∑HCH = 5.3, ∑DDT = 48, ∑CHL = 2.5, HCB = 1.1, ∑PCB = 140, ∑PBDE = 3.8	
Long-eared Owl (*Asio otus*)	10	∑HCH = 2.5, ∑DDT = 110, ∑CHL = 0.63, HCB = 4.9, ∑PCB = 120, ∑PBDE = 6.3	
Common Buzzard (*Buteo buteo*)	43	∑HCH = 1.9, ∑DDT = 9.4, ∑CHL = 2.6, HCB = 0.70, ∑PCB = 40, ∑PBDE = 2.2	
Sparrowhawk (*Accipiter nisus*)	11	∑HCH = 4.6, ∑DDT = 230, ∑CHL = 1.5, HCB = 1.0, ∑PCB = 160, ∑PBDE = 10	
Kestrel (*Falco tinnunculus*)	3	∑HCH = 1.2, ∑DDT = 110, ∑CHL = 2.1, HCB = 2.9, ∑PCB = 57, ∑PBDE = 4.4	
Common Buzzards (*Buteo buteo*)	15-16	Outer vane: HCB = 0.87, ∑DDT = 8.18, ∑PCB = 41.3, PBDEs = 2.79 Inner vane: HCB = 1.48, ∑DDT = 12.5, ∑PCB = 60.7, PBDEs = 3.01 Outer shaft: HCB = 0.44, ∑DDT = 2.71, ∑PCB = 15.5, PBDEs = 0.47 Inner shaft: HCB = 1.1, ∑DDT = 5.23, ∑PCB = 37.5, PBDEs = 0.75	(Jaspers *et al.*, 2007a)
Common Magpie (*Pica pica*)	30	∑DDT = 3.07-27.2, ∑PCB = 4.61-140, HCB = bellow LOQ, ∑PBDE = 0.28-0.41	(Jaspers *et al.*, 2008)

Table 11. *Levels of polyhalogenated compounds in feathers of different species as per literature (continued).*

Polyhalogenated compounds in feathers of different species			
Species	**n**	**Mean/median concentrations (ng/g)**	**Reference**
Grey Heron (*Ardea cinerea*)	10	PFOS = 247, PFOA = ND, PFNA = ND	(Meyer *et al.*, 2009)
Eurasian Sparrowhawk (*Accipiter nisus*)	10	PFOS = 102, PFOA = ND, PFNA = ND	
Herring Gull (*Larus argentatus*)	10	PFOS = 79, PFOA = ND, PFNA = ND	
Eurasian Collared Dove (*Streptopelia decaocta*)	10	PFOS = 48, PFOA = ND, PFNA = ND	
Eurasian Magpie (*Pica pica*)	10	PFOS = 31, PFOA = ND, PFNA = ND	
Greater Spotted Eagle (Aq*uila clanga*)	3	∑HCH = 21, ∑DDT = 26, HCB = 5, ∑PCB = 17	(Behrooz *et al.*, 2009a)
Sparrowhawk (*Accipiter nisus*)	1	∑HCH = 33, ∑DDT = 81, HCB = < LOQ, ∑PCB = 55	
Kestrel (*Falco tinnunculus*)	3	∑HCH = 48, ∑DDT = 20, HCB = 48, ∑PCB = 41	
Merlin (*Falco columbarius*)	1	∑HCH = 82, ∑DDT = 24, HCB = < LOQ, ∑PCB = 80	
Hobby (*Falco subbuteo*)	2	∑HCH = 23, ∑DDT = 11, HCB = 12, ∑PCB = < LOQ	
Peregrine Falcon (*Falco peregrinus*)	1	∑HCH = 60, ∑DDT = 13, HCB = 10, ∑PCB = 42	
Little Owl (*Athene noctua*)	3	∑HCH = 46, ∑DDT = 73, HCB = 32, ∑PCB = 21	
European Scops Owl (*Otus scops*)	1	∑HCH = 212, ∑DDT = 295, HCB = < LOQ, ∑PCB = 182	
Short-eared Owl (*Asio flammeus*)	1	∑HCH = 28, ∑DDT = 62, HCB = 24, ∑PCB = 42	
Chukar (*Alectoris chukar*)	2	∑HCH = 18, ∑DDT = 3, HCB = 29, ∑PCB = < LOQ	
Black Francolin (*Francolinus francolinus*)	3	∑HCH = 22, ∑DDT = 2, HCB = 5, ∑PCB = 6	
Collared Dove (*Streptopelia decaocto*)	3	∑HCH = 65, ∑DDT = 15, HCB = 22, ∑PCB = 8	
Hoopoe (*Upupa epops*)	3	∑HCH = 18, ∑DDT = 15, HCB = 6, ∑PCB = 6	
Raven (*Corvus corax*)	3	∑HCH = 16, ∑DDT = 4, HCB = 2, ∑PCB = 10	
Magpie (*Pica pica*)	1	∑HCH = 76, ∑DDT = 11, HCB = 19, ∑PCB = 25	
European Roller (*Coracias garrulus*)	4	∑HCH = 18, ∑DDT = 12, HCB = 12, ∑PCB = 10	
Houbara Bustard (*Chlamydotis undulata*)	1	∑HCH = 32, ∑DDT = 15, HCB = 8, ∑PCB = 22	
Common Cuckoo (*Cuculus canorus*)	1	∑HCH = 18, ∑DDT = 14, HCB = 46, ∑PCB = 18	
Pied Kingfisher (*Ceryle rudis*)	2	∑HCH = 25, ∑DDT = 96, HCB = 42, ∑PCB = 108	(Behrooz *et al.*, 2009b)
White-breasted Kingfisher (*Halcyon smyrnensis*)	1	∑HCH = 40, ∑DDT = 19, HCB = 30, ∑PCB = 67	
Night Heron (*Nycticorax nycticorax*)	3	∑HCH = 46, ∑DDT = 24, HCB = 7, ∑PCB = 72	
Little Bittern (*Ixobrychus mintus*)	3	∑HCH = 49, ∑DDT = 37, HCB = 48, ∑PCB = 29	
Coot (*Fulica atra*)	3	∑HCH = 26, ∑DDT = 5, HCB = 22, ∑PCB = 10	
Cattle Egret (*Bubulcus ibibs*)	1	∑HCH = 24, ∑DDT = 9, HCB = 46, ∑PCB = 11	
Bittern (*Botaurus stellaris*)	1	∑HCH = 39, ∑DDT = 7, HCB = 10, ∑PCB = 53	
Purple Gallinule (*Prophyrio porphyrio*)	3	∑HCH = 32, ∑DDT = 22, HCB = 16, ∑PCB = 25	
Slender-billed Gull (*Larus genei*)	4	∑HCH = 55, ∑DDT = 11, HCB = 8, ∑PCB = 32	
White Pelican (*Pelecanus onocrotalus*)	1	∑HCH = 32, ∑DDT = 15, HCB = 10, ∑PCB = 34	
Teal (*Anas crecca*)	4	∑HCH = 15, ∑DDT = 20, HCB = 3, ∑PCB = 28	
Mallard Duck (*Anas platyhychos*)	2	∑HCH = 12, ∑DDT = 10, HCB = 7, ∑PCB = 27	
Wigeon (*Anas penelope*)	3	∑HCH = 31, ∑DDT = 11, HCB = 10, ∑PCB = 17	
Shoveler (*Anas clypeata*)	2	∑HCH = 50, ∑DDT = 39, HCB = 20, ∑PCB = 60	
Ferruginous Duck (*Aythya nyroca*)	2	∑HCH = 5, ∑DDT = 85, HCB = 15, ∑PCB = 72	

Table 11. *Levels of polyhalogenated compounds in feathers of different species as per literature (continued).*

Polyhalogenated compounds in feathers of different species			
Species	**n**	**Mean/median concentrations (ng/g)**	**Reference**
Common Magpie (*Pica pica*)	UA=12	∑DDT = 3.07, ∑PCB = 140, HCB = bellow LOQ, ∑PBDE = 0.41	(Jaspers *et al.*, 2009)
	RA=13	∑DDT = 34.2, ∑PCB = 4.25, HCB = bellow LOQ, ∑PBDE = 0.27	
Mallard Duck (*Anas platyrhynchos*)	10	Non-washed barbs: ∑HCH = 86.43, ∑Heptachlor = 23.37, ∑DDT = 66.49, ∑Drins = 1325.9, ∑Endosulfan = 196.56 Washed barbs: ∑HCH = 28.95, ∑Heptachlor = 11.23, ∑DDT = 52.96, ∑Drins = 326.22, ∑Endosulfan = 197 Non-washed shaft: ∑HCH = 37.08, ∑Heptachlor = 19.02, ∑DDT = 66.67, ∑Drins = 633.2, ∑Endosulfan = 79.66 Washed shaft: ∑HCH = 11.55, ∑Heptachlor = 8.24, ∑DDT = 27.3, ∑Drins = 177.14, ∑Endosulfan = 52.92 ng/g	(Espín *et al.*, 2010a) Chapter II
Goshawk (*Accipiter gentilis*)	18	β-HCH = 0.30, DDE = 43.9, HCB = 0.90, ∑PCB = 39.7, ∑PBDE = 3.54	(Eulaers *et al.*, 2011a)
Sea Eagle (*Haliaeetus albicilla*)	5	β-HCH = 0.19, DDE = 8.30, HCB = 0.60, ∑PCB = 34.8, ∑PBDE = 2.21	
Golden Eagle (*Aquila chrysaetos*)	15	β-HCH = 0.28, DDE = 33.3, HCB = 0.54, ∑PCB = 32.3, ∑PBDE = 1.18	
Cattle Egret (*Bubulcus ibis*)	-	∑PBDE = 0.34-0.86	(Malik *et al.*, 2011)
Little Egret (*Egretta garzetta*)	-	∑PBDE = 1.11-1.30	
Razorbill (*Alca torda*)	50	Total: ∑HCH = 174.99, ∑DDT = 323.56, ∑Heptachlor = 196.27, ∑Drins = 61.14, ∑Endosulfan = 114.52 Young birds (n=23): ∑HCH = 68.79, ∑Heptachlor = 209.46, ∑DDT = 155.07, ∑Drins = 72.73, ∑Endosulfan = 83.76 Old birds (n=27): ∑HCH = 265.45, ∑Heptachlor = 185.04, ∑DDT = 467.09, ∑Drins = 51.27, ∑Endosulfan = 140.71 ng/g	(Espín *et al.*, 2012) Chapter IV
Mallard Duck (*Anas platyhychos*)	3	p,p'-DDT= 1.4, o,p'-DDT= 7, p,p'-DDD= 1.3, p,p'-DDE= 14.3, o,p'-DDE= 0.2, α-HCH= ND, β-HCH= 15, γ-HCH= 6, HCB= 0.4, PCB28= 29.7,PCB52=48, PCB101=<LOQ, PCB118=1.2, PCB153=2, PCB138=4, PCB180=ND	(Rajaei *et al.*, 2011)
Common Teal (*Anas crecca*)	4	p,p'-DDT= 1, o,p'-DDT= 40.5, p,p'-DDD= 3.2, p,p'-DDE= 50.5, o,p'-DDE= 2, α-HCH= 1, β-HCH= 25, γ-HCH= 21.5, HCB= 6, PCB28= 26,PCB52=9, PCB101=3, PCB118=2, PCB153=3, PCB138=6.5, PCB180=3	
Pintail (*Anas acuta*)	3	p,p'-DDT= 0.5, o,p'-DDT= 40, p,p'-DDD= 2.4, p,p'-DDE= 17, o,p'-DDE= 0.04, α-HCH= 1, β-HCH= 5, γ-HCH= 12, HCB= 2, PCB28= 9.6,PCB52=19, PCB101=0.7, PCB118=1, PCB153=2, PCB138=2, PCB180=ND	
Common Gull (*Larus canus)*	3	p,p'-DDT= 1.8, o,p'-DDT= 7.9, p,p'-DDD= 0.7, p,p'-DDE= 28, o,p'-DDE= 0.4, α-HCH= 6, β-HCH= 21, γ-HCH= 7.5, HCB= 4.5, PCB28= 8,PCB52=12, PCB101=0.2, PCB118=1.4, PCB153=2, PCB138=,2.5 PCB180=36	
Little Gull (*Larus minutus*)	5	p,p'-DDT= 5.8, o,p'-DDT= 50.2, p,p'-DDD= 12.3, p,p'-DDE= 96, o,p'-DDE= 12, α-HCH= <LOQ, β-HCH= 17, γ-HCH= 4, HCB= 1, PCB28= 59,PCB52=41, PCB101=0.7, PCB118=11.8, PCB153=14.2, PCB138=25, PCB180=27	
Black-headed Gull (*Larus ridibundus*)	8	p,p'-DDT= 6.7, o,p'-DDT= 10.5, p,p'-DDD= 2, p,p'-DDE= 99, o,p'-DDE= 0.5, α-HCH= 4.70, β-HCH= 9, γ-HCH= 11, HCB= 1.6, PCB28= 48,PCB52=40, PCB101=0.9, PCB118=5, PCB153=4, PCB138=7, PCB180=18	
Little Grebe (*Tachybaptus ruficollis*)	3	p,p'-DDT= 15, o,p'-DDT= 1, p,p'-DDD= 5, p,p'-DDE= 13, o,p'-DDE= <LOQ, α-HCH= <LOQ, β-HCH= 2.5, γ-HCH= 4.5, HCB= 39, PCB28= 8,PCB52=16, PCB101=<LOQ, PCB118=1, PCB153=<LOQ, PCB138=2.5, PCB180=<LOQ	
Black-necked Grebe (*Podiceps nigricollis*)	3	p,p'-DDT= 1.3, o,p'-DDT= 10.3, p,p'-DDD= 3.4, p,p'-DDE= 69, o,p'-DDE= 0.3, α-HCH= 8.4, β-HCH= 9, γ-HCH= 26, HCB= 4, PCB28= 47,PCB52=26, PCB101=1, PCB118=2.7, PCB153=4, PCB138=7, PCB180=6	
Great crested Grebe (*Podiceps cristatus*)	6	p,p'-DDT= 1, o,p'-DDT= 1, p,p'-DDD= 1, p,p'-DDE= 89, o,p'-DDE= 0.2, α-HCH= <LOQ, β-HCH= 4, γ-HCH=7, HCB= 2, PCB28=17,PCB52=13, PCB101=1.3, PCB118=3.3, PCB153=1.6, PCB138=5	
Great Cormorant (*Phalacrocorax carbo*)	8	p,p'-DDT= 2.3, o,p'-DDT= 16, p,p'-DDD= 4, p,p'-DDE= 21, o,p'-DDE= 0.5, α-HCH= 1, β-HCH= 13, γ-HCH= 7, HCB= 2, PCB28= 22,PCB52=17, PCB101=0.5, PCB118=3.7, PCB153=7, PCB138=2, PCB180=24	

Table 11. *Levels of polyhalogenated compounds in feathers of different species as per literature (continued).*

Polyhalogenated compounds in feathers of different species			
Species	**n**	**Mean/median concentrations (ng/g)**	**Reference**
White-tailed Eagles (*Haliaeetus albicilla*)	15	Tail feather (left): ∑PCB=34, HCB=2.2, p,p'-DDE=27, ∑Chls=8.6, ∑HCH=1.7, ∑PBDE=0.69 Tail feather (right): ∑PCB=55, HCB=1.5, p,p'-DDE=34, ∑Chls=9.4, ∑HCH=2.6, ∑PBDE= 0.71 Body feather: ∑PCB=420, HCB=3.2, p,p'-DDE=250, ∑Chls=73, ∑HCH=12, ∑PBDE=6.3 Primary feather (2nd): ∑PCB=28, HCB=1.7, p,p'-DDE=19, ∑Chls=6.3, ∑HCH=2.8, ∑PBDE=0.34 Primary feather (5th): ∑PCB=20, HCB=1.5, p,p'-DDE=14, ∑Chls=3.5, ∑HCH=1.1, ∑PBDE= 0.47 Primary feather (8th): ∑PCB=20, HCB=1.1, p,p'-DDE=12, ∑Chls=3.0, ∑HCH=1.1, ∑PBDE=0.23	(Jaspers *et al.*, 2011)
White-tailed Eagles (*Haliaeetus albicilla*)	14	∑PCB = 16.9, HCB = 0.37, DDE = 6.59, ∑CHL = 0.85, ∑PBDE = 3.37	(Eulaers *et al.*, 2011b)
Barn Owl (*Tyto alba*)	13	PFOS=15.8, PFOA=37.1, PFHxS=<1.9	(Jaspers *et al.*, 2013)

n = number of samples, UA = Urban Area, RA = Rural area, ND = Not detected, LOQ = Limit of quantification, Ref=Reference.
CHL =chlordanes, DDT = dichlorodiphenyltrichloroethane, DDE = dichlorodiphenyldichloroethylene, DDD = dichlorodiphenyldichloroethane, HCB =hexachlorobenzene, HCH = hexachlorocyclohexane, OxC =oxychlordane, PCBs = polychlorinated biphenyls, PBDE = polybrominated diphenyl ethers, PBB = polybrominated biphenyls, PFOS = perfluorooctane sulfonate, PFOA = perfluorooctanoic acid, PFNA = perfluorononanoic acid, PFHxS = perfluorohexane sulfonate, TN =trans-nonachlor.

4.1. Body distribution pattern and correlations between concentrations of polyhalogenated compounds in feathers and internal tissues of birds

Regarding the distribution pattern, the highest concentrations of these lipophilic contaminants are expected to be found in adipose tissue, followed by liver and muscle. Fat tissue has a higher percentage of triglycerides than the rest of the body and these compounds have a higher affinity for this type of lipid (Cockcroft *et al.*, 1989). In addition, lipidic content is higher in liver than in feathers. Accordingly, Dauwe *et al.* (2005) and Espín *et al.* (2010b, 2012) (see Chapters III and IV) observed higher levels of persistent organic pollutants in fat tissue than in feathers of the Great Tit and Razorbill, and several studies have also shown higher concentrations of these contaminants in liver or muscle than in the feathers of several bird species (Espín *et al.*, 2010b, 2012 (see Chapters III and IV); Jaspers *et al.*, 2006, 2011; Rajaei *et al.*, 2011; Summers *et al.*, 2010).

However, less persistent PHCs are more easily metabolized (Juan *et al.*, 2002), and they may be found at low concentrations in fat or liver and in higher levels in the bloodstream for a limited time (Dauwe *et al.*, 2005), e.g. due to lipid mobilization. Therefore, these compounds could enter feathers during their growth period. In this sense, several studies have found that feathers had proportionally higher levels of less

chlorinated PCB congeners (tri-, tetra-, and pentachlorinated biphenyls) than did fat, liver or muscle (Dauwe *et al.*, 2005; Jaspers *et al.*, 2007b; Rajaei *et al.*, 2011). Espín *et al.* (2010b, 2012) (see Chapters III and IV) also found higher levels of ΣHCH in feathers than in the liver of Razorbills, which is consistent with the fact that more polar compounds may be more easily deposited during feather formation. External contamination could be another explanation of the higher levels of lower-chlorinated PCB congeners in feathers, which will be explained in detail in the following section (4.2.External contamination).

As to the relationship between concentrations of PHCs in feathers and internal tissues, Table 12 presents the correlations between levels of these compounds in feathers and those of internal tissues in a range of published papers. Strong and significant correlations have been found by several authors (Eulaers *et al.*, 2011a; Jaspers *et al.*, 2007a, 2011; Rajaei *et al.*, 2011). Jaspers *et al.* (2007a,b) found higher correlation coefficients for feather-muscle than for feather-liver in several species, probably due to the higher turnover rate in liver compared to muscle. Later, Eulaers *et al.* (2011a) described strong correlations between plasma and feather concentrations in Northern Goshawk (*Accipiter gentilis*), White-tailed Eagle (*Haliaeetus albicilla*) and Golden Eagle (*Aquila chrysaetos*) nestlings, which was expected considering the feathers used in this study were still developing when they were collected and the blood sample was taken at the same time.

Table 12. *Correlations between polyhalogenated compound levels in feathers and internal tissues.*

Species	Tissue[1]	Observations and n	Pearson's correlation coefficients[2]	Reference
Great Tit (*Parus major*)	F	Winter (16)	*PCBs, DDE, DDD, DDT* = NS	(Dauwe *et al.*, 2005)
	F	Breeding season (9)	*PCBs = 0.92*, DDE = 0.78**, DDD = 0.78**, DDT* = NS	
Common Buzzard (*Buteo buteo*)	M	43	PCBs = 0.76*, PBDEs = 0.73*, DDT+DDE = 0.53*	(Jaspers *et al.*, 2006)
	L	43	PCBs = 0.60*, PBDEs = 0.43*, DDT+DDE = 0.35**	
Grey Heron (*Ardea cinerea*), Common Moorhen (*Gallinula chloropus*), Herring Gull (*Larus argentatus*), Barn Owl (*Tyto alba*), Long-eared Owl (*Asio otus*), Common Buzzard (*Buteo buteo*), Eurasian Sparrowhawk (*Accipiter nisus*), Kestrel (*Falco tinnunculus*)	(Meta-analysis) M	Aquatic (32)	PCBs = 0.62*, BDEs = 0.47**, DDE = 0.48*	(Jaspers *et al.*, 2007b)
		Terrestrial (73)	PCBs = 0.71*, BDEs = 0.70*, DDE = 0.59*	
	(Meta-analysis) L	Aquatic (9)	PCBs, BDEs, DDE = NS	
		Terrestrial (73)	PCBs = 0.57*, BDEs = 0.47*, DDE = 0.40*	
Common Buzzards (*Buteo buteo*)	M	15	PCBs = 0.90*, PBDEs = 0.78*, DDE = 0.73*	(Jaspers *et al.*, 2007a)
	L	15	PCBs = 0.57**, PBDEs, DDE = NS	
Grey Heron (*Ardea cinerea*), Herring Gull (*Larus argentatus*), Eurasian Sparrowhawk (*Accipiter nisus*), Eurasian Magpie (*Pica pica*), Eurasian Collared Dove (*Streptopelia decaocta*)	L	No significant correlation in any of the species individually (50)	PFOS = 0.62* (pooled of five species)	(Meyer *et al.*, 2009)

Table 12. *Correlations between polyhalogenated compound levels in feathers and internal tissues (continued).*

Species	Tissue[1]	Observations and n	Pearson's correlation coefficients[2]	Reference
Goshawk (*Accipiter gentilis*)	P	18	PCBs = 0.86**, PBDEs = 0.87** , DDE = 0.86**, HCB = NS	(Eulaers *et al.*, 2011a)
Sea Eagle (*Haliaeetus albicilla*)	P	5	PCBs = 0.89**, PBDEs = 0.94**, DDE, HCB = NS	
Golden Eagle (*Aquila chrysaetos*)	P	15	PCBs = 0.93*, PBDEs = 0.95*, DDE, HCB = NS	
Razorbill (*Alca torda*)	ScF	49	*∑HCH = 0.46*, ∑Heptachlor, ∑Drins, ∑Endosulfan, ∑DDT = NS*	(Espín *et al.*, 2010b, 2012) Chapter III and IV
	AbF	48	*∑HCH, ∑Heptachlor, ∑Drins, ∑Endosulfan = NS, ∑DDT = 0.36***	
	L	50	*∑HCH, ∑Heptachlor, ∑Drins, ∑Endosulfan, ∑DDT = NS*	
	B	50	*∑HCH = -0.35**, ∑Heptachlor, ∑Drins, ∑Endosulfan = NS, ∑DDT = -0.4**	
White-tailed Eagles (*Haliaeetus albicilla*)	M	15	Tail feather:∑PCB=0.81-0.86, HCB=0.63-0.70, p,p'-DDE=0.82-0.88, Chls=0.74-0.86, HCH=NS-0.73, BDE=0.67-0.83(all**)	(Jaspers *et al.*, 2011)
			Body feather: ∑PCB=0.81, HCB=NS, p,p'-DDE=0.84, Chls=0.68-0.70, HCH=NS-0.67, BDE=0.67-0.76(all**)	
			Primary feather: ∑PCB=0.84-0.85, HCB=0.63-0.70, p,p'-DDE=0.86-0.89, Chls=0.78-0.85, HCH=NS -0.73, BDE=0.70-0.84(all**)	
	PO	13	Tail feather:∑PCB=0.84-0.86, HCB=0.68-0.76, p,p'-DDE=0.82-0.85, Chls=0.67-0.83, HCH=NS-0.67, BDE=0.58-0.77(all**)	
			Body feather: ∑PCB=0.82, HCB=NS, p,p'-DDE=0.79, Chls=0.70-0.71, HCH=NS-0.68, BDE=0.63-0.75(all**)	
			Primary feather: ∑PCB=0.86-0.87, HCB=0.68-0.83, p,p'-DDE=0.83-0.85, Chls=0.70-0.87, HCH=NS-0.79, BDE=0.67-0.84(all**)	
Gulls and Great Cormorant	L and M	-	*Organochlorine pesticides and PCBs=0.6-0.9**	(Rajaei *et al.*, 2011)
White-tailed Eagles (*Haliaeetus albicilla*)	P	14	∑PCB = 0.66**, HCB, DDE, ∑CHL, ∑PBDE = NS	(Eulaers *et al.*, 2011b)
	PO	14	∑PCB = 0.75*, HCB = 0.73*, DDE = 0.79*, β-HCH, ∑CHL, ∑PBDE = NS	
Barn Owl (*Tyto alba*)	L	12	*PFOS = 0.78*, PFOA=NS*	(Jaspers *et al.*, 2013)
	M, F, PO	13, 7, 4	*PFOS = NS*	

**p<0.01, **p<0.05. n = number of samples. [1]Tissue type: F= Fat, ScF= Subcutaneous fat, AbF= Abdominal fat, M= Muscle, L= Liver, B= Brain, PO= Preen Oil, P =Plasma. [2]In italics Spearman's correlations. NS = no significant correlation. CHL =chlordanes, DDT = dichlorodiphenyltrichloroethane, DDE = dichlorodiphenyldichloroethylene, DDD = dichlorodiphenyldichloroethane, HCH = hexachlorocyclohexane, HCB =hexachlorobenzene, PCBs = polychlorinated biphenyls, PBDE = polybrominated diphenyl ethers, PFOS = perfluorooctane sulfonate, PFOA = perfluorooctanoic acid.*

Van Den Steen *et al.* (2007) provided the first experimental evidence that feathers could be a useful tool for nondestructive organic compound biomonitoring. In this study, European Starlings were exposed to three different concentrations of PCB 153 over 15 weeks via silastic implants. They found significant positive correlations between concentrations in newly grown feathers and muscle (r = 0.76, p = 0.02), liver (r = 0.79, p = 0.01), brain (r = 0.72, p = 0.04), and blood (r = 0.73, p = 0.0013). However, other authors have found low significant correlation coefficients between pollutant

concentrations in feathers and those of internal tissues in several bird species (Table 12) (Dauwe *et al.*, 2005; Espín *et al.*, (2010b, 2012) (see Chapters III and IV); Jaspers *et al.*, 2006, 2007b). These ambiguous findings could be influenced by several factors such as changes in diet, time elapsed between the previous molt period and sampling, sample size or external contamination. Jaspers *et al.* (2007b) suggested that in some cases sample size is an important factor for obtaining significant correlations. They also established (more) significant correlations by increasing the sample size via meta-analysis, combining several aquatic and terrestrial bird species. However, on using a larger sample size (fifty individuals) according to data reported in Razorbills by Espín *et al.* (2010b, 2012) (see Chapters III and IV), few and low significant correlation coefficients were obtained. These findings suggest that the time elapsed between molting and sampling could be one of the greater limiting factors of feathers as a biomonitoring tool, since there was a significant time lapse between the time of molt and the time of death and sampling in the aforementioned work by Espín *et al.* (2010b, 2012) (see Chapters III and IV).

4.2. External contamination

The correlation between organic pollutant contamination in feathers and internal tissues can be misleading as a result of external contamination by organic pollutants on the surface of feathers (Jaspers *et al.*, 2008). This external contamination can be caused by both exogenous (such as atmospheric deposition), and endogenous sources (preening of the feathers with oil from the preen gland). Although this data may be indicative of the environmental quality of the habitat in which the bird resides, it may give a misleading indication of actual food chain uptake, ingestion and transfer of pollutants to the animal (Cardiel *et al.*, 2011). External contamination of feathers by heavy metals has been widely investigated (Cardiel *et al.*, 2011) and its influence on the concentrations in feathers has been demonstrated. Nevertheless, the chemical properties of heavy metals and organic pollutants differ considerably (Jaspers *et al.*, 2008) such that external contamination by organic compounds can affect the levels found in feathers in different ways. According to Vorhees *et al.* (1997), the atmospheric deposition of organic pollutants may be important in modifying contaminant feather profiles, and it is expected that more volatile compounds could contribute most of all to this type of external pollution. In addition, Greichus and Greichus (1974) noted that endogenous sources should be taken into account in the case of external contamination of feathers by preen gland oil in Double-crested Cormorant (*Phalacrocorax auritus*), Mallard Duck (*Anas platyrhynchos*) and White Leghorn (*Gallo*

gallo), since organic pollutants are lipophilic chemicals which accumulate in high concentrations in oily secretions (Van Den Brink, 1997; Yamashita *et al.*, 2007). Several studies have found that preen gland oil is relatively richer in lower-chlorinated PCB congeners (di-, tri-, and tetra-CB) compared to fatty tissue (Larsson and Lindegren, 1987; Yamashita *et al.*, 2007). In this sense, and as cited above, several studies have found that feathers also have proportionately higher levels of less-chlorinated PCB congeners than internal tissues in different bird species (Dauwe *et al.*, 2005; Jaspers *et al.*, 2007b; Rajaei *et al.*, 2011). The preening of feathers could explain the similar congener composition in preen gland oil to that of feathers (Yamashita *et al.*, 2007). Moreover, it is possible that this uropygial oil acts as an adhesive for external contamination originating from the air (Jaspers *et al.*, 2007b), and lower chlorinated PCB congeners are assumed to contribute more to external contamination from atmospheric deposition (Vorhees *et al.*, 1997). Taking this into account, it is possible that birds ingest both endogenous and exogenous pollutants while preening.

Nonetheless, some authors have suggested that the external deposition of these organic compounds does not occur in feathers, or only minimally so. Dauwe *et al.* (2005) observed that PCB and DDT concentrations in feathers did not differ greatly between young and old feathers of the Great Tit. In this regard, Jaspers *et al.* (2007a) concluded that external contamination seemed of little importance in Common Buzzards for a number of reasons. They predicted that, as the vane covers most of the feather's surface (Figure 9C), the influence of external contamination would be greater for the vane, thus leading to weaker correlations with internal tissues when compared to the shaft. However, no significant differences were found for prominent PCB congeners between the vane and the shaft, and the resultant correlations with internal tissues were not consistent. Moreover, these authors expected to find higher concentrations for the outer primaries, as these feathers are the most exposed to external contamination via the air. However, no differences in PBDE and PCB profiles were found between inner and outer primary feathers.

Despite all this, some studies have found that external contamination does occur in the feathers of several species such as White-tailed Eagle, Clapper Rail (*Rallus longirostris*), Common Magpie (*Pica pica*) and Mallard Duck (Espín *et al.*, 2010a (see Chapter II); Jaspers *et al.*, 2008, 2011; Summers *et al.*, 2010). Unfortunately, such surface contamination may be difficult to be removed effectively using the laboratory washing techniques tested to date. Espín *et al.* (2010a) (see Chapter II) compared levels between washed (with tap water, distilled water and Milli-Q water) and

nonwashed feathers of the Mallard Duck, as well as between vane and shaft. Organochlorine levels were significantly higher in unwashed samples than in washed ones for some compounds, in both vane and shaft, suggesting a possible interference by external contamination from atmospheric deposition or oil secreted by the preen gland. Moreover, higher levels of certain compounds in unwashed barbs than in unwashed shafts can be explained by the higher probability of organic pollutant deposition in barbs than in shafts. Barbs have a large and structurally complex surface area subject to exposure (Cardiel *et al.*, 2011). In contrast, the rachis of a feather has a smooth structure and can easily and effectively be cleaned (Cardiel *et al.*, 2011). According to Cardiel *et al.* (2011) based on a range of studies showing higher concentrations of organic pollutants for vanes than for shafts (Espín *et al.*, 2010a (see Chapter II); Jaspers *et al.*, 2007a), the shaft can be more confidently used, and the potentially misleading data caused by external contamination may be avoided. However, for developing or very recently molted feathers, the external surface contamination would be expected to be minimal and thus barbs may remain valuable (Cardiel *et al.*, 2011).

Jaspers *et al.* (2008) used different washing procedures to compare their validity for removing external contamination from Common Magpie feathers. Their results revealed a significant effect on the concentrations measured in feathers when washing with acetone and surfactant solution, compared to the control feathers. Moreover, Jaspers *et al.* (2008) observed that concentrations in the washes were found highest for acetone and lowest for water. Therefore, they concluded that water is not suitable for removing preen oil secretions from feathers, while airborne particles and dust can be easily washed away with water. Nonetheless, the possibility that washing with acetone or surfactant solution resulted in the leaching of some internal concentrations cannot be dismissed. Results from Jaspers *et al.* (2008) indicate that preen oil is probably the main source of external contamination and that airborne contamination is probably of minor importance for organic compounds. Later, Jaspers *et al.* (2011) found in White-tailed Eagles strong and significant correlations between PHCs concentrations in feathers washed with water and concentrations in muscle or preen oil, similar to the correlations found for feathers washed with acetone. Then they suggested that the external contamination removed both by water and acetone washing was linked to dirt and dust particles, and other agents may be required to remove preen oil from the feathers. It is difficult to estimate the potential daily deposition from preening since preening frequency depends on several factors such as

species, season, environmental conditions and gender (Caldwell *et al.*, 2001; Greichus and Greichus, 1974; Iersel and Bol, 1958; Pap *et al.*, 2010). Therefore, additional information would be required in order to distinguish between internal and external contamination. Accordingly, the use of external contamination markers, such as certain metals with a low intestinal absorption rate and therefore, low endogenous deposition in feathers, could be useful toward this aim. In this sense, aluminum or titanium have been shown to be useful when interpreting Pb levels in faecal excreta and feathers (Beyer *et al.*, 1997; Cardiel *et al.*, 2011), and also could be useful for organic pollutants. Further research must be carried out on this matter in order to determine an organic external contamination marker.

When using feathers as a biomonitoring tool, it is important to provide information about which feathers could preferably be used and the variations resulting from different molting patterns, as the latter is closely related with the external contamination. Interpreting feather concentrations is complex, mainly due to the molt strategy. The influence of preening activity on feathers also depends on the age of the feather, being higher in older feathers (Jaspers *et al.*, 2011). Therefore, since it is supposed that contamination from preening oil cannot be completely removed via washing processes as explained above, the age of the feather should be taken into account when measuring PHCs. Jaspers *et al.* (2011) found higher levels of organic pollutants in the older primary feathers of White-tailed Eagles, and also found better statistical correlations with internal tissues when using older primary feathers. They suggest that this may also be explained by the higher concentration of preen oil in such feathers due to age. In order to avoid the influence of the asynchronous molting of flight feathers, Jaspers *et al.* (2011) proposed the use of body feathers, which are replaced every year in White-tailed Eagles. However, this type of feather is smaller and lighter and thus it would be necessary to collect larger amounts. Moreover, further studies are required on the influence of external contamination in these feathers. The suggestion of these authors would depend on the species, since, for instance, Razorbills have a complete molt involving all contour and flight feathers, whereby primary and secondary feathers are replaced synchronously or nearly so, thus resulting in a period of flightlessness (Espín *et al.*, 2012) (see Chapter IV). Therefore, in this species all flight feathers could be used for monitoring purposes since they have approximately the same age.

In view of the low number of papers published so far (Jaspers *et al.*, 2011), all feathers could possibly be used, but the ideal feather depends on the molting pattern of

the species and the end point of the monitoring study. Clearly, further experimental studies are needed in order to determine correct methods for discriminating between the internal and external contamination of feathers by organic pollutants and the suitability of each type of feather.

4.3. Intraspecific differences

Factors such as body condition, gender and age could influence contaminant concentrations in feathers (Espín *et al.*, 2012 (see Chapter IV); Jaspers *et al.*, 2007b). Body condition could be a determining factor in PHC levels. The mobilization of organochlorines from depleting fat stores and the consequent increase of concentrations of these compounds in the liver and other well irrigated organs have been reported for birds that had low lipid concentrations (Kenntner *et al.*, 2003; Malcolm *et al.*, 2003; Wienburg and Shore, 2004). Since the feather root is vascularized during its development, contaminants in the bloodstream may enter the feather via the root (Dauwe *et al.*, 2005).

Dauwe *et al.* (2005) observed that compound concentrations in feathers and fat tissue of the Great Tit collected in the winter were not correlated or were even significantly negatively correlated. However, samples collected in the breeding season were significantly positively correlated, except for p,p'-DDT, suggesting a better relationship between levels in feather and fat during the breeding season than in the winter. This could be caused by confounding effects such as changes in lipid reserves, diet or egg laying, as well as differences in the extent and timing of the molt. The likelihood of egg laying as a confounding factor affecting correlations between contaminant concentrations in feathers and internal organs may, in part, depend on whether birds are capital or income breeders. Income breeders fuel reproductive expenditure by simultaneous feeding, whereas capital breeders fuel reproduction from energy gained earlier, and stored prior to use (Bonnet *et al.*, 1998). Presumably, it is likely to be less of an issue for income breeders. Jaspers *et al.* (2006, 2007b) observed that when starved birds were excluded from the statistical study, correlations between levels in feathers and internal tissues were higher. These results indicate the importance of monitoring the condition of the birds.

The importance of fat mobilization for migration, egg laying or other factors on the relative levels of feather and internal tissue contamination partly depends on the time when samples are collected. This is the offset in time between when feathers were grown and typically when the bird died (and internal tissues were collected). If egg

laying, migration, starvation periods etc. occurred after the molt but prior to death, then this is likely to reduce any correlation between contaminant concentrations in feathers and internal tissues. There is a need to collect further quantitative data on the influence of this timing offset in correlations between feather and other tissue contaminant levels.

Espín *et al.* (2012) (see Chapter IV) and Jaspers *et al.* (2011) observed significant differences in feather concentrations for certain compounds between young and old birds in Razorbills and White-tailed Eagles, with higher residue concentrations in old birds. Studies have found a positive relationship between age and organochlorine compounds in breast muscle, fat, brain and liver samples (Borgå *et al.*, 2001; Donaldson *et al.*, 1997; Espín *et al.*, 2010b (see Chapter III); María-Mojica *et al.*, 2000; Vorkamp *et al.*, 2004). Higher levels in the tissues of old birds may reflect a longer period of exposure in these individuals (Espín *et al.*, 2010b) (see Chapter III).

In regard to gender, several studies have concluded that adult females have lower organochlorine levels in blood and internal tissues than males, probably due to the transference of these compounds from mother to egg (Bustnes *et al.*, 2008; Moss *et al.*, 2009). However, Dauwe *et al.* (2005) and Espín *et al.* (2012) (see Chapter IV) found no significant differences for polychlorinated biphenyls and organochlorine pesticide levels between genders in feathers of the Great Tit and Razorbills. This could be due to the fact that all Razorbills analyzed were collected after the breeding season. Jaspers *et al.* (2011) found significant differences in concentrations between sexes in White-tailed Eagles, with females showing higher concentrations than males for juvenile birds. In view of the potential influence of off-loading contaminants into eggs and the resultant potential decrease in internal tissue contaminant concentrations, feathers should preferably be monitored from male birds so as to eliminate this bias, especially in the breeding season. However, depending on the end point of the research this recommendation may not be appropriate, i.e. studies on the influence of egg laying or studies on differences in exposure according to sex.

4.4. Interspecific and spatial differences

Species habits such as diet or migratory behavior should be reflected in the pollutant concentrations found in feathers. In this sense, several authors have analyzed PHCs in feathers of species with different dietary habits. Behrooz *et al.* (2009a) investigated the concentrations of organic contaminants in 37 birds divided into four groups according to their dietary habits. In their research, raptors (carnivores) showed

the highest levels of DDTs and PCBs due to the biomagnification process, and herbivores showed the lowest levels (Table 11). In this regard, Malik *et al.* (2011) observed higher PBDE concentrations in feathers from the piscivorous Little Egret (*Egretta garzetta*) compared with the terrestrial insectivore Cattle Egret (*Bubulcus ibis*), suggesting that fish consumption is the primary exposure pathway for PBDEs in the aquatic food web.

Rajaei *et al.* (2011) observed that organic pollutant concentrations varied widely for birds occupying different trophic levels. Concentrations of organochlorine compounds decreased in the following order: Laridae > Podicipedidae > Anatidae > Phalacrocoracidae families. The Black Headed Gull (*Larus ridibundus*), which forages on agricultural lands and near drainage channels, had high levels of organochlorine pesticides. The scavenging habit of gulls may be the cause of high exposure to xenobiotics. On the other hand, Anatidae feeding on lower trophic levels had lower levels of organic pollutants. The lower organic pollutant levels in the feathers of Phalacrocoracidae (fish eating birds) than in Anatidae (usually herbivorous or granivorous birds) was an unexpected result. However, the Phalacrocoracidae family may have a poor uropygial secretion since these birds can frequently be seen with their open wings to dry their feathers after fishing. In this sense, if we assume that gland mass is a valid parameter for quantifying the degree of gland development, Montalti and Salibián (2000) determined gland mass relative to body weight in 49 bird families and found a higher mean relative gland weight for Podicipedidae than for Anatidae and higher for Anatidae than for Phalacrocoracidae. Montalti and Salibián (2000) found the largest mean relative gland weight in Sternidae, Podicipedidae and Procellariidae, and the smallest glands proportional to body weight were found in Ardeidae and Columbidae. According to the unexpected results found by Rajaei *et al.* (2011), preening oil may be an important origin of the organic pollutants present in bird feathers.

Jaspers *et al.* (2007b) observed higher levels of organic contaminants in Sparrowhawks (*Accipiter nisus*) compared to other terrestrial predatory birds (Table 11). They also explain this result as being due to dietary habit. The diet of Sparrowhawks consists almost entirely of small birds (up to 98%) which feed on seeds treated with seed dressings, so they are susceptible to accumulating pesticides. The other terrestrial birds studied feed mainly on small mammals and to a lesser extent on small birds (Snow and Perrins, 1998). Moreover, Sparrowhawks have also been found to be poor metabolizers of xenobiotics compared with other birds of prey and this may

also account for their high contaminant burdens (Crosse *et al.*, 2012). On the other hand, Common Moorhens (*Gallinula chloropus*) feed on varying proportions of animal and plant material (Snow and Perrins, 1998) and yet had a lower intake of organic pollutants thus showing lower levels of accumulation compared to predatory birds. The varying accumulation profiles of Herring Gulls (*Larus argentatus*) could possibly be explained by their highly opportunistic feeding habits, consuming not only fish but a variety of items, including garbage (Snow and Perrins, 1998). The diverse diet of Herring Gulls could be the cause of the lack of significant correlations between concentrations in feathers and muscle tissue (Jaspers *et al.*, 2007b). However, Jaspers *et al.* (2007b) found high and significant correlations in Common Moorhen which feed on a more specific diet (Snow and Perrins, 1998).

Migratory habits must also be considered when evaluating the exposure to and distribution of contaminants in the body, this being closely linked with the diet within the migratory range. For instance, Razorbills overwinter in the western Mediterranean Sea, a closed sea surrounded by highly industrialized countries where the use of organochlorine insecticides has been more intense than in their breeding area (Espín *et al.*, 2010b) (see Chapter III). As a result this may be the cause of the higher organochlorine pesticide concentrations found in Razorbill feathers and tissues (Espín *et al.*, 2010b, 2012) (see Chapters III and IV) than those of other Alcids collected in other areas (Baffin Bay and Barents Sea) (Borgå *et al.*, 2001; Buckman *et al.*, 2004). In this sense, Rajaei *et al.* (2011) also found that gulls from the coast of the Caspian Sea had high organochlorine pesticide levels, probably due to their spending summer in western Siberia where high levels of DDTs have been reported.

Regarding spatial differences, Jaspers *et al.* (2009) studied the usefulness of feathers for monitoring regional variations in contamination. They compared the concentrations of organic pollutants in feathers of the Common Magpie between urban and rural areas of Flanders, Belgium. The results showed that concentrations of dichlorodiphenyldichloroethylene (p,p'-DDE) were significantly higher in rural areas, while levels of PCBs were higher in urban areas, confirming their expectations, since DDT is a pesticide that used to be applied in the countryside, while PCBs were mainly produced and used by industry mostly located close to cities (e.g. Antwerp). Feathers appear to reflect regional variations in the concentration of these compounds, which reinforces their utility as a nondestructive biomonitor for organic contaminants (Jaspers *et al.*, 2009).

4.5. Analytical methods for organohalogenated pollutants in feathers

Several methods have been used for the analysis of organohalogenated pollutants in feathers. In 2005, Dauwe *et al.* (2005) adapted a technique from the method described by Covaci and Schepens (2001) for the determination of organic pollutants in human hair. This is the analytical method used in most studies published after 2005. A new methodology was developed in 2010 by Espín *et al.* (2010a) (see Chapter II) in order to provide a specific technique for a series of organochlorine pesticides that have been widely used in the past. This new technique was adapted from the method described by Martínez-López *et al.* (2009) for blood samples. Malik *et al.* (2011) described a technique for 26 PBDE congeners in 2011, and Meyer *et al.* (2009) and Jaspers *et al.* (2013) established an analytical method for PFCs.

Prior to analysis, feathers are typically washed with distilled water, dried at room temperature, and cut into small pieces of approximately 1 mm (Behrooz *et al.*, 2009a,b; Jaspers *et al.*, 2006, 2007a,b, 2009, 2011). Although some studies have investigated the possibility of removing external contamination using different washing agents (water, acetone, surfactant solution) as explained previously, contamination by preening oil cannot likely be washed away thoroughly. Therefore, further studies are needed to determine reliable methods for discriminating between the internal and external contamination of feathers by organic pollutants.

In the sample preparation procedure described by Dauwe *et al.* (2005) feathers (200 mg) are accurately weighed and incubated overnight at 40ºC in 4 ml of HCl (4 M) and 3 ml of hexane:dichloromethane (4:1, v/v). Extraction of analytes from the incubation medium is performed by a liquid–liquid procedure with 2 x 4 ml of hexane:dichloromethane (4:1, v/v). The combined fractions of organic solvents are purified with a 250-mg cartridge filled with 500 mg of acidified silica and topped off with 250 mg of anhydrous Na_2SO_4. The cartridge is prewashed with 2 ml of hexane:dichloromethane (1:1, v/v) and 2 ml of hexane. The cartridge is eluted with 4 ml of hexane:dichloromethane (1:1, v/v). The final eluate is concentrated to approximately 50 µl under a gentle nitrogen stream. Internal standards are PCB 46 and PCB 143 (100 pg/µl), ε-hexachlorocyclohexane (100 pg/µl), PBB 103 (20 pg/µl), and PBB 155 (5 pg/µl).

In the analytical method for extracting organochlorine pesticides developed by Espín *et al.* (2010a) (see Chapter II), feathers (200 mg) are weighed and incubated overnight at 37ºC in 4 ml of HCl (37%) and 15 ml of hexane:acetone (2:1, v/v).

Extraction is performed with 20 ml of hexane:acetone (3:1, v/v). The samples are homogenized, centrifuged and filtered using anhydrous sodium sulfate and the collected solvent is subsequently evaporated until dryness. After redisolution in 5 ml hexane, samples are cleaned up via Florisil column chromatography (SepPak, Waters), activated with 2 ml of hexane, using a petroleum ether:diethyl ether mix (21:4, v:v) as the elution solvent. The solvent collected is evaporated until dryness. The final volume is adjusted to 1 ml with n-hexane. Methoxychlor (1 mg/ml) is added as an internal standard in order to compare the results and check for repeatability in chromatograms.

Regarding the technique described by Malik *et al.* (2011), feathers (200-500 mg) are Soxhlet extracted for 16 h using dichloromethane. Extracts are evaporated and the solvent phase is changed to hexane to 1 ml. Chromatography glass columns (25 mm i.d.) are filled with 15 mm of acidified silica and 1 g of anhydrous sodium sulfate, washed with 60 ml of hexane, loaded with extracts, eluted with 250 ml of hexane and rotary evaporated to 0.5 ml. The eluted extracts are passed to gel permeation chromatography columns packed with 6 g Biobeads SX-3 and eluted with hexane and dichloromethane (1:1 v:v). The first 16 ml are discarded, and a fraction between 16 and 35 ml is collected and concentrated under a gentle stream of N_2 to 25 µl. Twenty-five µl of dodecane containing ^{13}C-labeled PCB congeners 141 and 208, and two PBDE congeners BDE-69 and -181 were used as internal standards.

In regards to the analytical method for PFCs, Meyer *et al.* (2009) digest the feathers with 10 ml HNO3 (69%), and 20 µl of each internal standard (^{13}C-PFOA/PFOS) are added and left for 48 h at room temperature. The pH of the mixture is increased by adding 40 ml of NaOH. The samples are filtrated under vacuum conditions through a glass fiber filter (142 mm) in order to remove any particles. Extraction is performed by solid phase extraction using Oasis HLB Plus SPE cartridges. Briefly, the whole system is rinsed with 20 ml acetonitrile. The samples are loaded and then extracted at a constant flow rate of approximately 2 drops/s. The SPE cartridge is then rinsed with 2 ml of acetonitrile/water (40/60) mixture and eluted with 4 ml acetonitrile. The eluted extract is concentrated to 1 ml at room temperature, and transferred to a microvial containing approximately 25 mg of activated carbon and 50 µl of glacial acetic acid. The sample is then mixed for 1 min. using a vortex mixer. Following centrifugation, 500 µl of the supernatant is transferred to a clean microvial.

In the methodology for perfluoroalkyl substances (PFASs) analysis in feathers described by Jaspers *et al.* (2013), the analytical method for PFASs in biological

matrices is modified (Powley *et al.*, 2005) by including a digestion step with a KOH/MeOH mixture to resolve bound PFASs from the feathers. Feathers are washed twice prior to analysis using distilled water and hexane. In this technique, 200-300 mg of homogenized feather sample is spiked with internal standard (^{13}C-PFOA/PFOS), and 2 ml 200 mM KOH in methanol is added. After 1 h, 10 ml methanol is added and samples are extracted three times in ultrasonic bath-vortex for 10 min. Samples are left to soak overnight. Then, 200 µl 2 M HCl in methanol is added and ultrasonic bath-vortex cycle is repeated. Following centrifugation, supernatant is concentrated to 1.5 ml and cleaned up with ENVI-carb and glacial acetic acid.

The techniques described above were developed for the analysis of a range of compounds. The method adapted by Dauwe *et al.* (2005) is for the analysis of hexachlorobenzene, oxychlordane, trans-nonachlor, DDTs, PBBs, PBDEs, and PCBs; and the recovery of compounds ranging between 72 and 80% (Dauwe *et al.*, 2005). However, the technique developed by Espín *et al.* (2010a) (see Chapter II) is specific for OCs including hexachlorocyclohexane isomers; compounds from endosulfan, DDT and heptachlor groups; aldrin, dieldrin and endrin. Mean recoveries in spiked samples ranged from 46.13 to 146.05% depending on the compound (Espín *et al.*, 2010a) (see Chapter II). In regards to the methodology described by Malik *et al.* (2011) for 26 PBDE congeners, the recoveries of ^{13}C-labeled PCBs and PBDEs were between 63 and 135% (Malik *et al.*, 2011). The method described by Meyer *et al.* (2009) for PFCs showed recovery rates ranging between 77 and 108%. Finally, the technique for PFASs analysis in feathers described by Jaspers *et al.* (2013) showed average recoveries of 88% and 48% for ^{13}C-PFOS and ^{13}C-PFOA, respectively.

4.6. Adverse effects: Interpreting feather concentrations

There are several works on brain PHC concentrations associated with toxic effects in birds. In this regard, Stickel *et al.* (1969) suggested that bird and mammal brain were more useful than other tissues in diagnosing death from insecticides. However, there is little data available on the relationship between PHCs in feathers and their effects. In an experimental study by Greichus *et al.* (1975), nestling White Pelicans were treated with a daily dietary intake of 144 and 72 ppm of PCB or DDT complex (DDT+DDD+DDE) respectively, for 10 weeks. Liver was the tissue which accumulated the highest concentrations of these compounds (PCBs 290 ppm, and DDTs 135 ppm, wet weight), followed by brain (PCBs 110 ppm, DDTs 35 ppm) and feathers (PCBs 120 ppm, DDTs 48 ppm). These authors suggested that feathers may be preferable to

other tissues for indicating concentrations of DDT, its metabolites and PCBs in the brain. In relation to their effects, no signs of gross intoxication were observed. However, several sublethal effects were evident for liver vitamin A, as well as potassium, calcium and protein levels in serum. Therefore, the threshold value of sublethal effects associated with DDT complexes and PCBs is probably lower than 48 ppm and 120 ppm in feathers, respectively. Despite this, in the present study feathers were not washed, so there was most likely some external contamination from preening with uropygial oil.

There are very few monitoring studies available which provide information on both feather and internal tissue concentrations (Table 12), and even fewer discuss the importance of feather concentrations in estimating adverse effects (Behrooz *et al.*, 2009a,b; Espín *et al.*, 2010b, 2012) (see Chapter III and IV). Espín *et al.* (2010b, 2012) (see Chapter III and IV) analyzed organochlorine pesticides in feathers and internal tissues in the same individuals. Therefore, they were able to conclude that there was no risk of adverse effects associated with the OC levels found in feathers (Espín *et al.*, 2012) (see Chapter IV) taking into account the concentrations found in liver in a previous study (Espín *et al.*, 2010b) (see Chapter III). Behrooz *et al.* (2009a,b) carried out a hazard evaluation comparing concentrations of OCs in feathers with those reported in internal tissues from other studies. These authors note that this comparison should be used with caution due to problems with extrapolating such data across tissues and species. Therefore, further experimental and field studies are required in order to determine a nonadverse-effect threshold value in feathers from different species.

5. Recommendations, key uncertainties and conclusions

- Further research should focus on the structure of the feather, its binding affinities for PHCs, and the stability of these compounds in feathers.
- The deposition rate (pollutant load that enters feathers daily) is proposed as a unit of measurement, which allows any part of a feather to be validly compared to a different part of the same or other feathers. In this regard, further studies are required in order to provide the growth rate of feathers in different wild bird species.
- Further studies with newly grown feathers and blood samples are required in order to clarify the relationship between feather and internal tissue contaminant

concentrations. It is also necessary to eliminate the bias of external contamination by preen oil not removed by washing techniques and avoid the bias of the time elapsed between the last molt period and the time of sampling.

- Several studies have found that external contamination does occur in feathers, and preen oil is probably the main source of external contamination. Further experimental studies are needed to determine methods for discriminating between the internal and external contamination of feathers by organic pollutants.
- The use of external contamination markers such as certain metals with a low absorption rate and therefore with low endogenous deposition in feathers may be useful for discriminating between internal and external contamination. Further research must be carried out on this matter in order to look for organic external contamination markers.
- Barb material is structurally too complex to be washed effectively using the techniques proven so far. Rachis may be a better indicator of internal organic pollutant deposition in feathers than barbs. However, in developing or very recently molted feathers, surface external contamination would be expected to be minimal and thus barbs may remain valuable.
- All feather types could possibly be used, but the ideal feather depends on the molting pattern of the species and the end point of the monitoring study. Further studies are needed on the suitability of each type of feather.
- We recommend monitoring male feathers during the breeding season in order to eliminate the potential bias of off-loading contaminants into eggs. However, depending on the end point this recommendation may not be appropriate, that is, studies on the influence of egg laying or studies on differences in exposure according to sex.
- The importance of fat mobilization for migration, egg laying or other factors in the relative levels of feather and internal tissue contamination partly depends on the time when samples are collected. There is a need to collect further quantitative data as to what degree this time may influence correlations between feather and other tissue contaminant levels.

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CHAPTER II

Desarrollo de un método analítico para la extracción de plaguicidas organoclorados en plumas

Development of an analytical method for extracting organochlorine pesticides from feathers

Photo: Laurie Campbell. From www.arkive.org

Silvia Espín, Emma Martínez-López, Pedro María-Mojica, Antonio J. García-Fernández. 2010. Desarrollo de un método analítico para la extracción de plaguicidas organoclorados en plumas. Anales de veterinaria (Murcia) 26: 77-90.

Resumen

Debido a los efectos adversos de los plaguicidas organoclorados (OC), estos compuestos han sido monitorizados en diferentes especies de seres vivos. En estos estudios de biomonitorización ambiental, las aves han jugado un importante papel debido a su sensibilidad a los cambios ambientales y a su elevada posición en la cadena trófica. En los últimos años existe un interés creciente en utilizar muestras no destructivas como alternativa a los tejidos internos. En este sentido, las plumas han sido ampliamente utilizadas en la monitorización de la contaminación ambiental por metales pesados y podrían proporcionar información de la concentración de OC en la sangre durante su desarrollo. Sin embargo, la información sobre su uso en la monitorización de OC es escasa. El objetivo general del presente estudio es comprobar la utilidad de la pluma como unidad de biomonitorización de la exposición a plaguicidas organoclorados. Para ello se desarrolla un método de extracción de 16 OC en plumas, incluyendo α-, β- y δ-HCH, lindano, aldrín, dieldrín, endrín, endrín aldehído, endosulfán I y II, endosulfán sulfato, p,p'-DDT, DDD, DDE, heptacloro y su epóxido. Además se evalúa la interferencia por contaminación externa en los niveles encontrados en plumas y se estudia la distribución de los compuestos entre partes de la pluma (barbas y ejes).

La contaminación externa parece tener influencia en los niveles encontrados en plumas para determinados compuestos, sin embargo, no parece ser la única causa de los mayores niveles observados en barbas en comparación con los ejes.

La pluma parece ser una prometedora herramienta no destructiva de plaguicidas organoclorados en aves. Futuros estudios deben ir encaminados en determinar correlaciones entre concentraciones en plumas y tejidos internos de aves. Además, se deben evaluar factores adicionales como la edad, sexo y estado nutricional de las aves para comprobar su efecto sobre los niveles de contaminantes en plumas.

Abstract

Due to the adverse effects of organochlorine pesticides (OC), these compounds have been widely monitored in several species of living beings. Birds have played an important role in monitoring environmental pollution due to their sensitivity to environmental changes and their position in the upper of the food chain. In recent years, many efforts have been attempted to look for useful samples obtained in a non-destructive way as alternative to the collection of internal tissues. Feathers can provide information of OC concentrations in the circulating blood at the time of their development. They have been widely used in monitoring of metal environmental pollution. However, information about their use in OC monitoring is scarce. The general aim of this study is the validation of the feather as OC biomonitoring tool. In this sense, we develop a method of extraction for 16 OC in feathers, including α-, β-and δ-HCH, lindane, aldrin, dieldrin, endrin, endrin aldehyde, endosulfan I and II, endosulfan sulfate, p,p'-DDT, DDD, DDE, heptachlor and its epoxide. Moreover, we assess the influence of external contamination and the distribution of compounds between parts of the feather (barbs and shaft).

External contamination seems to have influence on the levels found in feathers for some compounds. However, it does not seem to be the only cause of the high levels observed in barbs in comparison with those detected in shaft.

Feather could be considered as a promising non-destructive tool for organochlorine pesticides in birds. Future studies should be carried out to obtain correlations between concentrations in feathers and internal tissues of birds. Moreover, it is necessary to evaluate additional factors such as age, sex and nutritional status of the birds in order to check its effect on the OC levels in feathers.

1. Introducción

La biomonitorización, es decir, la medición de concentraciones de un contaminante en tejidos o de los efectos relacionados con su exposición en seres vivos, puede revelar la biodisponibilidad de los compuestos tóxicos (Beeby, 2001). El grado de impregnación de los contaminantes ambientales en tejidos, fluidos o productos de cualquier especie animal, incluido el hombre, refleja el estado de salud del ecosistema o ambiente en el que se desenvuelven, además de servir como parámetro de evaluación de la supervivencia de la especie (García-Fernández, 1994).

Debido a los efectos perjudiciales de los plaguicidas organoclorados, estos compuestos orgánicos se han monitoreado tanto en muestras ambientales como en la biota y continúan encontrándose en la actualidad (Espín *et al.*, 2010) (ver Capítulo III). En este sentido, las aves han desempeñado un papel importante en la evaluación de la contaminación ambiental por su sensibilidad a los cambios ambientales y su elevada posición en la cadena alimentaria, acumulando altos niveles de contaminantes en sus tejidos.

A pesar de que la medida directa de los contaminantes en tejidos internos y sangre de aves es el mejor indicador del grado y tipo de exposición a determinados compuestos como metales pesados y plaguicidas organoclorados (García-Fernández *et al.*, 1997; María-Mojica *et al.*, 2000), razones prácticas, éticas y de conservación, abogan por la búsqueda de otro tipo de muestras de obtención poco o nada cruenta para el animal. De esta forma, numerosos estudios se han centrado en técnicas no destructivas para la biomonitorización de contaminantes utilizando muestras como la sangre (García-Fernández *et al.*, 1996; Martínez-López *et al.*, 2005, 2009), los excrementos (Sun *et al.*, 2006), las egagrópilas (Mateo *et al.*, 1999), los huevos (Malik *et al.*, 2011; Martínez-López *et al.*, 2007), el aceite de la glándula uropigial (Johnston, 1976; Yamashita *et al.*, 2007), el cabello (Covaci *et al.*, 2008; D'Havé *et al.*, 2005) y las plumas (Dauwe *et al.*, 2005; Jaspers *et al.*, 2006, 2009; Van Den Steen *et al.*, 2007).

Aunque todavía son pocos los trabajos que han investigado la acumulación de contaminantes orgánicos persistentes en las plumas, los últimos hallazgos crean perspectivas valiosas para futuros estudios de seguimiento de estos contaminantes en las comunidades de aves, como se ha hecho con éxito para los metales pesados (Garitano-Zavala *et al.*, 2010; Malik y Zeb, 2009; Martínez-López *et al.*, 2002, 2005). Los compuestos organoclorados analizados en otros estudios han sido PCBs, DDTs y HCHs, siendo de importancia validar la pluma como método de biomonitorización de

otros compuestos que fueron ampliamente utilizados como aldrín, dieldrín, endrín, heptacloro y endosulfán.

Las plumas tienen una serie de ventajas respecto al resto de tejidos de obtención no cruenta, ya que pueden ser recolectadas independientemente de la temporada, la edad o el sexo. Además son fácilmente recogidas, transportadas y almacenadas a temperatura ambiente y se pueden recoger en pequeño número sin causar daño permanente al ave.

Al contrario que el pelo, que crece continuamente, las plumas sólo crecen durante un cierto periodo de tiempo, conectadas al torrente sanguíneo (Jaspers *et al.*, 2004). Los contaminantes orgánicos pueden llegar a las plumas durante su crecimiento a través de la sangre, produciéndose de esta forma una contaminación interna. Después de la maduración de la pluma, el suministro de sangre se atrofia y se convierten en plumas aisladas del resto del cuerpo (Burger, 1993). Por lo tanto, las plumas pueden contener información sobre las concentraciones circulantes en la sangre en el momento de su desarrollo. Sin embargo, la correlación con los niveles en los tejidos puede ser confusa debido a la contaminación externa de los contaminantes orgánicos en la superficie de las plumas (Jaspers *et al.*, 2007b). Esta contaminación externa puede producirse tanto por fuentes exógenas, como la deposición atmosférica ya sea en seco o húmedo, como endógenas, es decir, por el acicalamiento de las plumas con aceite de la glándula uropigial.

La contaminación externa por metales pesados en las plumas ha sido ampliamente investigada (Burger, 1993; Dauwe *et al.*, 2003; Jaspers *et al.*, 2004; Pilastro *et al.*, 1993), y ha demostrado tener una influencia importante en las concentraciones medidas en las plumas. El grado de contaminación externa varía según el metal que se examina y proviene principalmente de fuentes exógenas (Jaspers *et al.*, 2004). Sin embargo, las propiedades químicas de los metales pesados y contaminantes orgánicos difieren considerablemente, por lo que la contaminación externa por compuestos orgánicos puede influir de distinta manera sobre los niveles encontrados en plumas.

Recientemente se llevó a cabo un estudio para investigar el grado de contaminación externa de contaminantes orgánicos a través del aire, y resultó ser de pequeña importancia (Jaspers *et al.*, 2007a). Sin embargo, según Vorhees *et al.* (1997) la deposición atmosférica de contaminantes orgánicos podría ser importante en la modificación del perfil en plumas, esperándose que los compuestos más volátiles

contribuyan más a este tipo de contaminación externa. Según Greichus y Greichus (1974), habría que tener en cuenta, además, las fuentes endógenas en la contaminación externa de las plumas, es decir, la contaminación con aceite de la glándula uropigial, ya que los contaminantes orgánicos son productos químicos lipofílicos y tienen la capacidad de acumularse en altas concentraciones en las secreciones aceitosas (Van Den Brink, 1997; Yamashita *et al.*, 2007).

A priori, para obtener información útil acerca del grado de exposición de los individuos y la distribución interna de los plaguicidas organoclorados, con una correcta interpretación de los resultados, sería necesario llevar a cabo un proceso de lavado que consiga eliminar los compuestos de la superficie de la pluma.

El objetivo general del presente trabajo es comprobar la utilidad de la pluma como unidad de biomonitorización de plaguicidas organoclorados. Para ello se plantearon como objetivos específicos la puesta a punto de una técnica de extracción de plaguicidas organoclorados en plumas y la evaluación de la posible interferencia por contaminación externa en los niveles encontrados en las plumas, estudiando además las posibles diferencias entre partes de la pluma (barbas y ejes) en la distribución interna de los compuestos.

2. Material y Métodos

2.1. Muestras utilizadas

En el presente estudio se utilizaron plumas de Ánade real (*Anas platyrhynchos*). Se realizó una mezcla de plumas troceadas y se tomaron 25 alícuotas para la puesta a punto de la metodología analítica. A la hora de evaluar la contaminación externa y estudiar la distribución de los compuestos en el interior de la pluma se realizaron cuatro grupos de muestras, correspondientes a la mezcla troceada de barbas lavadas, barbas sin lavar, ejes lavados, y ejes sin lavar, tomándose 10 alícuotas de cada grupo para su procesado y análisis.

Las muestras fueron facilitadas por el personal del Centro de Recuperación de Fauna Silvestre de Santa Faz (Alicante, Generalitat Valenciana).

2.2. Plaguicidas organoclorados estudiados

Los 16 plaguicidas y metabolitos organoclorados objeto de estudio fueron α-HCH, β-HCH, δ-HCH y γ-HCH (Lindano), heptacloro y su epóxido, endosulfán (isómeros I y

II) y su metabolito endosulfán sulfato, aldrín, dieldrín, endrín y endrín aldehído, p,p'-DDT y metabolitos p,p'-DDD y p,p'-DDE.

2.3. Disolventes y reactivos

Los disolventes orgánicos hexano, acetona, éter de petróleo y éter etílico son calidad de residuos Pestiscan de la marca comercial Lab-Scan®, y el sulfato sódico anhidro granulado es calidad de residuos para análisis orgánico de trazas de Merck®. Las columnas de Florisil® (SEP-PAK, Classic) son de la marca comercial Waters®. El patrón de los 16 plaguicidas organoclorados objeto de análisis es de la firma comercial Supelco®, disuelto en 1ml de metanol:diclorometano 98:2. Dicho patrón original se disolvió en hexano (1:25) a las siguientes concentraciones: 10 µg/ml para α-HCH, β-HCH, δ-HCH, lindano, heptacloro, heptacloro epóxido y aldrín; 20 µg/ml para endosulfán I, endosulfán II, DDE, dieldrín y endrín; y 60 µg/ml para DDD, DDT, endrín aldehído y endosulfán sulfato.

Se utilizó metoxicloro (PolyScience®) para la preparación de un patrón interno de concentración 1 mg/ml.

2.4. Evaluación de los métodos analíticos

El gran número de plaguicidas organoclorados a identificar y las variaciones en su polaridad plantean problemas en el proceso de recuperabilidad. Con el fin de resolver dichos problemas, se prepararon distintas cantidades y mezclas de disolventes orgánicos para averiguar cuál de ellas era la más adecuada. De esta forma, se plantearon cuatro métodos de extracción de plaguicidas organoclorados en plumas (A, B, C y D), cuyas características generales se presentan en la Tabla 13. Sobre cada método propuesto se realizaron pruebas de recuperabilidad que permitieron elegir el método más idóneo sobre el cual llevar a cabo las pruebas de exactitud, linealidad y precisión, cuyas características se describen a continuación.

Exactitud del método:

Con el fin de comprobar la exactitud del método, se realizó un estudio de recuperación de los plaguicidas en muestras iguales de pluma de Ánade real. Se realizaron los correspondientes ensayos en blanco o control, para comprobar los niveles de residuos de los compuestos de interés en las plumas utilizadas. Para obtener los porcentajes de recuperación, las muestras fueron fortificadas a tres niveles, añadiendo 1, 2 y 3 ml de disolución patrón 1:25 (dicho patrón se preparó

disolviendo el original en hexano) antes de realizar la técnica. Tanto para los controles como para los tres niveles de enriquecimiento se realizaron muestras por quintuplicado. Posteriormente se extrajeron con el procedimiento descrito a continuación, y se inyectaron en el cromatógrafo para su determinación.

Una vez realizada la determinación analítica, los valores obtenidos fueron comparados con los de las disoluciones patrón utilizadas para la fortificación, que también fueron analizadas en la misma secuencia.

Finalmente se seleccionó el método con el que se obtenían mejores porcentajes de recuperación. Para el cálculo de dicho porcentaje se utilizó la fórmula: *Recuperación (%)=(Cm/Cp)x100,* donde Cm es la concentración de cada uno de los compuestos en la muestra, y Cp la concentración de cada compuesto en la disolución patrón.

Tabla 13. *Métodos de extracción de plaguicidas organoclorados evaluados en plumas.*

<table>
<tr><th></th><th colspan="4">DISOLVENTES</th></tr>
<tr><th>MÉTODO</th><th>Incubación</th><th>Extracción</th><th>Redilución</th><th>Elución</th></tr>
<tr><td>Método A</td><td rowspan="2">3 ml de hexano:diclorometano (4:1)</td><td rowspan="2">4 ml de hexano:diclorometano (4:1)</td><td>5 ml hexano</td><td>25 ml de éter de petróleo:éter etílico (21:4)</td></tr>
<tr><td>Método B</td><td>5 ml hexano:diclorometano (4:1)</td><td>15 ml de hexano</td></tr>
<tr><td>Método C</td><td rowspan="2">15 ml de acetona:hexano (1:2)</td><td rowspan="2">20 ml de acetona:hexano (1:3)</td><td rowspan="2">5 ml hexano</td><td>15 ml de hexano</td></tr>
<tr><td>Método D</td><td>25 ml de éter de petróleo:éter etílico (21:4)</td></tr>
</table>

<u>*Linealidad del método:*</u>

La linealidad sirve para determinar la proporcionalidad entre la concentración del principio activo y su respuesta. La selección del rango y del número de puntos experimentales para obtener la linealidad está estrictamente relacionada con la aplicación del método. En el presente estudio se decidió tomar un punto control de plumas sin fortificar, y tres niveles de fortificación con concentraciones de 0,4-2,4 μg/g, 0,8-4,8 μg/g y 1,2-7,2 μg/g, según el compuesto. Para cada uno de los puntos de calibración se realizaron cinco repeticiones.

El ajuste lineal de los datos a una curva de regresión se evaluó mediante el método de mínimos cuadrados, aceptando la linealidad de respuesta entre las concentraciones inyectadas siempre que el coeficiente de correlación lineal fuera

superior a 0,95 para los factores de respuesta. La recta de regresión no fue forzada a pasar por el origen.

Precisión del método:

En cuanto a la precisión del método, la repetibilidad se estudia para comprobar que un método es capaz de dar resultados semejantes o alrededor de un valor medio, al realizar el proceso de extracción de forma repetida para un mismo tipo de muestra. Permite determinar la concordancia entre los resultados de mediciones obtenidas de forma independiente, para un mismo tipo de muestra, bajo unas mismas condiciones experimentales, y por un mismo operador. Se evalúa mediante la obtención del coeficiente de variación (CV). Para el cálculo de este parámetro se utilizó el mismo lote empleado en la linealidad, es decir, se calculó el CV de las cinco repeticiones para cada uno de los niveles de fortificación y para cada compuesto. El criterio de aceptación fue que el CV obtenido entre las repeticiones a cada nivel de fortificación fuese ≤ 20%.

La fórmula utilizada para su cálculo es: $C.V.(\%)=(S/Xm)x100$, donde S es la desviación típica de la serie de mediciones (5 repeticiones), cuya media es Xm.

La reproducibilidad permite determinar el grado de concordancia entre los resultados de mediciones obtenidas de forma independiente, para un mismo tipo de muestra, bajo las mismas condiciones experimentales pero a distinto tiempo o por distintos operadores, ya que puede suponerse que, a lo largo del tiempo, ha podido haber cambios en material o instrumentación que afectarán al resultado de los análisis. Para este cálculo, se analizaron en diferentes tiempos cinco muestras de plumas iguales fortificadas con 1 ml de disolución patrón 1:25. Se aceptó la reproducibilidad intra-laboratorio como válida cuando el CV fue ≤ 20%.

Para el estudio de dichos parámetros se utilizaron plumas de Ánade real previamente lavadas con agua del grifo, agua destilada y agua milli-Q, para evitar posibles interferencias por contaminación externa.

Influencia de la contaminación externa:

Una vez puesto a punto el método, se evaluó la influencia de la contaminación externa y se estudiaron las posibles diferencias de acumulación dentro de la estructura de la pluma. Para ello se tomaron plumas de vuelo de Ánade real y se separaron en barbas y ejes, realizando una mezcla de barbas sin lavar (n=10) y barbas lavadas

(n=10), y una mezcla de ejes sin lavar (n=10) y ejes lavados (n=10). Estas muestras se procesaron y analizaron independientemente.

2.5. Metodología analítica

Una vez estudiados los resultados, se optó por la metodología que se describe a continuación.

Se cortan 0,2 g de plumas al menor tamaño posible, aproximadamente 1 mm, previamente lavadas con agua del grifo, agua destilada y agua Milli-Q, para evitar posibles interferencias por contaminación externa. Una vez secadas a temperatura ambiente, se llevan a un tubo tipo Falcon con 4 ml de HCl (37%) y 15 ml de acetona:hexano (1:2). La mezcla se mantiene en baño térmico a 37ºC durante 13 horas. Posteriormente se realiza la extracción con 20 ml de acetona:hexano (1:3) y se homogeneíza la mezcla. El extracto se centrifuga a 3000 rpm durante 3 minutos, haciendo pasar el sobrenadante por un embudo de placa porosa con sulfato sódico anhidro a un matraz de 100 ml. Se concentra a vacío en el rotavapor y el extracto seco se redisuelve en 5 ml de n-hexano. La purificación se realiza a través de una microcolumna de Florisil® (SEP-PAK, Waters®), que debe ser activada previamente con 2 ml de n-hexano. Se pasan los 5 ml del extracto de hexano que se dejan caer por gravedad y posteriormente se prepara una elución con la mezcla éter de petróleo-éter etílico (21:4), recogiendo todo el solvente en el mismo matraz de 100 ml, que volverá a ser desecado a vacío en el rotavapor. Este último extracto seco se redisuelve en 5 ml de n-hexano, y se pasa a un tubo de vidrio con tapón de corcho, conservándolo en la cámara frigorífica hasta su análisis cromatográfico.

Finalmente, para el análisis de las muestras, el contenido de los tubos de vidrio se evapora a sequedad con nitrógeno y se redisuelve en 1 ml de hexano, haciéndose pasar a viales de los cuales se pinchará 1 µl en el cromatógrafo de gases (Shimadzu® GC 17A), con detector de captura de electrones (ECD). La columna usada es de tipo capilar SPB (SPB-608, Supelco®) con un grosor de 0.25 µm, longitud de 30 m y diámetro interno de 0.25 mm, específicamente recomendada por la EPA para el análisis de los 16 plaguicidas organoclorados objeto de estudio.

El tratamiento de los datos cromatográficos se realizó mediante el software Shimadzu GC Solution. Se utilizó helio como gas portador y nitrógeno en el *make-up*. El inyector se configuró en modo splitless, y las temperaturas del inyector y detector fueron 290ºC y 330ºC respectivamente. El programa de temperatura de la columna fue

el siguiente: 2 min 50ºC, de 50 a 150ºC a 40ºC/min, 2 min 150ºC, de 150 a 290ºC a 8ºC/min, 10 min 290ºC.

La identificación y cuantificación se realizó mediante el patrón externo de 16 OCs de Supelco® diluido en hexano al 1:25.

El límite de detección para cada materia activa se calculó como la menor concentración de disolución patrón detectada en las condiciones experimentales del instrumento utilizado, teniendo en cuenta que la respuesta presentara una relación señal/ruido mayor de 3. Los límites de detección se encuentran en el intervalo 0,03-0,54 ng/g. Se utilizó metoxicloro (PolyScience®) (1 mg/ml) como patrón interno. Se añadió un volumen de 10 µl a las muestras y los patrones con el fin de comparar resultados y comprobar la repetibilidad de los cromatogramas. Las concentraciones de OC se expresan en ng/g.

2.6. Análisis estadístico

El análisis estadístico de los datos se llevó a cabo con el paquete estadístico SPSS v.15.0 y la hoja de cálculo Excel 2007. Se realizaron análisis de estadística descriptiva, representándose los valores obtenidos como media ± desviación típica, mediana y rango (mínimo-máximo). Puesto que los datos de concentraciones de plaguicidas no se ajustan a una distribución normal, para determinar las posibles diferencias entre las variables estudiadas (plumas lavadas y sin lavar, y barbas y ejes) se utilizó el test no paramétrico de Mann-Whitney. Los resultados se consideraron significativos a $p < 0,05$.

3. Resultados y Discusión

3.1. Puesta a punto de la metodología analítica

La puesta a punto de un método analítico tiene como objetivo caracterizar su rendimiento en cuanto a su alcance, especificidad, exactitud, repetibilidad y reproducibilidad (SANCO, 2007).

La exactitud o porcentaje de recuperación de los analitos de la matriz estudiada mediante el método de extracción, se evalúa mediante la realización de fortificaciones. En la Tabla 14 se presentan los porcentajes de recuperación medios para cada uno de los métodos evaluados. El método D presenta los mayores porcentajes de recuperación. Finalmente se seleccionó dicho método como el más adecuado para la extracción de plaguicidas organoclorados en plumas, con un rango de valores medios

de recuperación para los 16 compuestos objeto de estudio entre 46,13 y 146,05%, a excepción del endrín aldehído (347,11%).

Tabla 14. *Validación del método de extracción de plaguicidas organoclorados en plumas.*

	VALORES MEDIOS DE RECUPERACIÓN (%)*				PARÁMETROS VALIDACIÓN MÉTODO D		
COMPUESTO	Método A	Método B	Método C	Método D	Linealidad (R)	CV Repetibilidad(%)*	CV Reproducibilidad(%)**
α-HCH	28,67	9,14	5,85	57,67	0,981	7,79	12,15
Lindano	14,10	7,68	9,69	57,50	0,976	8,75	13,33
β-HCH	51,22	13,47	7,29	52,98	0,979	14,14	12,43
Heptacloro	24,63	6,77	7,95	46,74	0,977	8,91	12,03
δ-HCH	43,67	10,66	10,21	53,62	0,960	11,40	18,22
Aldrín	28,97	7,05	4,55	46,13	0,973	15,81	18,55
Heptacloro Epóxido	26,95	9,13	13,27	81,60	0,987	9,12	16,84
Endosulfán I	9,09	1,06	3,13	68,47	0,968	11,74	24,15
DDE	23,08	14,42	19,12	122,23	0,989	14,17	13,29
Dieldrín	13,44	4,52	12,56	98,53	0,974	16,17	17,77
Endrín	10,08	0,00	0,49	48,87	0,964	8,70	17,59
DDD	15,05	18,68	28,20	138,15	0,974	17,86	9,19
Endosulfán II	2,99	0,00	0,10	146,05	0,969	15,56	14,45
DDT	18,75	12,03	29,77	135,56	0,989	17,22	12,79
Endrín aldehído	45,40	31,97	84,25	347,11	0,965	29,91	20,64
Endosulfán Sulfato	9,11	0,00	12,16	118,72	0,980	17,73	17,64

*R=Coeficiente de correlación de la linealidad, CV=Coeficiente de variabilidad, *Media de los tres niveles de fortificación, **Media de 5 determinaciones a un nivel de fortificación.*

Se debe tener en cuenta que al tratarse de un método analítico laborioso, con varias etapas, como extracción y purificación, y al ser una técnica para un gran número de plaguicidas con variaciones de polaridad, determinados compuestos pueden presentar problemas en la recuperabilidad, con menores porcentajes de recuperación, ya que los disolventes orgánicos no son capaces de extraer de la misma manera a todos los compuestos. Sin embargo, con el método D se obtienen recuperaciones bastante aceptables para los compuestos objeto de estudio.

La linealidad se utiliza para estimar la concentración de cada uno de los plaguicidas y el área resultante del análisis cromatográfico, demostrando la capacidad del método de obtener resultados lineales. Los valores obtenidos muestran una buena correlación concentración del plaguicida/área del pico cromatográfico, para cada uno de los compuestos, con valores del coeficiente de regresión (R) mayores a 0,96 (Tabla 14).

Finalmente, en relación a la precisión del método, los coeficientes de variabilidad medios obtenidos para la repetibilidad y reproducibilidad son indicativos de la buena precisión del método, con valores por debajo del 20% en todos los casos, umbral que hemos establecido en la validación del método analítico, con la única excepción del endrín aldehído, con un porcentaje de variabilidad medio del 29,91% para la repetibilidad, y el endosulfán I con un valor de variabilidad medio de 24,15% para la reproducibilidad.

Por tanto, consideramos que la técnica puesta a punto en el presente trabajo es aceptable como metodología de extracción de plaguicidas organoclorados en plumas.

3.2. Influencia de la contaminación externa de la pluma

A la hora de evaluar la influencia de la contaminación externa de la pluma se compararon las medias de plaguicidas organoclorados en barbas y ejes de plumas lavadas y sin lavar de Ánade real. Todos los compuestos se detectaron, al menos, una vez, aunque el endrín aldehído y el endosulfán sulfato fueron los que alcanzaron mayores concentraciones (Tabla 15). Los niveles de organoclorados fueron significativamente mayores en las muestras sin lavar que en las muestras lavadas para algunos compuestos, tanto en barbas como en ejes (endrín aldehído, endrín, lindano, DDE).

El hecho de que las concentraciones de organoclorados fueran mayores en muestras sin lavar que en lavadas para determinados compuestos sugiere una posible interferencia de la contaminación externa por deposición atmosférica o aceite secretado por la glándula uropigial. Si bien es cierto que en algunos casos los niveles son mayores en muestras lavadas que en muestras sin lavar, no existen diferencias significativas, y podría estar relacionado con los bajos niveles detectados en dichos casos, con valores de mediana próximos e incluso iguales a 0 (Tabla 15).

Por tanto, se recomienda un proceso de lavado previo a la técnica de extracción de los compuestos estudiados, evitando así que los residuos procedentes del exterior modifiquen los niveles procedentes del torrente sanguíneo. Sin embargo, dicha contaminación externa no parece tener tanta importancia como el caso de los metales pesados, dónde el 50-98% de las concentraciones de Pb pueden deberse a la contaminación externa (Dauwe *et al.*, 2002; Weyers *et al.*, 1988).

Tabla 15. *Niveles de plaguicidas organoclorados en barbas y ejes lavados y sin lavar.*

Niveles de plaguicidas organoclorados (ng/g) en plumas				
Compuesto	**Barbas lavadas (N=10)**	**Ejes lavados (N=10)**	**Barbas sin lavar (N=10)**	**Ejes sin lavar (N=10)**
α-HCH	1,2±2,02**	2,25±0,99	0,42±1,34**	5,05±3,64
	0,04 (0-4,97)	2,08 (0,48-3,56)	0 (0-4,26)	6,65 (0-9,21)
Lindano	23,59±15,32**[a]	7,49±2,95 [a]	79,91±52,39*	27,85±6,71
	29,72 (3,77-43,48)	7,73 (2,45-11,61)	59,22 (25,98-155,55)	27,23 (18,30-37,41)
β-HCH	2,15±2,79**	0,32±1,03	2,83±3,73	1,17±2,51
	0,55 (0-7,24)	0 (0-3,26)	0 (0-7,88)	0 (0-6,71)
δ-HCH	2,01±2,26	1,49±1,24	3,27±3,21	3,01±2,34
	1,19 (0-5,80)	1,5 (0-3,36)	3,55 (0-8,68)	3,67 (0-5,41)
Heptacloro	3,84±5,15	5,44±2,66	nd	5,71±6,29
	0,72 (0-12,87)	5,36 (1,15-9,23)		4,10 (0-15,17)
Heptacloro Epóxido	7,39±7,63	2,80±3,08 [a]	23,37±20,67	13,31±7,60
	5,11 (0-18,82)	2,07 (0-7,52)	23,13 (0-59,66)	14,57 (0-21,10)
Aldrín	25,03±15,66**	6,59±3,91	34,07±32,44	15,64±17,05
	30,52 (0-45,95)	5,99 (1,45-12,36)	34,91 (0-98,56)	10,95 (0-38,12)
Dieldrín	3,49±5,18	1,61±1,41	nd	nd
	0,26 (0-14,16)	1,42 (0-4,35)		
Endrín	27,64±29,78** [a]	52,92±19,83 [a]	138,89±57,85	90,41±25,66
	21,48 (0-85,13)	52,10 (26,90-88,88)	140,31 (43,37-208,54)	91,93 (52,73-128,59)
Endrín aldehído	270,06±233,96** [a]	116,02±64,69 [a]	1152,94±262,49*	527,15±152,05
	225,96 (0-784,02)	137,26 (2,40-196,49)	1202,78 (683,86-1547,18)	504,64 (345,46-817,93)
Endosulfán I	1,13±3,59 [b]	nd	12,36±16,05	nd
	0 (0-11,37)		4,7 (0-47,83)	
Endosulfán II	nd	0,54±1,29	nd	nd
		0 (0-3,99)		
Endosulfán Sulfato	195,87±101,39*	52,38±26,74	184,20±47,91*	79,66±35,66
	217,69 (0-316,28)	50,9 (12,54-106,16)	172,48 (119,75-276,99	81,69 (32,69-137,66)
DDE	16,86±18,31** [a]	2,03±2,10 [a]	38,41±11,86	29,85±12,92
	15,43 (0-61,72)	1,33(0-6,28)	40,37(15,99-58,34)	30,13(13,64-58,60)
DDD	nd	0,65±2,08	nd	nd
		0 (0-6,58)		
DDT	36,10±77,33	24,62±29,70	28,08±50,42	36,82±77,62
	0 (0-248,45)	6,34 (0-67,22)	0 (0-148,22)	0 (0-184,53)

*Media ± Desviación estándar, mediana (mínimo-máximo). N=número de muestras, nd=no detectado. Diferencias significativas entre barbas y ejes: *p<0,01, **p<0,05. Diferencias significativas entre mismo tipo de muestras lavadas y sin lavar: [a]p<0,01, [b]p<0,05.*

3.3. Distribución de compuestos organoclorados en el interior de la pluma

Algunos trabajos han encontrado diferencias en la acumulación de contaminantes según la parte de la pluma analizada. En estudios con metales pesados se han observado concentraciones en las barbas hasta 51 veces superiores a las del eje en Cárabo común (*Strix aluco*) y hasta 120 veces mayor en Gavilán (*Accipiter nisus*) (Dauwe *et al.*, 2003). Dichos autores atribuyen estos resultados a la contaminación externa, la cual puede variar dependiendo del metal, desde prácticamente ausente en el caso del Hg hasta 120 veces los niveles internos para el Pb (Dauwe *et al.*, 2003; Ek *et al.*, 2004; Weyers *et al.*, 1988). Jensen *et al.* (2002) encontraron niveles significativamente mayores de Pt y Pd en barbas en comparación con los ejes de aves rapaces, lo que también atribuían a la influencia de la contaminación externa. Jaspers *et al.* (2007a) encontraron niveles significativamente mayores de HCB, DDE, PCBs y PBDEs en barbas en comparación con eje en Busardo ratonero (*Buteo buteo*), sugiriendo que la contaminación externa podría ser importante. Puesto que las barbas cubren la mayor parte de la superficie de la pluma, se espera que la influencia de la contaminación externa sea mayor en estas en comparación con el eje.

Para conocer las posibles diferencias de acumulación en la estructura de la pluma se compararon las medias de plaguicidas organoclorados entre ejes y barbas de plumas de Ánade real. Estas pruebas se realizaron con plumas sin lavar, para comprobar la influencia de la contaminación externa, y con plumas lavadas para evitar precisamente dicha influencia y comprobar el patrón de distribución de los plaguicidas organoclorados en el interior de las mismas.

En la Tabla 15 se recogen las concentraciones de plaguicidas organoclorados detectados en barbas y ejes de plumas lavadas y no lavadas. Se observaron mayores niveles de endrín aldehído, endosulfán sulfato y lindano en barbas sin lavar comparados con ejes sin lavar. En cuanto a las plumas lavadas, cinco de los compuestos también presentaron los mayores niveles en barbas.

Los mayores niveles de determinados compuestos en barbas sin lavar comparados con ejes sin lavar pueden explicarse por la mayor probabilidad de que se depositen partículas con contaminantes orgánicos asociados en barbas que en eje, ya que la vaina de la pluma tiene mayor superficie de contacto con el exterior y, por lo tanto, se encuentra más expuesta. Además, las aves acuáticas acicalan sus plumas con aceite de la glándula uropigial de manera intensa, contribuyendo a una mayor impregnación de las barbas. Por tanto, estos resultados muestran que la

contaminación externa por estos compuestos ha de tenerse en cuenta. Dauwe *et al.* (2005) y Jaspers *et al.* (2007a) encontraron resultados similares para otros contaminantes orgánicos.

Del mismo modo, en el caso de las plumas lavadas algunos compuestos también presentaron mayores niveles en barbas, lo que sugiere que las diferencias no se deben solo a la contaminación externa, sino a una combinación de factores. En este sentido, Jaspers *et al.* (2007a) sugirieron que los mayores niveles en barbas podrían ser debidos a diferencias en la capacidad de unión a diferentes estructuras químicas presentes en las plumas. Dauwe *et al.* (2003) también plantearon esta posibilidad para explicar las diferencias en la acumulación de Hg en plumas. Sin embargo, no se ha llevado a cabo ningún estudio sobre la estructura química específica y las capacidades de unión de compuestos orgánicos en pelo o plumas. Otra posible hipótesis es que las barbas sean el final de la ruta del compuesto, y que el eje sea un canal de transporte (Jaspers *et al.*, 2007a).

En nuestro caso, estas diferencias sólo ocurren para determinados compuestos, precisamente aquellos que se detectan a mayores concentraciones. Ésta y otras razones técnicas nos llevan a considerar más adecuado el análisis de la pluma completa a la hora de su uso como unidad de biomonitorización.

En definitiva, la pluma parece ser una prometedora herramienta no destructiva de plaguicidas organoclorados en aves. Futuros estudios deben ir encaminados a determinar la existencia de correlaciones entre concentraciones en plumas y tejidos internos de aves con el fin de validarlas como muestras alternativas a los tejidos internos. Además, se deben evaluar factores adicionales como la edad, sexo y estado nutricional de las aves para comprobar su efecto sobre los niveles de contaminantes en plumas.

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CHAPTER III

Assessment of organochlorine pesticide exposure in a wintering population of Razorbills (*Alca torda*) from the Southwestern Mediterranean

Photo: Thomas Graham. From www.arkive.org

Silvia Espín, Emma Martínez-López, Pilar Gómez-Ramírez, Pedro María-Mojica, Antonio J. García-Fernández. 2010. Assessment of organochlorine pesticide exposure in a wintering population of Razorbills (Alca torda) from the Southwestern Mediterranean. Chemosphere 80: 1190-1198.

Abstract

Sixteen organochlorine pesticides (OC) were analyzed in several tissue types (abdominal and subcutaneous fat, liver and brain) from juvenile (n=14), immature (n=9), subadult (n=7) and adult (n=20) Razorbill (*Alca torda*) collected from the southwestern Mediterranean coastline, in the East of Spain (La Marina, Elche, Alicante, Spain). These Razorbills had drowned in fishing nets (most probably) while searching for food. The objective was to assess the exposure to organochlorine pesticide residues in this wintering population of marine birds. This paper presents, as far as we are aware, the first published data on OC concentrations in Razorbills. The highest levels were found in abdominal fat followed by subcutaneous fat, liver and brain. A significant positive relationship was found between age and OC levels in tissues, and with the highest levels in adults. The group of ∑Drins had the highest concentrations, followed by ∑DDT, ∑Endosulfan, ∑HCH and ∑Heptachlor, with endrin aldehyde being the compound which reached the highest levels. The p,p'DDE/p,p'DDT ratio in fatty tissues suggests exposure to non-degradated DDT and thus is present in the environment despite its prohibition. The OC levels detected were higher than those found in other studies on Alcidae, which may be explained by the Mediterranean habitat in which the birds were found. However, these levels are below concentrations for which any observable effect has been described.

1. Introduction

Marine ecosystems are continuously threatened by contaminants, eutrophication, and the overuse of resources, while more recent concerns involve climate change and substances causing endocrine disruption, reproductive failure, and developmental problems (Hylland, 2006). Organochlorine pesticides have been identified as one of the main groups of environmental contaminants. These compounds and their metabolites have been spread across all geographical regions as a result of agricultural and industrial activities. The southwestern Mediterranean is mainly an agricultural region and these contaminants have frequently been detected at high levels in previous studies from this area.

Recent contaminant studies have demonstrated that OC accumulation is not uniform among seabird species, but that it varies with differences in diet and biotransformation abilities (Moisey *et al.*, 2001). Frequently, OC concentrations detected in different bird tissue samples are not considered directly responsible for organism death. Nevertheless, these compounds produce chronic effects that lead them to be considered hormone disruptors, immunosuppressants, and the cause of adverse effects on the nervous and reproductive systems (Denneman and Douben, 1993; Furness *et al.*, 1993; Martínez-López, 2005).

Razorbill (*Alca torda*) is an Alcidae seabird species that lives in high latitudes. At least 70% of the world's Razorbill population breeds in Iceland with 20% in the British Isles. They are migratory seabirds and they winter in the Mediterranean area (Figure 10). From 2005 to 2009, 1842 Razorbills have been recorded in the Mediterranean area (Trektellen, 2009), and in Alicante (Spain), 824 Razorbills were recorded in 2007 by the staff of a non-governmental organization, Friends of the Wetlands from South of Alicante (AHSA, 2007). These data correspond to the months from November to March, coinciding with winter of this species on the Spanish coastline. Migration to industrialized latitudes may also result in the accumulation of contaminants in seabird body burden. Furthermore, the Razorbill is a species of particular interest, considering their possibly declining population densities (Ribeiro *et al.*, 2009). However, environmental contaminant studies on Razorbills are scarce (Malcolm *et al.*, 2003; Ribeiro *et al.*, 2009) or inexistent in the case of organochlorine compounds.

In Spain, most organochlorine insecticides have been banned (Orden 4 de Febrero de 1994, Decision 2000/801/EC, 2005/864/EC). However, they are still frequently

found in tissues or fluids samples from several species (Piqué *et al.*, 2006; Martínez-López *et al.*, 2007, 2009; Van Drooge *et al.*, 2008).

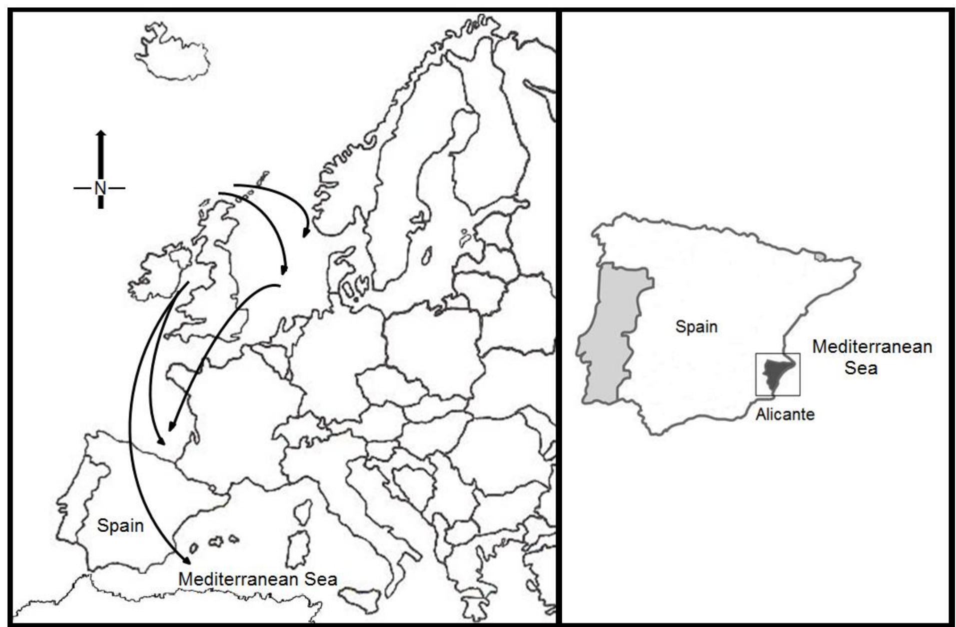

Figure 10. *Map showing Razorbill migration routes to Spain (Brown, 1985) and the geographical location of the Razorbill mortality incidents.*

The aim of this study was to determine concentrations of organochlorine pesticides in a homogeneous population of *Alca torda* from the Mediterranean area which had drowned in fishing nets, in order to assess the pattern of organochlorine pesticide residue distribution taking into account the age, sex and nutritional status of the specimen analyzed. The present work provides the first available data on levels of organochlorine compounds in the tissues of Razorbill.

2. Material and methods

2.1. Species and sample size

Fifty Razorbills were used in this study. These animals were found dead along the Occidental Mediterranean coastline in the East of Spain (La Marina, Elche, Alicante) (Figure 10), having drowned in fishing nets in February of 2007. They were collected by the staff of “Santa Faz” Wildlife Recovery Center (Alicante, Spain), and taken to the laboratory under refrigerated conditions.

A total of 197 samples of liver (n=50), brain (n=50), subcutaneous fat (n=49) and abdominal fat (n=48) were collected via necropsy. Two birds did not have sufficient fat

for collection and analysis. After collection, the samples were packed in Eppendorf tubes and frozen until their analysis.

During the necropsy, some parameters such as mass measurements (g), organ weights (g) and bill development were determined for all seabirds, and sex, age and physical condition were registered.

2.2. Age and physical condition of the seabirds

Three criteria were used to age the Razorbills: bill development (Anker-Nilssen *et al.*, 1998), size and appearance of the gonads (Van Franeker, 2004; Camphuysen and Franeker, 2007), and size and presence of the bursa Fabricii, one of the glands in the endocrine system of birds (Camphuysen and Franeker, 2007).

Razorbills develop a white vertical band on the bill during the second winter. In front of the white band, grooves will be developed in the following years. Generally, birds were classified into four age groups according to the structure of the bill. When no white bands or grooves on the bill are present they are considered juveniles; immature birds have the white band but no grooves; subadult and adult birds have one and two grooves in front of the white band, respectively, as described by Anker-Nilssen *et al.* (1998).

Internally, Razorbills were examined for sexual organ development. For testis in male birds, length and width were measured and compared with Camphuysen and Franeker's (2007) results. In general, males with a testis index (length x width) greater than 30 mm^2 were classified as adults, and those with a testis index lower than 10 mm^2 were classified as juveniles. For females, we used the follicle-oviduct index as suggested by Van Franeker (2004). The ovary appearance and development of the oviduct was described scoring 1 = the ovary has no structure (amorphous) and the oviduct is thin and straight (juvenile), 2 = the ovary with tiny follicles are visible and the oviduct is thicker and straight (immature), 3 = the ovary has visible follicles and the oviduct is thicker still and slightly twisted (subadult), or 4 = the ovary has clearly visible follicles of different sizes and the oviduct is swollen and twisted (adult).

Finally, all birds were examined for the presence or absence of the "bursa of Fabricius", and when present it was measured. Juvenile seabirds have a large bursa, immature ones may have a much reduced bursa, while subadult and adult seabirds normally have no trace of a bursa. Hence, the presence of a large bursa in combination with non-developed gonads is a fine indication that the bird in question is a juvenile.

The ratio of female:male was 3:1, approximately (76:24%). With regard to age, the majority of birds were adult (40%), followed by juvenile (28%), immature (18%) and subadult (14%). Relating sex with age, for females 47% were adult, 11% subadult, 16% immature and 26% juvenile. In males, 17% were adult, 25% subadult, 25% immature and 33% juvenile.

Furthermore, physical condition could also be a factor accounting for some of the variability in residues. According to Van Franeker (1983, 2004) three characters (subcutaneous fat, abdominal fat and pectoral muscle) were scored on a four-point scale (0-3) and the overall condition index was calculated based on the sum of scores of fat stores and breast muscles (0-9): score 0-1 = mortally emaciated; 2-3 = critically emaciated; 4-6 = moderate body condition; and 7-9 = good body condition. Only 4% of Razorbills analyzed had serious nutrition problems, while 40% had moderate body condition and 56% good body condition.

2.3. Organochlorine analysis

Liver, brain, subcutaneous and abdominal fat samples were analyzed for a series of organochlorine pollutants including hexachlorocyclohexane (HCH) isomers (α-HCH, β-HCH, γ-HCH or lindane and δ-HCH), endosulfan I, endosulfan II, endosulfan sulfate, aldrin, dieldrin, endrin, endrin-aldehyde, dichlorodiphenyltrichloroethane (p,p'-DDT), dichlorodiphenyldichloroethane (p,p'-DDD), dichlorodiphenyldichloroethylene (p,p'-DDE), heptachlor and heptachlor-epoxide.

2.4. Chemicals and standards

All reagents used for the analysis were of a trace analysis grade. Hexane, acetone, petroleum ether, diethyl ether were supplied by Lab-scan Analytical Sciences and anhydrous sodium sulfate by Merck Co. (Darmstadt). SepPak®Classic, Florisil® cartridges were supplied by Waters®. Pesticide standard (EPA Pesticide Mix 48858 dissolved in methanol:methylene chloride 98:2) was procured from Supelco (USA). Prior to analytical procedures, all glassware was rinsed several times with distilled water, hexane and acetone.

2.5. Analytical procedure

Samples were analyzed according to a method described by María-Mojica *et al.* (2000) and slightly modified by Martínez-López *et al.* (2009). A volume of 0.2 g of tissue was homogenized using hexane:acetone (3:1, v:v) as an extract solvent. The

samples were filtered using anhydrous sodium sulfate and then the solvent collected was evaporated until dryness. After redisolution in 5 ml hexane, samples were cleaned up via Florisil column chromatography (SepPak, Waters®), activated with 2 ml of hexane, using a petroleum ether:diethyl ether mix (21:4, v:v) as an elution solvent. The solvent collected was evaporated until dryness.

The final volume was adjusted to 1 ml with n-hexane. One microlitre was injected into a gas chromatograph with electron capture (GC-ECD 17 Shimadzu) for the detection of OCs. The SPB-608 capillary column (Supelco®) was 30 m long, 0.25 mm i.d. with a 0.25 µm film thickness, specifically recommended by the EPA for the 16 organochlorine pesticides studied. Helium was used as the carrier gas. The injector was set at the splitless mode; the injector temperature was 290ºC. The column program was: 2 min 50ºC, from 50 to 150ºC at 40ºC/min, 2 min 150ºC, from 150 to 290ºC at 81ºC/min, 10 min 290ºC. The detector temperature was 330ºC and the make-up gas was nitrogen.

Identification and quantification were based on an external standard. The standard solution marked in mixture was prepared by dissolving the reference substances in n-hexane (1:25) at the following concentrations: 10 µg/ml for α-HCH, β-HCH, δ-HCH, lindane, heptachlor, heptachlor epoxide and aldrin; 20 µg/ml for endosulfan I, endosulfan II, DDE, dieldrin and endrin; and 60 µg/ml for DDD, DDT, endrin aldehyde and endosulfan sulfate. Detection limits ranged from 0.03 to 0.54 ng/g. Methoxychlor (1 mg/ml) was used as an internal standard, and was supplied by PolyScience®. A volume of 10 µl was added to samples and standards in order to compare results and check the repeatability in the chromatograms. Mean recoveries in spiked samples ranged from 85.8% to 146.0%. Concentrations of OC were expressed as ng/g.

2.6. Statistical analysis

All analyses were carried out using the SPSS v.15.0 statistical package. Reported OC values represent the mean ± standard deviation, median and range.

Since the concentrations of organochlorine pesticides were not distributed normally, the data was log-transformed. ANOVA and Tukey's tests were performed to elucidate significant differences between tissues.

Generalized Linear Models (GLM) were used to analyze the concentrations of organochlorine pesticides. For each tissue, the OC Group was the response variable. We used GLMs with an identity function and Gaussian errors. The three explanatory

variables considered were age, sex and body condition. We followed a forward stepwise procedure, testing the statistical significance of each explanatory variable (*F*-test) and retaining those that contributed to the largest change in deviance from the null model until all the variables with a significant effect at $p<0.05$ had been included in the model.

The total concentrations of organochlorine pesticides (ΣOC) were calculated as the sum of individual compound concentrations. The group of DDT and metabolites (ΣDDT) represented the sum of p,p-DDE, p,p-DDD and p,p-DDT, hexaclorocyclohexanes (ΣHCH) included α, β, δ and γ-isomers, the group of heptachlor (ΣHeptachlor) was formed by heptachlor and its epoxide, ΣDrins represented the sum of endrin, aldrin, dieldrin and endrin aldheyde, and finally ΣEndosulfan incorporated endosulfan and endosulfan sulfate.

Pearson's correlation coefficient was used in order to calculate correlations between variables.

3. Results and discussion

3.1. Tissues

The highest organochlorine levels were found in abdominal fat followed by subcutaneous fat, liver and brain (Table 16). We found significant differences in concentrations of OC group between fat tissues and liver ($p<0.020$ for all OC groups), fat tissues and brain ($p<0.000$ for all OC groups), and between liver and brain ($p<0.000$ for all OC groups except ΣHeptachlor, $p=0.066$).

Coinciding with this study, Buckman *et al.* (2004) found that OC concentrations (wet weight) were greater in fat than liver for all OC groups across three species of Alcidae (*Alle alle*, *Cepphus grylle* and *Uria lomvia*) from Baffin Bay, which may be explained by the higher percentage of triglycerides in fat and the affinity of OC for this type of lipids (Cockcroft *et al.*, 1989). Although the higher lipid content in fat is the main cause of lower concentrations in liver compared to fat, biotransformation processes may also influence this, since the liver is an active biotransformation site. This occurs for example with HCH, as seabirds are known to readily biotransform both α- and γ-HCH (Moisey *et al.*, 2001). In this sense, we found negative correlations between fatty tissues and liver for δ-HCH ($r=-0.391$, $p<0.01$), DDE ($r=-0.288$, $p<0.05$) and heptachlor epoxide ($r=-0.484$, $p<0.01$).

Table 16. *Concentrations of organochlorines (ng/g, wet weight) in Razorbill tissues. Values are presented as mean ± standard deviation, median and range (min-max).*

Concentrations of OC (ng/g wet weight) in Razorbill (*Alca torda*) tissues				
Compound	**Abdominal fat (n=48)**	**Subcutaneous fat (n=49)**	**Liver (n=50)**	**Brain (n=50)**
α-HCH	64.95 ± 68.20	69.28 ± 71.60	5.58 ± 18.37	nd
	33.75 (nd-270.33)	36.86 (nd-256.64)	0 (nd-101.52)	
Lindane	85.07 ± 98.39	36.91 ± 43.99	15.31 ± 44.21	4.51 ± 8.98
	42.15 (nd-447.97)	23.85 (nd-190.49)	0 (nd-216.25)	0 (nd-40.97)
β-HCH	144.77 ± 161.00	174.37 ± 185.87	48.41 ± 166.54	nd
	93.81 (nd-723.87)	110.62 (nd-928.08)	0 (nd-972.39)	
δ-HCH	122.64 ± 187.25	96.82 ± 118.48	24.41 ± 84.11	0.30 ± 2.12
	36.21 (nd-826.48)	37.11 (nd-548.02)	0 (nd-462.60)	0 (nd-14.96)
∑ HCH	417.44 ± 323.79	377.38 ± 328.31	93.71 ± 308.38	4.81 ± 9.89
	307.55 (31.34-1274.44)	272.04 (71.21-1476.1)	7.17 (nd-1654.54)	0 (nd-40.97)
Heptachlor	122.30 ± 208.07	127.65 ± 209.43	16.04 ± 63.03	0.54 ± 2.00
	12.35 (nd-854.96)	26.01 (nd-1133.83)	0 (nd-317.74)	0 (nd-9.83)
Heptachlor epoxide	280.51 ± 297.50	214.56 ± 247.02	68.16 ± 228.38	0.12 ± 0.84
	178.52 (nd-1277.09)	158.99 (nd-1349.57)	0 (nd-1127.12)	0 (nd-5.92)
∑ Heptachlor	402.81 ± 443.20	342.21 ± 368.38	84.20 ± 278.47	0.66 ± 2.27
	251.83 (nd-1692.81)	200.86 (17.64-1695.04)	0 (nd-1171.72)	0 (nd-9.83)
Aldrin	92.03 ± 95.48	70.00 ± 91.28	23.29 ± 62.13	4.02 ± 17.49
	58.12 (nd-422.9)	41.03 (nd-557.9)	0 (nd-352.76)	0 (nd-99.63)
Dieldrin	504.56 ± 308.46	230.59 ± 194.04	70.08 ± 133.37	5.54 ± 18.01
	442.11 (144.23-1485.28)	167.44 (68.17-1213.7)	0 (nd-629.68)	0 (nd-99.48)
Endrin	1460.41 ± 943.78	1282.73 ± 662.06	544.92 ± 366.10	18.78 ± 68.57
	1289.4 (287.9-4307.41)	1150.13 (347.73-3287.99)	442.96 (nd-1633.82)	0 (nd-438.59)
Endrin aldehyde	3479.64 ± 1340.46	4363.39 ± 2166.49	901.82 ± 755.46	140.17 ± 241.72
	3502.97 (1300.28-6314.85)	3553.77 (1827.21-11361.62)	699.36 (161.36-3545.23)	45.31 (nd-1423.93)
∑ Drins	5536.65 ± 2187.87	5946.71 ± 2627.67	1540.11 ± 1030.05	168.50 ± 298.66
	4903.13 (1826.95-10095.49)	4926.13 (2513.7-14338.79)	1255.77 (355.97-5511)	55.98 (nd-1658.25)
Endosulfan I	42.85 ± 130.26	30.45 ± 79.74	60.44 ± 160.38	2.36 ± 12.65
	0 (nd-613.7)	0 (nd-336.28)	0 (nd-906.54)	0 (nd-83.06)
Endosulfan II	447.71 ± 355.78	255.28 ± 317.40	76.94 ± 238.50	nd
	342.72 (nd-1446.11)	140.12 (nd-1573.46)	0 (nd-1442.08)	
Endosulfan sulfate	876.14 ± 435.74	537.07 ± 453.26	189.04 ± 393.10	55.30 ± 98.71
	759.29 (128.84-2013.82)	359.87 (164.37-2575.62)	0 (nd-1633.56)	0 (nd-440.7)
∑ Endosulfan	1366.70 ± 785.73	822.80 ± 757.97	326.42 ± 741.74	57.66 ± 106.96
	1139.275 (134.25-3396.49)	530.57 (212.49-3913.72)	51.50 (nd-3365.13)	0 (nd-523.76)

Note: nd=not detected, n=number of samples

Table 16. *Concentrations of organochlorines (ng/g, wet weight) in Razorbill tissues. Values are presented as mean ± standard deviation, median and range (min-max) (continued).*

Concentrations of OC (ng/g wet weight) in Razorbill (*Alca torda)* tissues				
Compound	**Abdominal fat (n=48)**	**Subcutaneous fat (n=49)**	**Liver (n=50)**	**Brain (n=50)**
p,p'-DDT	1723.40 ± 675.97	1457.56 ± 672.66	174.75 ± 439.84	49.67 ± 123.55
	1636.42 (747.57-3361.94)	1379.97 (398.77-3443.95)	0 (nd-2122.6)	0 (nd-689.94)
p,p'-DDE	1370.80 ± 581.21	1064.50 ± 494.23	251.31 ± 291.10	33.38 ± 89.16
	1294.63 (421.73-3030.79)	991.45 (379.74-3000.96)	160.24 (nd-1235.09)	0 (nd-454.44)
p,p'-DDD	1036.92 ± 650.39	479.68 ± 381.23	160.03 ± 348.21	47.87 ± 141.88
	871.84 (72.6-2901.5)	334.66 (139.92-2111.99)	45.81 (nd-1633.54)	0 (nd-867.63)
∑ DDT	4131.12 ± 1595.68	3001.75 ± 1300.59	586.09 ± 1008.47	130.92 ± 323.34
	3967.29 (1560.69-7606.87)	2728.63 (1013.75-6933.54)	268.39 (nd-4176.76)	0 (nd-1537.67)
∑ OC	11854.72 ± 4517.59	10490.85 ± 4448.00	2630.53 ± 3121.63	362.54 ± 693.85
	11704.80 (4726.57-20096.20)	9632.98 (3868.32-24481.61)	1676.36 (495.01-14696.37)	96.41 (nd-3530)

Note: nd=not detected, n=number of samples

Regarding correlations between concentrations in brain and liver, a significant negative correlation was found for lindane (r=-0.359, p<0.01). More polar organochlorines such as lindane, tend to accumulate in tissues containing, proportionately, higher concentrations of phospholipids, such as brain (Kawai *et al.*, 1988), consequently, a lindane mobilization to the brain is a possible interpretation for the decrease in liver.

Finally, regarding correlations between fatty tissues, significant positive correlations were found for most compounds between abdominal and subcutaneous fat (r=0.399-0.722, p<0.01). Both fatty tissues have similar behaviour, with no significant differences in OC accumulation (p>0.692 for all OC groups). However, the organochlorine levels were highest in abdominal fat. This fact is logical since during periods of distress (such as migration), subcutaneous fat stores are metabolized firstly and these compounds are mobilized and distributed throughout the bloodstream to highly metabolically active organs, *e.g.,* the liver (Hela *et al.*, 2006).

3.2. Sex

Several studies have concluded that females have lower organochlorine concentrations than males, probably due to the transference of these compounds from mother to egg (Bustnes *et al.*, 2008; Moss *et al.*, 2009). In our study, no significant differences between sexes were found, except for ∑Drins in brain, with the highest levels in females (p=0.0449, ∑Drins in brain of female=208.57 ng/g, ∑Drins in brain of

male=41.61 ng/g), being the sex the best explanatory variable in the model of ∑Drins in brain (Table 17).

This could be due to the fact that all Razorbills analyzed were collected after the breeding season. Ólafsdóttir *et al.* (2005) reached the same conclusion in Black Guillemots.

Table 17. *Generalized Linear Models for the OC groups and tissues, and concentrations of organochlorines (ng/g, wet weight) according to age group. Values are presented as mean ± standard deviation, median and range (min-max).*

				Concentrations of OC (ng/g wet weight) by age group	
Tissue	**OC Group**	**Model**	***p***	**Young birds (n=23)**	**Old birds (n=27)**
Subcutaneous fat	∑ HCH	Age	0.0003	235.76 ± 233.39**	492.77 ± 352.42
				143.12 (71.21-1104.42)	399.43 (108.23-1476.1)
	∑ Heptachlor	Age	0.0002	189.18 ± 227.65**	466.90 ± 415.57
				104.01 (17.64-988.36)	352.48 (38.66-1695.04)
	∑ Drins	Age + Body condition	0.0004 / 0.0364	4862.19 ± 2247.05**	6830.39 ± 2619.65
				4501.18(2513.70-12759.96)	6425.91 (3545.44-14338.79)
	∑ Endosulfan	None	None	654.08 ± 453.20	960.25 ± 922.52
				483.67 (214.33-1773.62)	556.44 (212.49-3913.72)
	∑ DDT	Age	0.0000	2301.77 ± 879.24**	3572.09 ± 1321.38
				2062.55 (1013.75-4491.95)	3120.62 (1893.43-6933.54)
Abdominal fat	∑ HCH	Age	0.0409	314.13 ± 308.54*	497.78 ± 317.74
				187.4 (82.24-1274.44)	556.53 (31.34-1157.96)
	∑ Heptachlor	Age	0.0235	291.46 ± 458.06*	489.41 ± 419.37
				106.15 (nd-1692.81)	346.76 (7.35-1622.88)
	∑ Drins	Age	0.0006	4401.01 ± 1920.35**	6419.92 ± 1991.08
				3908.42 (1826.95-7928.23)	6558.45 (2378.37-10095.49)
	∑ Endosulfan	None	None	1425.18 ± 847.49	1321.21 ± 747.41
				1172.17 (316.03-3396.49)	1106.38 (134.25-2636.82)
	∑ DDT	Age	0.0086	3502.06 ± 1470.62**	4620.38 ± 1539.77
				2965.66 (1560.69-5799.58)	4355.98 (2061.24-7606.87)

*Note: Model: indicates the most influential factor (explanatory variable) in the response variable "OC group". None= Concentrations of OC group is not significantly influenced by any variable. nd=not detected. n=number of samples. Young birds=Juveniles + Immatures. Old birds=Subadults + Adults. Significant differences between young and old birds: **p<0.01,*p<0.05.*

Table 17. *Generalized Linear Models for the OC groups and tissues, and concentrations of organochlorines (ng/g, wet weight) according to age group. Values are presented as mean ± standard deviation, median and range (min-max) (continued).*

Tissue	OC Group	Model	*p*	Concentrations of OC (ng/g wet weight) by age group: Young birds (n=23)	Old birds (n=27)
Liver	∑ HCH	None	None	118.78 ± 303.64	72.34 ± 316.51
				5.95 (nd-1380.47)	7.35 (nd-1654.54)
	∑ Heptachlor	None	None	125.24 ± 330.07	49.23 ± 226.27
				0 (nd-1158.64)	0 (nd-1171.72)
	∑ Drins	None	None	1589.24 ± 961.20	1498.24 ± 1101.74
				1394.27 (451.44-3782.10)	1197.41 (355.97-5511.00)
	∑ Endosulfan	Age	0.0459	417.70 ± 788.89*	248.66 ± 704.71
				80.69 (nd-2848.81)	20.64 (nd-3365.13)
	∑ DDT	None	None	703.35 ± 1070.38	486.20 ± 961.60
				347.53 (nd-3725.81)	238.72 (nd-4176.76)
Brain	∑ HCH	None	None	6.93 ± 12.61	2.99 ± 6.49
				0 (nd-40.97)	0 (nd-20.34)
	∑ Heptachlor	Age	0.0237	1.42 ± 3.21*	nd
				0 (nd-9.83)	nd
	∑ Drins	Sex	0.0449	259.23 ± 410.44	91.21 ± 109.86
				46.21 (nd-1658.25)	65.75 (nd-349.23)
	∑ Endosulfan	None	None	72.77 ± 131.20	44.78 ± 81.36
				0 (nd-523.76)	0 (nd-309.86)
	∑ DDT	None	None	218.57 ± 440.54	56.25 ± 142.21
				0 (nd-1537.67)	0 (nd-479.2)

*Note: Model: indicates the most influential factor (explanatory variable) in the response variable "OC group". None= Concentrations of OC group is not significantly influenced by any variable. nd=not detected. n=number of samples. Young birds=Juveniles + Immatures. Old birds=Subadults + Adults. Significant differences between young and old birds: **p<0.01,*p<0.05.*

3.3. Age

Between juvenile and immature organochlorine levels, and between subadult and adult OC levels, there were no statistically significant differences. Therefore, two groups are discussed in this section: young birds (calculated by the sum of juvenile and immature organochlorine levels) and old birds (calculated by the sum of subadult and adult organochlorine levels). In general, the best explanatory variable for most OC groups was the age ($p<0.0459$) (Table 17).

Significant differences were observed between young and old birds in fatty tissues, with higher residue concentrations in old birds (Table 17). Several studies have found a positive relationship between age and organochlorine compounds in breast muscle, fat,

brain and liver samples (Borga *et al.*, 2001; Donaldson *et al.*, 1997; María-Mojica *et al.*, 2000; Vorkamp *et al.*, 2004). The highest levels in the fat tissues of old birds may reflect a longer period of exposure in these individuals and a low elimination rate of these compounds. However, this trend was not observed for brain and liver samples, where no significant differences were found between age groups for most compounds, except for ∑Endosulfan in liver (p=0.0459) and ∑Heptachlor in brain (p=0.0237) with higher levels in young than in old individuals (Table 17). In this regard, these findings do not agree with most studies. However, in Common kestrel (*Falco tinnunculus*), María-Mojica *et al.* (2000) found no significant differences in brain and liver concentrations of organochlorine insecticides among age groups (immature, juvenile and adult).

3.4. Diet and corporal condition

Organochlorine concentrations in seabirds depend, among other factors, on trophic level and feeding habits (Buckman *et al.*, 2004; Borga *et al.*, 2007). In studies with birds of different feeding habits it has been observed that the highest OC levels were found in piscivorous birds, followed by insectivores, omnivores and herbivores (Kunisue *et al.*, 2002). Changes in feeding behaviour can also influence OC exposure. For example, at certain times of the year, the Little Auk (*Alle alle*) diet is formed of zooplankton, which has lower OC concentrations than pelagic fish (Fisk *et al.*, 2001). The Alcidae diet, including that of Razorbill, consisting mainly of fish (80-90% by volume) and their migratory status are factors that increase OC exposure (Johnsgard, 1987; Kunisue *et al.*, 2002). In the present study, Razorbill tissues showed higher liver ΣDDT concentrations (586.09 ng/g, Table 16) than those of other Alcides collected by Buckman *et al.* (2004) and Borga *et al.* (2001) in other areas (Baffin Bay and Barents Sea). These authors found ΣDDT in liver of *Cepphus grylle* at 54.5 ± 11.0 ng/g wet weight and 715 ± 89 ng/g lipid weight (34.46 ng/g, wet weight), respectively.

These results can be only explained by the habitat, suggesting that the Mediterranean area presents higher OC levels than higher latitudes. The use of organochlorine compounds has been more intense in the Mediterranean area than at higher latitudes. Besides, the Mediterranean is a closed sea surrounded by highly industrialized countries, which constitutes a high-risk marine environment due to contamination by toxic compounds (Bacci, 1989; Meadows, 1992; Kuetting, 1994; Borrell *et al.*, 1997).

In this regard, several studies have determined the concentration of OC in the Barents Sea and Baffin Bay (∑HCH=0.46-1.42 ng/L, ∑DDT=0.003-0.015 ng/L) (Harner *et al.*, 1999; Strachan *et al.*, 2000), with levels below those detected in studies of the Mediterranean Sea (Lindane=200-400 ng/L, p,p'-DDT=<30 ng/L, p,p'-DDE=30-50 ng/L, p,p'-DDD=<10 ng/L) (Abbassy, 2000). Therefore, the species tested could be a good biomonitor of organochlorine concentrations achieved in this area.

Furthermore, body condition is also a determining factor in OC levels. Bustnes *et al.* (2008) found higher OC blood residues in Great black-backed gulls (*Larus marinus*) colonies where environmental conditions were poor. This variation was explained by differences in body condition among colonies. Birds with decreasing fat deposits had, in general, elevated concentrations of the lipophilic OCs in the liver. The mobilization of organochlorines from depleting fat stores and associated higher concentrations of these compounds in body organs *e.g.,* the liver (expressed on a wet-weight basis), have been reported for birds that had low lipid concentrations (Kenntner *et al.*, 2003; Malcolm *et al.*, 2003). In addition, the liver will decrease in size and chemical concentrations will rise in inverse proportion to the organ weight (Malcolm *et al.*, 2003). In our case, no significant differences were found comparing OC concentrations and body condition. This is probably due to most of Razorbills having good body condition, so population was fairly homogeneous and there were insufficient malnourished birds to observe such differences in our study.

The only exception was ∑Drins in subcutaneous fat, with age and body condition being the best explanatory variables, with p=0.0004 and p=0.0364, respectively (Table 17). This result shows higher ∑Drins concentrations in the subcutaneous fat of individuals with moderate body conditions (6,613.16 ng/g) than in individuals with good body conditions (5,519.05 ng/g). This result is not in agreement with most studies, since birds with decreasing fat deposits should have reduced concentrations of OC in fat tissues. This unexpected result could be due to isolated circumstances.

3.5. Study of the OC groups

Organochlorine concentrations in Razorbill tissues are summarized in Table 16.

Firstly, in all analyzed tissues, ΣDrins had the highest concentrations, followed by ∑DDT, ∑Endosulfan, ∑HCH and ∑Heptachlor (Table 16).

In the ΣDrins group, endrin aldehyde, endrin and dieldrin were detected in 100% fat tissues, and endrin aldehyde was the most frequently detected in liver and brain.

ΣDrins concentrations were the highest due to endrin aldehyde, which reached the highest levels of all pesticides and for all analyzed tissues (Table 16). Aldrin was detected at lower concentrations, which could be explained by a further transformation into dieldrin in humid areas, as it has been reported that certain aminoacids and humic acid in aquatic environments have the capacity to produce aldrin epoxidation under natural light (Ross and Crosby, 1985). Following ingestion of aldrin, epoxidation can occur in liver (Smith, 2004). As to endrin aldehyde, several studies (María-Mojica *et al.*, 2000; Martínez-López, 2005) also described high levels of this compound.

Regarding DDT pesticides, these were detected in 100% of the fatty tissues and, in agreement with previous studies (Takazawa *et al.*, 2004; Sakellarides *et al.*, 2006), DDE was the compound most frequently detected in brain and liver samples. As for the residue levels reached, this group had the second highest concentrations. ΣDDT concentrations followed the order of DDT>DDD>DDE in fat tissues (Table 16). Like other authors (Buckman *et al.*, 2004; Borga *et al.*, 2007), in our study the highest concentrations in liver were reached by DDE, meanwhile in brain tissues, this compound showed the lowest concentrations. The p,p'-DDT is metabolized in the liver, mainly to p,p'-DDE and p,p'-DDD (Gold and Brunk, 1982). Therefore, higher hepatic DDE concentrations could indicate the ability to convert DDT into DDE (Tanabe *et al.*, 1998). Fatty tissue ratios (p,p'DDE/p,p'DDT) of less than 1 indicate exposure to non-degraded DDT, and thus it was present in the environment despite its prohibition.

Respecting ΣEndosulfan, information in wildlife samples is limited. However, it is known that endosulfan I has a shorter half life than endosulfan II. Endosulfan is not considered a persistent compound in warm-blooded organisms, and presents an easy and fast metabolism excretion (Dorough *et al.*, 1978). It is partially biotransformed to dialcohol by hydrolysis and oxidized to endosulfan sulfate (Gorbach, 1966). In the present study, endosulfan sulfate is the most common compound found in all tissues and reached the highest concentrations (Table 16). In other studies it was also frequently detected and in high concentrations in blood samples (Martínez-López *et al.*, 2009).

In regard to hexachlorocyclohexanes, β-HCH and γ-HCH (lindane) isomers were the most frequently detected. The β-HCH isomer was also the most frequently detected in previous studies in forest raptors (Martínez-López *et al.*, 2009), being the most persistent compound (Li *et al.*, 1998). The β-HCH isomer reached the highest levels in all tissues analyzed except in brain samples, where the highest concentrations were

reached for lindane (Table 16). The β isomer is accumulated in fat tissue 10 to 30 times more than lindane and its metabolism is slower (Heeschen *et al.*, 1980). This isomer (β-HCH) has a greater stability against enzymatic degradation than the other isomers, and it has been demonstrated as having the lowest degradation ratio of the HCH group (WHO, 1992), which would explain its presence at higher concentrations. Kawai *et al.* (1988) found that the more polar organochlorines such as lindane, tend to accumulate in tissues like the brain, which contains high concentrations of phospholipids in proportion. Our results coincide with other Alcidae studies (Buckman *et al.*, 2004; Borga *et al.*, 2007), where the highest concentrations also corresponded to the β-isomer, both in liver and fat samples. However, the mean levels found for each isomer in our study were greater than those found by Buckman *et al.* (2004) at Baffin Bay and Borga *et al.* (2007) in the Barents Sea. Lindane has a short half-life in the environment compared with other compounds (2 years, approximately), and the ability of organisms to metabolize and excrete this metabolite (Blus *et al.*, 1985) is known. The difference between concentrations found in liver and brain could be due to the fast disappearance of lindane in liver of dead birds, as found experimentally by French and Jefferies (1968).

In the case of ∑Heptachlor, this OC group reached the lowest concentrations (Table 16), and both heptachlor and heptachlor epoxide were barely detected in brain and liver samples. Heptachlor epoxide concentrations were higher than heptachlor levels in all tissues except brain. When heptachlor is ingested, it is quickly metabolized to heptachlor epoxide (Melnikov, 1971), which is soluble in lipids and stored in body fat, which explains its presence at higher concentrations.

3.6. Effect assessment

Maximum residue levels for the OCs found in this study were all below the limits known to cause adverse effects in birds (Table 16). In Ardeidae species, levels of DDE in liver of 124,300 ng/g wet weight, were related to egg breakage (Pratt, 1972), while levels of 569,740 ng/g have been associated with bird death (Call *et al.*, 1976). In the present study, a mean concentration of DDE in liver of 251 ng/g was found, with a maximum concentration of 1,235 ng/g (Table 16), 100 times smaller than values found by Pratt (1972) and 460 times lower than those found by Call *et al.* (1976).

Only a few studies linking organochlorine levels in brain and clinical effects have been published. However, encephalic levels of DDTs exceeding 20,000 ng/g and dieldrin concentrations greater than 4,000 ng/g have been described as a cause of

mortality in birds (Stickel *et al.*, 1969, 1970). Both data are 13 and 40 times greater than the maximum concentrations reached in this study, respectively (Table 16).

Other studies have demonstrated the capacity of these compounds to cause behavioural abnormalities at concentrations above those detected in Razorbill tissues. Ringneck Dove (*Streptopelia risoria*) exposed to 10 ppm DDE in diet for 63 days, which showed residues of 2,900 ng/g in brain (87 times higher than the mean concentration of DDE in brain of the present study), showed alterations in reproductive behaviour (Haegale and Hudson, 1977). Similarly, Mallards (*Anas platyrhynchos*) receiving different dieldrin levels in their diet (4, 10 and 30 ppm) showed that behavioural changes were associated with a decrease in the levels of biogenic amines such as serotonin, dopamine and noradrenaline. Encephalic and hepatic levels detected in individuals exposed to 4 ppm in diet were 2,300 ng/g and 120 ng/g, respectively (Sharma *et al.*, 1976), 32 and 20 times higher than those detected in the present study (Table 16).

In a study of Cow birds (*Molothrus ater*) dosed with dieldrin, Heinz and Johnson (1981) showed that birds stopped feeding when the geometric mean of the brain residues was 6,800 ng/g, but that dead birds from the same dosing group had a mean brain dieldrin level of 16,300 ng/g, levels above those appearing in our data. Walker and Newton (1998, 1999) observed in Sparrowhawks (*Accipiter nisus*) and Kestrels (*Falco tinnunculus*) that the effect on an individual is lethal when the level of dieldrin residue in the liver exceeds 9,000 ng/g in wet weight, which is also higher than those described in our study.

During periods of distress, such as migration or breeding, the stored body fat is metabolized and the lipophilic organochlorines are mobilized and distributed through the blood stream to highly active organs, i.e., the liver, which exhibits only a modest change in lipid content (Meador *et al.*, 2002). Migration is a period of exceptional energy demand (Berthold, 1975; Blem, 1980; Alerstam, 1990) and birds deposit substantial fat stores to meet this high demand which may reach 50% of the total body mass in long-distance intercontinental migrants (Blem 1980, 1990). At worst, if we assumed that birds of this study mobilize all their fat reserves due to a stressful situation such as those mentioned above, 100% of the pesticides accumulated in fat would be released into the bloodstream. However, not all the organochlorine in the bloodstream would be distributed immediately to organs. Cole *et al.* (1970) and Lay *et al.* (1982), studied the elimination of 14C following intraperitoneal or intravenous

injection of 14C-dieldrin in male rats. These authors observed that between 70 and 80% of the total injected dose was excreted within 2 weeks post-dosing, primarily via feces. In a study on mallards dosed with 20 ppm of endrin during a large dietary experiment, Heinz and Johnson (1979) found a half-life for endrin of 3 days with 90% of residues eliminated at 33 days post-dosing. Therefore, it could be assumed that a mere 20% (approximately) of these compounds would be available for accumulation in organs. Applying this percentage to the sum of ∑Drins of both the abdominal and subcutaneous fat samples of the present study, we calculated a theoretical blood concentration of ΣDrines of approximately 2,300 ng/g, which is lower than those concentrations referred to as indicative of damage by most authors. In spite of this, a study by Sharma *et al.* (1976) showed that a similar level (2,300 ng/g) of dieldrin in mallard brains was associated with behavioral changes. However, we must keep in mind that organochlorine compounds accumulated in fat tissues would not be released at once into the bloodstream but would be gradually released over time during the stressful period. Moreover, we have evaluated the risk for the worst-case scenario, with the sum of aldrin, dieldrin, endrin and endrin aldehyde, such that if they are compared individually, the risk would be lesser. Therefore, according to the prediction made with data from other studies, the probability of risk associated to fat mobilization is low.

Moreover, we aimed to check the relationship between the presence of organochlorines and organ size. Different studies have observed an increase in hepatic weight due to organochlorine exposure (Ravinder *et al.*, 1990; Paul *et al.*, 1992; Ben Rhouma *et al.*, 2000; Kostka *et al.*, 2000; Tomiyama *et al.*, 2003, 2004), or smaller brains associated with higher DDT exposure levels (Iwaniuk *et al.*, 2006). In this study, concentrations of OC in brain and liver were related with the corresponding organ weights. Significant positive correlations between OC concentrations and liver weight were found for endosulfan sulfate ($r=0.698$, $p<0.01$), ∑Endosulfan ($r=0.524$, $p<0.05$), DDT ($r=0.463$, $p<0.05$) and DDD ($r=0.663$, $p<0.01$) in adult Razorbills. Correlations appear to be due to larger livers implying bigger birds which have eaten more food and, consequently, have greater OC accumulations, so the correlations do not seem to indicate hepatotoxic effects. We found significant negative correlations between OC levels and brain weight for lindane ($r=-0.696$, $p<0.05$), δ-HCH ($r=-0.691$, $p<0.05$), ∑HCH ($r=-0.721$, $p<0.05$) and aldrin ($r=-0.691$, $p<0.05$) in immature Razorbills. However, negative correlations do not appear to be due to a neurotoxic effect since these compounds were detected in few individuals (11-33%) and at low concentrations (Table 16).

4. Conclusions

This is the first study of organochlorine pesticides in *Alca torda* species. The OC levels in tissues were higher than those of other studies on Alcides, which is probably due to the habitat in which they were found. In spite of this, these concentrations were below the limits known to cause adverse effects in birds.

Respect to the OC concentrations, no differences were found between sexes, probably due to it being outside the Razorbills' breeding season. However, age does affect the concentration of these compounds in fatty tissues.

The p,p'DDE/p,p'DDT ratio in fatty tissues was lower than 1, which indicates exposure to non-degrading DDT, despite the ban on its use. In liver, the ratio greater than 1 indicates a greater concentration of DDE, which is explained by the liver's capacity of transforming DDT to DDE.

According to the results, the species tested could be a good biomonitor of organochlorine concentrations.

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CHAPTER IV

Razorbill (*Alca torda*) feathers as an alternative tool for evaluating exposure to organochlorine pesticides

Photo: Roger Powell. From www.arkive.org

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Abstract

The aim of this study was to explore the usefulness of feathers as a biomonitoring tool for organochlorine pesticides (OC) in a Razorbill population (*Alca torda*). Fifteen OC were analyzed in feathers, including α-, β- and δ- hexachlorocyclohexane, lindane, aldrin, dieldrin, endrin, endosulfan I and II, endosulfan sulfate, p,p'-DDT, DDD, DDE, heptachlor and its epoxide. The geometric mean concentrations observed in this study were ∑DDT 67.40 ng/g, ∑HCH 62.88 ng/g, ∑Heptachlor 61.75 ng/g, ∑Endosulfan 19.70 ng/g, and ∑Drins 10.17 ng/g. The higher OC levels found in this study compared with other studies are probably affected by the Razorbill diet and migration status. However, levels found in the feathers of the present study are related to concentrations in internal tissues below those which cause adverse reproductive and behavioral effects or other signs of organochlorine-pesticide poisoning in birds. Age does affect the concentration of OC pesticides in feathers. Thus, feathers would appear to be a promising tool for OC biomonitoring in seabirds, since it is possible to quantify OC compounds.

1. Introduction

Organochlorine pesticides (OC) have been identified as one of the main groups of environmental contaminants. OC can produce negative effects on the endocrine, immune, nervous and reproductive systems (Furness *et al.*, 1993). Although most of these compounds were banned in developed countries, they are still frequently found in tissues or fluid samples from many species (Espín *et al.*, 2010b; van Drooge *et al.*, 2008), especially in agricultural regions, such as the southwestern Mediterranean, where these contaminants were widely used in agricultural practices during the second half of the twentieth century.

Due to the sensitivity of birds to environmental changes and their position in the food chain, they can accumulate high levels of contaminants and thus are widely used in biomonitoring studies of environmental pollution (Furness *et al.*, 1993). Direct measurement of pollutants in the internal tissues of birds is the best indicator of the degree of exposure to accumulative compounds (García-Fernández *et al.*, 1997). However in recent years, for practical, ethical and conservation reasons, it has become necessary to look for alternative sampling methods to the collection of internal tissues.

Keratinized tissues have been used to measure persistent organochlorine pollutant accumulation in humans (Altshul *et al.*, 2004). These studies suggested that keratin could be use for the biomonitoring of persistent pollutants, and feathers are the main keratin-containing tissue in birds. There are few studies that have investigated the accumulation of organic contaminants in feathers, however recent papers have indicated great prospects for future pollutant-biomonitoring studies in avian populations, as has been done successfully with heavy metals (Burger, 1993).

Feathers only grow, connected to the bloodstream, for a certain period of time: during their initial development and molt period (Jaspers *et al.*, 2004). Organic pollutants can reach the feathers, during their growth period, via the blood. When they are mature, vascular connections undergo atrophy and compound concentrations remain stable (Burger and Gochfeld, 2000). Therefore, feathers can provide information on concentrations in the blood circulation at the time of their growth. No data are available in literature on the specific chemical structure and binding capacities of organic compounds to feathers. However, once incorporated, contaminants seem to be permanently retained, since it is possible to detect non-persistent pollutants (e.g., low chlorinated PCBs or α, δ and γ-hexachlorocyclohexane) in feathers over long periods of time. Even Behrooz *et al.* (2009) reported on organochlorine

contaminants in feathers from museum collections as an indication of OC and PCB concentrations from the past (1991–1996).

Studies which have analyzed organochlorine compounds in feathers have covered species from only two locations (Belgium and South-West Iran), determining PCB, DDT, HCB and HCH concentrations in both terrestrial and aquatic species (Behrooz *et al.*, 2009a,b; Dauwe *et al.*, 2005; Jaspers *et al.*, 2006a, 2007a, 2008, 2009). Therefore, the scientific literature on this subject is still scarce. Southeastern Spain is mainly an agricultural region and these contaminants have frequently been detected in biota, water and soil. The OC concentrations in the internal tissues of Razorbill (*Alca torda*) collected from this area are higher than those of other studies on Alcides, which is probably due to the habitat in which they were found (Espín *et al.*, 2010b). The Razorbill is a migratory seabird which winters in the Mediterranean area (Figure 10). Furthermore, this species is of particular interest due to their possibly declining population densities (Ribeiro *et al.*, 2009).

The aim of this study was to explore the usefulness of feathers as a biomonitoring tool for fifteen organochlorine pesticides in a Razorbill population. Moreover, additional factors such as the age and sex of the birds were examined in order to evaluate their influence on the concentration of pollutants in feathers. The present work provides the first available data on concentrations of organochlorine compound in Razorbill feathers.

2. Material and methods

2.1. Species and sample size

Primary wing feathers (p10) (n = 50) were collected from fifty Razorbills, which were found dead along the Western Mediterranean coastline in the East of Spain (La Marina, Elche, Alicante), having drowned in fishing nets in February of 2007 (Figure 10). They were collected by the staff of “Santa Faz” Wildlife Recovery Center (Alicante, Spain), and taken to the laboratory under refrigerated conditions.

The criteria used to establish age, sex and physical conditions are reported in Espín *et al.* (2010). Briefly, three criteria were used to age the Razorbills: bill development (Anker-Nilssen *et al.*, 1998), size and appearance of the gonads (Camphuysen and Van Franeker, 2007; van Franeker, 2004), and presence and size of the bursa Fabricii, one of the glands in the endocrine system of birds (Camphuysen and Van Franeker, 2007). Generally, the birds were classified into four age groups according to the structure of the bill. When no white bands or grooves on the bill are

present they are considered juveniles; immature birds have the white band but no grooves; subadult birds have a single groove in front of the white band while adults have two grooves in front of the white band. Internally, males with a testis index (length x width) greater than 30 mm^2 were classified as adults, and those with a testis index lower than 10 mm^2 were classified as juveniles. For females, the ovary's' appearance and the development of the oviduct was scored as 1 = ovary has no structure (amorphous) and the oviduct is thin and straight (juvenile), 2 = ovary with tiny visible follicles and a thicker and straighter (immature) oviduct, 3 = ovary has visible follicles and the oviduct is thicker still and slightly twisted (subadult), or 4 = ovary has clearly visible follicles of different sizes and the oviduct is swollen and twisted (adult). Finally, all birds were examined for the presence or absence of the "bursa of Fabricius", and when present it was measured. Juvenile seabirds have a large bursa, immature ones may have a much reduced bursa, while subadults and adults normally have no trace of a bursa. Hence, the presence of a large bursa in combination with non-developed gonads is a good indication that the bird in question is a juvenile.

The physical condition was defined according to Van Franeker (2004). Three characters (subcutaneous fat, abdominal fat and pectoral muscle) were scored on a four-point scale (0-3) and the overall condition index was calculated based on the sum of scores for fat stores and breast muscles (0-9): score 0-1 = mortally emaciated; 2-3 = critically emaciated; 4-6 = moderate body condition; and 7-9 = good body condition.

The ratio of female:male was 3:1. With regard to age, the majority of the birds were adult (40%), followed by juvenile (28%), immature (18%) and subadult (14%). Relating sex with age, for females 47% were adult, 11% subadult, 16% immature and 26% juvenile. In males, 17% were adult, 25% subadult, 25% immature and 33% juvenile. As regards physical condition, only 4% of Razorbills analyzed had serious nutrition problems, while 40% had a moderate body condition and 56% a good body condition.

2.2. Organochlorine analysis

Feather samples were analyzed for a series of organochlorine pollutants including hexachlorocyclohexane (HCH) isomers (α-HCH, β-HCH, γ-HCH or lindane and δ-HCH), endosulfan I, endosulfan II, endosulfan sulfate, aldrin, dieldrin, endrin, dichlorodiphenyltrichloroethane (p,p'-DDT), dichlorodiphenyldichloroethane (p,p'-DDD), dichlorodiphenyldichloroethylene (p,p'-DDE), heptachlor and heptachlor-epoxide.

All reagents used for the analysis were of a trace analysis grade. Hexane, acetone, petroleum ether, diethyl ether were supplied by Lab-scan Analytical Sciences and anhydrous sodium sulfate by Merck Co. (Darmstadt). Sep-Pak® Classic and Florisil® cartridges were supplied by Waters®. The pesticide standard (EPA Pesticide Mix 48858 dissolved in methanol:methylene chloride 98:2) was procured from Supelco (USA). Prior to analytical procedures, all glassware was rinsed several times with distilled water, hexane and acetone.

The samples were analyzed according to the method described by Espín *et al.* (2010a). In order to remove external contamination from the feather surface, prior to the analytical determination, a brief washing process was performed with tap water, distilled water and Milli-Q water, and two pairs of tweezers were used to separate the barbs of the vane. Thus, efficient washing within the barbs was assured (Jaspers *et al.*, 2007b). The feathers were subsequently dried at room temperature.

The feathers (0.2 g) were weighed and incubated overnight at 37ºC in HCl and hexane:acetone (2:1, v/v). Extraction was performed with hexane:acetone (3:1, v/v). The samples were homogenized, centrifuged and filtered using anhydrous sodium sulfate and then the solvent collected was evaporated until dryness. After redisolution in 5 ml hexane, samples were cleaned up via Florisil column chromatography (Sep-Pak, Waters®), activated with 2 ml of hexane, using a petroleum ether:diethyl ether mix (21:4, v:v) as the elution solvent. The solvent collected was evaporated until dryness.

The final volume was adjusted to 1 ml with n-hexane. One microlitre was injected into a gas chromatograph with electron capture (GC-ECD 17 Shimadzu) for the detection of OC. The SPB-608 capillary column (Supelco®) was 30 m long, 0.25 mm i.d. with a 0.25 µm film thickness, specifically recommended by the manufacturer (Supelco, 1997) for the 15 organochlorine pesticides studied and included in the US Environmental Protection Agency (EPA) Method 608 (US EPA, 1984). Helium was used as the carrier gas. The injector was set at the splitless mode; the injector temperature was 290ºC. The column program was: 2 min at 50ºC, from 50 to 150ºC at 40ºC/min, 2 min at 150ºC, from 150 to 290ºC at 81ºC/min and 10 min at 290ºC. The detector temperature was 330ºC and the make-up gas was nitrogen.

Identification and quantification was based on an external standard. The standard solution was prepared by dissolving the reference substances in n-hexane (1:25) at the following concentrations: 10 µg/ml for α-HCH, β-HCH, δ-HCH, lindane, heptachlor, heptachlor epoxide and aldrin; 20 µg/ml for endosulfan I, endosulfan II, DDE, dieldrin

and endrin; and 60 µg/ml for DDD, DDT and endosulfan sulfate. Detection limits ranged from 0.03 to 0.41 ng/g. Methoxychlor (1 mg/ml) was used as an internal standard, and was supplied by PolyScience®.

A volume of 10 µl was added to samples and standards in order to compare the results and check for repeatability in chromatograms. Mean recoveries in spiked samples ranged from 46.13% to 146.05%. A blank with hexane was incorporated every five samples, and samples were analyzed in duplicate. Concentrations of OC were expressed as ng/g.

2.3. Statistical analysis

All analyses were carried out using the SPSS v.15.0 statistical package. Reported OC values provide the geometric mean; arithmetic mean ± standard deviation, median and range. Since the concentrations of organochlorine pesticides were not distributed normally, the data was log-transformed. Under log-normality, it is suitable the use of geometric mean (Smothers *et al.*, 1999), however, because several authors describe their results using the arithmetic mean, we have provided, in this study, both geometric and arithmetic means for comparisons. Generalized Linear Models (GLM) were used to analyze the concentrations of organochlorine pesticides. The OC compound was the response variable. We used GLMs with an identity function and Gaussian errors. The two explanatory variables considered were age and sex. We followed a forward stepwise procedure, testing the statistical significance of each explanatory variable (F-test) and retaining those that contributed to the largest change in deviation from the null model until all the variables with a significant effect at $p<0.05$ had been included in the model.

The total concentrations of organochlorine pesticides (ΣOC) were calculated as the sum of individual compound concentrations. Five groups of OC compounds were considered. The group of DDT and metabolites (ΣDDT) represented the sum of p,p-DDE, p,p-DDD and p,p-DDT, the group of hexaclorocyclohexanes (ΣHCH) included α, β, δ and γ-isomers, the group of heptachlor (ΣHeptachlor) was formed by heptachlor and its epoxide**,** ΣDrins involved the sum of endrin, aldrin and dieldrin, and finally ΣEndosulfan included endosulfan and endosulfan sulfate. Spearman's correlation coefficient was used in order to calculate correlations between variables. The level of significance for these tests was set at $\alpha=0.05$.

3. Results and discussion

3.1. OC levels in feathers

Organochlorine concentrations in *A. torda* tissues are summarized in Table 18. All of the organochlorine pesticides examined were detected in Razorbill feathers. ΣDDT had the highest geometric mean concentrations, followed by ΣHCH, ΣHeptachlor, ΣEndosulfan and ΣDrins (Table 18). The compounds that reached the highest geometric mean levels were DDE, heptachlor epoxide, lindane and heptachlor, respectively. Moreover, DDE was one of the compounds most frequently detected, a finding similar to that in brain and liver tissues from the same individuals (Espín *et al.*, 2010b). As in research by Dauwe *et al.* (2005) into persistent organic pollutants in great tit feathers (*Parus major*), the ΣDDT-group concentrations followed the order of DDE>DDD>DDT (Table 18). The substance p,p'-DDT is metabolized by the liver, mainly to p,p'-DDE, for which p,p'-DDD is an intermediate (Gold and Brunk, 1982). Moreover, DDE can be found in the environment, as a result of aerobic degradation, abiotic dehydrochlorination and the photochemical decomposition of DDT. It has also appeared as a contaminant in commercial-grade DDT (Thomas *et al.*, 2008). Therefore, higher DDE concentrations in feathers could be explained by the ability of the organism to convert DDT into DDE (Tanabe *et al.*, 1998), as well as direct DDE inputs from the environment and prey and/or DDE transfer to feathers.

The hexachlorocyclohexane isomer γ-HCH (lindane) has a short half-life in the environment compared with other organochlorine compounds, and is metabolized and excreted by organisms relatively rapidly (Blus *et al.*, 1985). Therefore, the higher levels of γ-HCH in feathers probably reflect an exposure to lindane during feather growth or a release into the bloodstream by fat mobilization during feather growth. Moreover, this result shows the capacity of feathers as an excretion route for this compound. Behrooz *et al.* (2009a,b) studied organic compounds in various bird species from Iran, and also found that lindane was the most predominant HCH isomer in feathers, which they felt was due to recent exposure of birds to γ-HCH.

***Table 18.** Concentrations of organochlorine pesticides (ng/g) in feathers of Alca torda and Generalized Linear Models for organochlorine compounds.*

Concentrations of OC (ng/g) in feathers of *Alca torda*

Organochlorine Compound	Total (n=50)	Detection rate (%)	Young birds (n=23)	Old birds (n=27)	Model	*p*
α-HCH	5.46; 52.25±81.93 0 (nd-293.93)	40	10.44; 49.26±59.00** 33.20 (nd-203.43)	2.97; 54.79±98.43 0 (nd-293.93)	Age	0.048
Lindane	10.46; 101.65±127.58 0 (nd-401.71)	46	0.48; 9.37±35.32* 0 (nd-163.71)	64.43; 180.26±125.18 191.99 (nd-401.71)	Age	0.000
β-HCH	1.14; 10.62±24.81 0 (nd-103.28)	20	1.47; 10.17±21.03 0 (nd-71.77)	0.89; 11.01±28.03 0 (nd-103.28)	None	None
δ-HCH	0.59; 10.47±32.46 0 (nd-130.88)	10	nd**	1.36; 19.38±42.50 0 (nd-130.88)	Age	0.022
∑ HCH	62.88; 174.99±177.87 101.45 (nd-683.56)	84	30.57; 68.79±77.62 59.49 (nd-367.15)	115.45; 265.45±189.87 272.92 (nd-683.56)	None	None
Heptachlor	9.43; 108.11±156.95 0 (nd-542.37)	44	32.35; 163.30±163.82* 145.51 (nd-500.65)	2.88; 61.09±136.84 0 (nd-542.37)	Age	0.002
Heptachlor epoxide	16.43; 88.16±96.48 59.45 (nd-332.14)	62	5.33; 46.16±82.14* 0 (nd-332.14)	40.30; 123.94±94.58 128.58 (nd-273.57)	Age	0.010
∑ Heptachlor	61.75; 196.27±174.92 174.27 (nd-624.98)	80	62.65; 209.46±192.79 173.99 (nd-599.65)	61.00; 185.04±161.03 187.15 (nd-624.98)	None	None
Aldrin	6.06; 47.52±70.99 0 (nd-272.74)	44	21.57; 61.40±55.85* 43.81 (nd-174.01)	1.63; 35.69±80.88 0 (nd-272.74)	Age	0.000
Dieldrin	0.44; 8.32±41.44 0 (nd-281.31)	10	0.50; 4.63±18.07 0 (nd-86.47)	0.40; 11.46±54.20 0 (nd-281.31)	None	None
Endrin	0.47; 5.30±18.46 0 (nd-94.38)	10	0.66; 6.69±18.94 0 (nd-77.43)	0.32; 4.12±18.32 0 (nd-94.38)	None	None
∑ Drins	10.17; 61.14±79.60 23.05 (nd-281.31)	54	33.37; 72.73±57.71* 78.95 (nd-210.09)	3.29; 51.27±94.36 0 (nd-281.31)	Age	0.000
Endosulfan I	1.17; 9.62±23.01 0 (nd-86.78)	24	1.61; 9.27±20.80 0 (nd-86.78)	0.86; 9.91±25.13 0 (nd-83.09)	None	None
Endosulfan II	2.58; 22.58±41.99 0 (nd-155.38)	34	nd*	9.59; 41.81±49.89 30.90 (nd-155.38)	Age	0.000
Endosulfan sulfate	5.61; 82.33±134.29 0 (nd-451.84)	36	6.03; 74.49±131.92 0 (nd-451.84)	5.27; 88.99±138.42 0 (nd-355.55)	None	None
∑ Endosulfan	19.70; 114.52±154.44 54.08 (nd-500.02)	66	11.19; 83.76±135.48 16.86 (nd-451.84)	31.50; 140.71±166.96 63.90 (nd-500.02)	None	None
p.p'-DDT	2.50; 90.60±269.01 0 (nd-1457.65)	24	7.25; 94.59±193.31* 0 (nd-678.48)	0.68; 87.19±323.62 0 (nd-1457.65)	Age	0.003
p.p'-DDE	19.45; 132.21±179.21 58.36 (nd-974.70)	60	1.27; 20.17±67.48* 0 (nd-315.71)	131.95; 227.65±190.03 213.56 (nd-974.70)	Age	0.000
p.p'-DDD	3.58; 100.76±193.68 0 (nd-821.13)	26	0.64; 40.31±171.63* 0 (nd-821.13)	9.99; 152.25±199.45 0 (nd-566.73)	Age	0.005
∑ DDT	67.40; 323.56±372.68 175.85 (nd-1596.24)	76	12.92; 155.07±288.22* 9.23 (nd-1136.85)	264.45; 467.09±380.73 423.13 (nd-1596.24)	Age	0.000
∑ Cyclodienes	208.08; 371.93±286.60 330.55 (nd-1103.22)	96	178.98; 365.96±315.88 314.69 (nd-1103.24)	236.55; 377.02±265.17 353.77 (nd-878.68)	None	None
∑ OC	583.46; 870.48±614.48 796.10 (nd-2104.05)	98	444.24; 589.82±442.02 497.84 (107.84-1909.77)	735.90; 1109.56±645.94 1129.72 (nd-2104.05)	None	None

*Note: Values are presented as geometric mean; arithmetic mean ± standard deviation, median and range (min–max).Model: indicates the most influential factor (explanatory variable) in the response variable "Organochlorine compound". None = concentration of organochlorine compound is not significantly influenced by any variable. nd=not detected. n=number of samples. Young birds = juveniles + immatures. Old birds = subadults + adults. Significant differences between young and old birds: *p<0.01, **p<0.05.*

Levels found in Razorbill feathers are much higher than those found in the feathers of both aquatic and terrestrial bird species for ΣHCH and ΣDDT (Behrooz *et al.*, 2009a,b; Dauwe *et al.*, 2005; Jaspers *et al.*, 2006b, 2007a,b, 2008, 2009), except an individual of European scops owl (*Otus scops*) (∑HCH = 212 ng/g; ∑DDT = 295 ng/g) and eleven individuals of Sparrowhawks (*Accipiter nisus*) (∑DDT arithmetic mean = 230 ng/g) which have similar levels (Behrooz *et al.*, 2009b; Jaspers *et al.*, 2007a). The Razorbill diet, consisting mainly of fish (80–90% volume), and their migratory status are factors that increase OC exposure (Johnsgard, 1987; Kunisue *et al.*, 2002). In studies of birds with different feeding habits, it has been observed that the highest OC levels were found in piscivorous birds (Kunisue *et al.*, 2002). In the same way, the feeding habits of carnivorous species (small mammals or small birds) such as the European scops owl and sparrowhawks have a high impact on the contaminant levels in predatory birds (Jaspers *et al.*, 2006b; van Drooge *et al.*, 2008).

Migratory habits must also be considered when evaluating the exposure and distribution of contaminants in the body. For instance, the European scops owl migrates to the north of Iran and therefore may incorporate a significant contaminant load from these areas (Mansoori, 1999). The bulk of the Razorbill world population, perhaps 60–70%, breeds in Iceland. This species also breeds in Greenland, the British Isles, Norway, the Baltic Sea and Barents Sea regions, among others (Lavers *et al.*, 2009). Regarding winter range, Razorbills from Britain and Ireland mainly overwinter from the Irish Sea south to the Bay of Biscay and Portugal, although some move as far as Morocco, and into the west Mediterranean Sea (Humple *et al.*, 2007; Lloyd, 1974; Mead, 1974). The use of organochlorine insecticides has been more intense in the Mediterranean area than at higher latitudes (Sánchez-Gelabert *et al.*, 2008). Besides, the Mediterranean is a closed sea surrounded by highly industrialized countries, which constitutes a high-risk marine environment due to contamination by toxic compounds (Kuetting, 1994).

However, interpreting feather concentrations is complex, mainly due to the molt strategy. Specifically, Razorbills have a complete post-nuptial molt (also called prebasic molt) in August-September or October, prior to migration, involving all contour and flight feathers (Bédard, 1985). Primary and secondary feathers are replaced synchronously or nearly so, resulting in a period of flightlessness; primaries may be shed in two concurrent waves that move rapidly both distally and proximally from a central point on the wing (Thompson *et al.*, 1998). In February-May there is a pre-

nuptial molt (also called prealternate molt) that includes most, if not all feathers of the head and throat but none of the back, rump, or wings (Lavers *et al.*, 2009).

In periods of distress, such as migration, breeding and molt, the stored body fat is metabolized and the lipophilic organochlorines are mobilized and distributed throughout the blood stream (Perkins and Barclay, 1997). As the blood OC concentrations are transported into the growing feathers during molt, the OC concentrations detected in primary feathers were deposited in their Northern nesting location during the post-nuptial molt, and thus, feather concentrations would indicate both OC exposure from their Northern ranges and OC incorporated during the previous winter on the Mediterranean coast albeit not yet excreted. It is feasible to assume that not all the organochlorine body burden would be mobilized during the migration. Moreover, a percentage of the OC mobilized will not be excreted and would be available for accumulation again. In this sense, Cole *et al.* (1970) and Lay *et al.* (1982) studying the elimination of 14C following intraperitoneal or intravenous injection of 14C-dieldrin in male rats, observed that between 70% and 80% of the total injected dose was excreted within 2 weeks post-dosing, and therefore, 20-30% approximately would be accumulated again.

Nonetheless, there is little data available on the relationship between organochlorine pesticides in feathers and their effects. Behrooz *et al.* (2009b) concluded that the average concentrations of p,p′-DDE in their study ranged from 0.5 to 248 ng/g feather in the range of bird species they investigated, which would relate to a maximum concentration of 24500 ng/g muscle, this being only slightly lower than concentrations at which side-effects have been observed. Levels found in the present study are related to maximum concentrations in liver from Espín *et al.* (2010b) of 1654 ng/g for ∑HCH, 1172 ng/g for ∑Heptachlor, 1966 ng/g for ∑Endrin, 3365 ng/g for ∑Endosulfan and 4177 ng/g for ∑DDT. These levels of exposure rarely result in adverse reproductive effects, behavioral abnormalities, or other signs of organochlorine pesticide poisoning in birds (Pratt, 1972; Sharma *et al.*, 1976). Therefore, considering that this species undergoes a complete molt annually, we can assume there is no risk of adverse effects associated with the OC feather levels found in the present study.

3.2. Age and sex

Although birds were classified into four age groups, OC levels showed no statistically significant differences ($p>0.05$ for most compounds) between juvenile and immature individuals, and between subadult and adult ones. Therefore, two age groups

are discussed in this section, young birds (calculated as the sum of juvenile and immature OC levels) and old birds (calculated as the sum of subadult and adult OC levels). The best explanatory variable for most OC compounds was age ($p < 0.048$) (Table 18). Significant differences for some compounds were observed between young and old birds in feathers, with the highest residue concentrations residing in old birds for some compounds (Table 18). Espín *et al.* (2010b) also found the highest levels in fatty tissues of old birds, which may reflect a longer period of exposure in these individuals. Significant positive correlations were found between age and the levels of some compounds in feathers (r=0.36-0.78, p=0.000-0.009) for lindane, heptachlor epoxide, endosulfan II, DDE, DDD and δ-HCH. However, heptachlor, aldrin and DDT showed higher levels in young rather than old bird feathers, with significant negative correlations between age and levels of aldrin ($r = -0.48$, $p = 0.000$) and DDT ($r = -0.43$, $p = 0.002$). Precisely, heptachlor and DDT are compounds with a high bioconcentration factor ($\log K_b = 3.83$ and 6.11, respectively), thus they have a higher affinity for fatty tissues, and are therefore excreted with more difficulty than other compounds.

On the other hand, several studies have concluded that females have lower organochlorine concentrations than males in blood and internal tissues, probably due to the transference of these compounds from mother to egg (Bustnes *et al.*, 2008). Dauwe *et al.* (2005) found no differences between sexes in feathers from great tits. These authors explain their results as due to the small sample size (n=16). Nor were any significant differences found in the present study, as did occur for the internal tissues of this population (Espín *et al.*, 2010b). Sex as an explanatory variable in the model could not explain OC compound concentrations, whether alone or in combination with age (Table 18). A likely explanation for this is that the Razorbills analyzed were collected after the breeding season.

4. Conclusions

This is the first study of organochlorine pesticides in *A. torda* feathers. The OC concentrations in Razorbill feathers were higher than those observed in the feathers of other bird species from Belgium or Iran, which is probably influenced by the Razorbill diet and migration status. In spite of this, levels found in the feathers of the present study are related to concentrations in internal tissues below those that provoke adverse reproductive and behavioral effects, and other signs of organochlorine pesticide poisoning in birds.

Age does affect the concentration of OC pesticides in feathers. However, no differences were found between OC concentrations by gender. Feathers appear to be a promising tool for OC biomonitoring in seabirds since it is possible to quantify OC compounds.

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CHAPTER V

Razorbills (*Alca torda*) as bioindicators of mercury pollution in the southwestern Mediterranean

Photo: Wild Wonders of Europe. From www.arkive.org

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Abstract

Levels of mercury (Hg) were analyzed in the tissues of 50 Razorbills (*Alca torda*), from the Mediterranean area, which had drowned in fishing nets. The mercury distribution pattern in tissues was similar to those of other studies (liver>feather vane>kidney>muscle>brain>feather shaft), with mercury concentrations of 2.85 ± 0.90, 2.66 ± 1.60, 2.23 ± 0.87, 1.54 ± 0.54, 1.48 ± 0.54 and 1.30 ± 0.76 mg/Kg (dry weight), respectively. It could be considered that Razorbills in the Southwestern Mediterranean were chronically exposed to relatively low levels of MeHg, probably below 0.5 ppm, via dietary intake. We have proposed prediction equations for brain and kidney Hg concentrations using feather shafts as non-invasive samples. This work provides a solid understanding of Razorbill Hg exposure both in their wintering and breeding grounds, and shows that this species can be useful for assessing marine environmental health in the Mediterranean area.

1. Introduction

Mercury (Hg) is a persistent, toxic and non-essential heavy metal of special concern due to its bioaccumulation and biomagnification along the food chain, as well as its association with negative effects such as immune system suppression, compromised cardiovascular health, and neurological and reproductive impacts on both humans and animals (Evans *et al.*, 1982; Finley and Stendell, 1978). Mining is an important anthropogenic source of Hg with cinnabar being the main mercury-containing ore. The world's most abundant deposits are located in the Mediterranean region, and mercury from sites such as Almaden (Spain), Idrja (Slovenia) and Monte Amiata (Italy) has been exploited since ancient times (Berg and Barrett, 2004).

Aquatic environments are especially at high risk of Hg contamination since much of the atmospheric deposition and all industrial water-runoff culminates in these ecosystems (Zamani-Ahmadmahmoodi *et al.*, 2010). Furthermore, inorganic mercury is efficiently biotransformed into organic forms (methylmercury, MeHg) and accumulated in biota (Bryan, 1979). The Mediterranean is a closed sea surrounded by highly industrialized countries, which constitutes a high-risk marine environment due to contamination by toxic compounds (Bacci, 1989) (Figure 11). Seabirds are often used as biomonitors of marine ecosystem health (Espín *et al.*, 2010, 2012) and have frequently been used to monitor Hg in marine environments via both internal tissues and non-destructive sources of tissue samples such as feathers, eggs and blood (Bond and Diamond, 2009a,b; Kim *et al.*, 1998; Kojadinovic *et al.*, 2007b). These species are advantageous as sentinels of the health of marine ecosystems since they are large, wide-ranging, conspicuous, abundant, long-lived, as well as easily observed and monitored. Furthermore, they are of interest to the public, and are often at the top of the food chain (Burger and Gochfeld, 2004). Since many species of seabirds return to the same nest and colony sites each year, and travel over substantial distances to obtain food, these birds accumulate contamination over time and space (Burger, 1993; Walsh, 1990).

The Razorbill (*Alca torda*) is an Alcidae seabird species that breeds in high latitudes, mainly in Iceland (probably 60–70% of the world population). This species also breeds in Greenland, the British Isles, Norway, the Baltic Sea and Barents Sea regions, among others. Regarding their winter range, Razorbills from Britain and Ireland mainly overwinter from the Irish Sea south to the Bay of Biscay and Portugal, although some migrate as far as Morocco and into the west Mediterranean Sea (Lavers

et al., 2009). Migration to industrialized latitudes may result in the accumulation of contaminants in the body of these specimens.

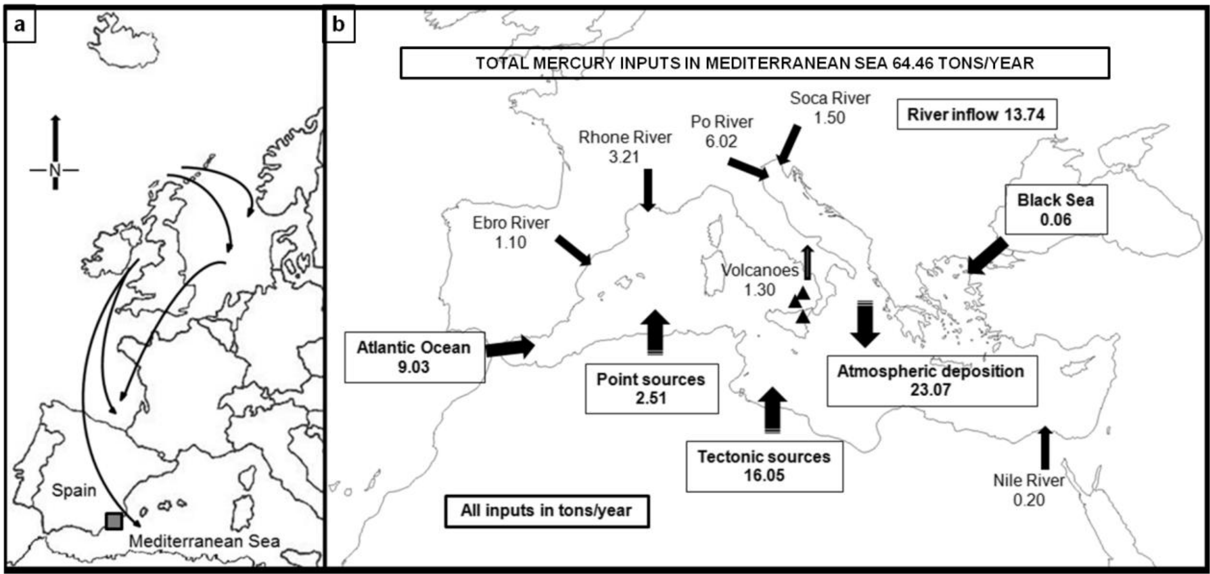

Figure 11. *(a) Map showing Razorbill migration routes to Spain (Brown, 1985) and the geographical location of the Razorbill mortality incident. (b) Mercury inputs (tons/year, based on data from 2005) in Mediterranean Sea (based on Rajar et al., 2007).*

Different reasons lead us to consider that the Razorbill could be a valuable sentinel species for mercury pollution in the southwestern Mediterranean. The Razorbill is a common wintering species in the studied area. In Alicante (Spain), 824 Razorbills were recorded in 2007 (AHSA, 2007) from November to March, coinciding with the wintering of this species on the Spanish coastline. Furthermore, it is generally assumed that seabirds can tolerate higher Hg concentrations, considering the range of processes which may contribute to Hg detoxification, such as the moulting process (Honda *et al.*, 1986), induction of synthesis and binding to metallothionein (MT) (Scheuhammer, 1987), as well as demethylation and the formation of Se–Hg complexes (Scheuhammer *et al.*, 2008; Thompson, 1996). On the other hand, this diving species has a specific diet (mainly fish, 80-90% by volume), and may be of great interest since their regular prey are different from those of other more commonly monitored species such as gulls. Moreover, top predators have higher Hg levels and are at a higher risk of suffering the consequences of its accumulation. However, there are few environmental contaminant studies on Razorbills (Bond and Diamond, 2009a,b; Espín *et al.*, 2010, 2012 (see Chapters III and IV); Ribeiro *et al.*, 2009; Savinov *et al.*, 2003).

Mediterranean Sea constitutes a high-risk marine environment due to its semi-closed condition and being surrounded by industrialized countries. Moreover, data on mercury concentrations in seabirds in this area are scant. Therefore, the aim of this study was to provide data on mercury residue concentrations in Razorbills from the Mediterranean area which had drowned in fishing nets, in order to assess Hg exposure, evaluate the influence of age and sex of the specimens analyzed, and to develop prediction equations for estimating Hg levels in target organs (brain or kidney) using the Hg concentrations in feathers.

2. Material and methods

2.1. Species and sample size

Fifty Razorbills were used in this study. These animals were found dead along the Occidental Mediterranean coastline in the southeast of Spain (La Marina, Elche, Alicante) at 38°N and 0°W, having drowned in fishing nets in February of 2007 (Figure 11). They were collected by the staff of ‘‘Santa Faz” Wildlife Recovery Center (Alicante, Spain), and taken to the laboratory under refrigerated conditions.

A total of 300 samples of liver (n=50), brain (n=50), kidney (n=50), muscle (n=50) and the 9th primary wing feathers (p9) (numbering outwards) separated in vane (n=50) and shaft (n=50) were collected via necropsy. Following this, internal tissues were packed in Eppendorf tubes and the feathers placed in sterile plastic bags and frozen until analysis. During the necropsy, certain parameters such as mass measurements (g), organ weights (g) and bill development were determined for all seabirds and sex, age and physical condition were registered.

Three criteria were used to age the Razorbills: bill development (Anker-Nilssen *et al.*, 1998), size and appearance of the gonads (Camphuysen and Franeker, 2007; Franeker, 2004), and presence and size of the bursa Fabricii, one of the glands in the endocrine system of birds (Camphuysen and Franeker, 2007). If all criteria would not agree with each other, size and appearance of the gonads take priority, followed by bill development (for further details see Espín *et al.*, 2010 (Chapter III).

The ratio of female:male was 3:1. In respect to age, the majority of birds were adult (40%), followed by juvenile (28%), immature (18%) and subadult (14%). Relating sex with age, for females 47% were adult, 11% subadult, 16% immature and 26% juvenile. In males, 17% were adult, 25% subadult, 25% immature and 33% juvenile.

Furthermore, mercury body burden may be affected by physical condition. According to Franeker (2004) three characters (subcutaneous fat, abdominal fat and pectoral muscle) were scored on a four-point scale (0-3) and the overall condition index was calculated based on the sum of scores for fat stores and breast muscles (0-9): score 0-1 = mortally emaciated; 2-3 = critically emaciated; 4-6 = moderate body condition; and 7-9 = good body condition. Only 4% of Razorbills analyzed had serious nutritional problems, while 40% had a moderate body condition and 56% a good body condition.

2.2. Mercury analysis

Total mercury was analyzed in a Milestone DMA-80 direct mercury analyzer by atomic absorption spectrophotometry with a detection limit of 0.005 ng. Samples (0.1 g wet weight for internal tissues and 0.01 g dry weight for vane and shaft, approximately) were loaded in a nickel boat and analyzed. The calibration curve was calculated with eleven points (in duplicate) from 0 to 1004 ng of mercury.

The precision and accuracy of the method were tested using certified reference material (CRM) (Mercury Standard for AAS, Fluka, 1000 mg/L Hg in 12% nitric acid, prepared with high purity Hg metal, HNO_3TraceSELECT® and water TraceSELECT®Ultra). Recovery of total mercury from seven replicates of CRM diluted to 1 ppm was 98.14±3.52% (mean±standard deviation). The coefficient of variation for repeatability was 3.58%.

In order to remove external contamination from the surface of feathers, which otherwise could alter the results for mercury, a washing process was performed prior to analytical determination using tap water, distilled water, Milli-Q water and acetone, while two pairs of tweezers were used to separate the barbs from the vane. This way, efficient washing between the barbs was assured (Jaspers *et al.*, 2007). The feathers were subsequently dried at room temperature overnight.

Mercury concentrations were referred to dry weight (dw). Internal tissues were dried over 24h at 80 ºC, and concentrations in dry weight were calculated taking into account the water content of each sample. The mean percentage for water content was 68.76% ± 2.89 for liver, 71.46% ± 1.98 for kidney, 72.43% ± 1.95 for muscle and 78.56% ± 1.14 for brain.

2.3. Statistical analysis

All analyses were carried out using the SPSS v.15.0 statistical package. Reported Hg values represent the mean ± standard deviation. The data were tested for normality using a Kolmogorov–Smirnov test, and mercury concentrations were distributed normally. Generalized Linear Models (GLMs) were used to analyze the concentrations of mercury in each tissue. We used GLMs with an identity function and Gaussian errors. The Hg concentration in each tissue was the response variable. The explanatory variables considered were age and sex. Furthermore, to assess the use of a non-invasive sample (feather) for predicting Hg levels in the target organ (brain or kidney), GLMs were used with brain Hg or kidney Hg as dependent variables, and feather shaft Hg as a covariate, with sex and age as factors. We followed a backward stepwise procedure to select the final models, excluding the predictor variables when they had no significant effects. Significant differences in Hg concentrations between tissues were tested with Tukey tests. Pearson's correlation coefficient was used in order to calculate correlations between variables. The level of significance for these tests was set at α=0.05.

3. Results and discussion

3.1. Mercury levels in Razorbill and diet habits

Mercury concentrations in Razorbill tissues are detailed in Table 19, and the scarce information regarding this metal in other Alcidae studies is summarized in Table 20. In general, mercury levels in liver and muscle (Table 19) were higher than those found in alcids from other areas such as Barents Sea, Svalbard or Greenland (Table 20), except for Hg concentrations in Razorbills from Portugal with higher levels, and for Hg concentrations in alcids from Aleutian archipelago of Alaska and Galicia with similar concentrations (Pérez-López *et al.*, 2006; Ribeiro *et al.*, 2009; Ricca *et al.*, 2008). No information on Hg levels in the brain of alcids has been previously published, however, mercury levels in brain were similar to concentrations in goosander (*Mergus merganser*) wintering in Poland (1.3 mg/Kg dw) (Kalisińska *et al.*, 2010). Regarding the mercury levels in whole feathers, concentrations found in the present study were also higher than those described in other alcids (Table 20).

Table 19. *Concentrations of mercury (mg/Kg dry weight) in Razorbill tissues and Generalized Linear Models. Values are presented as mean ± standard deviation.*

Mercury concentrations in tissues of *Alca torda* (mg/Kg dry weight)									
Tissue	**Total Hg (n=50)**	**Juvenile (n=14)**	**Immature (n=9)**	**Subadult (n=7)**	**Adult (n=20)**	**Female (n=38)**	**Male (n=12)**	**Model**	***p***
Liver	**2.85** ± 0.90[a,b,c,d]	**2.68** ± 0.86	**2.54** ± 0.63	**3.18** ± 1.29	**3.00** ± 0.87	**2.79** ± 0.84	**3.04** ± 1.08	**None**	**None**
Kidney	**2.23** ± 0.87[a,e,f,g]	**1.8** ± 0.65	**1.94** ± 0.79	**2.44** ± 1.22	**2.54** ± 0.82	**2.21** ± 0.91	**2.30** ± 0.77	**Age**	**0.008**
Muscle	**1.54** ± 0.54[b,e, h]	**1.38** ± 0.49	**1.32** ± 0.53	**1.71** ± 0.68	**1.68** ± 0.51	**1.51** ± 0.54	**1.63** ± 0.56	**Age**	**0.022**
Brain	**1.48** ± 0.54[c,f,i]	**1.22** ± 0.40	**1.32** ± 0.34	**1.66** ± 0.81	**1.67** ± 0.52	**1.46** ± 0.55	**1.54** ± 0.52	**Age**	**0.004**
Vane	**2.66** ± 1.60 [h,i,j]	**1.03** ± 0.59	**1.90** ± 1.57	**3.78** ± 1.13	**3.76** ± 0.97	**2.74** ± 1.63	**2.41** ± 1.54	**Age**	**0.001**
Shaft	**1.30** ± 0.76 [d,g,j]	**0.48** ± 0.15	**0.87** ± 0.65	**1.63** ± 0.34	**1.95** ± 0.44	**1.37** ± 0.77	**1.10** ± 0.72	**Age**	**0.001**

Note: n=number of samples. Significant differences between tissue mean concentrations: [a]p=0.014, [b]p<0.001, [c]p<0.001, [d]p<0.001, [e]p=0.003, [f]p=0.001, [g]p<0.001, [h]p<0.001, [i]p<0.001, [j]p<0.001. Concentrations on dry weight were calculated taking into account the water content of each sample, with a mean of 68.76% ± 2.89 for liver, 71.46% ± 1.98 for kidney, 72.43% ± 1.95 for muscle and 78.56% ± 1.14 for brain. Model: indicates the most influential factor (explanatory variable) in the response variable "Mercury concentration". None=concentration of mercury is not significantly influenced by any variable.

Mercury concentrations in Razorbills from this study were 1.3-2.1 times higher than levels in Razorbills and other piscivore Alcidae species from higher latitudes, probably due to differences in their dietary habits as a consequence of migratory processes during the winter (Figure 11). Fish species prominent in their summer diet include sandlance (*Ammodytes*), capelin (*Mallotus*), Atlantic herring (*Clupea harengus*), and Atlantic cod (*Gadus morhua*). Although data on the winter eating habits of Razorbills are scarce (Freethy, 1987), it is well known that the prey preferred by this species have a body length between 50 and 140 mm and always below 250 mm (Bradstreet and Brown, 1985). Some fish species included in their diet during the breeding season, such as sardines (*Sardina pilchardus*), are common in the Mediterranean Sea. In this sense, certain studies have detected mercury levels between 0.077 and 0.830 mg/kg dw in a pooling fish of similar size (Arcos *et al.*, 2002), or 0.700 mg/kg ww in muscle (Storelli *et al.*, 2003) from fish collected in the Mediterranean area with body lengths between 69 and 196 mm, thus being potential prey of Razorbills. In contrast, mercury levels in prey from Svalbard, Barents Sea and Iceland were 0.010 mg/kg ww in muscle, 0.049 mg/kg dw in muscle, and 0.030-0.049 mg/kg ww in flesh, respectively (Jæger *et al.*, 2009; Joiris *et al.*, 1995; Matís, 2008). Bacci (1989) explained the elevated mercury levels in Mediterranean biota in terms of the higher mercury methylation in these waters due to the elevated water temperature (Mediterranean deep waters are approximately 10°C warmer than the Atlantic waters at the same depth). Therefore, wintering habitats may be an important factor in the contaminant body burden. Guitart *et al.* (2003) also observed different Hg loads between two Ebro Delta Common Tern

(*Sterna hirundo*) colonies, which they attributed to variations in diet since they found differences in egg yolk fatty acid composition, a valuable means of detecting variations in feeding habits between groups.

Table 20. *Literature mean mercury levels (mg/Kg) in Alcidae tissues from different areas. Note: L: Liver, K: Kidney, M: Muscle, B: Brain, F: Feathers, V: Vane, S: Shaft.*

Species	Mean Mercury levels (mg/Kg)	Sampling area	Year	Reference
Dry weight				
Razorbill (*Alca torda*)	**L: 2.85, K: 2.23, M: 1.54, B: 1.48, V: 2.66, S: 1.30**	**Alicante coast (SE Spain)**	**2007**	**Present study**
Razorbill (*Alca torda*)	F: 1.29 (Adults), F: 1.40 (Chicks)[a]	Machias Seal Island, New Brunswick, Canada	2005-2006	Bond and Diamond 2009a
Atlantic Puffin (*Fratercula arctica*)	F: 1.41 (Adults), F: 0.99 (Chicks)			
Common Murre (*Uria aalge*)	F: 1.65 (Adults), F: 1.14 (Chicks)			
Common Murre (*Uria aalge*)	F: 0.99	Machias Seal Island, Bay of Fundy, Canada	2006	Bond and Diamond 2009b
Razorbill (*Alca torda*)	F: 1.40			
Atlantic Puffin (*Fratercula arctica*)	F: 1.81			
Razorbill (*Alca torda*)	L: 6.09, K: 3.94, M: 2.67, F: 2.39 (Total)	Central coast of Portugal	2005-2007	Ribeiro *et al.* 2009
	L: 5.00, K: 2.99, M: 1.63, F: 0.79 (Juveniles)			
	L: 4.89, K: 3.77, M: 2.14, F: 2.06 (Immatures)			
	L: 9.63, K: 4.90, M: 4.54, F: 4.10 (Adults)			
Tufted puffin (*Fratercula cirrhata*)	L: 2.9[b]	Aleutian archipelago of Alaska	2000-2001	Ricca *et al.* 2008
Common Murre (*Uria aalge*)	L: 4.17			
Pigeon Guillemot (*Cepphus columba)*	L: 6.36			
Brünnichs guillemot (*Uria lomvia*)	M: 0.38	West Greenland	2003	Rigét *et al.* 2007
Razorbill (*Alca torda*)	L: 2.28	Galician coast (NW Spain)	2002-2003	Pérez-López *et al.* 2006
Common Murre (*Uria aalge*)	L: 1.21			
Atlantic Puffin (*Fratercula arctica*)	L: 1.77			
Brünnichs guillemot (*Uria lomvia*)	L: 0.33-1.61, M: 0.15-0.60	Barents Sea and Norwegian Sea	1991-1992	Savinov *et al.* 2003
Common Murre (*Uria aalge*)	L: 1.08-1.09, M: 0.33-0.50			
Atlantic Puffin (*Fratercula arctica*)	L: 1.12-1.37, M: 0.31-0.46			
Black guillemot (*Cepphus grylle*)	L: 0.76-1.12, M: 0.27-0.43			
Little auk (*Alle alle*)	L: 0.24-0.51, M: 0.10-0.32			
Razorbill (*Alca torda*)	L: 1.71, M: 0.88			
Brünnichs guillemot (*Uria lomvia*)	L: 1.11, M: 0.33	Barents Sea	1992-1993	Wenzel and Gabrielsen 1995
Common Murre (*Uria aalge*)	L: 1.88, M: 0.42			
Black guillemot (*Cepphus grylle*)	L: 2.20, M: 0.73	Greenland	1984-1986	Nielsen and Dietz 1989
Brünnichs guillemot (*Uria lomvia*)	L: 2.63, M: 0.73			
Little auk (*Alle alle*)	L: 1.68	Svalbard	1980	Norheim 1987

Note: L: Liver, K: Kidney, M: Muscle, B: Brain, F: Feathers, V: Vane, S: Shaft. All values are presented as arithmetic mean except [a]= Estimated mean marginal and [b]=Geometric mean.

Table 20. *Literature mean mercury levels (mg/Kg) in Alcidae tissues from different areas. Note: L: Liver, K: Kidney, M: Muscle, B: Brain, F: Feathers, V: Vane, S: Shaft (continued).*

Species	Mean Mercury levels (mg/Kg)	Sampling area	Year	Reference
Wet weight				
Razorbill (*Alca torda*)	**L: 0.89, K: 0.64, M: 0.43, B: 0.32**	**Alicante coast (SE Spain)**	**2007**	**Present study**
Little auk (*Alle alle*)	L: 0.26, M: 0.06	Svalbard	2005-2006	Jæger *et al.* 2009
Brünnich's guillemot (*Uria lomvia*)	L: 0.37, M: 0.11			
Dovekie (*Alle alle*)	L: 0.27, M: 0.08	Northwater Polynia, Baffin Bay	1998	Campbell *et al.* 2005
Black guillemot (*Ceppus grylle*)	L: 1.17, M: 0.34			
Brünnichs guillemot (*Uria lomvia*)	L: 1.17, M: 0.33			

Note: L: Liver, K: Kidney, M: Muscle, B: Brain, F: Feathers, V: Vane, S: Shaft. All values are presented as arithmetic mean except [a]= Estimated mean marginal and [b]=Geometric mean.

Some studies have found that levels in large predators such as seabirds (alcids included) are ten times higher than levels in medium-sized fish, the latter being components of the former's diet (Atwell *et al.*, 1998; Jæger *et al.*, 2009). Using this argument and the fact that Razorbills in the present study had mean mercury concentrations of 1.54 mg/kg dw in muscle, thus we could estimate mercury concentrations in their Mediterranean prey as being ten times lower (approximately 0.154 mg/kg dw). This is consistent with the observations of Arcos *et al.* (2002) who recorded mercury levels of 0.140-0.156 mg/kg dw (whole fish) in three different fish species from the Mediterranean Sea with lengths between 74-196 mm, thus being potential prey of Razorbills as mentioned above.

3.2. Body condition, sex and age

Mercury tissue concentrations have been found to vary with body condition (Kalisińska *et al.*, 2010; Kojadinovic *et al.*, 2007b), but body condition was generally good in the Razorbills in the present study and no effect of nutritional status on tissue mercury concentrations was detectable. There was also no significant difference in Hg accumulation between males and females (Table 19). This is consistent with the findings of some studies (Jæger *et al.*, 2009; Zamani-Ahmadmahmoodi *et al.*, 2010) but others have found higher levels in males than females (Nielsen and Dietz, 1989; Savinov *et al.*, 2000), thought to be due to transfer of body burdens into eggs by laying females. However, in the present study, Razorbills were collected after the breeding season, and the amount eliminated into eggs is usually small compared to the amount transferred into feathers during the moult (Furness, 1993).

There were significant differences between age groups in Hg kidney, muscle, brain and feather concentrations ($p < 0.022$; Table 19) with residues being greater in older birds. Other studies on alcids have likewise observed that older individuals have a tendency to accumulate greater mercury levels in internal tissues (Dietz *et al.*, 1990; Ribeiro *et al.*, 2009), although others have reported no effect of age (Thompson *et al.*, 1991). Age-dependent increment of mercury in feathers has been reported in Razorbills and other seabirds (Burger *et al.*, 2008; Kojadinovic *et al.*, 2007a; Ribeiro *et al.*, 2009). Greater accumulation of Hg by older individuals could occur if they eat larger, more contaminated prey and/or may simply reflect an accumulation of mercury in their tissues over a longer period of time (Kojadinovic *et al.*, 2007a). Although feathers act as an excretion route for about 90% of the mercury (Burger, 1993), a certain percentage of the body burden is not evacuated into the plumage (Lewis and Furness, 1991), and this can lead to an increase of mercury with age in the internal tissues of some species.

3.3. Exposure assessment

The distribution pattern of mercury was liver>feather-vane>kidney>muscle>brain>feather-shaft (Table 19). We found significant differences in concentrations of mercury between internal tissues except for muscle and brain (Table 19). Supporting our results, other authors have also described this distribution model (Kenow *et al.*, 2007; Norheim and Frøslie, 1978; Ribeiro *et al.*, 2009; Wolfe and Norman, 1998). The chronic daily exposure to Hg implies a balance between compartment concentrations. This may explain the distribution pattern observed and the strong correlations in Hg levels between internal tissues ($r > 0.80$, $p < 0.001$) (Figure 12).

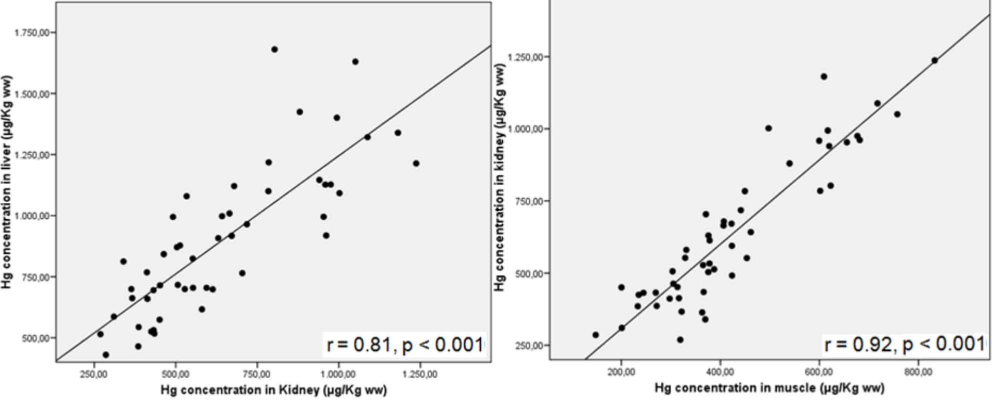

Figure 12. *Relationship between mercury concentrations in Razorbill tissues.*

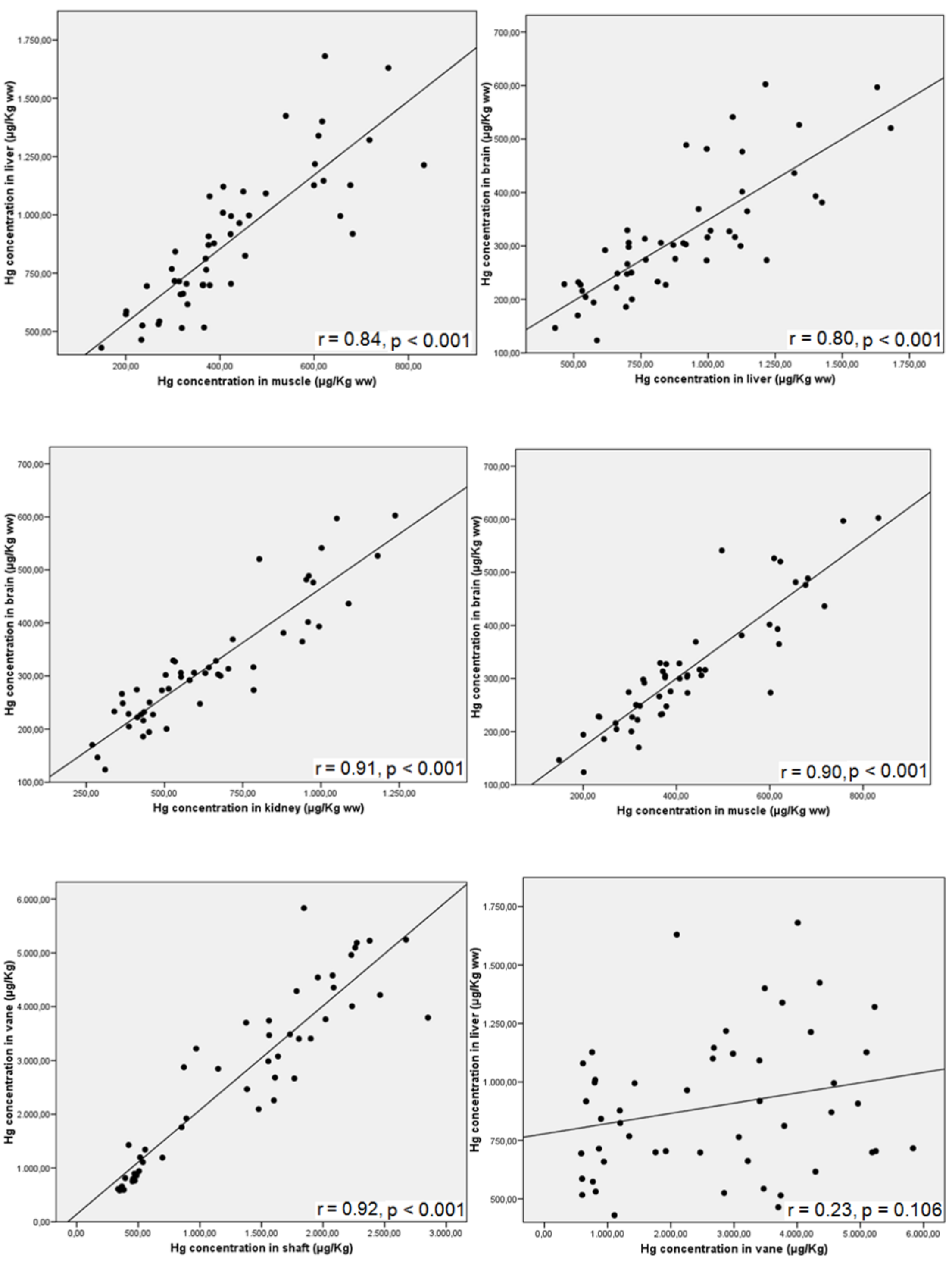

Figure 12. *Relationship between mercury concentrations in Razorbill tissues (continued).*

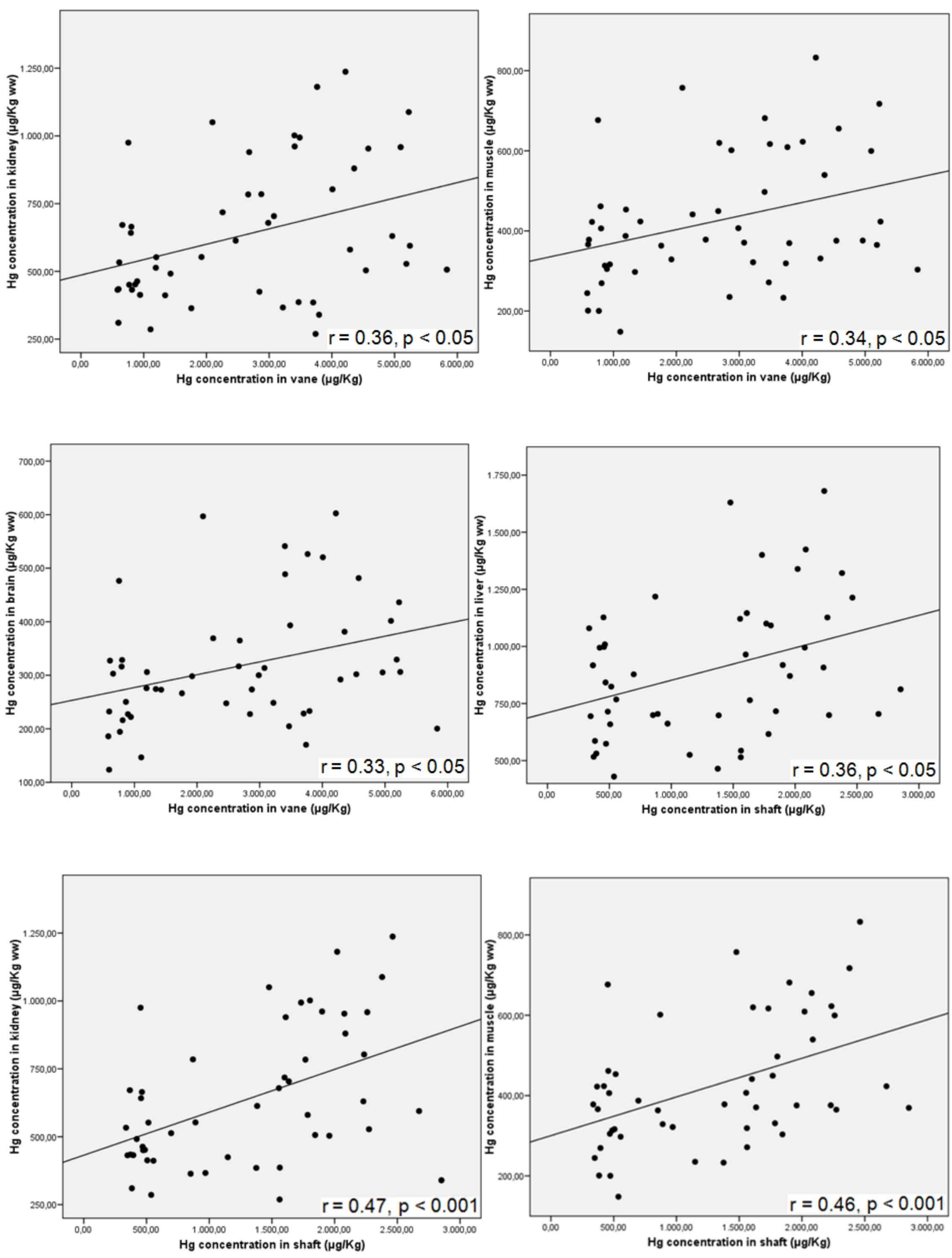

Figure 12. *Relationship between mercury concentrations in Razorbill tissues (continued).*

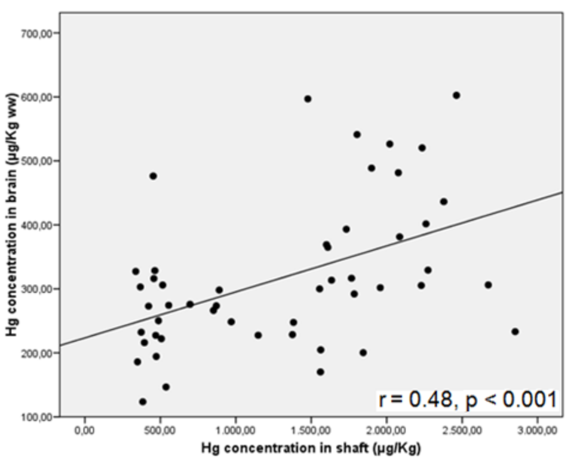

Figure 12. *Relationship between mercury concentrations in Razorbill tissues (continued).*

The Hg kidney:liver ratio has been proposed as a means of distinguishing chronic MeHg from inorganic Hg exposure (Scheuhammer, 1987); kidney:liver ratios much greater than 1 reflecting exposure to inorganic Hg, whereas ratio closer to 1 (and <2) being characteristic of exposure to MeHg. In the present study, the mean kidney:liver ratio in the Razorbills was 0.78 and so probably reflected exposure to MeHg via dietary intake. This is consistent with the fact that almost all (close to 100%) of the total Hg detected in the muscle of several fish species is MeHg (Scheuhammer, 1987).

The total mercury concentrations in this study were within ranges cited as background levels in wild birds (1-10 mg/Kg ww in liver; Fimreite, 1974), with a maximum concentration of 1.7 mg/Kg ww in liver. These results seem to indicate that the exposure to mercury in this species is low. In this sense, in experimental studies, birds treated with dietary Hg (0.1-0.56 ppm) showed tissue Hg levels 1.1-4.5 times higher than those observed in our study (Heinz 1976, 1979; Kenow *et al.*, 2007; Wolfe and Norman, 1998).

3.4. Effect assessment

Several authors have described sublethal toxic effects in birds related to dietary exposure and tissue concentrations of Hg, including effects on growth, development, reproduction, blood and tissue chemistry, metabolism, and behavior (Evans *et al.*, 1982; Finley and Stendell, 1978; Zillioux *et al.*, 1993). The kidney levels of Hg in the present study were 25 times lower than those reported as causing reproductive alterations and brain lesions in American black ducks (*Anas rubripes*) (16.0 ppm ww in kidney) (Finley and Stendell, 1978). In addition, the brain levels were 37 to 50 times lower than those described as causing behavioral changes in pigeons (12-16 ppm ww

in brain) (Evans *et al.*, 1982). Therefore, it is likely that renal and brain Hg concentrations found in the present study were low and insufficient to cause adverse effects in these birds. Despite this, Hg concentrations in liver (2.85 ppm dw, 0.89 ppm ww) were close to the critical level associated with high embryo/duckling mortality and brain lesions described by Zillioux *et al.* (1993) (1-2 ppm ww in liver). Nonetheless, it should be noted that the total mercury concentration, would probably not be the best indicator of toxic effects (Pérez-López *et al.*, 2006; Savinov *et al.*, 2003). On the contrary, more importance should be given to organic mercury as it seems to be considerably more toxic than inorganic Hg to animals at high trophic levels (Wolfe *et al.*, 1998). In this sense, no data are available on the ratio between the organic (methyl) and the inorganic form of mercury in Razorbills from the Mediterranean area.

3.5. The role of feathers in the mercury distribution pattern

If we consider the whole feather instead of the separate mercury levels in vane and shaft, feather Hg concentrations were higher than levels for internal tissues in adult birds. This is consistent with other studies (Braune and Gaskin, 1987; Kenow *et al.*, 2007). During the feather growth, liver Hg is transferred to feathers, thus reducing Hg amounts in the liver (Furness *et al.* 1986; Lewis *et al.* 1993). In this sense, Burger (1993) considered that > 90% of a bird's total mercury body burden may be sequestered into feathers during the moult, where mercury binds with feather keratin in the form of methylmercury (Thompson and Furness 1989a,b).

The mean concentration in vanes was twice as high as for shafts, this difference being statistically significant ($p<0.001$). Similar findings were described by Dauwe *et al.* (2003) who observed mean concentrations 1.18 times higher in the vanes of sparrowhawks (*Accipiter nisus*) and tawny owls (*Strix aluco*). Several hypotheses could be used to explain these differences: i) the influence of external contamination, in spite of the exhaustive washing process carried out prior to the analytical determination; ii) influence of the chemical structure of the vanes and shafts and their Hg-binding capacity, as suggested by Dauwe *et al.* (2003); and/or iii) mercury accumulates in the barbs after passing through the shaft (Jaspers *et al.*, 2007).

The higher correlation coefficients between internal tissues and shafts than those between internal tissues and vanes (Figure 12) may corroborate the hypothesis that vanes may accumulate some external Hg (adsorbed to feather waxes) that is not removed by the washing method performed. Therefore, despite vanes having higher Hg levels than shafts, the latter may be a better indicator of internal tissue levels.

Therefore, we can use feather shafts, as a model of a non-invasive sample for predicting accumulated Hg levels in target organs (brain and/or kidney). The prediction equations for Hg concentrations, obtained via GLMs, are described below for both dry and wet weight (Eq. 1 and Eq. 2). Sex and age were not significant when included as factors. Finally, all models were statistically significant ($p<0.001$).

Brain Hg (µg/Kg dry weight) = 1050+0.34*Shaft Hg (µg/Kg) (Eq. 1)

Kidney Hg (µg/Kg dry weight) = 1567+0.51*Shaft Hg (µg/Kg) (Eq. 2)

On the other hand, seabirds eliminate Hg during the moulting process (Braune and Gaskin, 1987; Monteiro and Furness, 2001), with smaller proportions of the total body Hg burden excreted into guano or eggs (Monteiro and Furness, 1995, 2001). Therefore, internal tissue concentrations would reflect the mercury levels accumulated from the moment in which the last feather finished growing to the moment in which internal tissues were sampled, following necropsy (Arcos *et al.*, 2002). Assuming that concentrations in feathers reflect circulating concentrations in the body at the time of their formation (Jaspers *et al.*, 2006), collection time may have an influence on the correlations between internal tissues and feathers. Razorbills usually arrive to the Spanish Mediterranean coastline in October, where they remain until April (Bédard, 1985; Freethy, 1987). Since the Razorbills were found on the Mediterranean coastline in February 2007, the Hg concentrations found in internal tissues most likely correspond to the concentrations accumulated by the birds up until that date, and would reflect the most recent exposure during the previous overwintering. However, the levels in feathers reflect the blood concentrations during feather growth, after the breeding moult (July to September 2006) in breeding grounds prior to migration to Spain (Pyle, 2009). Therefore, feather Hg concentrations are related to those in internal tissues, but for the year prior to collection (from October 2005 to September 2006). Accordingly, Hg concentrations in feathers and internal tissues do not reflect the same period of Hg exposure.

4. Conclusions

According to the results, the Razorbill could be a good sentinel species for Hg pollution in the Mediterranean area. The higher levels in their tissues compared to concentrations in other Alcidae species from higher latitudes are probably due to their dietary habits over the winter in that area, although the concentrations found do not seem to be associated with risks for the Razorbill's health. It is feasible to assume that

Razorbills in the southwestern Mediterranean were chronically exposed to relatively low levels of MeHg via dietary intake, probably below 0.5 ppm. Results indicate that feathers are an excellent non-destructive tool for monitoring mercury levels in Razorbills, and feather shafts may be a better indicator of internal tissue levels than feather vanes. We have proposed prediction equations for brain and kidney Hg concentrations using feather shafts as non-invasive samples. However, the time between the moult and the moment of sample collection should be considered. Finally, this work provides a solid understanding of Razorbill Hg exposure both in their wintering and breeding grounds, and shows that this species could be useful for assessing marine environmental health in the Mediterranean area.

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CHAPTER VI

Factors influencing mercury concentrations in nestling Eagle Owls (*Bubo bubo*)

Photo: José Alfonso Lacalle Martínez

Silvia Espín, Emma Martínez-López, Mario León-Ortega, José F. Calvo, Antonio J. García-Fernández. Factors influencing mercury concentrations in nestling Eagle Owls (Bubo bubo).

Abstract

Mercury (Hg) is a global pollutant that bioaccumulates and biomagnifies through the food chain, and is associated with adverse effects both in humans and wildlife. Because little is known about the bioaccumulation potential of Hg in terrestrial food chains, new information about Hg levels in terrestrial species is necessary. The Eurasian Eagle Owl (*Bubo bubo*) could be a suitable biomonitor of Hg in the terrestrial ecosystem since it is a top predator in food chains, it has a long life span and it is a resident and territorial species. Hg levels detected in blood of Eagle owl chicks from Southeastern Spain (Murcia) can be considered low (mean Hg concentration in blood from 2006 to 2012 was 36.09 ± 142.93 µg/L wet weight, n=623), and it is unlikely that Hg pollution can negatively affect the breeding performance. A unique growing back feather of nestling Eagle owl may be enough to estimate Hg concentrations in blood of this bird species ($r = 0.339$, $p < 0.001$, $n = 229$). We provide a regression equation that could be helpful to estimate blood Hg levels analyzing Hg concentrations in back feathers. Blood Hg concentrations in Eagle owls have shown positive correlations with Hg levels in muscle of rabbits, which is more evident in the nests from the Northern area ($r= 0.489$, $p < 0.001$, $n=51$), where rabbits are the Eagle owl main prey. Although the studied region is not considered Hg polluted, higher Hg levels in Eagle owls and European rabbits were found in area near the ancient mine site comparing with the rest of the study region. This result supports the fact that spatial differences in Hg concentrations of Eagle owls appear to be mostly related to local contamination, and probably the differences in diet composition among areas plays a role of less extent. Rainfalls could be the main cause of the differences in Hg concentrations found in blood of nestling Eagle owls between years.

1. *Introduction*

Mercury (Hg) is a global pollutant that bioaccumulates and biomagnifies through the food chains, and is associated with adverse effects such as neurological, immunological and reproductive impacts in both humans and wildlife (Evans *et al.*, 1982; Burger and Gochfeld, 1997). Primary sources of anthropogenic Hg emissions include the combustion of fossil fuels, mining and reprocessing of ores (gold, copper, lead and zinc), iron, steel and cement production, operation of chloralkali plants, and waste incineration and disposal (Driscoll *et al.*, 2007; Pacyna *et al.*, 2006). The persistence of this metal in the atmosphere and its ability to travel great distances has allowed it to become a global pollutant (Seewagen, 2010).

Inorganic Hg is converted into organic forms (methylmercury, MeHg), especially in aquatic ecosystems. This organic form of Hg is the most harmful and able to bioaccumulate in food chains (Thompson and Furness, 1989a; Driscoll *et al.*, 2007). Due to the methylation and bioaccumulation of MeHg in the aquatic systems, much of the effort involving Hg investigations has disproportionately focused on particular taxa (waterbirds), foraging guilds and trophic levels (piscivores) and habitat types (aquatic ecosystems) (Seewagen, 2010). However, these are not necessarily the only groups of birds and ecosystems impacted. Although it appears to be smaller than in aquatic systems (Lindqvist, 1991), evidence suggests that methylation may also occur in terrestrial systems (Rimmer *et al.*, 2005, 2010; Driscoll *et al.*, 2007). Some studies have found large accumulations of this contaminant in birds of prey that feed at the top of terrestrial trophic chains (Broo and Odsjö, 1981; Palma *et al.*, 2005). In this sense, Zolfaghari *et al.* (2007) found significant differences in feather Hg concentrations in relation to trophic level in 18 bird species from southwest Iran, and raptors (including Eagle owl) that feed from vertebrates (except fish) showed the highest level of Hg.

The Eurasian Eagle Owl (*Bubo bubo*) is a large nocturnal raptor which could be a sensitive biomonitor of Hg in the terrestrial ecosystem since it is a long-lived top predator in food chains and it is resident and territorial, thus being able to indicate the local environmental contamination. Eurasian Eagle Owl in our study area feeds primarily on European Rabbit (*Oryctolagus cuniculus*), showing geographical variation in diet composition depending on local habitat conditions (León *et al.*, 2008). Several studies have shown that diet composition has an important influence on the concentration of contaminants in top predators, independently of spatial or temporal variation in environmental contamination (Lindberg and Odsjö, 1983; Mañosa *et al.*,

2003; Palma *et al.*, 2005). Palma *et al.* (2005) found that Bonelli's eagle (*Hieraaetus fasciatus*) Hg levels showed great spatial variation, related to diet composition and food chain biomagnification. Therefore, it is necessary to consider the effect of diet composition on the pollutant burdens of birds of prey feeding on terrestrial food chains to interpretate properly spatial or temporal changes in environmental contamination (Lourenço *et al.*, 2011; Mañosa *et al.*, 2003; Palma *et al.*, 2005).

In this study, we have evaluated Hg levels in blood and feathers of Eurasian Eagle Owl chicks, collected during seven breeding seasons (2006-2012), from two areas of Murcia (Southeastern Spain), one of them possibly influenced by an ancient mine site and an industrial complex. Analysis of Hg in muscle samples from carcasses of its main prey, the European Rabbit, was also carried out. We tested local contamination and diet composition as factors affecting Hg concentrations in European Eagle owl chicks.

2. *Materials and methods*

2.1. Study area and species

The study area is located in the east of Murcia Region (37º45' N, 0º57' W) (Figure 13). The climate is meso-arid Mediterranean with 275–400 mm of annual rainfall and a high average annual temperature of 19ºC. Since the study area is relatively large and some differences in land use are known, it was subdivided in two sub-areas. Northern subarea comprises the mountains Escalona, Altaona, Monte el Valle and Columbares. In this subarea, land is mainly dedicated to citrus and dry farming, and the European Rabbit is abundant, accounting for 71% of the prey consumed by Eagle Owls (León *et al.*, 2008). In Southern subarea, the European Rabbit is less abundant (35% of the Eagle Owls' diet), and the raptor consumes a similar proportion of rats (*Rattus rattus* and *Rattus norvergicus*) (23% of the diet), apart from pigeons (*Columba spp.*), partridges (*Alectoris rufa*), hedgehogs (*Erinaceus europeus* and *Atelerix algirus*) and yellow-legged gulls (*Larus michahellis*) (León *et al.*, 2008). This subarea is delimited by Sierra Minera Cartagena-La Union, La Muela-Cabo Tiñoso and Almenara. Irrigation farming is predominant and it is remarkable the fact that some ancient mining sites (Sierra Minera de Cartagena-La Unión) is found in this subarea (Figure 13). In our study area, the Eagle Owl prefers low to medium cliffs, especially in the Northern subarea. This species is the biggest nocturnal raptor in Spain, where it is sedentary and highly territorial during the whole year, occupying territories which size and foraging area depend on the prey availability. Eagle Owl population in Murcia Region is

abundant and has been estimated in approximately 240–270 pairs (Martínez and Calvo, 2006; Martínez and Zuberogoitia, 2003). However, this data is most probably underestimated, and the number of pairs may be above 300 (León, personal communication).

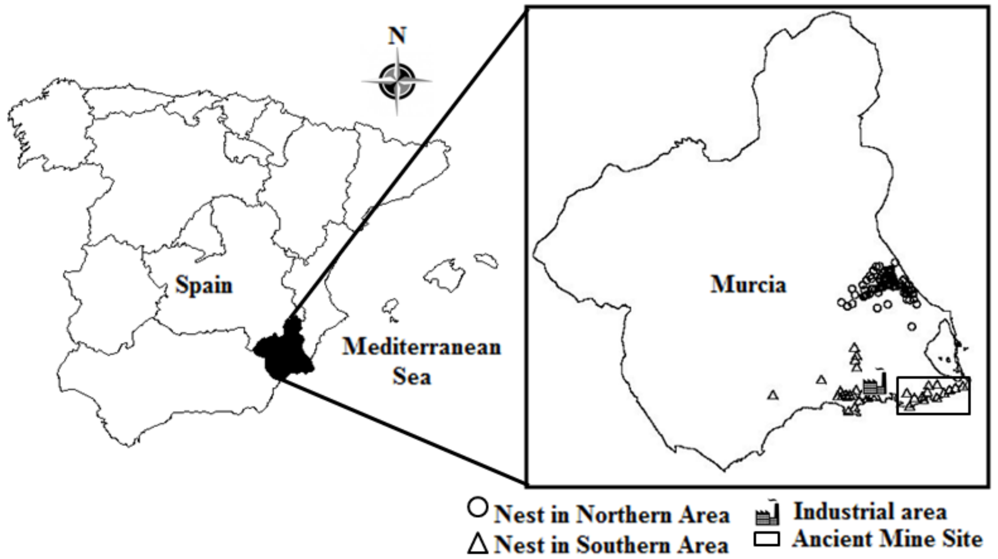

Figure 13. *Map with the location of the study area (Murcia, Spain). Circles and triangles represent nests in Northern and Southern areas respectively. Industrial area is represented by a factory figure and area under mining influence is framed by a square.*

2.2. Sampling method

A total of 623 blood samples from 2006 to 2012 (435 from Northern area and 188 from Southern area), and 229 back feathers from 2006 to 2008 (174 from Northern area and 55 from Southern area) were analyzed for Hg concentrations in Eurasian Eagle Owl chicks of approximately 30-days old from Murcia, Southeastern Spain (Figure 13). In addition, we obtained 40 muscle samples from carcasses of European Rabbit from 2009, 2011 and 2012 collected at the sampling nests (25 from Northern area and 15 from Southern area). Preys were returned to the nest after the sampling.

Blood samples were obtained by puncturing brachial vein with a 23G needle and a syringe. We collected 3-5 ml of blood, depending upon the stage of development of the nestling. Growing feathers were plucked from the back of chicks. We used body feathers because their sampling does not affect the future chick flight performance. After sampling, the nestlings were returned to their nests. Feathers were stored in sterile plastic bags at room temperature, and whole blood samples were stored in

Eppendorf tubes at -80 °C until analysis. The sampling in Murcia was authorized by the General Directorate of Natural Patrimony and Biodiversity from the Autonomous Community of Murcia Region.

2.3. Mercury analysis

Total Hg was analyzed in a Milestone DMA-80 direct Hg analyzer by atomic absorption spectrophotometry with a detection limit of 0.005 ng. Samples (0.1 g wet weight for blood, 0.01 g dry weight for feathers and 0.2-0.3 g wet weight for muscle of rabbit, approximately) were loaded in a nickel boat and analyzed. Calibration curve was done with ten points (in duplicate) from 0 to 1004 ng of Hg.

Precision and accuracy of the method were tested using certified reference material (CRM) (Hg Standard for AAS, Fluka, 1000 mg/L Hg in 12% nitric acid, prepared with high purity Hg metal, HNO_3TraceSELECT® and water TraceSELECT®Ultra). Recovery of total Hg from 5 replicates of CRM diluted to 1 ppm was 104.2±11.8% (mean±standard deviation). The coefficient of variation for the repeatability was 11.4%.

In order to remove external contamination on the feather surface which could alter the results of the Hg sequestrated in the feathers, prior to the analytical determination a washing process was performed with tap water, distilled water, Milli-Q water and acetone. The feathers were subsequently dried at room temperature overnight.

2.4. Statistical analysis

All analyses were carried out using the SPSS v.15.0 statistical package. Reported Hg values represent the mean ± standard deviation, median and range. The data were tested for normality using a Kolmogorov–Smirnov test. Since the concentrations of Hg were not normally distributed, the data was log-transformed. ANOVA and Student t-test were performed to elucidate significant differences between variables. We used simple linear regression to evaluate the relationship between Hg concentrations in blood and back feathers. Generalized Linear Models (GLMs) with a normal distribution and an identity function were performed to study the effect of area and year in the concentrations of Hg in blood of Eagle owls and in muscle of European rabbit. Hg concentrations in owls or in rabbits were the response variable, and the explanatory variables considered were area and year. A backward stepwise procedure was used to select the final model. Pearson's correlation coefficient was used in order to calculate correlations between variables. The level of significance was set at α=0.05.

3. *Results and discussion*

3.1. Mercury levels in blood and feathers of nestling Eagle owls

Table 21 shows Hg concentrations in blood and feathers of Eagle owl. There is no data available about Hg concentrations in blood of Eagle owls. However, levels found were much lower than those reported for nestlings of fish-eating raptors such as Bald eagles (*Haliaeetus leucocephalus*) and Ospreys (*Pandion haliaetus*) (Jagoe *et al.*, 2002; Langner *et al.*, 2012). Regarding Hg concentration in feathers, few studies have analyzed them in owls, and more specifically in Eagle owl (Broo and Odsjö, 1981; Dauwe *et al.*, 2003; Lourenço *et al.*, 2011; Ortego *et al.*, 2006; Zolfaghari *et al.*, 2007). Table 22 provides a review of Hg concentrations in feathers of Strigiformes. Mean Hg concentrations in back feathers of Eagle owl chicks in the present study were slightly higher than those reported by Ortego *et al.* (2006) in Eagle owl chicks from Central Spain; and similar to those reported in nestling Long-eared Owl (*Asio otus*) from Finland (Solonen and Lodenius, 1990). However, Hg concentrations found in the present study were lower than those reported by Lourenço *et al.* (2011) in adult Eagle owls (Table 22). In this sense, adult individuals are expected to have higher Hg concentrations, as observed in several bird species (Espín *et al.*, 2012 (see Chapter V); Kojadinovic *et al.*, 2007). Greater accumulation of Hg by older individuals could occur if they eat larger, more contaminated prey and/or may simply reflect an accumulation of mercury in their tissues over a longer period of time (Kojadinovic *et al.*, 2007). Other authors have also found higher Hg levels in Eagle owl than those from this study, probably related to the use of alkyl Hg as a seed-dressing agent in terrestrial habitats in their studied area between 1940 and 1966 (Broo and Odsjö, 1981; Odsjö and Olsson, 1975) (Table 22). According to this and comparing with other studies analyzing Hg in chick feathers of other bird species (Barata *et al.*, 2010; Goutner *et al.*, 2011), Hg levels detected in Eagle owl chicks from Southeastern Spain can be considered low.

Besides, Hg levels found in Eagle owls were below those related to toxic effects. The highest Hg concentrations in feathers were found in three chicks sampled in 2007 (1.3% of total studied population), with levels between 3 and 5 mg/Kg, close but below the critical level described by NAS (1978) (5 mg/Kg in feathers) associated with reproductive impairment (lower clutch and egg size, reduced hatching rate and decreased chick survival). Hence, for the overall population it is unlikely that Hg pollution can negatively affect the breeding performance.

On the other hand, significant higher Hg concentrations were found in feathers than in blood (p<0.001) of nestling Eagle owls (Table 21). This could be due to the fact that feathers represent an important excretion route for Hg in birds (Kenow *et al.*, 2007). In this sense, Burger (1993) considered that > 90% of Hg total body burden may be sequestered into feathers during molt, and Hg binds with feather keratin as methylmercury (Thompson and Furness, 1989a; Thompson and Furness, 1989b).

In the present study, feathers were still growing when they were sampled. Experimental studies have shown that Hg levels in feathers reflect the Hg concentrations in the blood at the time of feather growth (Lewis and Furness, 1991). Therefore, as expected, a positive correlation between Hg concentrations in blood of chicks and in back feathers was found (r = 0.339, $p = 0.001$, and $n = 229$) (Figure 14).

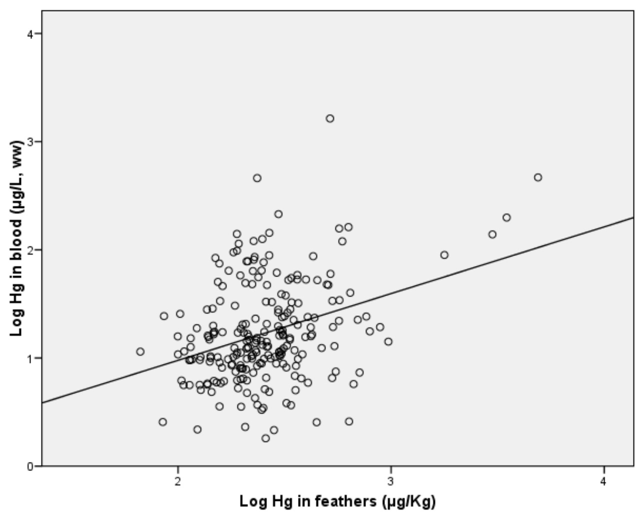

Figure 14. *Relationship between mercury blood levels (µg/L, ww) and mercury feather levels (µg/Kg) in Eurasian Eagle Owls (r = 0.339, p < 0.001, and n = 229). Log Hg in blood (µg/L, ww) = -0.255 + 0.617 * Log Hg in feathers (µg/Kg).*

As a result, a unique growing back feather of Eagle owl could be enough to estimate Hg concentrations in blood of this terrestrial species. We provide the equation estimated by simple linear regression calculated considering Hg concentrations in blood and feathers of 229 nestling Eagle owls from Southeastern Spain: Log Hg in blood (µg/L, ww) = -0.255 + 0.617 * Log Hg in feathers (µg/Kg). Taking into account that there is no published data on Hg concentrations in blood of Eagle owls, this equation may be helpful to estimate Hg concentrations in blood of this species in those studies where only growing back feathers are analyzed.

Table 21. *Concentrations of mercury in blood and feathers of Eagle Owl (Bubo bubo) and muscle of rabbit (Oryctolagus cuniculus).*

Concentrations of Hg in Eagle Owl (*Bubo bubo*) and their main prey (*Oryctolagus cuniculus*)						
	Chick Blood (µg/L, ww)		**Chick Feathers (µg/Kg)**		**Rabbit muscle (µg/Kg, ww)**	
	Mean ± SD, median (range)	***n***	**Mean ± SD, median (range)**	***n***	**Mean ± SD, median (range)**	***n***
Total	36.09 ± 142.93, 14.61 (1.45-2886.20)	623	328.88 ± 447.15, 238.09 (66.50-4891.62)	229	13.71 ± 12.83, 9.26 (3.24-72.81)	40
Year						
2006	18.10 ± 22.52, 11.20 (3.82-140.09)	71	243.58 ± 144.24, 210.73 (85.89-970.31)	69	-	
2007	50.88 ± 75.98, 20.38 (3.31-466.71)	93	409.38 ± 670.11, 246.51 (66.50-4891.62)	93	-	
2008	41.41 ± 198.69, 10.16 (1.81-1635.98)	67	304.97 ± 161.28, 257.74 (84.77-887.342)	67	-	
2009	60.45 ± 270.69, 23.84 (3.08-2886.20)	116	-		15.04 ± 11.75, 8.57 (5.57-40.48)	12
2010	32.21 ± 91.98, 14.53 (2.33-840.48)	87	-		-	
2011	19.28 ± 38.60, 10.56 (1.45-384.36)	123	-		12.47 ± 7.23, 9.58 (6.34-28.18)	9
2012	21.53 ± 22.97, 14.57 (5.64-125.03)	66	-		13.46 ± 15.71, 7.85 (3.24-72.81)	19
Area						
North	33.77 ± 148.90, 12.36 (1.81-2886.20)	435	305.68 ± 368.36, 229.27 (66.50-3485.57)	174	11.32 ± 7.14, 9.21 (3.24-32.57)	25
South (mine area included)	41.46 ± 128.26, 18.44 (1.45-1635.98)	188	402.25 ± 634.26, 295.12 (85.90-4891.62)	55	17.70 ± 18.54, 10.02 (4.22-72.81)	15
Only ancient mine site	57.03 ± 182.76, 27.49 (4.85-1635.98)	80	378.29 ± 147.74, 350.35 (188.89-696.87)	30	24.98 ± 12.76, 24.70 (10.02-40.48)	4

***Table 22**. Literature mercury concentrations in feathers of Strigiformes from different areas.*

Literature mercury levels (µg/g) in feathers of Strigiformes from different areas					
Species	n	Mean mercury levels (µg/g)	Sampling area	Year	References
Eagle Owl (*Bubo bubo*)	229	0.32. Chicks.	SE Spain	2006-2008	Present study
Eagle Owl (*Bubo bubo*)	32	0.12 (year 2002) and 0.09 (year 2003) (BF, Chicks 20-30 days old)	Toledo, C Spain	2002-2003	(Ortego *et al.*, 2006)
Eagle Owl (*Bubo bubo*)	61	1.29 (BF, Adult birds)	SW Iberian Peninsula	2003-2007	(Lourenço *et al.*, 2011)
Barn owl (*Tyto alba*)	13	1.22 (BF)			
Tawny owl (*Strix aluco*)	3	0.48 (BF)			
Little owl (*Athene noctua*)	15	0.64 (BF)			
Little owl (*Athene noctua*)	7	0.12 (P9)-0.36 (P3)[a]	Belgium	2001	(Dauwe *et al.*, 2003)
Barn owl (*Tyto alba*)	5	0.77 (P4)-0.90 (P2)[b]			
Little owl (*Athene noctua*)	3	0.50 (SF) and 1.10 (TF)	SW Iran	2005	(Zolfaghari *et al.*, 2007)
Tawny owl (*Strix aluco*)	2	0.56 (SF) and 0.85 (TF)			
Eagle Owl (*Bubo bubo*)	3	0.30 (SF) and 0.71 (TF)			
Eagle Owl (*Bubo bubo*)	39	3.20 (IP) and 6.51 (CP). Adult birds.	SW Sweden	1971-1976	(Broo and Odsjö, 1981)
Eagle Owl (*Bubo bubo*)	9	2.84 (IP) and 1.23 (CP). Young birds, 1-3 months old.		1975-1977	
Eagle Owl (*Bubo bubo*)	29	4.08 (IP) and 8.00 (CP). Adult birds.	SE Sweden	1970-1973	(Odsjö and Olsson, 1975)
Eagle Owl (*Bubo bubo*)	-	2.14 (IP) and 5.80 (CP). Young birds, 1-3 months old.		1970-1974	
Eagle Owl (*Bubo bubo*)	10	2.50 (TF) and 1.30 (InP)	Sweden	1829-1933	(Berg *et al.*, 1966)
Eagle Owl (*Bubo bubo*)	4	0.70. (SC, Chicks).	Southern Finland	1984-1987	(Solonen and Lodenius, 1990)
Tawny owl (*Strix aluco*)	16	0.50. (SC, Chicks).			
Ural Owl (*Strix uralensis*)	4	0.60. (SC, Chicks).			
Great Grey Owl (*Strix nebulosa*)	1	0.60. (SC, Chicks).			
Long-eared Owl (A*sio otus*)	3	0.30. (SC, Chicks).			
Boreal Owl (*Aegolius funereus*)	3	1.50. (SC, Chicks).			
Little owl (*Athene noctua*)	7	17.00 (CF) and 10.00 (RS)[c]	The Netherlands	-	(Van den Brink *et al.*, 2003)

[a]P1 is the innermost, first molted primary and P10 is the outermost, last molted primary; [b]P1 is the innermost, last molted feather and P6 is the first molted feather; [c]Geomean. IP=inland population, CP=coastal population, TF=Tail feathers, SF=Secondary feathers, InP=Inner primaries, BF=Body feathers, SC=Secondary coverts, CF=Contaminated floodplains, RS=Reference site.

3.2. Spatial variations in mercury concentrations. Are they affected by Eagle owl diet habits or by the contamination sources?

When studying spatial variations, we found significant differences in Eagle owl blood Hg concentrations ($p < 0.001$, n=623) between Northern and Southern areas, with Hg levels of 33.77 ± 148.90 µg/L in the North and 41.46 ± 128.26 µg/L in the South (Table 21). Other authors (Lourenço *et al.*, 2011) have noted that spatial differences in Hg concentrations in nestling Eagle owls appear to be largely related to

diet composition rather than to local contamination. In this sense, León *et al.* (2008) observed that in Northern area the rabbit is very abundant and therefore, it is the main prey of Eagle owls (71% of the diet), with partridges (6.74%) and pigeons (2.81%) as other preys. By contrast, in the Southern area the rabbit comprises only 35% of the diet, with rats (23%), pigeons (14%), partridges (5.26%), hedgehogs (5.26%) and yellow-legged gulls (3.16%) as other preys (León *et al.*, 2008). Even Stone curlew (*Burhinus oedicnemus*) and Peregrine Falcon (*Falco peregrinus*) has been described as preys in the Southern area (León *et al.*, 2008). In the present study, we found a significant positive correlation between Hg levels in blood of Eagle owl and muscle of rabbits in the Northern area (r= 0.489, p < 0.001) where the availability of rabbit is higher, but no significant correlation was found in the south (Figure 15), which may be due to the differences in diet composition described by León *et al.* (2008).

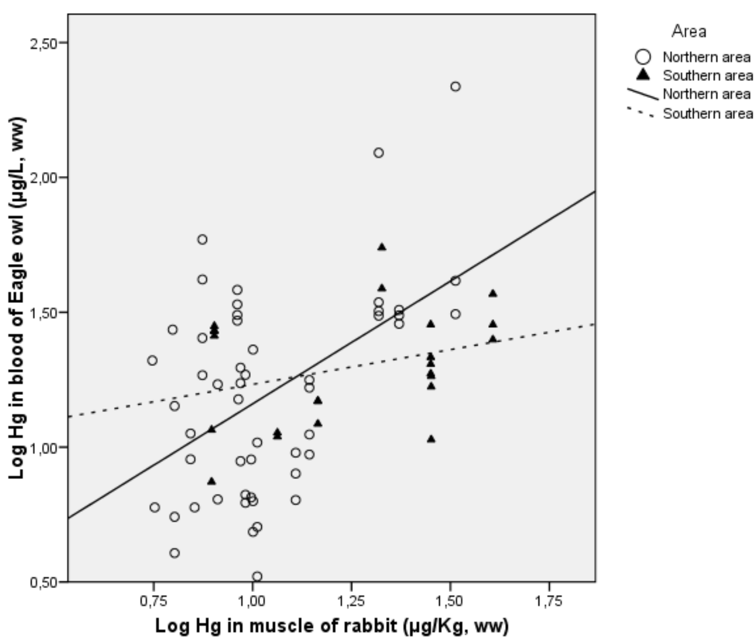

Figure 15. *Relationship between mercury blood levels (µg/L, ww) in nestling Eagle owl and mercury concentrations in muscle of rabbit (µg/Kg, ww) in Northern (r= 0.489, p < 0.001, n=51) and Southern areas (no significant correlation were found).*

Species such as rabbits, partridges and pigeons feed on plants, and they are classified as primary consumers (Lourenço *et al.*, 2011). However, omnivorous species such as rats, hedgehogs and gulls could be classified as secondary consumers (Lourenço *et al.*, 2011). These prey, although less important in terms of ingested biomass, are expected to have higher Hg levels than primary consumers. Accordingly, Lourenço *et al.* (2011) found that primary consumers had significantly lower Hg

concentrations than secondary consumers, and mesopredators had significantly higher Hg levels than secondary consumers when studying Hg levels in Eagle owl and its prey. Palma *et al.* (2005) found that the highest Hg concentrations were recorded in Bonelli's eagles (*Hieraaetus fasciatus*) incorporating a high dietary proportion of secondary consumers, whereas much lower Hg levels were found for eagles feeding almost exclusively on herbivores such as rabbits, pigeons and partridges. Following this reasoning, it seems logical that Eagle owls in the Southern area, with a higher proportion of secondary consumers (31.58%) and even mesopredators in their diet, are expected to have higher Hg concentrations in their tissues.

However, local contamination sources may also contribute to the highest concentrations found in Eagle owl from the south. In the Southern area there is a subarea corresponding to an ancient mine site (Figure 13). When Eagle owls from this mine area (n=80) were excluded, no significant differences were found between Northern and Southern areas for Hg concentrations in blood of Eagle owls, with Hg levels of 33.77 ± 148.90 µg/L in the North (n=435) and 29.93 ± 61.44 µg/L in the South (n=108). Hence, it seems that the diet composition is not able to explain the higher Hg concentrations in Eagle owls from the Southern area in comparison with the Northern area. However, mean Hg level in blood of chicks from the ancient mine site (57.03 ± 182.76 µg/L, n=80) was significantly higher ($p < 0.001$) than the mean concentration in chicks from the rest of the sampled population (33.01 ± 136.02 µg/L, n=543) (Table 21). Similarly, no location-related differences were found for Hg concentrations in muscle of European rabbit (Table 21), but when rabbits from the ancient mine site were excluded, mean Hg levels in muscle of rabbit were significantly lower (12.46 ± 12.38 µg/Kg ww, $p = 0.020$) than those found in rabbits from the mine area (24.98 ± 12.76 µg/Kg ww) (Table 21). In this regard, the southeast of the Iberian Peninsula has traditionally suffered a great extraction of their mineral resources, thus high amounts of wastes have remained within mining areas. These materials are strongly enriched in heavy metals, such as lead, copper and zinc, being reported also, cadmium, arsenic and mercury (Faz Cano *et al.*, 2001). Moreover, many factories manufacturing chemical products were constructed 50 years ago near the city of Cartagena (Figure 13). As a consequence, soils in this area have also been affected by industrial wastes, and displayed a rather high metal pollution level, mainly of copper, zinc, cadmium, lead, and mercury (Faz Cano *et al.*, 2001). Besides the large amounts of metals and other contaminants present in this environment, geographical and climatic factors could avoid the efficient dispersion of these contaminants (García-Fernández *et al.*, 1995). In

this sense, a study conducted in the mid 90s reported that the highest concentrations of metals were found in blood of wild birds (Eagle owl included) from Cartagena in comparison with the rest of Murcia Region (García-Fernández *et al.*, 1995). Moreover, Eagle owl chicks from the mining area “Sierra Minera Cartagena-La Unión” or their surroundings had higher lead concentrations than the rest of the population in the period from 2003 to 2007 (Gómez-Ramírez *et al.*, 2011).

Furthermore, precipitation along the Southern area (annual precipitation 318 mm) is slightly higher than in the Northern area (annual precipitation 292 mm) (SIGA, 2012), which may contribute to a higher Hg removal from the atmosphere and local wet deposition.

Therefore, although the studied area is not considered Hg polluted, these results support the fact that spatial differences in Hg concentrations in Eagle owls appear to be mostly related to local contamination, and probably diet composition plays a role of less extent.

3.3. Temporal variations in mercury concentrations in Eagle Owl

Significant differences in blood Hg concentrations between years were found in Eagle owls ($p < 0.001$, n = 623) (Table 21, Figure 16). However, Hg concentrations found in muscle of European rabbit were very similar between years (Table 21). As explained above, although rabbits are the main prey of Eagle owls in the studied area, when faced with prey scarcity, this generalist predator will diversify its diet to include other preys and predators (Lourenço *et al.*, 2011). This fact could explain the differences found between years in Hg blood concentrations, since it seems logical that birds did not fed on exactly the same proportion of rabbits over the studied period. However, meteorological conditions may play a major role in these results.

Rainfalls in Murcia Region were variable during the seven years of the study (CREM, 2013), and they may contribute to a higher Hg removal from the atmosphere and local wet deposition. Moreover, stormwater runoff also washes off surfaces that may contain Hg and contributes to its transport. The two years with the highest Hg levels in blood of Eagle owls were 2007 and 2009, precisely two years with high rainfalls (397 and 402 L/m^2, respectively) (CREM, 2013) (Figure 16). The year 2010 also had high rainfalls (458 L/m^2) (CREM, 2013). However, the previous year was rainy too, so it is possible that less Hg was available for mobilization and deposition, and consequently lower concentrations were found in blood of Eagle owls in 2010. The lack

of differences in rabbit Hg levels between years seems to conflict with this explanation. However, the number of rabbit samples was small and only samples of 3 years were available to be analyzed, making it difficult to observe a trend.

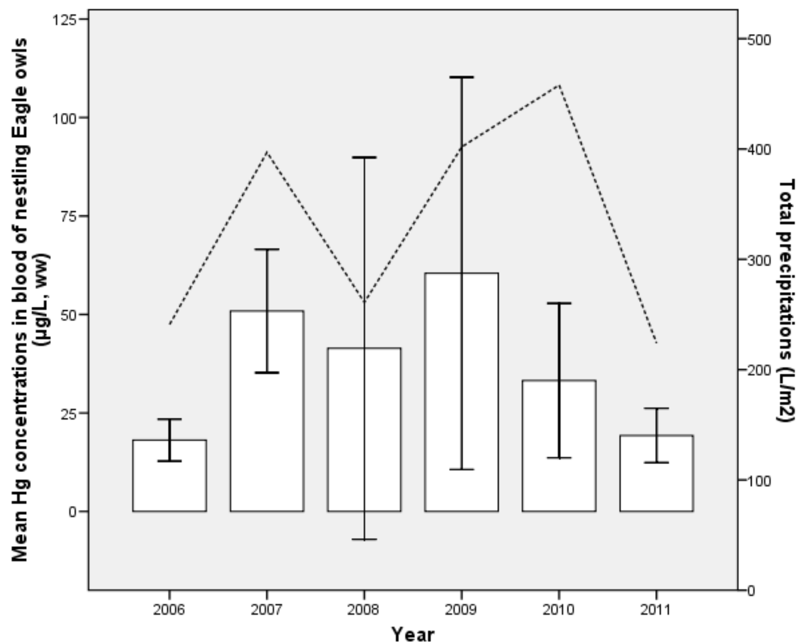

Figure 16. *Temporal trends in mercury levels (µg/L, ww) in blood of Eurasian Eagle Owls (p < 0.001, n = 623) and total precipitations (L/m2) in Murcia Region. Bars represent Confidence Intervals (CI) stated at the 95% confidence level. The dashed line represents the total annual rainfall.*

Positive correlations between Hg concentrations in blood of nestling Eagle owls and in muscle of their prey were found in samples collected in 2009 (r= 0.380, p = 0.029), 2011 (r= 0.614, p = 0.001) and 2012 (r= 0.556, p = 0.020). Moreover, Hg levels in blood of Eagle owls and in muscle of rabbit were very similar in samples collected in 2011 and 2012 (Table 21). However, significant differences were found (p=0.005) in samples collected in 2009, with the highest concentrations in blood of chicks (Table 21). This is because almost all nests sampled in 2009 containing carcasses of rabbits were from the Northern area, and owls from this area have lower Hg concentrations than in Southern area, as discussed above. These results suggest that blood Hg is greatly influenced by Hg ingested through the consumption of rabbit.

Hence, blood Hg concentrations in Eagle owls reflect Hg levels in muscle of rabbits, which is more evident in the nests from the Northern area, where rabbits are the main prey of Eagle owl. As explained above, both area and year variables

separately have a significant effect on Hg levels in blood of Eagle owls. Moreover, when Generalized Linear Models were performed, both variables combined also have a significant effect on Hg concentrations in blood of nestling Eagle owls ($p<0.001$, $n=623$). In this sense, local contamination seems to be the main cause of differences between areas, and rainfalls could have a major role in the differences found in blood Hg concentrations of Eagle owls between years.

4. *Conclusions*

Hg levels in Eagle owl chicks from Southeastern Spain can be considered low, and it is unlikely that Hg pollution can negatively affect the breeding performance. A unique growing back feather of nestling Eagle owl may be enough to estimate Hg concentrations in blood of this terrestrial species.

Blood Hg concentrations in Eagle owls reflect Hg levels in muscle of rabbits, which is more evident in the Northern area, where rabbits are the main prey. Although the studied region is not considered Hg polluted, the mining influence and the industrial zone seem to contribute to the higher Hg levels in Eagle owls and European rabbits from the area near the ancient mine site comparing with the rest of the studied region. This result supports the fact that spatial differences in Hg concentrations in Eagle owls appear to be mostly related to local contamination, and probably diet composition plays a role of less extent. Rainfalls could be the main cause of the temporal differences in blood Hg concentrations of nestling Eagle owls.

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CHAPTER VII

Effects of heavy metals exposure on oxidative stress biomarkers in Eurasian Eagle owl (*Bubo bubo*)

Photo: José Alfonso Lacalle Martínez

Silvia Espín, Emma Martínez-López, Mario León-Ortega, José E. Martínez, Antonio J. García-Fernández. Effects of heavy metals exposure on oxidative stress biomarkers in Eurasian Eagle owl (Bubo bubo).

Abstract

The main aim of the present study is the assessment of oxidative stress related to metal exposure in the Eurasian eagle owl (*Bubo bubo*) from two areas of Murcia, Southern Spain. Northern area is considered unpolluted while Southern area includes an ancient mine site. Mean metal concentrations in blood were Cd=0.07, Pb=3.27, Cu=10.62, Zn=311.47, Hg=2.32 µg/dl. Although individuals from Southern area have significant higher Pb and Hg concentrations (Pb=5.39 and 1.24, Hg=3.07 and 1.61 µg/dl in Southern and Northern area, respectively), and significant lower glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activities, the lack of differences in oxidative damage to membrane lipids (TBARS) suggests that the antioxidant capacity of both populations is able to deal with oxidant species and maintain TBARS levels in the same amount.

Despite the low levels of metals, several oxidative stress measures correlated with contaminant concentrations. Negative relationships between Cd or Pb levels and GPx or catalase (CAT) activities were found. In the Southern area, TBARS was positively correlated with Pb and Hg, and when only Eagle owls from the mining area were selected, a negative relationship between Pb concentrations and total glutathione (tGSH) levels was found. Moreover, results suggest a possible protective response by an increase of antioxidant enzymes (CAT and superoxide dismutase, SOD) activities under exposure of Hg in Eagle owls from the Northern area.

This study provides threshold concentrations at which metals cause effects on antioxidant system in Eagle owls. Blood Cd concentrations greater than 0.3 µg/dl inhibit CAT (25%) and GPx (32%) activities in red blood cells of Eagle owls. A depletion of 16% in tGSH levels was associated with Pb concentrations higher than 15 µg/dl in Eagle owls from the mining area. Finally, Pb concentrations above 10 µg/dl produced a TBARS induction of 28%, and Hg concentrations higher than 10 µg/dl produced a TBARS induction of 107% in Eagle owls from the Southern area.

1. ***Introduction***

The ability of metals to induce reactive oxygen species (ROS) has been suggested as one of the mechanisms involved in metal toxicity (Ercal *et al.*, 2001). ROS are unstable and very reactive molecules produced in oxidation-reduction reactions (Dowling and Simmons, 2009), presenting damaging effects on lipids, proteins and DNA (Rotilio *et al.*, 1995). In this sense, redox-active metals such as iron and copper catalyse Fenton reactions, which generate reactive hydroxyl radicals (Stohs and Bagchi, 1995). However, the primary route for redox-inactive metals such as lead, cadmium and mercury is to induce oxidative stress indirectly by depleting the major antioxidants of cells, such as glutathione and other thiol-containing antioxidants and protein-bound thiol groups (Stohs and Bagchi, 1995). However, they are also capable of inducing ROS formation indirectly (Lund *et al.*, 1991; Monteiro *et al.*, 1989; Ribarov and Bochev, 1982; Watanabe *et al.*, 2003).

To protect themselves against negative effects of ROS, living beings have developed an important mechanism able to prevent, neutralize and remove harmful toxicants from the body, the antioxidant defence (Koivula and Eeva, 2010). It consists of both endogenous and dietary antioxidants such as glutathione (GSH), ascorbate and vitamin E and different antioxidant enzymes, which operate in association with each other forming an integrated antioxidant defence system (Halliwell and Gutteridge, 1999). GSH is an endogenously produced antioxidant with an important role in the protection of cells against oxidative stress, because it participates in binding with ROS and metals through the sulfhydryl (SH) group, and it is also involved in enzymatic detoxification reactions for ROS as a cofactor or a coenzyme for enzymes such as glutathione S-transferase (GST) and glutathione peroxidase (GPx) (Gurer and Ercal, 2000). Other important components of the antioxidant system are enzymes such as superoxide dismutase (SOD), catalase (CAT) and GPx, that can detoxify peroxides, O_2 and H_2O_2, respectively (Gurer and Ercal, 2000). The imbalance between the production of ROS and the antioxidant molecules is defined as oxidative stress, so that the defence is overcome by radical formation (Halliwell and Gutteridge, 1999). An excess of ROS can react with biomolecules and induce lipid, protein and DNA oxidation leading to oxidative damage (Valavanidis *et al.*, 2006).

Since metal related oxidative stress has been demonstrated in experimental studies with birds (Hoffman *et al.*, 2005; Mateo *et al.*, 2003), levels of antioxidant molecules and activities of antioxidant enzymes could be useful biomarkers of metal

exposure and effect in birds. However, few works have studied oxidative stress related to metals in free-living birds (Berglund *et al*., 2007; Koivula *et al*., 2011; Martínez-Haro *et al*., 2011). Because of the close cooperation of the antioxidant defence system and the interspecific differences in the use of antioxidants against ROS, it is essential to use different biomarkers, enzymatic and non-enzymatic, when measuring oxidative stress (Berglund *et al*., 2007; Costantini and Verhulst, 2009). Moreover, to make inferences about oxidative stress, it is necessary to measure at least a marker of oxidative damage (Costantini and Verhulst, 2009).

The aim of the present study was to assess the concentrations of lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu) and zinc (Zn) in blood samples of Eurasian eagle owl (*Bubo bubo*) from two areas of Murcia, Southeastern Spain. Besides, effects induced by these metals on Eagle owl oxidative stress biomarkers have been studied. For this purpose, total GSH content, antioxidant enzymes activities (GPx, SOD, CAT and GST) and lipid peroxidation were analyzed in red blood cells.

2. Material and methods

2.1. Study area and species

The study area is located in the east of Murcia Region, in Southeastern Spain (37º45' N, 0º57' W) (Figure 17). Since the study area is relatively large and some differences in land use are known, it was subdivided in two subareas. In Northern subarea (mountains Escalona, Altaona, Monte el Valle and Columbares) land is mainly dedicated to citrus and dry farming, and the European Rabbit is abundant, accounting for 71% of the prey consumed by Eagle Owls (León *et al*., 2008). In Southern subarea, the European Rabbit is less abundant (35% of the Eagle Owls' diet), and the raptor consumes a similar proportion of rats (*Rattus rattus* and *Rattus norvergicus*) (23% of the diet), apart from pigeons (*Columba spp*.) (14%), partridges (*Alectoris rufa*) (5.26%), hedgehogs (*Erinaceus europeus* and *Atelerix algirus*) (5.26%) and yellow-legged gulls (*Larus michahellis*) (3.16%) (León *et al*., 2008). This subarea is delimited by Sierra Minera Cartagena-La Union, La Muela-Cabo Tiñoso and Almenara, irrigation farming is predominant and it is remarkable the fact that some ancient mining sites (Sierra Minera de Cartagena-La Unión) is found in this subarea (Figure 17). The climate is mesoarid Mediterranean with 275–400 mm of annual rainfall and a high average annual temperature of 19ºC (Gómez-Ramírez *et al*., 2011).

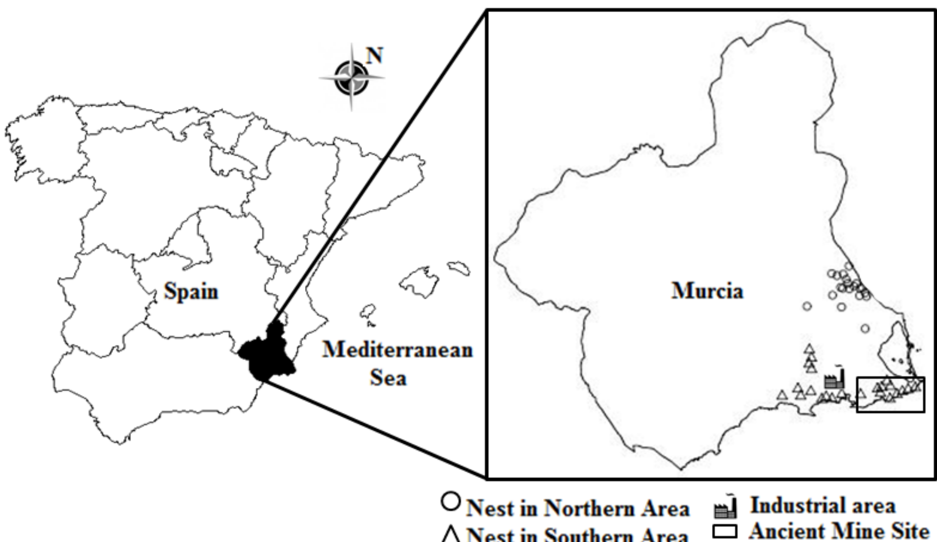

Figure 17. *Map showing the location of the study area (Murcia, Spain). Circles and triangles represent nests in Northern and Southern areas respectively. Industrial area is represented by a factory figure and area under mining influence is framed by a square.*

Eagle Owl prefers low to medium cliffs, especially in the Northern subarea. This species is the biggest nocturnal raptor in Spain, where it is sedentary and highly territorial during the whole year, occupying territories which size and foraging area depend on the prey availability. Eagle Owl population in Murcia Region is abundant and has been estimated in approximately 240–270 pairs (Martínez and Calvo, 2006; Martínez and Zuberogoitia, 2003). However, this data is most probably underestimated, and the number of pairs may be above 300 (León, personal communication).

2.2. Sample size

A total of 141 blood samples from 2011(n=71) and 2012 (n=70) (72 from Northern area and 69 from Southern area) were analyzed for metal concentrations in Eurasian Eagle Owl chicks (n=133) of approximately 30-days old and adult females (n=8) from Murcia (Figure 17). A total of 140 red blood cell (RBC) samples were analyzed for oxidative stress biomarkers.

Blood samples were collected by puncturing brachial vein with a 23G needle and a syringe, and stored in heparinised Eppendorf tubes under refrigerated conditions until processed in the laboratory. It was collected approximately 3-5 ml of blood, depending upon the stage of development of the nestling. After sampling, the nestlings were returned to their nest. Adult Eagle owls were captured in their territories with a mist net placed strategically close to the nest. One Eppendorf tube with whole blood was separated and another Eppendorf tube with blood was used to separate plasma and

red blood cell (RBC) fractions (10,000 rpm, 5 minutes). Plasma was separated in a new tube and RBC samples were washed with saline solution and centrifuged again at 10,000 rpm during 5 minutes. Hematocrit was recorded using a capillary tubes reader after centrifugation at 5,000 rpm during 5 minutes. Finally, three Eppendorf tubes with whole blood, plasma and RBC were stored at -80ºC until analysis. The sampling was authorized by the General Directorate of Natural Patrimony and Biodiversity from the Autonomous Community of Murcia Region.

The health status of the birds was evaluated clinically by a veterinarian prior to blood sampling. Besides, a plasma biochemistry analysis was done in every individual to check the normal health status and ensure that birds did not suffer any subclinical pathology. An A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain) was used to determine plasma biochemistry with commercial kits from BioSystems S.A. The plasma enzyme activities analyzed were alkaline phosphatase (ALP; Enzyme Commission number (EC) 3.1.3.1), aspartate aminotransferase (AST; EC 2.6.1.1), butyrylcholinesterase (CHE; EC 3.1.1.8), creatine kinase (CK; EC 2.7.3.2), gamma-glutamyltransferase (g-GT; EC 2.3.2.2), and lactate dehydrogenase (LDH; EC 1.1.1.27). The plasma constituents analyzed were albumin, total protein, cholesterol, glucose, triglycerides, uric acid, calcium and phosphorus.

2.3. Blood metals analysis

Cd, Pb, Cu and Zn levels were analyzed in blood samples following the method described by García-Fernández *et al.* (1995). The impurities of the samples were eliminated to prevent interferences with the results by a high temperature digestion with a mixture of acids following the method described by García-Fernández *et al.* (1995). A volume of 0.2 ml of whole blood was placed in a quartz digestion tube, to which 0.5 ml of an acid mixture (nitric:perchloric:sulfuric, 8:8:1) was added. The sample was then submitted to a progressive thermal treatment and, once dried, was left to cool down. Tetradistilled purified water was added and transferred to the measuring vessel, adjusting the final volume to 10 ml. Prior to anodic stripping voltammetry (ASV), 50 µl of hydrochloric acid were added to the measuring vessel as an electrolyte support. The pH of the final solution was between 1 and 2. The anodic stripping voltammeter (VA-757 Computrace Workstation; Methrohm, Switzerland) used was equipped with three standard electrodes: working electrode (hanging Hg drop), reference electrode (Ag/AgCl; KCl, 3 mol/l), and auxiliary electrode (platinum).

We used the differential pulse normal technique with an electrolysis time of 120 s and modulation amplitude of 50 mV. The concentration of each metal in the digested sample was calculated after twice adding dilutions prepared from standard solutions of Cd, Pb, Cu and Zn, respectively (Sigma, St. Louis, MO). Mean recoveries, which approached 96%, were calculated analysing 10 identical samples of reconstituted lyophilized blood (European Union Reference Standards CRM195). Detection limits were 0.05 and 0.1 µg/L for Cd and Pb, respectively, and 0.3 and 0.04 mg/L for Zn and Cu, respectively. All the reagents used were Suprapur quality from Merck (Darmstadt, Germany). The quartz tubes used for the wet digestion were previously washed with 2% nitric acid for 48 h and then rinsed twice with tetradistilled water and dried in an oven at 100ºC.

Total Hg was analyzed in a Milestone DMA-80 direct Hg analyzer by atomic absorption spectrophotometry with a detection limit of 0.005 ng. Blood samples (100 µl wet weight) were loaded in a nickel boat and analyzed. Calibration curve was done with ten points (in duplicate) from 0 to 1004 ng of Hg. Precision and accuracy of the method were tested using certified reference material (CRM) (Hg Standard for AAS, Fluka, 1000 mg/L Hg in 12% nitric acid, prepared with high purity Hg metal, HNO_3TraceSELECT® and water TraceSELECT®Ultra). Recovery of total Hg from five replicates of CRM diluted to 1 ppm was 104.2±11.8% (mean±standard deviation). The coefficient of variation for the repeatability was 11.4%.

2.4. Biomarker analyses in red blood cells (RBC)

Oxidative stress parameters (total glutathione, glutathione peroxidase, superoxide dismutase, catalase, glutathione-S-transferase and thiobarbituric acid-reactive substances) were analyzed in RBC, after homogenization (1:10 w/v) in a stock buffer (1.15% KCl in 0.01 M PBS (pH 7.4) with 0.02 M EDTA). Lipid peroxidation, estimated as thiobarbituric acid-reactive substances (TBARS), was assessed following the methodology described by Alonso-Alvarez *et al.* (2008) with a spectrophotometer (UV-1603, Shimadzu). Levels of total glutathione (tGSH) were obtained as described by Reglero *et al.* (2009) with an automated spectrophotometer A25-Autoanalyzer (BioSystems). The activities of glutathione peroxidase (GPx; EC1.11.1.9) and superoxide dismutase (SOD; EC1.15.1.1) were determined spectrophotometrically (A25-Autoanalyzer, BioSystems) using the Ransel and Ransod kits (Randox Laboratories), respectively, following descriptions of Reglero *et al.* (2009) with some modifications for RBC. Homogenized samples were diluted by 1:20 and 1:25 (v:v) with

Ransel diluting agent and Ransod sample diluents (Randox Laboratories), for GPx and SOD determinations respectively. GPx and SOD results were expressed as Units per gram of protein.

Catalase (CAT; EC 1.11.1.6) activity was assayed following the methodology described by Clairbone (1985), based on the decomposition of hydrogen peroxide (H_2O_2) in molecular oxygen and water by this enzyme. The rate of enzymatic decomposition of H_2O_2 was determined as absorbance decrements at 240 nm with a spectrophotometer (UV-1603, Shimadzu). The assay mixture consisted of 950 µl of potassium phosphate buffer (0.05M, pH 7.0), 500 µl of H_2O_2 (0.03M) and 50 µl of sample. Results were expressed as µmol H_2O_2 consumed per minute per milligram of protein.

The activity of glutathione-S-transferase (GST; EC 2.5.1.18) was determined by the method described by Habig *et al.* (1974), based on the measurement of the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione. This reaction is catalyzed by GST, and it is determined as absorbance increments at 340 nm. The assay mixture consisted of 1850 µl of potassium phosphate buffer (0.2M, pH 7.9), 50 µl of CDNB solution (8.17mM) (Sigma) diluted in ethanol:water (1:1.5), 50 µl of GSH solution (8.17mM) (Sigma) and 50 µl of sample. The results were expressed as nanomoles produced per minute per milligram of protein.

Because enzyme activities were expressed relative to grams of protein in the homogenates, total protein contents in these homogenates were measured spectrophotometrically at 595 nm following the method of Bradford (1976), using bovine serum albumin as standard protein.

2.5. Statistical analysis

All analyses were carried out using the SPSS v.15.0 statistical package. Reported metal concentrations and oxidative stress biomarker values represent the mean ± standard deviation, median and range. The data were tested for normality using a Kolmogorov-Smirnov test and when necessary, data were normalized using log-transformation. Differences in metal concentrations and biomarker activities values between areas and age groups were calculated by ANOVA. Correlations between metals and between biomarkers were checked using Pearson's correlation coefficient. Simple linear regression was performed to evaluate the effect of each metal on the oxidative stress biomarkers. The level of significance for these tests was set at $\alpha=0.05$.

Because of the limited amount of sample from some birds, sample sizes were not the same for all the parameters analyzed. Generalized Linear Models (GLMs) with a normal distribution and an identity function were performed to study the effects of studied area and age on the concentrations of metals and on the oxidative stress parameters. GLMs were also performed to study combined effects of metals on the biomarkers. Biomarker value was the response variable, metal concentrations were selected as covariates and studied area and age were selected as factors. A backward stepwise procedure was used to select the final models, and predictor variables were retained when they significantly improved model fit ($p<0.05$).

3. Results and discussion

In general, plasma biochemistry parameters were similar to those described as baseline data in Eagle owls by several authors (García-Rodríguez *et al.*, 1987; Gómez-Ramírez, 2011; Jennings, 1996), and are indicative of normal health status.

3.1. Metal concentrations in blood

Metal concentrations in blood of Eagle owl, metal levels according to area and age, and Generalized Linear Models for metal concentrations are presented in Table 23.

Table 23. *Metal concentration (µg/dl) in blood of Eagle owl, Generalized Linear Models for metal concentrations, and concentrations of metals according to area and age.*

Metal concentrations in blood of Eagle owl and Generalized Linear Models							
			Cd (µg/dl)	**Pb (µg/dl)**	**Cu (µg/dl)**	**Zn (µg/dl)**	**Hg (µg/dl)**
All individuals (N=141)		Mean±SD	0.07±0.21	3.27±5.21	10.62±4.77	311.47±67.14	2.32±3.83
		Median (range)	0.006 (0.006-1.94)	1.31 (0.05-31.23)	10.63 (3.36-34.20)	299.20 (62.50-610.52)	1.5 (0.15-38.44)
Model			**None**	**Area***	**Age***	**None**	**Area**+Age*****
Area	North (N=72)	Mean±SD	0.08±0.26	1.24±1.12*	10.35±4.19	318.30±78.90	1.61±1.48**
		Median	0.006	0.92	10.46	307.22	1.20
	South (N=69)	Mean±SD	0.06±0.13	5.39±6.75	10.90±5.32	304.35±51.75	3.07±5.17
		Median	0.006	3.32	10.68	293.76	1.75
Age	Nestling (N=133)	Mean±SD	0.07±0.21	3.38±5.33	10.29±4.56*	310.38±67.41	2.31±3.93
		Median	0.006	1.36	10.50	297.30	1.44
	Adult (N=8)	Mean±SD	0.006±0.01	1.39±0.95	16.14±5.05	329.59±63.69	2.49±0.74
		Median	0.006	1.19	14.63	316.21	2.31

*Note: Model: indicates the most influential factor (explanatory variable) in the response variable "Metal concentrations". None = concentrations of metals are not significantly influenced by any variable. N = number of samples. *p<0.001, **p<0.01, ***p<0.05.*

Mean Pb levels were lower than those found by García-Fernández *et al.* (1995) (mean=8.3 ± 6.7 μg/dl) and García-Fernández *et al.* (1997) (mean=7.6 μg/dl, median=6.5 ± 2 μg/dl) in Eagle owls from Murcia Region, and similar to those found by Gómez-Ramírez *et al.* (2011) (3.7 ± 4.2 μg/dl) in nestling Eagle owls from the same studied region. Studies in Murcia show that Pb exposure has decreased in the last 10 years (García-Fernández *et al.*, 2008). Our study confirms a decrease in Pb exposure in the last 20 years in Eagle owls from Murcia Region, which could be explained by the closure of mines in 1991 and the ban on leaded petrol in 2001.

In general, Pb concentrations were similar to those found in raptor species from non-polluted areas (Henny *et al.*, 1994; Martínez-López *et al.*, 2004). In fact, García-Fernández *et al.* (1995) suggested that raptors in the region of Murcia were chronically exposed to rather low Pb levels, mainly through food consumption. Only four individuals in this study presented Pb concentrations higher than 20 μg/dl, which is the minimum blood level for considering physiological effects in Falconiformes according to Franson (1996). However, more recent studies have found that Pb concentrations below 15 μg/dl in blood are enough to cause sublethal effects, such as inhibition of δ-aminolevulinic acid dehydratase (ALAD) activity in raptors in the field (Gómez-Ramírez *et al.*, 2011; Martínez-López *et al.*, 2004).

Significant differences were found in Pb concentrations between areas, with higher levels in Eagle owls from the Southern area (Table 23). When GLMs were performed, the area was included in the model (Table 23). Local contamination sources have probably contributed to the highest concentrations detected in Eagle owl from the south. In the Southern area, there is an important industrial zone near the city of Cartagena (Figure 17), including electric power plants, and explosives and shipbuilding factories (García-Fernández *et al.*, 1995). Large amounts of metals and other contaminants were present in this environment, and geographical and climatic factors could avoid the efficient dispersion of these pollutants (García-Fernández *et al.*, 1995). In this sense, these authors found higher concentrations of Pb and Cd in tissues of wild birds (Eagle owl included) from Cartagena in comparison with the rest of Murcia Region. Moreover, in the Southern area there are some nests located close to an ancient mine site called "Sierra Minera Cartagena-La Unión" (Figure 17). In fact, coinciding with previous studies (Gómez-Ramírez *et al.*, 2011), when only individuals from this ancient mine site were selected, mean Pb concentrations of 7.64±7.11 μg/dl (n=29, median=4.93, range=1.08-27.65) were obtained, significantly higher ($p<0.001$) than the levels found in the rest of the population (mean Pb levels=2.14±3.90 μg/dl,

n=112, median=1.08, range=0.051-31.23) and close to those described 15 years ago by García-Fernández *et al.* (1997). The mining district of Cartagena-La Unión suffered an intensive mining activity until 1991, mainly of lead, zinc, copper, tin, iron, manganese and silver, for more than 2,500 years (Pavetti *et al.*, 2006). This area was the main source for Pb and Zn in Spain during the nineteenth century (Estevan-Senís, 1967). The low concentrations found in the Northern area were expected, since nests are located far from potential sources for heavy metals.

Although no significant differences were found between age groups, adult individuals had lower Pb concentrations than nestlings (Table 23). However, it may be noted that the majority of adult individuals (7 out of 8) were captured in the Northern area, and these individuals presented Pb concentrations between 0.30 and 1.62 µg/dl, while the only adult from the Southern area had Pb levels of 3.53 µg/dl.

Regarding Hg concentrations, there is few data available about its levels in blood of Eagle owls (see Chapter VI). However, concentrations found were much lower than those reported for fish-eating raptors (Jagoe *et al.*, 2002; Langner *et al.*, 2012). Significant differences were found in Hg concentrations between areas. GLMs showed that the best explanatory variables were area and age (Table 23). A previous study in the same studied area (see Chapter VI) analyzed Hg concentrations in blood and feathers of Eagle owl and in muscle of their main prey, the European rabbit, from 2006 to 2011. As explained in Chapter VI, the region is not considered Hg polluted. However, spatial differences in Hg concentrations in Eagle owls appear to be mostly related to local contamination (ancient mine site and industrial zone), and probably diet composition plays a role of less extent. Besides, in Chapter VI, significant differences in blood Hg concentrations between years were found in Eagle owls from Murcia Region. This result is probably due to variable rainfalls during the seven years of study, which may contribute to a higher Hg removal from the atmosphere and local wet deposition in years with the highest rainfalls (see Chapter VI). On the other hand, higher Hg levels in adult individuals may occur if they eat larger, more contaminated prey and/or may simply reflect an accumulation of Hg in their tissues over a longer period of time (Kojadinovic *et al.*, 2007).

Cd was detected above the detection limit only in 26% of the samples. Cd concentrations were similar to those found by García-Fernández *et al.* (1995) and Gómez-Ramírez (2011) (0.1 µg/dl), and they were within the range considered as low exposure levels in birds (0.01 to 0.28 µg/dl) (García-Fernández *et al.*, 1996). Taking

into account these low Cd levels in Eagle owls from Murcia in the last 20 years (García-Fernández *et al.*, 1995; Gómez-Ramírez, 2011), it could be suggested that Eagle owls are being exposed to low Cd levels over time, and most probably through dietary ingestion (García-Fernández *et al.*, 1996). Non significant differences in Cd concentrations were found according to area and age (Table 23). The lack of differences between Northern and Southern areas probably shows that there are no important Cd emissions in the studied areas.

Finally, concentrations of the essential metals Zn and Cu were similar to those found by Gómez-Ramírez (2011) (median levels of 328 and 14 µg/dl for Zn and Cu, respectively). In general, Zn and Cu concentrations were within the range of physiological levels in several health bird species (163-495 µg/dl for Zn and 13-120 µg/dl for Cu), including raptors (García-Fernández *et al.*, 2005).

3.2. Oxidative stress

3.2.1. Oxidative stress biomarkers in Eagle owls from two areas of Murcia (Southeastern Spain)

Enzyme activities, tGSH levels and lipid peroxidation in red blood cells of Eagle owls are presented in Table 24. Table 24 also shows GLMs for oxidative stress biomarkers, and results according to area and age. Both SOD activity and TBARS concentrations showed significant differences between age groups, with higher SOD activity and TBARS levels in adult individuals than in chicks (Table 24). The best model for SOD activity was only constructed with age (X^2=12.45, p<0.001). Similarly, Oropesa *et al.* (in press) described the highest SOD activity in adult White storks (*Ciconia ciconia*). Apparently, oxidative damage increases with age (Koivula and Eeva, 2010), which has been related with increased production and susceptibility to ROS at older age (Hulbert *et al.*, 2007).

Table 24. *Enzyme activities, glutathione levels and lipid peroxidation in red blood cells of Eagle owls, and values according to area and age.*

Enzyme activities, glutathione levels and lipid peroxidation in red blood cells								
			GPx[a]	**SOD[b]**	**CAT[c]**	**GST[d]**	**tGSH[e]**	**TBARS[f]**
All individuals		N	140	139	140	140	139	135
		Mean±SD	617.12±208.73	686.38±179.26	21.19±7.24	10.28±2.97	8.63±2.07	0.051±0.026
		Median (range)	641.61 (105.13-1423.43)	697.82 (267.59-1092.66)	23.70 (8.74-36.99)	10.17 (3.77-25.29)	8.43 (1.43-14.62)	0.047 (0.009-0.213)
Model			**Area*****	**Age***	**None**	**Area***	**Area******	**Age****
Area	North	N	71	71	71	71	71	68
		Mean±SD	649.23±203.66	678.42±187.63	21.98±6.91	11.24±3.20*	8.32±2.20	0.054±0.027
		Median	653.58	670.66	23.83	11.29	8.20	0.047
	South	N	69	68	69	69	68	67
		Mean±SD	584.07±210.17	694.68±171.08	20.38±7.53	9.29±2.34	8.94±1.87	0.049±0.025
		Median	611.36	724.32	22.17	9.52	8.59	0.047
Age	Nestling	N	133	132	133	133	131	127
		Mean±SD	617.44±211.07	674.34±171.56*	21.29±7.32	10.19±3.00	8.67±2.08	0.050±0.025**
		Median	631.16	692.17	23.83	10.11	8.47	0.047
	Adult	N	7	7	7	7	8	8
		Mean±SD	611.03±170.46	913.39±182.01	19.33±5.44	11.98±1.63	7.82±1.73	0.068±0.028
		Median	689.48	983.44	19.46	12.02	8.00	0.061

Note: [a]Glutathione peroxidase (U/g protein), [b]Superoxide dismutase (U/g protein), [c]Catalase (µmol/min/mg protein), [d]Glutathione-S-Transferase (nmol/min/mg protein), [e]total Glutathione (µmol/g), [f]Lipid peroxidation, estimated as thiobarbituric acid-reactive substances (µmol/g).
*Model: indicates the most influential factor (explanatory variable) in the response variable "Oxidative stress biomarker". None = oxidative stress biomarker is not significantly influenced by any variable. N = number of samples. *p<0.001, ** p<0.05, ***p=0.06, ****p=0.07.*

Studies published up to now showed that metals can induce oxidative stress, but the response varies notoriously depending on the concentration of metals, duration of exposure and species studied (Hoffman *et al.*, 2000a; Ji *et al.*, 2006; Mateo and Hoffman, 2001). Several authors have found an enhanced activity of antioxidant enzymes, and higher levels of GSH and lipid peroxidation in birds from polluted areas compared with reference sites (see Chapter VIII; Berglund *et al.*, 2007; Ji *et al.*, 2006; Kamiński *et al.*, 2009). However, in the present study significant location-related differences were found only for GST activity, with lower activity in the Southern area (Table 24). When GLMs were performed, area was a variable with significant effect on the model of GST, and with marginal effect on the model of GPx and GSH (Table 23). However, when adult individuals were excluded from the analysis to avoid the possible influence of age, significant differences were also found in GPx activity (p=0.049; GPx Northern area (n=65)= 654.3 ± 206.2; GPx Southern area (n=68)= 582.2 ± 211.2 U/g

protein. GST is an enzyme used to catalyse the conjugation of GSH with cytotoxic aldehydes produced during lipid peroxidation (Halliwell and Gutteridge, 1999) and GSH conjugation with pollutants, and some GST isozymes posess non-Se-dependent GPx activity (Prohaska and Ganther, 1977). GPx enzyme catalyses the transformation of H_2O_2 to H_2O by oxidizing GSH (Koivula and Eeva, 2010), and requires selenium (Se) as a cofactor (ExPASy, 2012). An experimental study with Mallards (*Anas platyrhynchos*) has shown a reduction in GST and GPx activities in Pb-treated individuals (Mateo *et al.*, 2003). In this sense, it is possible that the higher concentrations of Pb and Hg in owls from the Southern area in comparison to the Northern area could induce a depletion of GST and GPx in this population. No differences were found in TBARS concentrations in red blood cells of Eagle owl between areas (Table 24). Although individuals from Southern area have significant higher Pb and Hg concentrations, significant lower GST and GPx activities, and marginal higher tGSH levels; the lack of differences in oxidative damage to membrane lipids (TBARS) suggests that metal levels in the Southern area are not high enough to produce significantly higher TBARS in comparison with the Northern area.

3.2.2. Effect of metal concentrations in oxidative stress biomarkers

Simple linear regression analysis was conducted to search for biomarker response on single metal concentrations in Eagle owl (Table 25), and GLMs were also performed to study combined effects of metals, studied area and age on the biomarkers. We conducted the statistical analysis with all individuals to increase the number of samples, and also with separate populations.

Despite the low levels of metals, several oxidative stress biomarkers correlated with contaminant concentrations. When only Eagle owls from the Northern area were selected, positive relationships were found between CAT activity and Hg concentrations ($r=0.343$, $F=9.23$, $p=0.003$), SOD activity and Hg concentrations ($r=0.313$, $F=7.485$, $p=0.008$) (Figure 18), and GST activity and Cd levels ($r=0.374$, $F=11.254$, $p=0.001$). These results suggest a possible protective response by an increase of antioxidant enzymes activities in individuals from the Northern area due to Cd and Hg exposure. In this sense, Hussain *et al.* (1999) reported increased CAT activity in mice exposed to Hg, which was explained as a possible compensatory mechanism to scavenge ROS levels produced as a result of Hg accumulation. Moreover, Hg stimulates the activity of Cu-ZnSOD (Gurer and Ercal, 2000), probably as a protective effect. In this sense, changes in active scavengers of free radicals such

as SOD enzyme are dependent on duration of exposure and Hg concentrations (Ji *et al.*, 2006), thus low-dose Hg exposure would result in increased levels of SOD as a counteractive response of the redox-defense system (Elia *et al.*, 2003). In Chapter VIII, SOD activity also depended mostly on Hg concentrations in Griffon vultures (*Gyps fulvus*). Besides, Cd also caused an increase of 17% in liver GST activity in rats (Jurczuk *et al.*, 2006). As suggested by Jurczuk *et al.* (2006), GST may be induced by this metal, since GST catalyzes the conjugation of Cd with GSH.

Table 25. *Linear regression analysis of biomarker response on single metal concentrations in Eagle owl.*

Biomarker response (Y)[a]	Metal (X)	*r*	*F*	*p*	Intercept (*a*)	Regression coefficient (*b*)
GPx	LogCd	-0.223	7.26	0.008	486.501	-69.581
GPx	LogPb	-0.154	3.35	0.069	628.508	-61.249
LogCAT	LogCd	-0.355	19.95	<0.001	1.139	-0.085
LogCAT	LogPb	-0.244	8.73	0.004	1.312	-0.074
LogCAT	Cu	-0.24	8.47	0.004	1.384	-0.008
LogTBARS	LogHg	0.232	7.54	0.007	-1.349	0.118

Regressions follow the model Y=a+bx. r=Pearson´s correlation coefficient. [a]GPx=Glutathione peroxidase, SOD=Superoxide dismutase, CAT=Catalase, GST=Glutathione-S-Transferase, TBARS=Thiobarbituric acid-reactive substances.

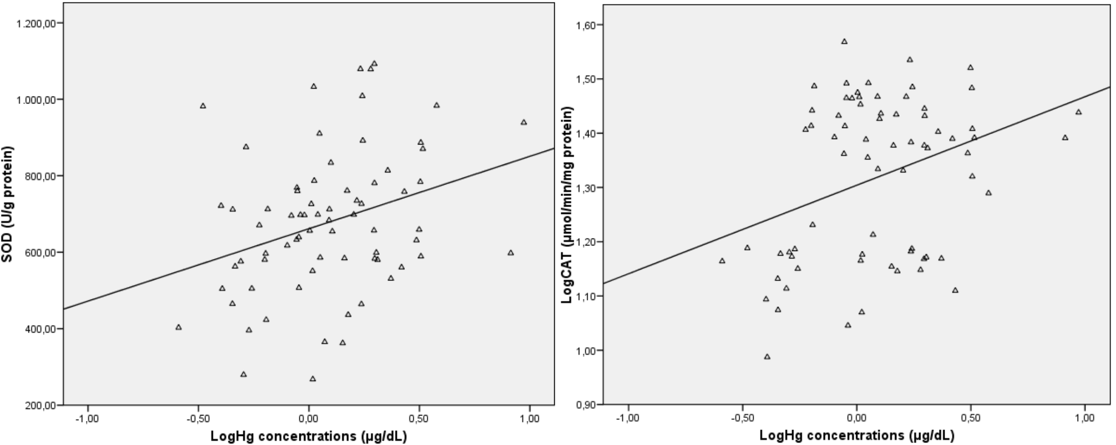

Figure 18. *Relationship between Log Hg concentrations (µg/dl) in blood and SOD (U/g protein) (r=0.313, p=0.008, n=71), and between Log Hg levels and Log CAT (µmol/min/mg protein) (r=0.343, p=0.003, n=71) in red blood cells of Eagle owls from the Northern area.*

Glutathione peroxidase (GPx)

GPx activity was inversely correlated with Cd concentrations, and a marginally inverse correlation between GPx activity and Pb concentration was found (Table 25). When adult individuals were excluded of the analysis because of the possible effect of

age, the inverse correlation between GPx activity and Pb concentrations was significant (r=-0.171, p=0.049). When GLMs were performed, the best model for GPx activity was constructed only with Cd (X^2=7.17, p=0.007). The GPx activity and Cd concentration inverse relationship was still true and even stronger when only the individuals from the Southern area were selected (r=-0.508, F=23.29, p=<0.001). Cd concentrations ≥ 0.3 µg/dl in blood produced an inhibition of 32% in GPx activity in red blood cells of Eagle owls. In this regard, several studies have found that rats treated with Cd showed decreased GPx activity (Gambhir and Nath, 1992; Jamall and Smith, 1985; Omaye and Tappel, 1975), and increased dietary Se prevented the inhibition of GPx activity (Jamall and Smith, 1985). GPx activity also showed a 47% decrease in liver of starlings (*Sturnus vulgaris*) treated with Cd, due to inhibition of the Se-dependent fraction of the enzyme (Congiu *et al.*, 2000). GPx reduces peroxides in cells, such as the transformation of H_2O_2 to H_2O by oxidizing GSH (Koivula and Eeva, 2010), and requires Se as a cofactor (ExPASy, 2012). Hence, the formation of Cd-Se complex as a protective effect of Se against Cd toxicity (Gambhir and Nath, 1992) could be the reason of the negative correlation found between GPx activity and Cd levels in the present study. Antagonistic effects between Pb and Se have also been described (Schrauzer, 1987), resulting in decreased Se uptake that may affect GPx activity. In this sense, several authors have found an inhibition of GPx activity in Pb exposed birds (see Chapter VIII; Mateo *et al.*, 2003; Somashekaraiah *et al.*, 1992). In the present study, Pb concentrations ≥ 10 µg/dl in blood produced an inhibition of 6.3% in GPx activity in red blood cells of Eagle owls chicks.

Catalase (CAT)

Regarding CAT activity, it was inversely related with concentrations of the single metals Cd, Pb and Cu (Table 25). The best explanatory variables when GLMs were constructed were Cd and Pb (X^2=23.12, p<0.001). In the Southern area CAT activity was negatively correlated with both Cd (r=-0.5, F=22.33, p=<0.001) and Cu (r=-0.27, F=5.25, p=0.025) concentrations, however in the Northern area CAT activity was related with Pb levels (r=-0.284, F=6.05, p=0.016). CAT enzyme catalyses H_2O_2 to H_2O and molecular oxygen (Koivula and Eeva, 2010). CAT activity has been inhibited following both *in vivo* and *in vitro* exposure to Cd in rats and several fish species (Koizumi and Li, 1992; Palace *et al.*, 1993; Pruell and Engelhardt, 1980; Roméo *et al.*, 2000). Palace *et al.* (1993) suggested a direct structural alteration of the enzyme and depression of CAT synthesis by Cd. In the present study, Cd levels ≥ 0.3 µg/dl in blood produced an inhibition of 25% in CAT activity in red blood cells of Eagle owls.

Moreover, CAT enzyme has heme as the prosthetic group (ExPASy, 2012) which contains iron (Fe), and it is known that Pb reduces the absorption of Fe in the gastrointestinal tract and inhibits the heme biosynthesis (Gurer and Ercal, 2000). In fact, several authors have found inhibition of CAT activity in Pb-exposed animals (Sandhir and Gill, 1995; Sandhir *et al.*, 1994).

Total glutathione (tGSH)

Regarding tGSH levels, no relationship was found with any single metal and no significant models were constructed with the studied variables. However, linear regression analysis was performed selecting only the individuals living in the ancient mine site (n= 29, Figure 17) in order to elucidate if the significant higher levels of Pb in this subarea (7.64±7.11 µg/dl) in comparison with the rest of the population could have an effect on tGSH concentrations. In this sense, a significant negative relationship between blood Pb concentrations and tGSH levels in red blood cells of Eagle owls from the mining area was found (r=-0.392, F=4.729, p=0.039; Figure 19).

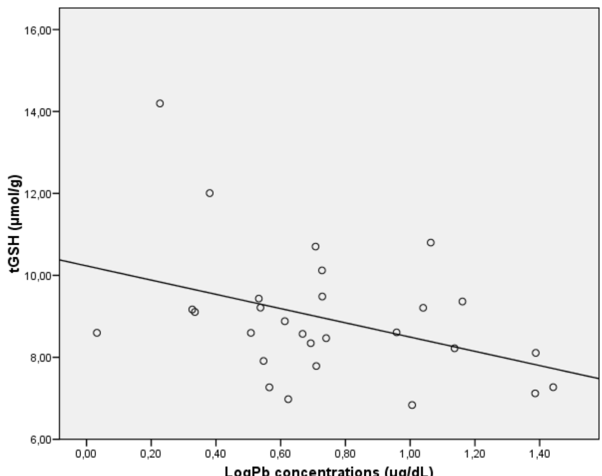

Figure 19. *Relationship between Log Pb concentrations (µg/dl) in blood and total glutahione (µmol/g) in red blood cells of Eagle owls from an ancient mine site (r=-0.392, p=0.039, n=28).*

A depletion of 16% in tGSH levels were associated with Pb concentrations ≥ 15 µg/dl in Eagle owls from the ancient mine site. Several studies have found a reduction in GSH concentrations in Pb-exposed rats (Korsrud and Meldrum, 1988) and birds (Mateo *et al.*, 2003; Somashekaraiah *et al.*, 1992). This may be explained by GSH role in the excretion of this metal through Pb binding to GSH due to its affinity for sulfhydryl groups (Sharma *et al.*, 2011).

Lipid peroxidation

TBARS levels were positively related with Hg concentrations (Table 25, Figure 20). The explanatory variables in the best model were age and Hg concentrations (X^2=11.44, p=0.003). When only Eagle owls from the Southern area were selected, the relationship between TBARS and Hg levels was stronger (r=0.434, F=15.096, p=<0.001), and a new relationship between TBARS levels and Pb concentrations was found (r=0.259, F=4.687, p=0.034) (Figure 20).

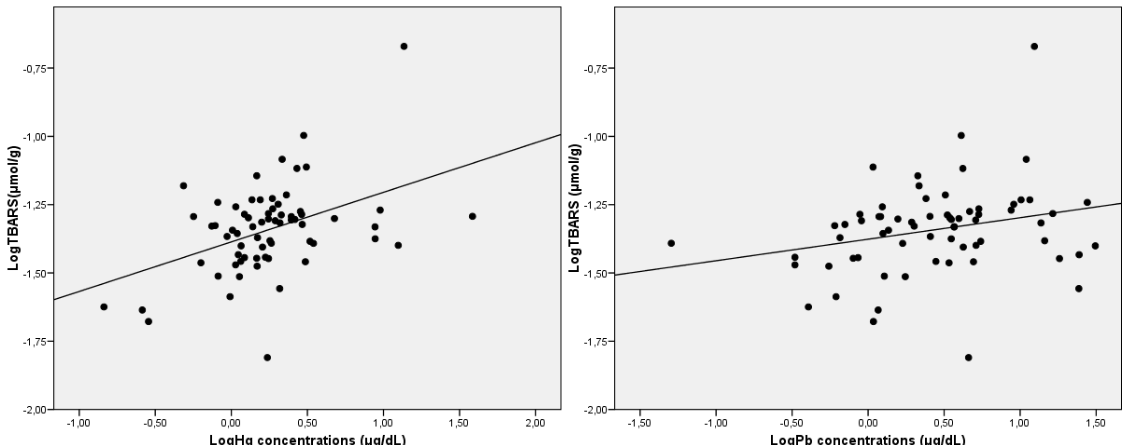

Figure 20. *Relationship between Log TBARS (µmol/g) in red blood cells and Log Hg concentrations (µg/dl) in blood (r=0.434, p<0.001, n=67), and between Log TBARS (µmol/g) in red blood cells and Log Pb concentrations (µg/dl) in blood (r=0.259, p=0.034, n=67) of Eagle owls from the Southern area*.

An increase in lipid peroxidation after Pb (Hoffman *et al.*, 2000a,b; Mateo and Hoffman, 2001; Mateo *et al.*, 2003; Somashekaraiah *et al.*, 1992) and Hg exposure (Hoffman *et al.*, 2005; Huang *et al.*, 1996) has been found in birds and rats in several studies. In Chapter VIII, a correlation between Pb and Hg concentrations and TBARS levels was also found in Griffon vultures. The correlations found are indicative of an effect of Hg and Pb on lipid peroxidation, particularly in the Southern area. Pb concentrations ≥ 10 µg/dl produced a TBARS induction of 28%, and Hg concentrations ≥10 µg/dl resulted in a TBARS induction of 107% in Eagle owls from the Southern area. In this sense, several mechanisms may be responsible for lipid peroxidation by these metals. Both Hg and Pb may induce generation of ROS, associated with lipid peroxidation in membranes (Lund *et al.*, 1991; Monteiro *et al.*, 1989; Ribarov and Bochev, 1982; Verity *et al.*, 1975). Moreover, as explained above, both metals can alter levels of GSH (Flora *et al.*, 2008) and the activity of antioxidant enzymes (Gstraunthaler *et al.*, 1983; Sandhir and Gill, 1995; Schrauzer, 1987; Zalups and Lash, 1996), which interfere in the protection against lipid peroxidation.

3.2.3. Correlations among oxidative stress biomarkers

Correlations between oxidative stress biomarkers were also conducted, and some of them were correlated with each other. The positive correlation found between GPx and CAT enzymes (r=0.644, p=<0.001, n=140) was most probably due to their collaboration in the decomposition of H_2O_2 (Halliwell and Gutteridge, 1999). We also found positive correlations between GST with GPx (r=0.343, p=<0.001, n=140), and GST with CAT (r=0.250, p=0.003, n=140), which may be interpreted as a collaboration of GST with these enzymes since GST also removes H_2O_2 from the cells through the GSH oxidation (Koivula and Eeva, 2010). The negative correlation found between CAT and TBARS (r=-0.242, p=0.004, n=134) suggests that CAT may play a role in the protection of the cell against the lipid peroxides, as suggested by other authors for GST enzyme (Isaksson *et al.*, 2009). Furthermore, one of the functions of GSH is the reduction of lipid peroxides by GPx (Kidd, 1997), which may explain the positive correlation found between tGSH and TBARS levels (r=0.315, p=<0.001, n=135).

3.2.4. Relationship between oxidative stress biomarkers and size of the brood

Koivula *et al.* (2011) found a positive relationship between GST activity and the size of the brood. The higher GST activity in larger broods in Great tit (*Parus major*) was explained by an increase within the brood competition for food and space may cause increased oxidative stress among nestlings (Koivula *et al.*. 2011). However, in the present study we found negative relationships between the number of nestlings in each nest and GPx activity (r=-0.316, p<0.001, n=133) or CAT activity (r=-0.201, p=0.021, n=133) (Figure 21), showing lower enzyme activities in larger broods. Several studies have found increased oxidative stress in nestlings from larger broods in Common kestrels (*Falco tinnunculus*) and Common starlings (*Sturnus vulgaris*) (Costantini *et al.*, 2006, 2010), and nestling Common starlings raised in experimentally enlarged broods had lower total antioxidant capacity (Bourgeon *et al.*, 2011).

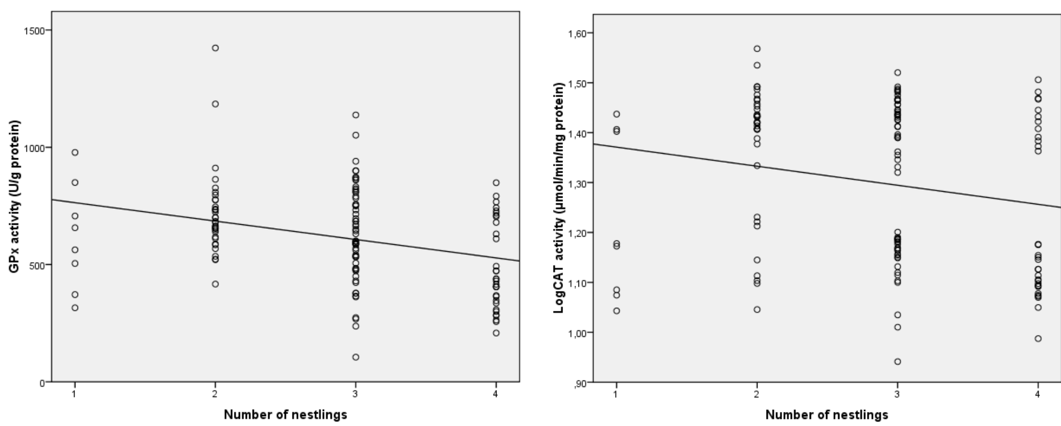

Figure 21. *Relationship between number of nestlings and glutathione peroxidase activity (U/g protein) (r=-0.316, p<0.001, n=133) or catalase activity (µmol/min/mg protein) (r=-0.201, p=0.021, n=133).*

4. Conclusions

The present study provides information about oxidative stress in Eagle owls exposed to different levels of several metals. Although individuals from Southern area (around an ancient mine site) had significant higher Pb and Hg concentrations, significant lower GST and GPx activities, and marginal higher tGSH levels; the lack of differences in oxidative damage to membrane lipids (TBARS) suggests that metal levels in the Southern area were not high enough to produce significantly higher TBARS in comparison with the Northern area. Despite the low levels of metals, several oxidative stress biomarkers were correlated with contaminant concentrations. Negative relationships between Cd and Pb levels and GPx or CAT activities were found. In the Southern area, TBARS were positively correlated with Pb and Hg, and when only Eagle owls from the mining area were selected, a negative relationship between Pb concentrations and tGSH levels was found. Finally, results suggested a possible protective response in Eagle owls from the Northern area, since the low exposure levels of Hg in Eagle owls resulted in increased activities of antioxidant enzymes CAT and SOD.

In addition, this study provides threshold concentrations at which metals cause effects on antioxidant system in Eagle owls. Blood Cd concentrations greater than 0.3 µg/dl produced an inhibition of 25% in CAT activity and 32% in GPx activity in red blood cells of Eagle owls. A depletion of 16% in tGSH levels was associated with Pb concentrations higher than 15 µg/dl in Eagle owls from the mining area. Finally, Pb concentrations above 10 µg/dl produced a TBARS induction of 28%, and Hg

concentrations higher than 10 µg/dl resulted in a TBARS induction of 107% in Eagle owls from the Southern area.

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CHAPTER VIII

Effects of heavy metals exposure on oxidative stress biomarkers in Griffon Vulture (*Gyps fulvus)*

Photo: Pedro Jiménez Montalbán.

Silvia Espín, Emma Martínez-López, Pedro María-Mojica, Pedro Jiménez, Antonio J. García-Fernández. Effects of heavy metals exposure on oxidative stress biomarkers in Griffon Vulture (Gyps fulvus).

Abstract

Induction of reactive oxygen species (ROS) has been suggested as one of the mechanisms involved in the metal toxicity. This ROS formation results in metal-related oxidative stress that can lead to oxidative damage to membrane lipids, DNA and proteins. The aim of this study was to assess the concentrations of lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu) and zinc (Zn) in blood samples of Griffon vultures (*Gyps fulvus*) from two areas of Valencian Community (Alcoy and Cinctorres), in the East of Spain. Moreover, we elucidate the effects of these metals on Griffon vulture oxidative stress biomarkers. Since sampling in the present study was done at the beginning of the hunting season, the low Pb levels found in Griffon vultures from Alcoy and Cinctorres (median=12.37 and 16.26 µg/dl, respectively) are suggested to be the normal or background levels usually found in vultures feeding on porcine origin carcasses during the whole year. On the other hand, it is possible that ingestion of game meat with bullet fragments in carcasses or Pb shot embedded in their flesh could be the cause of the high blood Pb concentrations found in three Griffon vultures from Cinctorres (83, 290 and 362 µg/dl). Griffon vultures feeding in Cinctorres had enhanced CAT and GST activities, and tGSH concentrations and lipid peroxidation. These results may be interpreted as a protective response against raised amount of ROS. Several metal-related effects were observed in antioxidant enzymes of Griffon vultures. Inverse relationships between Pb and GPx or CAT activity were found. Besides, direct correlations between Cd and GPx or CAT, and Hg and SOD were observed. Pb had a significant effect on lipid peroxidation in Griffon vultures. The positive correlations found between some oxidative stress biomarkers prove that antioxidant defence operates as a balanced and coordinated system.

In addition, the present study provides threshold concentrations at which metals caused effects on the antioxidant system in Griffon vultures. Blood Cd concentrations greater than 0.05 µg/dl produced an induction of 33% in GPx activity and of 44% in CAT activity in red blood cells of vultures from Alcoy. Hg concentrations in blood higher than 3 µg/dl produced an induction of 10% in SOD activity. Concentrations of Pb upper than 15 µg/dl in blood were able to produce an inhibition of 12.5% in GPx activity and 11.3% in CAT activity, and a TBARS induction of 10.7% in red blood cells of Griffon vultures.

1. Introduction

Although several essential metals form a crucial part in normal biological functioning of cells (Flora *et al.*, 2008), metal induced toxicity in birds altering reproductive success, behaviour, immune response and biochemical processes is well reported in the literature (Frederick and Jayasena, 2010; Mateo *et al.*, 2003a; Snoeijs *et al.*, 2004). Some of these elements are present in the environment mainly as a result of human activity, and their ubiquity, persistence and accumulation in organisms imply continuous exposure in living beings (García-Fernández *et al.*, 2005a).

It has been suggested that one of the mechanisms involved in metal toxicity is the induction of reactive oxygen species (ROS) by metals (Ercal *et al.*, 2001), highly reactive oxygen-containing molecules produced in oxidation-reduction reactions (Dowling and Simmons, 2009). This ROS formation results in metal-related oxidative stress, a state where there is an imbalance between antioxidant defence and the production of ROS, so that the defence is overcome by radical formation (Halliwell and Gutteridge, 2007). An excess of radicals can cause oxidative damage to membrane lipids, DNA and proteins, and their oxidation may ultimately lead to cellular dysfunction and tissue injury (Hoffman *et al.*, 1998; Valavanidis *et al.*, 2006). Multiple mechanisms may be responsible for the metal-induced oxidative stress. Among them, direct or indirect generation of ROS, depletion of glutathione (GSH) and other thiol-containing antioxidants, and inhibition of antioxidant enzymes, are well-known for all redox active (iron and copper) and inactive (lead, cadmium and mercury) metals (Ercal *et al.*, 2001; Koivula and Eeva, 2010).

Aerobic organisms have developed an antioxidant defence system with the ability to inhibit free radical generations and reduce oxidation and damage caused by the radicals (Koivula and Eeva, 2010). The main antioxidants are GSH, carotenoids, flavonoids, α-tocopherol, vitamin C, uric acid and different antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) (Koivula and Eeva, 2010). The tripeptide GSH (L-gamma-glutamyl-L-cysteinylglycine) is one of the most abundant sulfhydryl (SH)-containing compounds in most organisms, and has an important role in binding with ROS and in the elimination of metals (Klaassen *et al.*, 1985). The antioxidant enzymes catalyze the breakdown of free radicals (GPx, CAT, SOD) and support the antioxidant defence system indirectly by catalyzing the conjugation of

pollutants with GSH (GST) or reducing the oxidised form of GSH (GSSG) back to GSH (GR) (Gurer and Ercal, 2000).

Since metal-induced oxidative stress in birds has been found in experimental studies (Mateo and Hoffman, 2001; Mateo *et al.*, 2003a), the levels of antioxidant molecules and activities of antioxidant enzymes could be interesting biomarkers of metal exposure and effect in birds. Because several different antioxidants are needed to protect against ROS and antioxidant defence may respond differently depending on species, it is essential to use several biomarkers to detect oxidative stress (Berglund *et al.*, 2007; Halliwell and Gutteridge, 1999; Koivula and Eeva, 2010). Moreover, in order to make inferences about oxidative stress, it is necessary to measure a marker of antioxidant capacity and at least a marker of oxidative damage (Costantini and Verhulst, 2009).

The measurement of metal concentrations in blood is a good indicator of recent exposure, and some papers about metal concentrations in vultures have been published (Gangoso *et al.*, 2009; García-Fernández *et al.*, 2005a; Hernández and Margalida, 2009; Shlosberg *et al.*, 2012). However, few studies have been conducted on the effects of heavy metals on oxidative stress biomarkers in free-living birds exposed to metals under natural conditions (Berglund *et al.*, 2007; Koivula *et al.*, 2011; Martínez-Haro *et al.*, 2011), and differences among bird species are still unclear (Koivula and Eeva, 2010).

The aim of this study was to assess the concentrations of lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu) and zinc (Zn) in blood samples of Griffon vultures (*Gyps fulvus*) from two areas of Valencian Community, in the east of Spain. Besides, effects induced by these metals on Griffon vulture oxidative stress biomarkers have been studied. For this purpose, a battery of biomarkers was analyzed including total GSH content, antioxidant enzymes activities (GPx, SOD, CAT and GST) and lipid peroxidation.

2. Material and methods

2.1. Study area, species and sample size

Sixty-six Griffon vultures were caught in baited cage traps at two different feeding stations located in Valencian Community, in the East of Spain (Figure 22). These two feeding stations are places where supplementary food, mainly of porcine origin, is provided for vultures. The first sampling was conducted in the feeding station of

Cinctorres (n=30), in the province of Castellón (40º35’ N, 0º12’ W), on 27 September and 3 October 2011. In Castellón, vulture population has grown since 1972. Only three pairs were found in 1972, while in 2008, 236 breeding pairs were found in this area (93% of the breeding pairs in Valencian Community) (Del Moral, 2009). In Cinctorres, food is only provided for the trapping during approximately 6 weeks every year. However, there are several feeding stations in Castellón (Zorita del Maestrazgo and Vallibona) where food is provided once a week during the whole year. On the other hand, the second sampling was conducted in the feeding station of Alcoy (n=36), in the province of Alicante (38º42’ N, 0º28’ W), on 13 November 2011. In Alicante, Griffon vulture breeds since 2005 as a result of a reintroduction program conducted by the FAPAS-Alcoi NGO in 2000 (“Projecte Canyet”) (Del Moral, 2009). In 2008, 19 breeding pairs were found in the north of Alicante (Del Moral, 2009). Food is provided normally once a week throughout the year in Alcoy.

Figure 22. *Map showing the geographical location of the studied areas, Cinctorres (Castellón) and Alcoy (Alicante), in Valencian Community (Spain).*

Griffon vulture is a large bird of prey from Accipitridae family that belongs to the Old World vultures. It is a scavenger, feeding mostly from carcasses of dead domestic livestock and, to a lesser extent, of wild species dead in the field (Donázar, 1993). The world population of Griffon vulture extends from North Africa, by several South European countries, to Central Asia; and an important population is concentrated in Spain (Del Moral, 2009). This species is considered sedentary across most of its breeding area, except for young and immature birds which often disperse or migrate from north to south (Ferguson-Lees and Christie, 2001).

Blood samples were collected by puncturing brachial vein with a 23G needle and a syringe, and stored in heparinised Eppendorf tubes under refrigerated conditions until processed in the laboratory. One tube with whole blood was separated and another tube with blood was centrifuged at 10,000 rpm during 5 minutes to separate plasma and red blood cell (RBC) fractions. Plasma was separated in a new tube and RBC samples were washed with saline solution and centrifuged again at 10,000 rpm during 5 minutes. Hematocrit was recorded using a capillary tubes reader after centrifugation at 5,000 rpm during 5 minutes. Finally, three Eppendorf tubes with whole blood, plasma and RBC were stored at -80ºC until analysis.

The health status of the birds was evaluated clinically by a veterinarian prior to blood sampling. Besides, a plasma biochemistry analysis was done in every individual to check the normal health status and ensure that birds did not suffer any subclinical pathology. An A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain) was used to determine plasma biochemistry with commercial kits from BioSystems S.A. The plasma enzyme activities analyzed were alkaline phosphatase (ALP; Enzyme Commission (EC) number 3.1.3.1), aspartate aminotransferase (AST; EC 2.6.1.1), butyrylcholinesterase (CHE; EC 3.1.1.8), creatine kinase (CK; EC 2.7.3.2), gamma-glutamyltransferase (g-GT; EC 2.3.2.2), and lactate dehydrogenase (LDH; EC 1.1.1.27). The plasma constituents analyzed were albumin, total protein, cholesterol, glucose, triglycerides, uric acid, calcium and phosphorus.

2.2. Blood metals analysis

Total Hg was analyzed in a Milestone DMA-80 direct Hg analyzer by atomic absorption spectrophotometry with a detection limit of 0.005 ng. Blood samples (100 µl wet weight) were loaded in a nickel boat and analyzed. Calibration curve was done with ten points (in duplicate) from 0 to 1004 ng of Hg. Precision and accuracy of the method were tested using certified reference material (CRM) (Hg Standard for AAS, Fluka, 1000 mg/L Hg in 12% nitric acid, prepared with high purity Hg metal, HNO_3TraceSELECT® and water TraceSELECT®Ultra). Recovery of total Hg from five replicates of CRM diluted to 1 ppm was 107.06 ± 13.23% (mean ± standard deviation). The coefficient of variation for the repeatability was 12.36%.

Cd, Pb, Cu and Zn levels were analyzed in blood samples following the method described by (García-Fernández *et al.*, 1995). The samples were prepared for analysis eliminating impurities that might interfere with the results by a complete digestion ensured using high temperature digestion with a mixture of acids following the method

described by (García-Fernández *et al.*, 1995), in which a volume of 0.2 ml of whole blood was placed in a quartz digestion tube, to which 0.5 ml of an acid mixture (nitric:perchloric:sulfuric, 8:8:1) was added. The sample was then submitted to a progressive thermal treatment and, once dried, was left to cool. Tetradistilled purified water was added and transferred to the measuring vessel, adjusting the final volume to 10 ml. Prior to anodic stripping voltammetry (ASV), 50 µl of hydrochloric acid was added to the measuring vessel as an electrolyte support. The pH of the final solution was between 1 and 2. The anodic stripping voltammeter (VA-757 Computrace Workstation; Methrohm, Switzerland) used was equipped with three standard electrodes: working electrode (hanging Hg drop), reference electrode (Ag/AgCl; KCl, 3 mol/l), and auxiliary electrode (platinum).

We used the differential pulse normal technique with an electrolysis time of 120 s and modulation amplitude of 50 mV. The concentration of each metal in the digested sample was calculated after twice adding dilutions prepared from standard solutions of Cd, Pb, Cu and Zn, respectively (Sigma, St. Louis, MO). Mean recoveries, which approached 96%, were calculated analysing 10 identical samples of reconstituted lyophilized blood (European Union Reference Standards CRM195) (García-Fernández, 1994). Detection limits were 0.05 and 0.1 µg/L for Cd and Pb, respectively, and 0.3 and 0.04 mg/L for Zn and Cu, respectively. All the reagents used were Suprapur quality from Merck (Darmstadt, Germany). The quartz tubes used for the wet digestion were previously washed with 2% nitric acid for 48 h and then rinsed twice with tetradistilled water and dried in an oven at 100ºC.

2.3. Biomarker analyses in red blood cells (RBC)

Several oxidative stress parameters (total glutathione, glutathione peroxidase, superoxide dismutase, catalase, glutathione-S-transferase and thiobarbituric acid-reactive substances) were analyzed in RBC, after homogenization (1:10 w/v) in a stock buffer (1.15% KCl in 0.01 M PBS (pH 7.4) with 0.02 M EDTA). Lipid peroxidation, estimated as thiobarbituric acid-reactive substances (TBARS), was assessed following the methodology described by (Alonso-Alvarez *et al.*, 2008) with a spectrophotometer (UV-1603, Shimadzu). Levels of total glutathione (tGSH) were obtained as described by (Reglero *et al.*, 2009) with an automated spectrophotometer A25-Autoanalyzer (BioSystems). The activities of glutathione peroxidase (GPx; EC1.11.1.9) and superoxide dismutase (SOD; EC1.15.1.1) were determined spectrophotometrically (A25-Autoanalyzer, BioSystems) using the Ransel and Ransod kits, respectively

(Randox Laboratories), following descriptions of (Reglero *et al.*, 2009) with some modifications for RBC. Homogenized samples were diluted by 1:20 and 1:25 (v:v) with Ransel diluting agent and Ransod sample diluents (Randox Laboratories), for GPx and SOD determinations respectively. GPx and SOD results were expressed as Units per gram of protein.

CAT (EC 1.11.1.6) activity was assayed following the methodology described by (Clairbone, 1985), based on the decomposition of hydrogen peroxide (H_2O_2) in molecular oxygen and water by this enzyme. The rate of enzymatic decomposition of H_2O_2 was determined as absorbance decrements at 240 nm with a spectrophotometer (UV-1603, Shimadzu). The assay mixture consisted of 950 µl of potassium phosphate buffer (0.05M, pH 7.0), 500 µl of H_2O_2 (0.03M) and 50 µl of sample. Results were expressed as µmol H_2O_2 consumed per minute per milligram of protein.

The activity of GST (EC 2.5.1.18) was determined by the method described by (Habig *et al.*, 1974), based on the measurement of the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione. This reaction is catalyzed by GST, and it is determined as absorbance increments at 340 nm. The assay mixture consisted of 1850 µl of potassium phosphate buffer (0.2M, pH 7.9), 50 µl of CDNB solution (8.17mM) (Sigma) diluted in ethanol:water (1:1.5), 50 µl of GSH solution (8.17mM) (Sigma) and 50 µl of sample. The results were expressed as nanomoles produced per minute per milligram of protein.

As enzyme activities were expressed relative to grams of protein in the homogenates, total protein contents in these homogenates were measured spectrophotometrically at 595 nm following the method of (Bradford, 1976), using bovine serum albumin as standard protein.

2.4. Statistical analysis

All analyses were carried out using the SPSS v.15.0 statistical package. Reported metal concentrations and oxidative stress biomarker values represent the mean ± standard deviation, median and range. The data were tested for normality using a Kolmogorov-Smirnov test and when necessary, data were normalized using log-transformation. Differences in metal concentrations and biomarker values between areas were calculated by ANOVA. Correlations between metals and between biomarkers were checked using Pearson's correlation coefficient. Simple linear regression was performed to evaluate the effect of each metal on the oxidative stress

biomarkers in each studied area and with pooled data of all individuals. The level of significance for these tests was set at α=0.05. Because of the limited amount of plasma from some birds, sample sizes were not the same for all the parameters analyzed. Generalized Linear Models (GLMs) with a normal distribution and an identity function were performed to study combined effects of metals and their interactions on the biomarkers. Biomarker value was the response variable, metal concentrations were selected as covariates and studied area (when the two populations were pooled together) were selected as factors. A backward stepwise procedure was used to select the final models. Several criteria can be used to select the best regression models (Murtaugh, 2009). In the present study predictor variables and interaction terms are retained when they significantly improved model fit ($p<0.05$). However, some authors suggest that Akaike´s information criterion (AIC), that selects the model that fits the data best and also has the smallest number of parameters (i.e., simplicity and parsimony), is remarkably superior in model selection (Mazerolle, 2006). However, the best model according to the AIC is usually large and complex, and often includes variables, and even interactions, with small effects (Murtaugh, 2009). Therefore, we also provide the best model according to AIC criteria to show other variables that may also affect to the biomarkers but do not have a significant effect ($p<0.05$) on the model.

3. Results and discussion

In general, plasma biochemistry parameters were similar to those described as baseline data in Egyptian vultures (*Neophron percnopterus*), Griffon vultures and Black vultures (*Aegypius monachus*) by several authors (Dobado-Berrios *et al.*, 1998; Polo *et al.*, 1992; Villegas *et al.*, 2002), and are indicative of normal health status.

3.1. Metal concentrations in blood

Concentrations of metals in Griffon vulture are shown in Table 26. In Table 27 a review of metal concentrations in tissues of vultures from Accipitridae family is presented.

Mean blood Pb concentrations in Griffon vultures from Alcoy (15.3 ± 8.3 µg/dl) were similar to those found in Griffon vultures from Israel and Egyptian vultures from Canary Islands (Donázar *et al.*, 2002; Shlosberg *et al.*, 2012), but higher than those found in Pyrenean bearded vultures (*Gypaetus barbatus*) from Iberian Peninsula and France (Gangoso *et al.*, 2009; Hernández and Margalida, 2009) (Table 27). Regarding mean blood Pb concentrations in vultures from Cinctorres (41.4 ± 79.5 µg/dl), they

were significantly higher than those found in Alcoy in the present study and by other authors in vultures from different areas (Gangoso *et al.*, 2009; Hernández and Margalida, 2009; Shlosberg *et al.*, 2012), but similar to those found in Griffon vultures from Cazorla Natural Park (Southern Spain) outside hunting season and from Murcia, Southeastern Spain (García-Fernández *et al.*, 1995, 2005a) (Table 27). However, Pb concentrations in both populations were lower than those found in Griffon vultures from Cazorla Natural Park during hunting season (García-Fernández *et al.*, 2008) (Table 27).

Table 26. *Metal concentration in blood samples (µg/dl), enzyme activities, glutathione levels and lipid peroxidation in red blood cells of Griffon vulture.*

Metal concentrations in Griffon vulture blood samples (µg/dl)						
Metal	**Alcoy, Alicante**			**Cinctorres, Castellón**		
	n	**Mean±SD**	Median (range)	n	**Mean±SD**	Median (range)
Cd	36	**0.018±0.027**	0.006 (0.006-0.1610)	30	**0.025±0.043**	0.006 (0.006-0.217)
Pb	36	**15.32±8.28****	12.37 (7.03- 45.61)	30	**41.44±79.50**	16.26 (9.31-362.13)
Cu	36	**20.39±5.92**	19.31 (13.77-44.41)	30	**26.85±23.11**	19.58 (9.89-134.70)
Zn	36	**332.16±65.40**	327.48 (248.55-629.82)	30	**347.12±80.89**	351.22 (146.57-497.95)
Hg	36	**2.27±2.24**	1.53 (0.55-10.03)	30	**1.72±1.35**	1.17 (0.62-6.75)
Enzyme activities, glutathione levels and lipid peroxidation in red blood cells of Griffon vultures						
Biomarker	**Alcoy**			**Cinctorres, Castellón**		
	n	**Mean±SD**	Median (range)	n	**Mean±SD**	Median (range)
GPx[a]	36	**415.56±91.45**	408.22 (246.57-626.86)	29	**483.09±239.80**	425.88 (217.60-1247.78)
SOD[b]	36	**929.92±202.78**	911.26 (588.39-1445.87)	30	**827.72±314.55**	782.99 (411.48-2058.73)
CAT[c]	36	**0.55±0.15***	0.55 (0.18-0.84)	30	**1.18±0.39**	1.12 (0.60-2.13)
GST[d]	36	**7.26±1.59***	7.07 (4.67-10.82)	29	**9.79±2.24**	9.37 (6.73-16.41)
tGSH[e]	36	**4.56±0.99***	4.49 (1.86-7.05)	30	**5.42±0.93**	5.24 (4.11-7.16)
TBARS[f]	36	**0.0338±0.0074***	0.032 (0.024-0.052)	30	**0.0499±0.0038**	0.0499 (0.0438-0.0565)

*Note: Significant differences between areas: *p<0.001, ** p<0.01, *** p<0.05. [a]Glutathione peroxidase (U/g protein), [b]Superoxide dismutase (U/g protein), [c]Catalase (µmol/min/mg protein), [d]Glutathione-S-Transferase (nmol/min/mg protein), [e]Glutathione total (µmol/g), [f]Lipid peroxidation, estimated as thiobarbituric acid-reactive substances (µmol/g).*

Since sampling in the present study was done in September-November at the beginning of the hunting season in Valencian Community (Orden 1, 2011), and concentrations detected were much lower than those found in Griffon vultures sampled during hunting season (García-Fernández *et al.*, 2008) (Table 27), it is possible that Pb levels in Griffon vultures from Alcoy and Cinctorres (median=12.37 and 16.26 µg/dl, respectively) are normal or background levels in vultures feeding on porcine origin carcasses during the whole year.

However, high Pb concentrations were found in three individuals from Cinctorres (83, 290 and 362 µg/dl). The blood levels of Pb found in these vultures probably indicate recent exposure to large amounts of this metal (García-Fernández *et al.*, 1995). In this sense, these high Pb concentrations have been related to ingestion of the metallic form of this metal (García-Fernández *et al.*, 2008; Hoffman *et al.*, 1981). Several authors have demonstrated that Pb ammunition can produce hundreds of small fragments contaminating animal carcasses and discarded viscera that serve as food for scavengers (Hunt *et al.*, 2006; Knopper *et al.*, 2006). In Spain, the use of Pb shot has been banned only in wetlands included on Ramsar's list due to the risk for waterfowl (Royal Decree 581, 2001). However, Pb ammunition is still used in big- and small-game hunting. In Valencian Community, big-game hunting is allowed and regulated for Iberian wild goat (*Capra pyrenaica*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), wild boar (*Sus scrofa*), mouflon (*Ovis aries*) and barbary sheep (*Ammotragus lervia*) (Orden 1, 2011). There are also many species allowed for small-game hunting such as partridge (*Alectoris rufa*), European rabbit (*Oryctolagus cuniculus*), red fox (*Vulpes vulpes*) and several pigeon species (*Columba sp.*) (Order 1, 2011). Although the diet of Griffon vultures from this study is mainly based on porcine origin carcasses provided in feeding stations, they can also feed from wild species dead in the field (Donázar, 1993). Therefore, the ingestion of game meat with bullet fragments in carcasses or Pb shot embedded in their flesh is probable. This may be the major cause of the blood Pb concentrations found in the three individuals from Cinctorres with high blood Pb concentrations.

Twenty-seven vultures from Alcoy (75% of total studied population) and 18 vultures from Cinctorres (60% of total studied population) had Pb levels below 20 µg/dl, mentioned by Franson (1996) as the minimum blood Pb level necessary in Falconiformes for considering physiological effects. Besides, 9 individuals from Alcoy (25% of total population) and 8 from Cinctorres (27% of total population) had Pb concentrations between 20 and 50 µg/dl, considered as threshold value for physiological effects (Franson, 1996). Finally, 4 individuals from Cinctorres (13% of total population) had Pb levels higher than 50 µg/dl, with 2 vultures (290.48 and 362.13 µg/dl) presenting concentrations higher than those considered by Franson (1996) as threshold value in individuals with probable clinical symptoms (100 µg/dl). In spite of the threshold of 20 µg/dl set by Franson (1996), more recent studies have found that Pb concentrations below 15 µg/dl in blood can cause sublethal effects such as inhibition of δ-aminolevulinic acid dehydratase (ALAD) activity in raptors and waterbirds

in the field (Gómez-Ramírez *et al.*, 2011; Martínez-Haro *et al.*, 2011; Martínez-López *et al.*, 2004). Therefore, according to the concentrations found in the present study, some individuals could be susceptible to suffer sublethal effects due to Pb exposure.

Table 27. *Literature metal concentrations in blood (µg/dl) and other tissues (µg/g dry weight) of vultures (Accipitridae family).*

Species[a]	Mean metal concentrations in blood (µg/dl) and tissues (µg/g dry weight)[b]	Observations	Sampling area	Year	Reference
G fu	B(n=36): Pb= 15.32, Cd= 0.018, Cu= 20.39, Zn= 332.16, Hg=2.27	Alcoy	Spain	2011	Present study
	B(n=30): Pb= 41.44, Cd= 0.025, Cu= 26.85, Zn= 347.12, Hg=1.72	Cinctorres			
G fu	B(n=23): Pb=43.07	Outside hunting season	Spain	2003	(García-Fernández *et al.*, 2005a)
G fu	B(n=26): Pb=123	During hunting season	Spain	2006	(García-Fernández *et al.*, 2008)
G fu	B(n=6): Pb= 37.9, Cd= 0.11		Spain	1993	(García-Fernández *et al.*, 1995)
G fu	B(n=9): Pb= 10.4, As= <1, Cd= <1, Hg= <2, Se= 46.2	Juvenile	Israel	2007	(Shlosberg *et al.*, 2012)
	B(n=7): Pb= 14.2, As= <1, Cd= <1, Hg= 1.2, Se= 36.5	Subadult			
	B(n=9): Pb= 8.4, As= <1, Cd= <1, Hg= 1.3, Se= 42.4	Adult			
G fu	BN(n=4): Pb=5.54*, As=18**		Spain	1998-2001	(Mateo *et al.*, 2003b)
G fu	BN(n=20): Pb=9.30		Spain	-	(Cardiel *et al.*, 2011)
	F(n=20): Pb=0.208(rachis), 1.919(barbs), Al=<LOD[c](rachis), Al=1978(barbs)				
G ba	B(n=40): Pb= 2.33*, BN(n=12): Pb= 1.46*, L(n=10): Pb= 0.57*	Fledgling	Spain and France	2008	(Hernández and Margalida, 2009)
	B(n=15): Pb= 4.56*, BN(n=3): Pb= 2.71*, L(n=3): Pb= 0.83*	Juvenile			
	B(n=20): Pb= 4.20*, BN(n=11): Pb= 2.87*, L(n=5): Pb= 1.15*	Subadult			
	B(n=26): Pb= 5.45*, BN(n=17): Pb= 3.16*, L(n=12): Pb= 1.36*	Adult			
N pe	B(n=32): Pb= 0.56-21.73 range, BN(n=11): Pb= 6.17*	Iberian Peninsula	Spain	1999-2005	(Gangoso *et al.*, 2009)
	B(n=137): Pb= 0.51-178 range, BN(n=28): Pb=7.42*	Canary Islands			
	B: Pb (During hunting season, n=47)= 9.33*	Canary Islands			
	B: Pb (Outside hunting season, n=90)= 2.88*	Canary Islands			
N pe	B (n=26): Pb= 14.6, Cd= 0.113, Cu (n=11)= 0.047, Zn= 361.5		Spain	1998-2001	(Donázar *et al.*, 2002)
A mo	BN(n=3): Pb=11.13				
	F(n=3): Pb=<LOD[c](rachis), Pb=0.265(barbs), Al=<LOD[c](rachis), Al=<LOD[c](barbs)				
G fu	F(n=3): Hg= 0.93 (Secondary feather), Hg= 1.16 (Tail feather)		Iran	2005	(Zolfaghari *et al.*, 2007)

*[a]G fu=Gyps fulvus, Griffon vulture; A mo=Aegypius monachus, Cinereous vulture; G ba=Gypaetus barbatus, Pyrenean bearded vulture; N pe=Neophron percnopterus, Egyptian Vulture. [b]B=Blood, BN=Bone, L=Liver, F=Feathers, *Geometric mean, **Median, [c]LOD=Limit of detection.*

Regarding Hg, few papers have studied the concentrations of this metal in blood of terrestrial birds of prey (see Chapter VI; Shlosberg *et al.*, 2012) mainly due to the methylation and bioaccumulation of methylmercury in the aquatic systems. Only one report has documented Hg concentrations in blood of a vulture species (Shlosberg *et*

al., 2012), with concentrations slightly lower than those found in the present study (Table 26 and 27). Hg concentrations in blood of Griffon vultures in the present study were similar to those found in nestlings Eagle owls (*Bubo bubo*) from Southeastern Spain (see Chapter VI), and seem to be too low to cause any adverse effects on vultures (2.27 µg/dl in Alcoy and 1.72 µg/dl in Cinctorres). In fact, levels of Hg in blood considered as no observed adverse effect level in adult Common loon (*Gavia immer*) are two orders of magnitude larger (1 µg/ml) (Evers *et al.*, 2004).

As regards to Cd concentrations in blood of Griffon vultures in the present study, levels were lower than those found in Egyptian vultures by Donázar *et al.* (2002) and in Griffon vultures by García-Fernández *et al.* (1995) (Table 26 and 27). Levels producing sublethal effects are unknown for Cd, but concentrations were below those found in raptors from unpolluted zones (0.1 µg/dl) (García-Fernández *et al.*, 1995).

Finally, Zn concentrations in blood of Griffon vultures in the present study were similar to those found in Egyptian vultures by Donázar *et al.* (2002), although Cu levels were higher than those published by these authors. No commonly accepted toxicity threshold for sublethal effects exists for Cu and Zn in blood of birds, but concentrations found in the present study are in the range of Cu and Zn levels for healthy birds (García-Fernández *et al.*, 2005b) and seem to be too low to produce any adverse effects on vultures. Therefore, Zn and Cu concentrations found in the present study could be considered as physiological in this species.

3.2. Oxidative stress biomarkers

3.2.1. Oxidative stress biomarkers in Griffon vultures from two areas of Spain

Griffon vultures from Cinctorres displayed significantly higher CAT and GST activities, and higher concentrations of tGSH and TBARS in red blood cells compared to vultures from Alcoy (Table 26). However, no significant differences in GPx and SOD activities were found between vulture populations (Table 26). The general trend observed in the present study is an increase in enzymatic and non-enzymatic antioxidant mechanisms in Griffon vultures from Cinctorres. Antioxidant response of vultures from Cinctorres to ROS, while still operating, may be not sufficient to maintain oxidative damage at the same level of vultures from Alcoy, since TBARS concentrations are higher in vultures from Cinctorres (Table 26). The enhanced activities of CAT and GST, and concentrations of tGSH in vultures from Cinctorres may

be interpreted as a protective response against the higher TBARS levels, since these mechanisms may contribute together to the scavenging of ROS, and alleviate oxidative damage. In this sense, it seems that a mild exposure to oxidative attacks could result in a more permanent up-regulation of the antioxidant defence (Rattan, 2008). Besides, as explained above, Pb concentrations were significantly higher in blood of Griffon vultures from Cinctorres than in vultures from Alcoy. This may be related with the highest TBARS levels found in vultures from Cinctorres, since it is known that Pb may induce generation of ROS, which is associated with lipid peroxidation in erythrocytic membranes (Gurer and Ercal, 2000).

GSH is a major antioxidant in aerobic organisms with an important role in the protection of cells, since it binds to free radicals and many metals (Klaassen *et al.*, 1985), and an up-regulation of GSH concentrations may be interpreted as a protective response against metals and/or raised amount of ROS. Several studies have found enhanced total GSH levels in Pb-fed birds and rats (Hoffman *et al.*, 2000a; Hsu, 1981; Mateo and Hoffman, 2001; McGowan and Donaldson, 1986). It is also known that the enzyme γ-glutamylcysteine synthetase, involved in GSH synthesis, can be induced by heavy metals and oxidative stress (Griffith, 1999). Besides, the increased activity of antioxidant enzymes such as CAT, GST and GPx, and lipid peroxidation has also been previously described in different bird species from polluted sites compared with reference sites (Berglund *et al.*, 2007; Kamiński *et al.*, 2009). GST catalyses the conjugation of GSH with cytotoxic aldehydes produced during lipid peroxidation (Halliwell and Gutteridge, 1999) and GSH conjugation with pollutants, and some GST isozymes have non-Se-dependent GPx activity (Prohaska and Ganther, 1977). Hence, an induction of GST activity could be an indication of a detoxification process (Jemec *et al.*, 2007). In addition, the higher GST activity in vultures from Cinctorres could imply increased GSH concentrations due to higher GSH requirements for conjugation reactions of detoxification (Josephy, 1997). Regarding CAT and GPx, these enzymes could be enhanced to cope with and increment in H_2O_2 levels in the O_2- dismutation process (Gürer *et al.*, 1998; Shaikh *et al.*, 1999). We found a low CAT activity, which could be due to GPx being the main enzyme used by Griffon vulture for catalyse H_2O_2 as suggested by (Hernández-García, 2010) and (Koivula *et al.*, 2011) in other bird species. In fact, the normal rate of H_2O_2 production is mainly balanced by GPx that uses H_2O_2 to oxidise GSH, but CAT becomes more important at enhanced H_2O_2 formation because its ability to directly catalyse the transformation of H_2O_2 to H_2O and O_2 (Halliwell and Gutteridge, 1999).

Antioxidant defence responds differently depending on pollution levels and species (Berglund *et al.*, 2007; Ji *et al.*, 2006; Martínez-Haro *et al.*, 2011; Mateo and Hoffman, 2001). Therefore, it is necessary to use several biomarkers for oxidative stress as supported by several authors (Berglund *et al.*, 2007; Halliwell and Gutteridge, 1999). In the present study, it seems that increased lipoperoxidation in vultures from Cinctorres modifies antioxidant status causing an up-regulation of the antioxidant defence system (CAT and GST activity, and tGSH concentrations), as a possible protective response.

3.2.2. Effect of metal concentrations in oxidative stress biomarkers

Simple linear regression analysis was conducted (Table 28) to check for relationships between single metal concentrations in blood of Griffon vultures and biomarker response.

***Table 28**. Linear regression analysis of biomarker response on single metal concentrations.*

Biomarker response (Y)[a]	Metal (X)	*r*	*F*	*p*	Intercept (*a*)	Regression coefficient (*b*)
Alcoy, Alicante						
GPx	LogCd	0.370	5.39	0.026	588.66	88.56
GPx	Cu	0.371	5.42	0.026	298.82	5.727
SOD	Cu	0.397	6.36	0.016	652.68	13.6
SOD	LogHg	0.414	7.02	0.012	874.13	256.538
CAT	LogCd	0.336	4.34	0.045	0.8	0.129
CAT	Cu	0.403	6.58	0.015	0.343	0.01
CAT	LogPb	-0.323	3.97	0.055	0.818	-0.238
TBARS	Zn	0.541	14.08	0.001	0.013	6.14E-05
Cinctorres, Castellón						
LogGPx	LogPb	-0.404	5.27	0.03	2.908	-0.2
CAT	Zn	0.376	4.62	0.04	0.531	0.002
All individuals						
LogGPx	LogPb	-0.306	6.49	0.013	2.798	-0.143
SOD	LogHg	0.321	7.34	0.009	832.008	279.818
TBARS	Zn	0.308	6.71	0.012	0.027	4.28E-05
TBARS	LogPb	0.354	9.19	0.004	0.027	0.011
tGSH	LogCu	0.24	3.92	0.052	2.993	1.481

Regressions follow the model Y=a+bx. [a]GPx=Glutathione peroxidase, SOD=Superoxide dismutase, CAT=Catalase, tGSH=Total Glutathione, TBARS=Thiobarbituric acid-reactive substances.

In order to increase the number of samples, we also conducted linear regression with a pool of both vulture populations. Simple linear regression analysis showed that several metals are related to GPx, SOD, CAT, tGSH and TBARS. Besides, Generalized Linear Models (GLMs) (Table 29) were developed to evaluate combined effects of metals and their interactions on the biomarkers. Biomarkers were related to

one or several metals, and some of them were affected by area when all individual were pooled together.

Almost all the relationships found between metal concentrations and oxidative stress biomarker responses were found in vultures from Alcoy or when all samples were pooled together. However, in Griffon vultures from Cinctorres only two significant correlations were found (Table 28 and 29).

Table 29. *Generalized Linear Models (GLMs) evaluating combined effects of metals and their interactions on the oxidative stress biomarkers response.*

Biomarker response[a]	**Model[b]**	**AIC[c]**	**ΔAIC[d]**	**Akaike weight[e]**	**X^2**	***p***	**Model selection criteria[f]**
Alcoy, Alicante							
GPx	**Cu (0.017)**	**426.96**	**0.00**	**0.36**	**5.32**	**0.021**	p-value and AIC
SOD	**Cu (0.011), Hg (0.008)**	**478.92**	**4.30**	**0.05**	**12.70**	**0.002**	p-value
SOD	Cd (0.046), Cu (0.107), Cd*Cu (0.009), Hg (0.009)	474.62	0.00	0.47	20.99	<0.001	AIC
CAT	**Cu (0.008)**	**-37.25**	**0.49**	**0.20**	**6.37**	**0.012**	p-value
CAT	Pb (0.074), Cu (0.05), Zn (0.142)	-37.74	0.00	0.26	10.85	0.013	AIC
tGSH	**Cd (<0.001), Pb (0.004), Cu (0.004), Cd*Cu (0.003)**	**96.12**	**0.00**	**0.60**	**16.32**	**0.003**	p-value and AIC
TBARS	**Pb (0.002), Zn (<0.001), Hg (0.037)**	**-265.11**	**0.00**	**0.50**	**23.22**	**<0.001**	p-value and AIC
Cinctorres, Castellón							
GPx	**Pb (0.017)**	**-13.35**	**0.00**	**0.47**	**5.17**	**0.02**	p-value and AIC
CAT	**Zn (0.026)**	**30.45**	**3.41**	**0.06**	**4.58**	**0.032**	p-value
CAT	Zn (<0.001), Cd (0.006), Pb (0.147), Cu (0.252), Pb*Cu (0.074)	27.03	0.00	0.32	16.00	0.007	AIC
All individuals							
GPx	**Pb (0.010)**	**-66.06**	**2.65**	**0.10**	**6.37**	**0.012**	p-value
GPx	Area (0.034), Pb (0.002), Hg (0.077)	-68.72	0.00	0.36	13.03	0.005	AIC
SOD	**Hg (0.006)**	**920.41**	**0.00**	**0.36**	**7.16**	**0.007**	p-value and AIC
CAT	**Area (<0.001), Pb (0.048)**	**26.03**	**0.11**	**0.27**	**55.96**	**<0.001**	p-value
CAT	Area (<0.001), Pb (0.066), Zn (0.143)	25.92	0.00	0.29	58.07	<0.001	AIC
GST	**Area (<0.001)**	**272.36**	**0.00**	**0.40**	**24.05**	**<0.001**	p-value and AIC
tGSH	**Area (<0.001)**	**186.31**	**0.36**	**0.31**	**12.39**	**<0.001**	p-value
tGSH	Area (0.001), Cu (0.121)	185.96	0.00	0.37	14.75	0.001	AIC
TBARS	**Area (<0.001), Pb (0.039), Hg (0.046), Zn (<0.001)**	**-494.39**	**0.45**	**0.32**	**86.02**	**<0.001**	p-value
TBARS	Area (<0.001), Cd (0.114), Pb (0.075), Hg (0.032), Zn (<0.001)	-494.84	0.00	0.40	88.47	<0.001	AIC

[a]GPx=Glutathione peroxidase, SOD=Superoxide dismutase, CAT=Catalase, GST=Glutathione-S-Transferase, tGSH=Total Glutathione, TBARS=Thiobarbituric acid-reactive substances.[b]Model indicates the most influential explanatory variables (partial significance of each variable in the model) in the response variable. [c]AIC (Akaike´s information criterion) value. [d]$\Delta AIC=AIC_{min}-AIC_i$.[e]Akaike weight is the likelihood that a given model is the best among all candidate models. The model with the greatest AIC weight and lowest AIC value indicates the closest to unknown reality. [f]Model selection criteria: we select the best variables in a model according to p-value criteria and discuss the models obtained in this way (models in bold), but we also provide the best model according to AIC criteria.

Glutathione peroxidase (GPx)

In vultures from Alcoy, GPx activity was directly correlated with blood Cd and Cu concentrations (Table 28). GPx enzyme uses H_2O_2 as substrate, and Cd exposure has been shown to increase H_2O_2 levels in rat pituitary membrane (Pillai *et al.*, 2002), although its ROS generation is indirect (Price and Joshi, 1983). Concentrations of Cd ≥ 0.05 µg/dl in blood produced an induction of 33.3% in GPx activity. Besides, in vultures from Cinctorres, GPx activity was inversely related with blood Pb concentrations (Table 28, Figure 23). When both populations were pooled together, GPx was inversely correlated with Pb concentrations (Table 28), and the best-fitting GLM for GPx activity only included an effect of Pb (Table 29). GPx enzyme requires selenium (Se) as a cofactor (ExPASy, 2012), and Schrauzer (1987) indicated antagonistic effects between Pb and Se, resulting in reduced Se uptake that may affect GPx activity. In fact, Se has a protective effect against Pb attributed to the formation of inactive Se-Pb complex (Gurer and Ercal, 2000). Experimental studies with Pb-treated birds have found an inhibition of this enzyme (Mateo *et al.*, 2003a; Somashekaraiah *et al.*, 1992). In the present study, concentrations of Pb ≥ 15 µg/dl in blood were able to produce an inhibition of 12.5% in GPx activity, and Pb levels ≥ 25 µg/dl produced an inhibition of 16.7% in GPx activity in Griffon vultures. When only individuals from Cinctorres were selected, the inhibition of GPx was 18.3% and 25.2% with Pb concentrations ≥ 15 and 25 µg/dl, respectively (Figure 23).

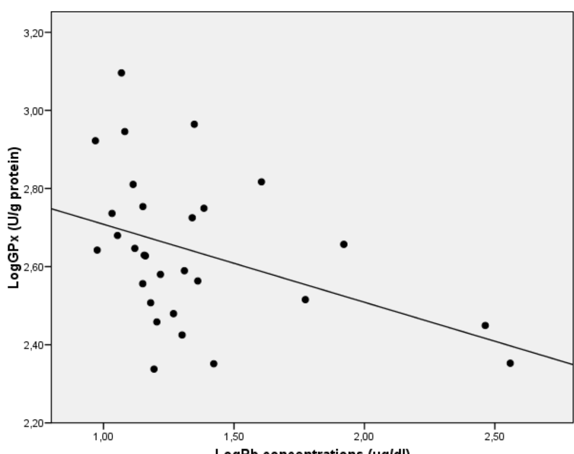

Figure 23. *Effect of blood lead concentrations on GPx activity in Griffon vultures from Cinctorres (r=-0.404, p=0.03, n=29).*

Superoxide dismutase (SOD)

Both Hg and Cu levels as single metals show a significant positive relationship with SOD activity in Griffon vultures from Alcoy (Table 28), population with higher, although not significantly, Hg concentrations (Table 26). When GLMs were performed both Hg and Cu became included in the top-ranked model (Table 29), and SOD activity seemed to depend mostly on Hg concentrations when all individuals were pooled together (Table 28 and 29). Results clearly show that Hg has an effect on this enzyme despite the low levels found in this study. Hg concentrations in blood ≥ 3 µg/dl can produce an induction of 10% in SOD activity. It is known that Hg stimulates the activity of Cu-ZnSOD (Gurer and Ercal, 2000). In this sense, changes in SOD enzyme are dependent on exposure time and level of Hg (Ji *et al.*, 2006), thus low-dose Hg exposure would result in increased levels of SOD as a protective response of the redox-defense system (Elia *et al.*, 2003). Moreover, the effect of Cu in SOD activity was expected since Cu is a cofactor of this enzyme (ExPASy, 2012).

Catalase (CAT)

CAT activity was positively correlated with Cd and Cu concentrations in blood of vultures (Table 28). In this regard, Cd concentrations ≥ 0.05 µg/dl in blood were able to induce CAT activity in 44%. As explained above, Cd exposure has been shown to increase H_2O_2 levels in rat pituitary membrane (Pillai *et al.*, 2002), and CAT catalyzes H_2O_2 to H_2O and oxygen (Koivula and Eeva, 2010). In addition, an almost significant negative relationship between Pb and CAT activity was found in vultures from Alcoy (Table 28). When all individuals were pooled together, the top-ranked model for CAT activity included an effect of area and Pb concentrations (Table 29). Catalase enzyme has heme as the prosthetic group (ExPASy, 2012), and Pb is known to reduce the absorption of iron, present in this group, in the gastrointestinal tract and to inhibit the heme biosynthesis (Gurer and Ercal, 2000). In fact, several authors have found CAT activity inhibition in Pb-exposed animals (Sandhir and Gill, 1995; Sandhir *et al.*, 1994). Concentrations of Pb ≥ 15 µg/dl in blood were able to produce an inhibition of 11.3% in CAT activity, while Pb levels ≥ 20 µg/dl resulted in a CAT inhibition of 15.8%.

Lipid peroxidation

TBARS concentrations were significantly positively correlated with Pb and Zn levels when all individuals were pooled together (Table 28, Figure 24). The best-fitting model for TBARS concentrations was constructed with Pb, Zn and Hg concentrations

as covariates in vultures from Alcoy, and the same variables including area when all individuals where pooled together (Table 29). Several authors have noted an increase in lipid peroxidation after Pb exposure in birds (Hoffman *et al.*, 2000a, 2000b; Mateo and Hoffman, 2001; Mateo *et al.*, 2003a; Somashekaraiah *et al.*, 1992); and after Hg exposure in birds and rats (Hoffman *et al.*, 2005; Huang *et al.*, 1996). Pb concentrations ≥ 15 μg/dl produced a TBARS induction of 10.7%, while Pb levels ≥ 30 μg/dl produced an induction of 13.4% in red blood cells of Griffon vultures. In Eagle owl, Pb concentrations ≥ 10 μg/dl produced a TBARS induction of 28% (see Chapter VII), suggesting that Griffon vulture is more resistant to sublethal effects of Pb than other species. In this sense, García-Fernández *et al.* (2008) found high Pb concentrations in Griffon vultures (750-1100 μg/dl) that not showed observable effects, suggesting that this species may be more tolerant to Pb exposure.

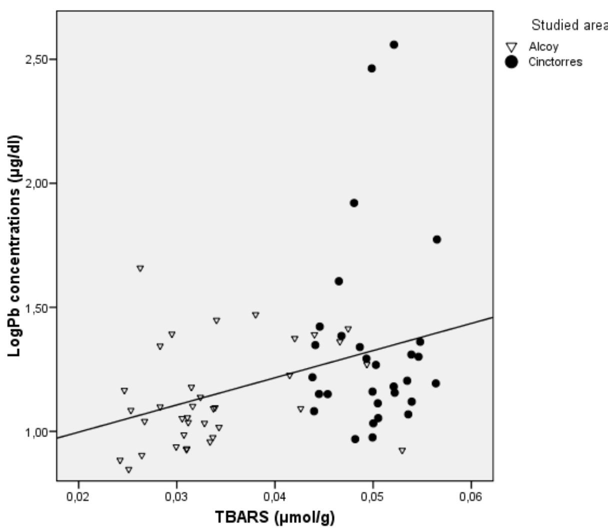

Figure 24. *Effect of blood lead concentrations on TBARS in Griffon vultures (r=0.354, p=0.004, n=66).*

Pb-induced lipid peroxidation has been associated with several mechanisms (Mateo and Hoffman, 2001). In this sense, Pb can produce ROS that attack membranes by its interaction with haemoglobin and by ALAD inactivation and the consequent accumulation of the pro-oxidant aminolevulinic acid in erythrocytes. Moreover, Pb inhibits GSH because the binding of this molecule with Pb or aldehydic products of lipid peroxidation, but can also inhibit antioxidant enzymes involved in the protection of the cell such as GPx, SOD or CAT. The effects on GSH and antioxidant enzymes reduce the protection of membranes to ROS attack and lipid peroxidation. Pb may also alter the membrane integrity and fatty acid composition increasing susceptibility of membranes to oxidative attack (Gurer and Ercal, 2000). As discussed

above, in the present study Pb concentrations have inverse relationships with GPx and CAT activity, two important scavengers of H_2O_2. These correlations together with the positive effect of Pb concentrations on TBARS levels suggest that the inhibition of antioxidant enzymes can play a role in the increase of lipid peroxidation.

Regarding the positive correlation between Zn and TBARS, which has also been found in other bird species (Berglund *et al.*, 2007), may be related to a protective effect by an increased amount of these essential metals. Several authors have proposed that one function of Zn is the maintenance of membrane structure and function (Bettger and O'Dell, 1981). In fact, dietary Zn deficiency was shown to increase the susceptibility to lipid peroxidation in rats (Sullivan *et al.*, 1980).

3.2.3. Correlations among oxidative stress biomarkers

Pearson correlations were conducted for oxidative stress biomarkers in Griffon vultures (Table 30). Some biomarkers were correlated with each other (tGSH-GPx, GPx-SOD, GPx-CAT, CAT-SOD) (Table 30). SOD, GPx and CAT are enzymes that collaborate together in the decomposition of H_2O_2 and O_2^- to less detrimental forms. In this sense, SOD catalyses the transformation of O_2^- into H_2O_2 and O_2, and then GPx and CAT catalyse the decomposition of H_2O_2 (Halliwell and Gutteridge, 1999). This important collaboration could explain the positive correlations found between these three enzymes in the present study (Table 30).

In addition, GPx oxidises GSH to GSSG, which supports the positive correlation between tGSH and GPx (Table 30). We also found positive correlations between SOD and CAT with GST (Table 30), which may be interpreted as a collaboration of GST with these enzymes since GST also removes H_2O_2 from the cells through the GSH oxidation (Koivula and Eeva, 2010).

The toxic lipid peroxides accumulated in the system are generally metabolized by cytosolic defence enzymes to prevent any damage to the biological membranes (Somashekaraiah *et al.*, 1992). In this regard, we found significant positive correlations between CAT, GST and tGSH with TBARS concentrations (Table 30), which also support the protective effect of the antioxidant system.

Table 30. *Pearson correlations for oxidative stress biomarkers among them from Griffon vultures.*

Pearson correlation for oxidative stress biomarkers									
	Alcoy, Alicante			**Cinctorres, Castellón**			**All individuals**		
Parameters[a]	n	r	p	n	r	p	n	r	p
GPx-SOD	36	0.650	0.001	29	0.370	0.047	65	0.390	0.001
GPx-CAT	36	0.600	0.001	29	0.470	0.011	65	0.360	0.004
GPx-tGSH				29	0.510	0.004	65	0.390	0.001
SOD-CAT	36	0.410	0.013	30	0.330	0.078			
SOD-GST				29	0.510	0.005			
SOD-tGSH				30	0.350	0.060			
SOD-TBARS				30	-0.350	0.058			
CAT-GST				29	0.360	0.052	65	0.570	0.001
CAT-tGSH				30	0.350	0.056	66	0.450	0.001
CAT-TBARS							66	0.570	0.001
GST-TBARS							65	0.420	0.001
tGSH-TBARS							66	0.290	0.020

Note: n=number of samples, r=Pearson´s correlation coefficient, p=significance. [a]GPx=Glutathione peroxidase, SOD=Superoxide dismutase, CAT=Catalase, GST=Glutathione-S-Transferase, tGSH=Total Glutathione, TBARS=Thiobarbituric acid-reactive substances.

4. Conclusions

Since sampling in the present study was done at the beginning of the hunting season, Pb levels found in Griffon vultures from Alcoy and Cinctorres could be considered normal or background levels in vultures feeding on carcasses of porcine origin during the whole year. However, the high blood Pb concentrations found in three Griffon vultures from Cinctorres could be due to ingestion of game meat with bullet fragments in carcasses or Pb shot embedded in their flesh.

The general trend observed in oxidative stress status is an increase in antioxidant mechanisms and oxidative stress products (CAT and GST activity, and tGSH and TBARS concentrations) in Griffon vultures from Cinctorres, which may be interpreted as a protective response against raised amount of ROS that may be generated by higher metal concentrations. Several metal-related effects were observed in antioxidant enzymes of Griffon vultures. Inverse relationships between Pb and GPx or CAT activity were found. Besides, direct correlations between Cd and GPx or CAT, and Hg and SOD were found. Pb had a significant effect on lipid peroxidation in Griffon vultures. The positive correlations found between some oxidative stress biomarkers prove that antioxidant defence operates as a balanced and coordinated system.

The present study provides threshold concentrations at which metals can cause effects on antioxidant system in Griffon vultures. Blood Cd concentrations greater than 0.05 µg/dl produced an induction of 33% in GPx activity and of 44% in CAT activity in red blood cells of vultures from Alcoy. Hg concentrations in blood higher than 3 µg/dl produced an induction of 10% in SOD activity. Concentrations of Pb above 15 µg/dl in blood were able to produce an inhibition of 12.5% in GPx activity and 11.3% in CAT activity, and a TBARS induction of 10.7% in red blood cells of Griffon vultures.

Our results suggest that antioxidant enzymes, particularly GPx, CAT and SOD, as well as lipid peroxidation as biomarker of oxidative damage, may function as useful biomarkers of metal induced effects on antioxidant system in Griffon vultures.

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DISCUSIÓN GENERAL

A continuación se presenta una discusión general que aborda los principales resultados de la tesis de forma global y proporciona algunas recomendaciones para futuros estudios.

Pluma como herramienta de biomonitorización de contaminantes ambientales persistentes

A pesar de que algunos estudios han encontrado correlaciones significativas entre los niveles de contaminantes orgánicos en plumas y tejidos internos (Eulaers *et al.,* 2011; Jaspers *et al.*, 2007, 2011; Rajaei *et al.*, 2011), existen dos importantes inconvenientes en el uso de la pluma que afectan a estas correlaciones. El primero de ellos es la contaminación externa. Resulta evidente que existe una influencia de contaminación externa por compuestos orgánicos en plumas, principalmente debida al aceite de la glándula uropígea (Jaspers *et al.*, 2008), como se ha observado en la presente tesis (Espín *et al.*, 2010a) (ver Capítulo II) además de en diversos estudios (Jaspers *et al.*, 2008, 2011). Para evitar la incertidumbre debida a la contaminación externa en plumas, es necesario buscar nuevos métodos de lavado, así como técnicas para diferenciar entre contaminación interna y externa de la pluma. En este sentido, el uso de marcadores externos con bajas tasas de absorción y por tanto poca deposición endógena podría ser una interesante herramienta para diferenciar entre contaminación interna y externa. Además, puede ser recomendable el uso del raquis cuando se pretende conocer los niveles de compuestos orgánicos que han llegado a la pluma por vía interna y se sospecha de la influencia de contaminación externa, ya que las barbas son más susceptibles a este tipo de contaminación por su mayor superficie de contacto y su mayor complejidad estructural que dificulta el lavado (García-Fernández *et al.*, in press) (ver Capítulo I).

El segundo inconveniente que resulta clave para el uso de la pluma en estudios de biomonitorización es el tiempo transcurrido entre la última muda del ave y el momento del muestreo. Por un lado, en este periodo de tiempo pueden producirse movilizaciones grasas debido a migraciones, puesta de huevos u otros factores (Perkins y Barclay, 1997), que pueden afectar a las concentraciones de estos compuestos en tejidos internos y por tanto disminuir las correlaciones con las concentraciones presentes en plumas. Además, cuanto mayor sea el tiempo transcurrido entre la última muda y la toma de muestras, probablemente mayor será la contaminación externa en la pluma (Jaspers *et al.*, 2011), lo que también afectará a las

correlaciones. Por tanto, son necesarios más datos que evalúen como puede afectar este período de tiempo a las correlaciones entre niveles de contaminantes orgánicos en plumas y tejidos internos (García-Fernández *et al.*, in press) (ver Capítulo I). En este sentido, en la presente tesis se evidencia el efecto de este lapso de tiempo, ya que se encontraron pocas y débiles correlaciones entre los niveles de plaguicidas organoclorados en plumas y tejidos internos de Alca común (Espín *et al.*, 2010b, 2012; García-Fernández *et al.*, in press) (ver Capítulos I, III y IV), probablemente debido a los meses transcurridos entre la última muda del ave (Julio-Septiembre 2006) y la toma de muestras (Febrero 2007).

Resultaría de gran ayuda el desarrollo de protocolos que ayuden a estandarizar metodologías en diferentes países y hacer así los estudios comparables entre sí. En lo que respecta a las plumas, se debe tener en cuenta algunos aspectos clave como el momento de la toma de muestras en relación a la muda, el tipo de pluma a muestrear y los métodos de lavado. A pesar de que todas las plumas pueden ser utilizadas, la pluma ideal dependerá del patrón de muda de cada especie y el objetivo del estudio. En este sentido, el programa “Investigación y monitorización para y con rapaces en Europa, EURAPMON” de la Fundación Europea para la Ciencia (ESF) presenta entre sus objetivos la creación de unas guías y protocolos de mejores prácticas que incluirán información sobre el muestreo, tejidos a analizar, preparación y análisis de muestras, y especies y contaminantes de interés, entre otros aspectos, debido a la necesidad de recopilar información a este respecto y estandarizar las prácticas.

En definitiva, es evidente que son necesarios más estudios que nos ayuden a interpretar las concentraciones de compuestos orgánicos en plumas. Sin embargo, la pluma ha demostrado ser una excelente herramienta para la biomonitorización de Hg. En la presente tesis se han encontrado correlaciones significativas entre los niveles de Hg en tejidos internos y plumas tanto de un ave marina, el Alca común (Espín *et al.*, 2012a), como de un ave terrestre, el Búho real (ver Capítulos V y VI). Incluso hemos podido proporcionar ecuaciones de predicción de la concentración de Hg en encéfalo y riñón en alcas, y en sangre en búhos, a partir de los niveles de Hg en plumas. En el estudio realizado en alcas (Espín *et al.*, 2012a) (ver Capítulo V), observamos que las concentraciones de Hg en barbas eran el doble de las encontradas en ejes. Los mayores coeficientes de correlación encontrados entre los niveles de Hg en tejidos internos y ejes, en comparación con los encontrados entre tejidos internos y barbas, podrían deberse a una mayor influencia de contaminación externa en barbas. Por tanto, los ejes podrían ser mejores indicadores de los niveles de Hg en tejidos internos

de alcas, por lo que las ecuaciones de predicción para este estudio se proporcionan en relación a las concentraciones de Hg en ejes.

Evaluación de la exposición a contaminantes ambientales persistentes en tres especies de aves

En los últimos años se ha observado que la concentración de plaguicidas organoclorados en sangre de aves terrestres que habitan zonas agrícolas en la Región de Murcia ha disminuido drásticamente debido a su prohibición, incluso no detectándose la mayoría de estos compuestos (Gómez-Ramírez, 2011; Martínez-López *et al.*, 2009). Sin embargo, a pesar de que los niveles de metales también han disminuido considerablemente en los últimos años debido a las prohibiciones y restricciones impuestas (García-Fernández *et al.*, 2003, 2008a), estos contaminantes siguen encontrándose en aves, ya sea por la presencia de antiguas zonas mineras o áreas industriales, o en el caso de aves carroñeras, por el consumo de cadáveres cazados con perdigones de Pb. Además, los plaguicidas organoclorados y el Hg son contaminantes ubicuos con capacidad de biomagnificación a través de la cadena trófica, especialmente en ambientes marinos (Braune *et al.*, 2005). Todo ello justifica nuestra decisión de analizar metales (Pb, Hg, Cd, Cu y Zn) en dos especies terrestres y plaguicidas organoclorados y Hg en una especie marina.

Respecto al Alca común (Espín *et al.*, 2010b, 2012a, 2012b) (ver Capítulos III, IV y V), las concentraciones de plaguicidas organoclorados y Hg fueron mayores que las observadas en otros álcidos muestreados en latitudes más altas (Borgå *et al.*, 2001; Buckman *et al.*, 2004; Savinov *et al.*, 2003), lo que puede deberse a una mayor exposición a estos compuestos durante la invernada en el área mediterránea. Sin embargo, los niveles observados son menores que las concentraciones causantes de efectos adversos a nivel reproductivo y de comportamiento en aves (Evans *et al.*, 1982; Finley y Stendell, 1978; Pratt, 1972; Sharma *et al.*, 1976). No se encontraron diferencias en las concentraciones de organoclorados y Hg según el sexo, probablemente debido a que el muestreo se llevó a cabo fuera de la época de cría. Sin embargo, la edad afecta a las concentraciones de estos compuestos en tejidos internos y plumas, encontrando mayores concentraciones de estos contaminantes en aves adultas.

En cuanto a las concentraciones de metales pesados en Búho real (ver Capítulos VI y VII), los niveles encontrados en sangre y plumas fueron bajos, y es improbable que puedan afectar negativamente al éxito reproductivo de la especie. Las

concentraciones de Pb y Hg en sangre fueron mayores en los individuos procedentes del área cercana a una antigua zona minera en comparación con el resto de la población estudiada. A pesar de que existen diferencias en la proporción de conejos en la dieta entre las distintas áreas estudiadas (León *et al.*, 2008), parece que la contaminación local es la principal causa de las diferencias espaciales encontradas en cuanto a la concentración de Hg (ver Capítulo VI). Las diferencias encontradas en los niveles de Hg entre años en el periodo 2006-2012 probablemente se deban a la variación en las precipitaciones en este periodo de tiempo (CREM, 2013), las cuales puede contribuir a la deposición de este metal en años más lluviosos. Las concentraciones de Pb encontradas en los años 2011 y 2012 en la presente tesis son menores que las observadas hace 20 años en búhos del mismo área, observándose un claro descenso en la exposición a este metal en Búho real de la Región de Murcia (García-Fernández *et al.*, 1995, 1997; Gómez-Ramírez *et al.*, 2011), probablemente gracias al cierre de las minas en el año 1991 y a la prohibición de la gasolina plomada en el año 2001. Respecto a las concentraciones de Cd, fueron bajas y similares a los niveles observados 20 años atrás (García-Fernández *et al.*, 1995), lo que indica que no hay una importante fuente de emisión de este metal en el área estudiada. Los niveles de Zn y Cu se encontraban dentro del rango considerado como niveles fisiológicos en aves sanas (García-Fernández *et al.*, 2005).

En lo que respecta a los niveles de metales en sangre de Buitres leonados muestreados en Castellón y Alicante (ver Capítulo VIII), puesto que el muestreo se llevó a cabo en el inicio de la temporada de caza y las concentraciones de Pb en sangre fueron menores que las encontradas en Buitre leonado durante época de caza en un estudio previo (García-Fernández *et al.*, 2008b), los niveles de Pb observados en la presente tesis podrían considerarse niveles normales en buitres cuya dieta consta en mayor medida de cadáveres de origen porcino proporcionados en comederos durante todo el año. Los altos niveles de Pb en sangre encontrados en tres individuos muestreados en Castellón podrían deberse a un consumo puntual de cadáveres de origen cinegético abandonados en el campo que presentaran perdigones de Pb. Las concentraciones de Cd fueron menores que las consideradas normales en zonas no contaminadas (García-Fernández *et al.*, 1995), y los niveles de Hg fueron bastante menores que los considerados como causantes de efectos adversos en otras especies (Evers *et al.*, 2004). Los niveles de Cu y Zn encontrados pueden considerarse niveles fisiológicos en esta especie (García-Fernández *et al.*, 2005).

Evaluación de efectos subletales en el sistema antioxidante relacionados con la exposición a metales en aves

En general, las correlaciones positivas encontradas entre algunos biomarcadores de estrés oxidativo en Búho real y Buitre leonado muestran que la defensa antioxidante opera como un sistema equilibrado y coordinado (Halliwell y Gutteridge, 2007), lo cual ha sido observado en otros estudios en aves (Koivula *et al.*, 2011).

En el caso del Búho real (ver Capítulo VII), aunque los individuos procedentes del área sur (cercana a la antigua zona minera) presentaron concentraciones de Pb y Hg significativamente mayores, y una actividad de GST y GPx significativamente menor que el resto de la población estudiada, el hecho de no encontrar diferencias en los niveles de peroxidación lipídica (TBARS) sugiere que los niveles de metales en el área sur no son lo suficientemente elevados como para provocar un aumento significativo en la peroxidación lipídica con respecto al área norte.

Con respecto al Buitre leonado (ver Capítulo VIII), la tendencia general observada en el estado oxidativo fue un aumento del mecanismo antioxidante y del daño oxidativo (actividad CAT y GST, GSH total y concentración de TBARS) en buitres muestreados en Castellón en comparación con los buitres muestreados en Alicante. Diferentes estudios han observado un aumento en la actividad de enzimas antioxidantes y en la peroxidación lipídica en aves que habitan zonas contaminadas por metales (Berglund *et al.*, 2007; Kamiński *et al.*, 2009). Los resultados del presente estudio pueden interpretarse como un efecto protector en los individuos procedentes de Castellón frente a mayores niveles de especies reactivas que pueden generarse por una mayor concentración de Pb (Gurer y Ercal, 2000).

En conjunto para las dos especies estudiadas, los resultados sugieren que las enzimas GPx y CAT son interesantes biomarcadores de exposición y efecto a Pb y Cd, mientras que la enzima SOD parece ser más sensible a la exposición a Hg. Además, los niveles de TBARS parecen ser buenos biomarcadores de daño oxidativo inducido por el Pb. A pesar de ello, los efectos sobre el sistema antioxidante difieren según la especie. De esta forma, niveles de Cd en sangre mayores de 0,3 µg/dl produjeron una inhibición del 25% en la actividad CAT y del 32% en la actividad GPx en eritrocitos de Búho real. Sin embargo, concentraciones de Cd mayores de 0,05 µg/dl en sangre de Buitre leonado provocaron una inducción del 44% en la actividad CAT y del 33% en la actividad GPx en eritrocitos. Diferentes estudios muestran que el Cd puede producir una inhibición de estas enzimas (Gambhir y Nath, 1992; Palace *et al.*, 1993), y puesto

que este metal puede generar H_2O_2 (Pillai *et al.*, 2002), también es posible una inducción de estas enzimas puesto que catalizan la descomposición del H_2O_2 (Koivula y Eeva, 2010). Los resultados opuestos observados en Búho real y Buitre leonado probablemente se deban a diferencias interespecíficas en la respuesta del sistema antioxidante ante la exposición a Cd, ya que los niveles de este metal a los que están expuestas las aves son bajos y similares.

En cuanto al Pb, concentraciones por encima de 10 µg/dl en sangre de pollos de Búho real provocaron una inhibición del 6,3% en la actividad GPx y una inducción del 28% en los niveles de TBARS en eritrocitos del conjunto de la población estudiada. En el caso del Buitre leonado, niveles de Pb en sangre superiores a 15 µg/dl produjeron una inhibición del 12,5% en la actividad GPx además de una inducción del 10,7% en los niveles de TBARS en eritrocitos. Diversos estudios han observado que el Pb inhibe la actividad GPx y aumenta la peroxidación lipídica en aves (Mateo *et al.*, 2003; Somashekaraiah *et al.*, 1992). Los resultados del presente estudio muestran que el Pb es capaz de inducir peroxidación lipídica a concentraciones en sangre menores de 20 µg/dl. Sin embargo, nuestros resultados sugieren que el Buitre leonado podría ser más resistente al daño oxidativo provocado por el Pb que el Búho real. En este sentido, estudios previos han sugerido que el Buitre leonado podría tener una mayor tolerancia a la exposición a Pb (García-Fernández *et al.*, 2008b).

En definitiva, esta información puede ayudar a mejorar el conocimiento sobre los niveles de no efecto para estos metales en diferentes especies de aves. Sin embargo, son necesarios nuevos estudios que proporcionen información acerca de niveles de parámetros del sistema antioxidante y de los efectos que estos metales son capaces de producir en los biomarcadores de estrés oxidativo celular en diferentes especies de aves silvestres.

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CONCLUSIONES GENERALES

Las conclusiones específicas del presente trabajo se presentan en cada uno de los capítulos que conforman la tesis. A continuación se detallan las conclusiones generales de la tesis doctoral.

- Las plumas son una excelente herramienta de biomonitorización de mercurio en aves. Sin embargo, en el caso de los compuestos organoclorados, son necesarios más estudios que evalúen la influencia de la contaminación externa y traten de evitar la incertidumbre que supone en la correcta interpretación de las concentraciones de estos contaminantes en plumas.
- Es necesario estudiar en profundidad como afecta el tiempo transcurrido entre la última muda del ave y la toma de muestras a las correlaciones entre las concentraciones de contaminantes en plumas y tejidos internos.
- Las concentraciones de plaguicidas organoclorados y mercurio encontradas en Alca común fueron mayores que las observadas en otros álcidos muestreados en latitudes más altas, lo que puede deberse a una mayor exposición a estos compuestos durante la invernada en el área mediterránea. Sin embargo, los niveles observados son menores que las concentraciones causantes de efectos adversos a nivel reproductivo y de comportamiento en aves.
- Las concentraciones de plomo en Búho real de la Región de Murcia han disminuido significativamente en los últimos años. A pesar de que los niveles de plomo y mercurio en sangre fueron mayores en los individuos procedentes del área cercana a una antigua zona minera en comparación con el resto de la población estudiada, es improbable que las concentraciones de metales observadas puedan afectar negativamente al éxito reproductivo de la especie.
- Aunque los niveles de plomo observados en Buitres leonados muestreados en Castellón y Alicante son mayores que los encontrados en Búho real, podrían considerarse niveles normales en buitres cuya dieta se basa principalmente de cadáveres de origen porcino proporcionados en comederos durante todo el año.
- Determinadas enzimas antioxidantes como la glutatión peroxidasa (GPx) y la catalasa (CAT) parecen ser buenos biomarcadores de exposición y efecto a plomo y cadmio, mientras que la enzima superóxido dismutasa (SOD) parece ser más sensible a la exposición a mercurio, tanto en Búho real como en Buitre leonado.

Además, los niveles de especies reactivas al ácido tiobarbitúrico (TBARS) parecen ser buenos biomarcadores de peroxidación lipídica inducida por el plomo.

- Se observan diferencias interespecíficas en la respuesta del sistema antioxidante ante la exposición a cadmio y plomo en Búho real y Buitre leonado. El Buitre leonado parece ser más resistente al daño oxidativo provocado por el plomo que el Búho real.

- Los resultados de la presente tesis pueden ayudar al establecimiento, o cuando menos aumentar el conocimiento, de niveles de referencia de no efecto en diferentes especies de vida silvestre. Consideramos que esta información es de gran utilidad en la gestión y conservación de las especies de vida silvestre.

RESUMEN

Durante las últimas décadas, se han ido desarrollando programas de biomonitorización con el objetivo de conocer los niveles de contaminantes ambientales presentes en el medio ambiente, el grado de exposición en los seres vivos y los efectos que pueden causar a largo plazo. Las aves marinas y las rapaces han sido ampliamente utilizadas como animales centinela en este tipo de programas por su posición en la cadena alimentaria, especialmente en el estudio de los contaminantes bioacumulables y biomagnificables a lo largo de la cadena trófica. Los metales pesados y los plaguicidas organoclorados (OC) destacan entre los contaminantes ambientales por su baja tasa de degradación, su persistencia en el medio y por los efectos que son capaces de producir en los seres vivos.

El uso de marcadores biológicos o biomarcadores es de gran importancia en la evaluación de riesgo tóxico por contaminantes. De esta forma, los biomarcadores de exposición permiten conocer las concentraciones de un determinado compuesto tóxico en el organismo mediante el análisis del mismo en tejidos o fluidos. En este sentido, razones prácticas, éticas, legales y de conservación abogan por la búsqueda de muestras de obtención poco o nada cruenta para el animal como la sangre y las plumas, estas últimas ampliamente utilizadas en estudios de biomonitorización de metales y más recientemente en la biomonitorización de compuestos orgánicos.

Por otro lado, aunque la medición de concentraciones de contaminantes en tejidos de aves es muy útil para conocer el grado de exposición, absorción y acumulación de los compuestos tóxicos, no proporciona información sobre las posibles alteraciones biológicas causadas por la exposición a dichos contaminantes. Por lo tanto, también es necesario buscar biomarcadores de respuesta o efecto para evaluar los cambios sufridos por los organismos como resultado de la exposición a los contaminantes. Diversos estudios experimentales han mostrado la capacidad de los metales y OC para inducir estrés oxidativo en aves, por lo que los niveles de moléculas antioxidantes, la actividad de determinadas enzimas antioxidantes, y los productos del daño oxidativo pueden ser interesantes biomarcadores de exposición y efecto a estos contaminantes. Sin embargo, pocos estudios evalúan los efectos de la exposición a estos contaminantes en biomarcadores de estrés oxidativo en aves silvestres.

El objetivo general de la presente tesis doctoral era evaluar la exposición a OC y metales, con especial atención al uso de la pluma como herramienta de biomonitorización, así como estudiar los efectos subletales inducidos por la exposición

a metales en el sistema antioxidante en diferentes especies de aves silvestres. Para ello se analizaron OC y Hg en tejidos internos y plumas de una especie marina, el Alca común (*Alca torda*), y metales (Hg, Pb, Cd, Cu y Zn) y biomarcadores de estrés oxidativo (catalasa, CAT; glutatión peroxidasa, GPx; superóxido dismutasa, SOD; glutatión-S-transferasa, GST; glutatión total, GSH y especies reactivas al ácido tiobarbitúrico, TBARS) en sangre y plumas de dos especies terrestres, el Búho Real (*Bubo bubo*) y el Buitre leonado (*Gyps fulvus*).

Las muestras de Alca común fueron recogidas por el personal del Centro de Recuperación de Fauna Silvestre de Santa Faz, Alicante, en Febrero de 2007. Las aves aparecieron muertas a lo largo de las costas de la Marina de Elche, Alicante, probablemente debido a un ahogamiento en artes de pesca. Se tomaron 447 muestras de hígado, encéfalo, grasa abdominal y subcutánea, riñón, músculo y plumas de 50 individuos de Alca común para el análisis de OC y Hg. Las muestras de Búho real se obtuvieron gracias a la colaboración del grupo de investigación “Ecosistemas Mediterráneos” de la Universidad de Murcia. Se tomaron 623 muestras de sangre en los años 2006 a 2012, 229 muestras de plumas de 2006 a 2008, y 40 muestras de músculo de conejo en 2009, 2011 y 2012 para el análisis de Hg. Además, se utilizaron 141 muestras de sangre de 2011 y 2012 para el análisis de Hg, Cd, Pb, Cu y Zn y biomarcadores de estrés oxidativo. Por último, se tomaron 66 muestras de sangre de Buitre leonado para el análisis de Hg, Cd, Pb, Cu y Zn y biomarcadores de estrés oxidativo en dos comederos de la Comunidad Valenciana, Cinctorres (Castellón) en Septiembre y Octubre de 2011 y Alcoy (Alicante) en Noviembre de 2011.

Se han encontrado correlaciones significativas entre los niveles de Hg en tejidos internos y plumas tanto de Alca común como de Búho real. Incluso hemos podido proporcionar ecuaciones de predicción de la concentración de Hg en encéfalo y riñón en alcas, y en sangre en búhos, a partir de los niveles de Hg en plumas. Nuestros resultados corroboran que la pluma es una excelente herramienta de biomonitorización de Hg, tanto en un ave marina como terrestre.

En el caso del uso de la pluma como herramienta de biomonitorización de compuestos organoclorados, algunos estudios han encontrado correlaciones significativas entre los niveles de contaminantes orgánicos en plumas y tejidos internos. Sin embargo, son necesarios más estudios que evalúen la contaminación externa, cuya influencia ha sido demostrada en la presente tesis y en diferentes estudios, y traten de evitar la incertidumbre que supone en la correcta interpretación

de las concentraciones de estos contaminantes en plumas. Además, es necesario estudiar en profundidad como afecta el tiempo transcurrido entre la última muda del ave y la toma de muestras en las correlaciones entre las concentraciones de contaminantes en plumas y tejidos internos. En este sentido, en la presente tesis se evidencia el efecto de este lapso de tiempo, ya que se encontraron pocas y débiles correlaciones entre los niveles de OC en plumas y tejidos internos de alcas, probablemente debido a los meses transcurridos entre la última muda del ave y la toma de muestras. Durante este periodo de tiempo pueden producirse movilizaciones grasas que pueden afectar a las concentraciones de estos compuestos en tejidos internos, así como contaminación externa en la pluma, lo que puede disminuir las correlaciones con las concentraciones presentes en plumas.

Respecto a la evaluación de la exposición a contaminantes ambientales, en Alca común las concentraciones medias de OC (∑OC=10,5 ± 4,4; 2,6 ± 3,1; 0,4 ± 0,7 y 0,9 ± 0,6 μg/g peso húmedo en grasa subcutánea, hígado, encéfalo y plumas, respectivamente) y Hg (Hg=2,9 ± 0,9; 2,7 ± 1,6; 2,2 ± 0,9; 1,5 ± 0,5; 1,5 ± 0,5 y 1,3 ± 0,8 μg/g peso seco en hígado, barbas, riñón, músculo, encéfalo y ejes, respectivamente) fueron mayores que las observadas en otros álcidos muestreados en latitudes más altas, lo que puede deberse a una mayor exposición a estos compuestos durante la invernada en el área mediterránea. Sin embargo, los niveles observados son menores que las concentraciones causantes de efectos adversos a nivel reproductivo y de comportamiento en aves.

En cuanto a las concentraciones de Pb en Búho real de la Región de Murcia (Pb=3,3 ± 5,2 μg/dl en sangre), se observa una disminución significativa en los últimos años, con respecto a estudios previos sobre la misma especie. A pesar de que los niveles de Pb y Hg en sangre fueron mayores en los individuos procedentes del área cercana a una antigua zona minera, es improbable que las concentraciones de metales detectadas puedan afectar negativamente al éxito reproductivo de la especie. Aunque los niveles medios de Pb observados en Buitres leonados muestreados en Castellón (Pb=41,4 ± 79,5 μg/dl en sangre) y Alicante (Pb=15,3 ± 8,3 μg/dl en sangre) son mayores que los encontrados en Búho real, podrían considerarse niveles normales en buitres cuya dieta se basa principalmente de cadáveres de origen porcino proporcionados en comederos durante todo el año. Los altos niveles de Pb en sangre encontrados en tres buitres muestreados en Castellón (83, 290 and 362 μg/dl) podrían deberse a un consumo puntual de cadáveres de origen cinegético abandonados en el campo que presentaran perdigones de Pb, puesto que el muestreo se llevó a cabo en

el inicio de la temporada de caza. Las concentraciones medias de Hg y Cd se consideran bajas (Hg=2,3 ± 3,8 y 2,0 ± 1,9 µg/dl; Cd=0,07 ± 0,2 y 0,02 ± 0,04 µg/dl en sangre de búhos y buitres, respectivamente) y los niveles de Zn y Cu observados pueden considerarse fisiológicos tanto en búhos como en buitres (Zn=311 ± 67 y 339 ± 73 µg/dl; Cu=10,6 ± 4,7 y 23,3 ± 16,4 µg/dl en sangre de búhos y buitres, respectivamente).

Finalmente, en cuanto al estudio de parámetros del sistema antioxidante, las correlaciones positivas encontradas entre algunos biomarcadores de estrés oxidativo en Búho real y Buitre leonado muestran que la defensa antioxidante opera como un sistema equilibrado y coordinado. Determinadas enzimas antioxidantes como la glutatión peroxidasa (GPx) y la catalasa (CAT) parecen ser buenos biomarcadores de exposición y efecto a Pb y Cd, mientras que la enzima superóxido dismutasa (SOD) parece ser más sensible a la exposición a Hg, tanto en Búho real como en Buitre leonado. Además, los niveles de especies reactivas al ácido tiobarbitúrico (TBARS) parecen ser buenos biomarcadores de peroxidación lipídica inducida por el Pb.

Se observan diferencias interespecíficas en la respuesta del sistema antioxidante ante la exposición a Cd y Pb en Búho real y Buitre leonado. De esta forma, niveles de Cd en sangre superiores a 0,3 µg/dl produjeron una inhibición de la actividad CAT y GPx del 25% y 32%, respectivamente, en eritrocitos de Búho real. Sin embargo, concentraciones de Cd mayores de 0,05 µg/dl en sangre de Buitre leonado provocaron una inducción de la actividad CAT y GPx del 44% y 33%, respectivamente. En cuanto al Pb, concentraciones menores de 20 µg/dl en sangre provocaron una inhibición de la actividad GPx en eritrocitos (6% y 12,5% en búhos y buitres, respectivamente) y una inducción de los niveles de TBARS (28% y 11% en búhos y buitres, respectivamente). Los resultados sugieren que el Buitre leonado podría ser más resistente al daño oxidativo provocado por el Pb que el Búho real.

En definitiva, esta información puede ayudar al conocimiento de niveles de no efecto para estos metales en diferentes especies de aves. Sin embargo, son necesarios nuevos estudios que proporcionen información acerca de los efectos que estos metales son capaces de producir sobre los biomarcadores de estrés oxidativo celular en diferentes especies de aves silvestres.

EXTENDED ABSTRACT

Title: **Biomonitoring of persistent environmental pollutants using feathers and assessment of sublethal effects using oxidative stress biomarkers in wildbirds.**

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INTRODUCTION

Organochlorine pesticides (OC) and heavy metals are global contaminants widely distributed within ecosystems. They are present in the environment mainly as a result of human activity, and its ubiquity and persistence imply continuous exposure in living organisms. Moreover, these pollutants are accumulative, and some of them may biomagnify through the food chain. These compounds produce chronic effects on the endocrine, immune, nervous and reproductive systems (Cortinovis *et al.*, 2008; Frederick and Jayasena, 2010; Mateo *et al.*, 2003). Therefore, biomonitoring programs are conducted in order to assess concentrations of environmental pollutants and to know the effects that contaminants may cause in living beings (García-Fernández and María-Mojica, 2000).

Due to the sensitivity of seabirds and raptor species to environmental changes and their position in the food chain, they can accumulate high levels of contaminants, in particular those with bioaccumulation and biomagnification capacities, and thus they have been widely used in biomonitoring studies of environmental pollution (Furness, 1993). Measuring pollutants in internal tissue samples from birds is the best known indicator of the degree of exposure to accumulative compounds (García-Fernández *et al.*, 1997). Moreover, the need to look for alternative samples to internal tissues has arisen due to practical, ethical and conservationist reasons. In this sense, samples such as blood, feces, eggs, oil secreted by urogygial gland and feathers have been used in several biomonitoring studies (Van Den Brink, 1997; García-Fernández *et al.*, 1995; Gómez-Ramírez *et al.*, 2012; Jaspers *et al.*, 2009). Feathers offer many advantages as a useful research material (Bortolotti, 2010), such as the collection in small number without causing permanent damage to the bird, collection regardless of the season, age or gender, easy transport and storage, and pickup following the molting season or from carcasses. The feather has been widely used in biomonitoring studies of metals (Burger, 1993; Espín *et al.*, 2012b (Chapter V); Martínez-López *et al.*,

2005), and most recently in studies of organochlorine pesticides (Espín *et al.*, 2012a (Chapter IV); Jaspers *et al.*, 2006, 2009).

Although measuring contaminant concentrations in birds is very useful, it is not able to offer any information about the effects related to pollution (Geens *et al.*, 2010). Therefore, it is also necessary to look for biomarkers in order to assess the health state of organisms in relation to environmental pollution (Peakall, 1992). It has been suggested that one of the mechanisms involved in OC and metal toxicity is a contaminant-induced reactive oxygen species (ROS) (Abdollahi *et al.*, 2004; Ercal *et al.*, 2001), highly reactive oxygen-containing molecules produced during oxidation-reduction reactions (Dowling and Simmons, 2009). ROS formation results in oxidative stress, a state where there is an imbalance between antioxidant defence and the production of ROS, so that the defence is overcome by radical formation (Halliwell and Gutteridge, 2007). An excess of radicals can cause oxidative damage to membrane lipids, DNA and proteins, and their oxidation may ultimately lead to cellular dysfunction and tissue injury (Valavanidis *et al.*, 2006). Aerobic animals have antioxidant molecules with the ability to inhibit free radical generations and reduce oxidation and damage caused by the radicals (Koivula and Eeva, 2010). The main antioxidants are glutathione (GSH), and different antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) (Koivula and Eeva, 2010).

Since metal induced oxidative stress in birds has been found in experimental studies (Mateo and Hoffman, 2001; Mateo *et al.*, 2003), the levels of antioxidant molecules and activities of antioxidant enzymes could be interesting biomarkers of metal exposure and effect in birds. It is essential to use several biomarkers to detect oxidative stress (Berglund *et al.*, 2007; Halliwell and Gutteridge, 1999; Koivula and Eeva, 2010). Moreover, to make inferences about oxidative stress, it is necessary to measure a marker of antioxidant capacity and at least a marker of oxidative damage (Costantini and Verhulst, 2009). However, few studies have been conducted on the effects of heavy metals on oxidative stress biomarkers in free-living birds exposed to metals under natural conditions (Berglund *et al.*, 2007; Koivula *et al.*, 2011; Martínez-Haro *et al.*, 2011), and differences among bird species are still unclear (Koivula and Eeva, 2010).

OBJECTIVES

This thesis includes 8 chapters, 5 of them are published papers (Espín *et al.*, 2010a, 2010b, 2012a, 2012b; García-Fernández *et al.*, Accepted), divided in 2 parts.

Part 1. Biomonitoring of persistent environmental pollutants in wild birds: feathers as a biomonitoring tool.

Chapter I. Feathers as a biomonitoring tool of polyhalogenated compounds: a review (García-Fernández *et al.*, Accepted).

This paper proposes a review on the polyhalogenated compounds (PHCs) in feathers and factors influencing the pollutant load. Special attention has been paid on external contamination, and the main analytical methods used to detect these compounds in feathers.

Chapter II. Development of an analytical method for extracting organochlorine pesticides from feathers (Espín et al., 2010a).

The general aim of this study was the validation of the feather as organochlorine pesticides (OC) biomonitoring tool. In this sense, we have developed a method of extraction for 15 OC in feathers. Moreover, we have assessed the influence of external contamination and the distribution of compounds between parts of the feather (barbs and shaft).

Chapter III. Assessment of organochlorine pesticide exposure in a wintering population of Razorbills (Alca torda) from the southwestern Mediterranean (Espín et al., 2010b).

The aim of this study was to determine concentrations of OC in a homogeneous population of *Alca torda* from the Mediterranean area, in order to assess the pattern of OC residue distribution taking into account the age, sex and nutritional status of the specimen analyzed. The present work provides the first available data on levels of organochlorine compounds in tissues of Razorbill.

Chapter IV. Razorbill (Alca torda) feathers as an alternative tool for evaluating exposure to organochlorine pesticides.

The aim of this study was to explore the usefulness of feathers as a biomonitoring tool for 15 OC in a Razorbill population. Moreover, additional factors such as the age and sex of the birds were examined in order to evaluate their influence on the

concentration of pollutants in feathers. The present work provides the first available data on concentrations of organochlorine compound in Razorbill feathers.

Chapter V. Razorbills (Alca torda) as bioindicators of mercury pollution in the southwestern Mediterranean (Espín *et al.*, 2012a).

The aim of this study was to provide data on mercury residue concentrations in Razorbills from the Mediterranean area, in order to assess Hg exposure, evaluate the influence of age and sex of the specimens analyzed, and to develop prediction equations for estimating Hg levels in target organs (brain or kidney) using the Hg concentrations in feathers.

Chapter VI. Factors influencing mercury concentrations in nestling Eagle Owls (Bubo bubo).

In this study, we have evaluated mercury levels in blood and feathers of Eurasian Eagle Owl (*Bubo bubo*) chicks, collected during seven breeding seasons (2006-2012), from two areas of Murcia (Southeastern Spain), one of them with a possible influence of an ancient mine site and an industrial complex. Analysis of mercury in muscle samples from carcass of its main prey, the European Rabbit, was also carried out. We tested local contamination and diet composition as factors affecting Hg concentrations in Eurasian Eagle owl chicks.

Part 2. Induction of oxidative stress in metal exposed wild birds.

Chapter VII. Effects of heavy metals exposure on oxidative stress biomarkers in Eurasian Eagle owl (Bubo bubo).

The present study has as main aim the assessment of oxidative stress related to metals in a terrestrial wild bird, the Eurasian Eagle owl, species widely distributed in Southeastern Spain. Concentrations of lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu) and zinc (Zn) in blood samples of Eurasian Eagle owl from two areas of Murcia, Southeastern Spain, have been evaluated. Besides, we have assessed the effects of these metals on total GSH content, antioxidant enzymes activities (GPx, SOD, CAT and GST) and lipid peroxidation.

Chapter VIII. Effects of heavy metals exposure on oxidative stress biomarkers in Griffon Vulture (Gyps fulvus).

The aim of this study was to assess the concentrations of lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu) and zinc (Zn) in blood samples of Griffon vultures (*Gyps fulvus*) from two areas of Valencian Community, in the east of Spain. Moreover, effects induced by these metals on Griffon vulture oxidative stress biomarkers have been studied. We have used a battery of biomarkers including total GSH content, antioxidant enzymes activities (GPx, SOD, CAT and GST) and lipid peroxidation.

MATERIAL AND METHODS

Study area and species of study

Razorbill (*Alca torda*) is an Alcidae seabird species that lives in high latitudes. At least 70% of the world's Razorbill population breeds in Iceland. They are migratory seabirds and are common wintering species in the Mediterranean area (824 Razorbills were recorded in 2007). The study area is the Occidental Mediterranean coastline in the Southeast of Spain (La Marina, Elche, Alicante) at 38ºN and 0ºW. Migration to industrialized latitudes such as Mediterranean area may result in the accumulation of contaminants in seabird body burden. Furthermore, the Razorbill is a species of particular interest, considering their possibly declining population densities (Ribeiro *et al.*, 2009).

The Eurasian Eagle Owl (*Bubo bubo*) is a large nocturnal raptor which could be a sensitive biomonitor of metals in the terrestrial ecosystem since is a long-lived top predator in food chains and it is resident and territorial. The study area is located in the east of Murcia Region, in Southeastern Spain (37º45' N, 0º57' W). The study area was subdivided in two sub-areas. In Northern subarea land is mainly dedicated to citrus and dry farming, and the European Rabbit is abundant, accounting for 71% of the prey consumed by Eagle Owls (León *et al.*, 2008). In Southern subarea, the European Rabbit is less abundant (35% of the Eagle Owls' diet), and the raptor consumes a similar proportion of rats (23% of the diet), apart from pigeons, partridges, hedgehogs and yellow-legged gulls (León *et al.*, 2008). In this subarea irrigation farming is predominant and it is remarkable the fact that some ancient mining sites (Sierra Minera de Cartagena-La Unión) is found in this subarea.

Griffon vulture (*Gyps fulvus*) is a large bird of prey from Accipitridae family that belongs to the Old World vultures. It is a scavenger, feeding mostly from carcasses of dead domestic livestock and, to a lesser extent, of wild species dead in the field (Donázar, 1993). The world population of Griffon vulture extends from North Africa, by

several South European countries, to Central Asia; and an important population is concentrated in Spain (Del Moral, 2009). This species is considered sedentary across most of its breeding area, except for young and immature birds which often disperse or migrate from north to south (Ferguson-Lees and Christie, 2001). The two study areas are located in Valencian Community, in the east of Spain. In this Community, Griffon vulture breeds in Castellón, with 236 breeding pairs in 2008 (93% of the breeding pairs in Valencian Community); and in the north of Alicante, with 19 breeding pairs in 2008 (Del Moral, 2009).

Sample collection

Fifty Razorbills were used in this study. These animals were found dead along the Occidental Mediterranean coastline in the East of Spain (La Marina, Elche, Alicante), having drowned in fishing nets in February of 2007. They were collected by the staff of “Santa Faz” Wildlife Recovery Center (Alicante, Spain). A total of 447 samples of liver (n=50), brain (n=50), subcutaneous fat (n=49), abdominal fat (n=48), kidney (n=50), muscle (n=50), 10th primary wing feathers (p10) (n=50) and 9th primary wing feathers (p9) (numbering outwards) separeted in vane (n=50) and shaft (n=50) were collected via necropsy. After collection, the samples were packed in Eppendorf tubes and frozen until their analysis. Liver, brain, subcutaneous fat, abdominal fat and 10th primary wing feathers were used for organochlorine pesticide analyses (Chapter III and IV). Liver, brain, kidney, muscle, and 9th primary wing feathers separeted in vane and shaft were used for mercury analyses (Chapter V). Sex, age and physical condition were registered.

Regarding Eurasian Eagle Owl, in Chapter VI a total of 623 blood samples from 2006 to 2012 (435 from Northern area and 188 from Southern area), and 229 back feathers from 2006 to 2008 (174 from Northern area and 55 from Southern area) were analyzed for mercury concentrations in Eagle Owl chicks (30-days old) from Murcia, Southeastern Spain. Moreover, we obtained 40 muscle samples from carcass of European Rabbit from 2009, 2011 and 2012 collected at the same nest where chicks were growing (25 from Northern area and 15 from Southern area). In Chapter VII a total of 141 blood samples from 2011(n=71) and 2012 (n=70) (72 from Northern area and 69 from Southern area) were analyzed for metal concentrations in Eurasian Eagle Owl chicks (n=133) and adults (n=8) from Murcia. Besides, a total of 140 red blood cell (RBC) samples were analyzed for oxidative stress biomarkers.

Blood samples (3-5 ml) were obtained by puncturing brachial vein with a 23G needle, and stored in heparinised Eppendorf tubes. Feathers were plucked from the back of chicks (they were still developing) and stored in plastic bags at room temperature. Adult Eagle owls were captured with nets in the nest. One Eppendorf tube with whole blood was separated and another Eppendorf tube with blood was used to separate plasma and RBC fractions (10,000 rpm, 5 minutes). Finally, Eppendorf tubes were stored at -80ºC until analysis. The sampling was authorized by the General Directorate of Natural Patrimony and Biodiversity from the Autonomous Community of Murcia Region.

Finally, 66 Griffon vultures were caught in baited cage traps at two different sites from Valencian Community (Chapter VIII). The sampling was done in the feeding station of Cinctorres (n=30), in the province of Castellón (40º35' N, 0º12' W), on 27 September and 3 October 2011; and in the feeding station of Alcoy (n=36), in the province of Alicante (38º42' N, 0º28' W), on 13 November 2011. These feeding stations are places where it is provisioned supplementary food for vultures, normally once a week and mainly of porcine origin. Blood samples collection and treatment is the same as explained above.

Analysis of organochlorines pesticides in internal tissues and feathers

Internal tissues in Chapter III were analyzed for 15 OC (α-HCH, β-HCH, δ-HCH, lindane, heptachlor, heptachlor epoxide, aldrin, dieldrin, endrin, endosulfan I, endosulfan II, endosulfan sulfate, DDT, DDE and DDD), according to a method described by Martínez-López *et al.* (2009). In this technique 0.2 g of tissue were extracted with organic solvents, and 1 µl was injected into a gas chromatograph with electron capture (GC-ECD 17 Shimadzu). The SPB-608 capillary column (Supelco®) was 30 m long, 0.25 mm i.d. with a 0.25 µm film thickness. Identification and quantification were based on an external standard. Mean recoveries in spiked samples ranged from 85.8% to 146.0%.

In Chapter II a mix of feathers from Mallard duck (*Anas platyrhynchos*) were used for the development of a method of extraction for 16 OC in feathers. Feathers from Mallard duck were provided by the staff of "Santa Faz" Wildlife Recovery Center (Alicante, Spain). Four methods with different solvents were tested, and finally, the method with a greater recovery was selected. The feathers (0.2 g) were weighed and incubated overnight at 37ºC in HCl and hexane:acetone or hexane:dichloromethane depending on the technique evaluated. Extraction was performed with different organic

solvents, and 1 µl was injected into a gas chromatograph with electron capture as explained above. Then, in Chapter IV feather samples were analyzed according to the method described by Espín *et al.* (2010a) (Chapter II). In order to remove external contamination from the feather surface, prior to the analytical determination a washing process was performed with tap water, distilled water and Milli-Q water.

Analysis of heavy metals in internal tissues, blood and feathers

Total mercury was analyzed in a Milestone DMA-80 direct mercury analyzer by atomic absorption spectrophotometry (Chapters V-VIII). Samples (0.1 g wet weight for internal tissues, 0.01 g dry weight for feathers, 100 µl wet weight for blood, and 0.2-0.3 g wet weight for muscle of rabbit) were loaded in a nickel boat and analyzed. Recovery of total mercury from replicates of certified reference material was approximately 99%. In order to remove external contamination from the surface of feathers, a washing process was performed prior to analytical determination using tap water, distilled water, Milli-Q water and acetone.

In Chapters VII and VIII, cadmium, lead, copper and zinc levels were analyzed in blood samples following the method described by García-Fernández *et al.* (1995), using an anodic stripping voltammeter (VA-757 Computrace Workstation; Methrohm, Switzerland). The samples (0.2 ml of whole blood) were prepared for analysis eliminating impurities by a complete high temperature digestion with a mixture of acids.

The concentration of each metal in the digested sample was calculated after twice adding dilutions prepared from standard solutions of Cd, Pb, Cu and Zn, respectively (Sigma, St. Louis, MO). Mean recoveries, which approached 96%, were calculated analysing 10 identical samples of reconstituted lyophilized blood (European Union Reference Standards CRM195) (García-Fernández *et al.*, 1995).

Analysis of oxidative stress biomarkers in red blood cells (RBC)

Several oxidative stress parameters were analyzed in RBC in Chapters VII and VIII, after homogenization (1:10 w/v) in a stock buffer (1.15% KCl in 0.01 M PBS (pH 7.4) with 0.02 M EDTA). Lipid peroxidation, estimated as thiobarbituric acid-reactive substances (TBARS), was assessed following the methodology described by Alonso-Alvarez *et al.* (2008) with a spectrophotometer (UV-1603, Shimadzu). Levels of total glutathione (tGSH) were obtained as described by Reglero *et al.* (2009) with an automated spectrophotometer A25-Autoanalyzer (BioSystems). The activities of glutathione peroxidase (GPx; EC1.11.1.9) and superoxide dismutase (SOD;

EC1.15.1.1) were determined spectrophotometrically (A25-Autoanalyzer) using the Ransel and Ransod kits, respectively (Randox Laboratories), following descriptions of Reglero *et al.* (2009) with some modifications for RBC. GPx and SOD results were expressed as Units per gram protein. CAT (EC 1.11.1.6) activity was assayed following the methodology described by Clairbone (1985). Results were expressed as µmol H_2O_2 consumed per minute per mg protein. The activity of GST (EC 2.5.1.18) was determined by the method described by Habig *et al.* (1974). The results were expressed as nanomoles produced per minute per mg protein. Enzyme activities were expressed relative to grams of protein in the homogenates. Total protein content in the homogenate was measured spectrophotometrically at 595 nm following the method of Bradford (1976).

Statistical analyses

All analyses were carried out using the SPSS v.15.0 statistical package. Reported contaminants and oxidative stress biomarker values represent the mean ± standard deviation, median and range. Data were tested for normality using a Kolmogorov–Smirnov test, and when concentrations of contaminants were not distributed normally, the data was log-transformed. ANOVA and Tukey's tests were performed to elucidate significant differences in contaminant concentrations and biomarker values between variables (tissues, areas, year, gender and age groups).

Generalized Linear Models (GLM) were used to analyze the concentrations of organochlorine pesticides or metals in each tissue. The OC compound/metal was the response variable. We considered several explanatory variables depending on the chapter, such as age and sex (Chapter IV and V); age, sex and body condition (Chapter III); area and year (Chapter VI); or age and area (Chapter VII). We used GLMs with an identity function and Gaussian errors. In Chapters III and IV we followed a forward stepwise procedure. It was tested the statistical significance of each explanatory variable (F-test) and was retained those that contributed to the largest change in deviance from the null model until all the variables with a significant effect at $p<0.05$ had been included in the model. In Chapters V, VI, VII and VIII, we followed a backward stepwise procedure to select the final models, excluding the predictor variables when they had no significant effects.

In Chapter VII and VIII, simple linear regression was performed to evaluate the effect of each metal on the oxidative stress biomarkers, and GLMs with a normal distribution and an identity function were performed to study combined effects of metals

on the biomarkers. Biomarker value was the response variable, metal concentrations were selected as covariates and studied area and age were selected as factors.

Furthermore, in Chapter V to assess the use of a non-invasive sample (feather) for predicting Hg levels in the target organ (brain or kidney), GLMs were used with brain Hg or kidney Hg as dependent variables, and feather shaft Hg as a covariate, with sex and age as factors.

Pearson's correlation coefficient was used in order to calculate correlations between variables. In Chapter IV Spearman's correlation coefficient was used. The level of significance for these tests was set at $\alpha=0.05$.

RESULTS AND DISCUSSION

PART 1. Biomonitoring of persistent environmental pollutants in wild birds: feathers as a biomonitoring tool.

Chapter I. Feathers as a biomonitoring tool of polyhalogenated compounds: a review.

Growth rate of feathers and deposition rate of pollutants in feathers.

Pollutants can reach feathers via the blood only during their growth periods (Burger, 1993), thus producing internal contamination. When they mature, vascular connections undergo atrophy and compound concentrations remain stable (Burger and Gochfeld, 2000). Therefore, feathers can provide information on concentrations in the blood circulation at the time of their growth. No data are available in the literature on the specific chemical structure and binding capacities of organic compounds to feather tissue. However, once incorporated, contaminants seem to be permanently retained, since it is possible to detect non-persistent pollutants in feathers over long periods of time (Behrooz *et al.*, 2009a).

Recently, Bortolotti (2010) has shown that different factors could affect the chemical analysis of feathers and, perhaps, the expression of results as mass of contaminant per mass of feather is not the most appropriate unit of measure (Bortolotti, 2010). One factor is the influence of feather mass. Bortolotti (2010) suggested that the variation in mass across feathers affects the interpretation of their contaminant concentrations. A given amount of contaminant deposited in a heavier feather will result in a diluted concentration. Other factor is the influence of feather growth rate.

Slower growing feathers are likely to have a higher contaminant load than faster growing feathers.

Therefore, the key factor could be the deposition rate as unit of measurement, which allows comparisons to be made between different parts of the same feather as well as between different feathers. To express the results in terms of deposition rate, we propose the following equation (Eq.1):

Deposition rate (ng contaminant /day) = mass contaminant (ng) / pairs of growth bars measured (day) (Eq.1)

We can use ptilochronology (the alternating pale and dark band which are indictive of daily growth; Grubb, 1989) to calculate this, since one pair of growth bars represents a 24-h period of growth. However, in certain bird species the growth bars are not visible, making this calculation impossible. In this case, a second option including growth rate data could be of interest (Eq.2):

Deposition rate (ng contaminant /day) = mass contaminant (ng) / feather length (mm) * growth rate (mm feather/ day) (Eq.2)

The latter option has two disadvantages. Firstly, it is necessary to know the growth rate of the different feathers in each species. On the other hand, it should be noted that not all individuals in a given species have the same feather growth rate, and it is possible to find different growth rates associated with different nutritional resources (Wolf *et al.*, 2003). Taking into account these statements, we suggest, whenever posible, the use of the equation 1 as complementary data in future studies.

Feathers and organic compounds.

Several PHCs have been analyzed in feathers including PCBs, OC and PBDEs in both terrestrial and aquatic species from a few locations such as Belgium, south-west Iran and south-east Spain.

Early studies used feathers to determine the concentration of PHCs in bird species treated with certain doses of these pollutants (Greichus and Greichus, 1974; Greichus *et al.*, 1975; Hall *et al.*, 1971; Ivie *et al.*, 1974). Some studies have evaluated the correlations between levels in feathers and internal tissues (Dauwe *et al.*, 2005; Espín *et al.*, 2010b, 2012a (Chapter III and IV); Jaspers *et al.*, 2007a, 2011; Rajaei *et al.*, 2011) as well as the influence of external contamination on organic contaminant levels

found in the feathers of several bird species (Van Den Steen *et al.*, 2007; Espín *et al.*, 2010a (Chapter II); Rajaei *et al.*, 2011; Summers *et al.*, 2010). Some studies have even reported on concentrations of organic pollutants in feathers from museum collections (Behrooz *et al.*, 2009a,b).

Body distribution pattern and correlations between concentrations of polyhalogenated compounds in feathers and internal tissues of birds.

Regarding the distribution pattern, the highest concentrations of these lipophilic contaminants are expected to be found in adipose tissue, followed by liver and muscle. Accordingly, Dauwe *et al.* (2005) and Espín *et al.* (2010a, 2012a) (Chapter III and IV) observed higher levels of persistent organic pollutants in fat tissue than in feathers, and several studies have also shown higher concentrations of these contaminants in liver or muscle than in the feathers of several bird species (Espín *et al.*, 2010b, 2012a (Chapter III and IV); Jaspers *et al.*, 2006, 2011; Rajaei *et al.*, 2011; Summers *et al.*, 2010).

However, less persistent PHCs are more easily metabolized (Juan *et al.*, 2002) and they may be found in low concentrations in fat or liver and in higher levels in the bloodstream for a limited time (Dauwe *et al.*, 2005) e.g. due to lipid mobilization. Therefore, these compounds could enter feathers during their growth period. In this sense, several studies have found that feathers had proportionally higher levels of less chlorinated PCB congeners than fat, liver or muscle (Dauwe *et al.*, 2005; Jaspers *et al.*, 2007a; Rajaei *et al.*, 2011).

As to the relationship between concentrations of PHCs in feathers and internal tissues, strong and significant correlations have been found by several authors (Eulaers *et al.*, 2011; Jaspers *et al.*, 2007b, 2011; Rajaei *et al.*, 2011). However, other authors have found low significant correlation coefficients between the pollutant concentrations in feathers and those of internal tissues in several bird species (Dauwe *et al.*, 2005; Espín *et al.*, 2010b, 2012a (Chapter III and IV); Jaspers *et al.*, 2006, 2007a). These ambiguous findings could be influenced by several factors such as changes in diet, time elapsed between the previous molt period and sampling, sample size or external contamination.

External contamination.

The correlation between organic pollutant contamination into feathers and levels in internal tissues can be confusing due to external contamination by organic pollutants

on the surface of feathers (Jaspers *et al.*, 2008). This external contamination can be caused by both exogenous (such as atmospheric deposition), and endogenous sources (preening of the feathers with oil from the preen gland). Some authors have suggested that the external deposition of these organic compounds onto the feather does not occur, or does only minimally (Dauwe *et al.*, 2005; Jaspers *et al.*, 2007b). Despite this, some studies have found that external contamination does occur in feathers of several species (Espín *et al.*, 2010a (Chapter II); Jaspers *et al.*, 2008, 2011; Summers *et al.*, 2010). Unfortunately, such surface contamination may be difficult to be effectively removed using the washing techniques in the laboratory tested to date. Jaspers *et al.* (2011) suggested that the external contamination removed after both water and acetone washing was linked to dirt and dust particles, and other agents may be required to remove preen oil from the feathers. Moreover, the influence of preening activity on feathers also depends on the age of the feather, being higher in older feathers (Jaspers *et al.*, 2011). Therefore, all feathers could possibly be used, but the ideal feather depends on the molting pattern of the species, closely related with the external contamination.

On the other hand, the barbs have a large and structurally complex surface area subject to exposure, while the rachis of a feather can be easily and effectively cleaned (Cardiel *et al.*, 2011). According to Cardiel *et al.* (2011), since some studies have found higher organic pollutant concentrations in vanes than in shafts (Espín *et al.*, 2010a (Chapter II); Jaspers *et al.*, 2007b), the shaft can be more confidently used. However, on developing or very recently molted feathers, external contamination of their surfaces would be expected to be minimal, so barbs may remain valuable (Cardiel *et al.*, 2011).

The use of external contamination markers, such as certain metals with a low intestinal absorption rate and therefore with low endogenous deposition in feathers, could be useful toward discriminate between internal and external contamination. In this sense, aluminum or titanium have been shown to be useful when interpreting Pb levels in faecal excreta and feathers (Beyer *et al.*, 1997; Cardiel *et al.*, 2011).

Intraspecific, interspecific and spatial differences.

Body condition could be a determining factor in PHC levels. The mobilization of organochlorines from depleting fat stores and the consequent increase of concentrations of these compounds in the liver and other well irrigated organs have been reported for birds that had low lipid concentrations (Kenntner *et al.*, 2003; Malcolm *et al.*, 2003; Wienburg and Shore, 2004). Since the feather root is

vascularized during its development, contaminants in the bloodstream may enter the feather via the root (Dauwe *et al.*, 2005). The importance of fat mobilization for migration, egg laying or other factors on the relative levels of feather and internal organ tissue contamination partly depends on the time when samples are collected. This is the offset in time between when feathers were grown and typically when the bird died (and internal tissues were collected). If egg laying, migration, starvation periods etc. occurred after the molt but prior to death, then this is likely to reduce any correlation between contaminant concentrations in feathers and internal tissues.

On the other hand, Espín *et al.* (2012a) (Chapter IV) and Jaspers *et al.* (2011) observed significant differences in feather concentrations for some compounds between young and old birds, with higher residue concentrations in old birds. Studies have found a positive relationship between age and organochlorine compounds in muscle, fat, brain and liver samples (Borgå *et al.*, 2001; Donaldson *et al.*, 1997; Espín *et al.*, 2010b (Chapter III); María-Mojica *et al.*, 2000; Vorkamp *et al.*, 2004). Higher levels in tissues of old birds may reflect a longer period of exposure in these individuals (Espín *et al.*, 2010b).

In regard to gender, in view of the potential influence of off-loading contaminants into eggs and the resultant potential decrease in internal tissue contaminant concentrations in adult females, feathers should preferably be monitored from male birds so as to eliminate this bias, especially in the breeding season. However, depending on the research end point this recommendation may not be appropriate, i.e. studies on the influence of egg laying or studies on differences in exposure according to sex.

Species habits such as diet or migratory behavior should be reflected in the pollutant concentrations found in feathers. In this sense, several authors have analyzed PHCs in feathers of species with different dietary habits (Behrooz *et al.*, 2009a; Malik *et al.*, 2011). Behrooz *et al.* (2009a) found that raptors (carnivores) showed the highest levels of DDTs and PCBs due to the biomagnification process, and herbivores showed the lowest levels. Regarding to this, Malik *et al.* (2011) observed higher PBDE concentrations in feathers from the piscivorous Little Egret (*Egretta garzetta*) compared with the terrestrial insectivore Cattle Egret (*Bubulcus ibis*), suggesting that fish consumption is the primary exposure pathway for PBDEs in the aquatic food web. Besides, Rajaei *et al.* (Rajaei *et al.*, 2011) found that gulls from the coast of the

Caspian Sea had high OC levels, probably due to their summer in western Siberia where high levels of DDTs have been reported (Rajaei *et al.*, 2011).

Finally, Jaspers *et al.* (2009) studied the usefulness of feathers for monitoring regional variations in contamination. The results showed that concentrations of p,p'-DDE were significantly higher in rural areas, while levels of PCBs were higher in urban areas, confirming their expectations, since DDT is a pesticide that used to be applied on the countryside, while PCBs were mainly produced and used by industry, mostly located close to cities. The feather appears to reflect regional variations in the concentration of these compounds (Jaspers *et al.*, 2009).

Analytical methods for organohalogenated pollutants in feathers.

Several methods have been used for the analysis of organohalogenated pollutants in feathers (Dauwe *et al.*, 2005; Espín *et al.*, 2010a; Jaspers *et al.*, 2013; Malik *et al.*, 2011; Meyer *et al.*, 2009).

Previous to the analytical method, in most studies feathers are washed with distilled water, dried at room temperature, and cut into small pieces of approximately 1 mm (Behrooz *et al.*, 2009a,b; Jaspers *et al.*, 2006, 2007a,b, 2009, 2011). Although some studies have investigated the possibility of removing external contamination using different washing agents (water, acetone, surfactant solution), contamination by preening oil cannot likely be washed away thoroughly. Therefore, further studies are needed to determine reliable methods for discriminating between the internal and external contamination of feathers by organic pollutants.

Adverse effects: Interpreting feather concentrations.

There are very few monitoring studies available which provide information on both feather and internal tissue concentrations, and even fewer discuss the importance of feather concentrations in estimating adverse effects (Behrooz *et al.*, 2009a,b; Espín *et al.*, 2010b, 2012b). Espín *et al.* (2010b, 2012a) (Chapter III and IV) analyzed organochlorine pesticides in feathers and internal tissues in the same individuals. Therefore, they could conclude that there was no risk of adverse effects associated with the OCs levels found in feathers (Espín *et al.*, 2012b) (Chapter IV) taking into account concentrations found in liver in a previous study (Espín *et al.*, 2010b) (Chapter III). Behrooz *et al.* (2009a,b) carried out an hazard evaluation comparing concentrations of OCs in feathers with levels reported in internal tissues from other studies. These authors note that this comparison should be used with caution because

of problems with extrapolating such data across tissues and species. Therefore, further experimental and field studies are required in order to determine a non-adverse-effect threshold value in feathers from different species.

Chapter II. Development of an analytical method for extracting organochlorine pesticides from feathers.

Selection of the analytical technique.

Within the four methods tested, the technique with a greater recovery was selected. Mean recoveries in spiked samples ranged from 46.13% to 146.05%.

In this technique, the feathers (0.2 g) were weighed and incubated overnight at 37ºC in HCl and hexane:acetone (2:1, v/v). Extraction was performed with hexane:acetone (3:1, v/v). The samples were homogenized, centrifuged and filtered using anhydrous sodium sulfate and then the solvent collected was evaporated until dryness. After redisolution in 5 ml hexane, samples were cleaned up via Florisil column chromatography (Sep-Pak, Waters®), activated with 2 ml of hexane, using a petroleum ether:diethyl ether mix (21:4, v:v) as the elution solvent. The solvent collected was evaporated until dryness. The final volume was adjusted to 1 ml with n-hexane, and 1 µl was injected into a gas chromatograph with electron capture as explained above.

External contamination.

In order to assess the influence of external contamination and the distribution of compounds between parts of the feather (barbs and shaft), we compared OC levels between washed (with tap water, distilled water and Milli-Q water) and non-washed feathers, as well as between vane and shaft, after the development of the analytical technique. In general, OC levels were significantly higher in unwashed samples than in washed ones for some compounds, in both vane and shaft, suggesting a possible interference by external contamination from atmospheric deposition or oil secreted by the preen gland. We recommend washing feathers with tap water, distilled water and Milli-Q water in order to remove external contamination before analysis. However, external contamination seems to be of less extent in comparison with external contamination by some metals such as Pb (Dauwe *et al.*, 2002).

Organochlorine pesticides distribution within feathers.

Higher levels of certain compounds in unwashed barbs than in unwashed shafts can be explained by the higher probability of organic pollutant deposition in barbs than

in shafts. Barbs have a large surface area subject to exposure by both depositon of particules with OC associated, or contamination by preening oil. Therefore, external contamination should be considered when analyzing OC in feathers.

Moreover, higher OC concentrations of certain compounds in washed barbs than in washed shafts suggest that differences in OC levels among parts of the feather are not only due to external contamination. In this sense, Jaspers *et al.* (2007b) suggested that higher levels in barbs could be due to differences in binding capacity to different chemical structures in the feathers. However, no data are available in the literature on the specific chemical structure and binding capacities of organic compounds to feather tissue. Another possible hypothesis is that the barbs are the end of the compound route, and the shaft is a transport channel (Jaspers *et al.*, 2007b). It could also simply be the artifact of dilution (Bortolotti, 2010), since the shaft is heavier than the barbs.

Chapter III. Assessment of organochlorine pesticide exposure in a wintering population of Razorbills (Alca torda) from the southwestern Mediterranean.

Organochlorine pesticides in tissues.

The highest organochlorine pesticide (OC) levels were found in abdominal fat followed by subcutaneous fat, liver and brain (∑OC= 11854, 10490, 2630 and 362 ng/g, respectively). Buckman *et al.* (2004) also found that OC concentrations were greater in fat than liver for all OC groups across three species of Alcidae. Although the higher lipid content in fat is the main cause of lower concentrations in liver compared to fat (Cockcroft *et al.*, 1989), biotransformation processes may also influence this, since the liver is an active biotransformation site. In this sense, we found negative correlations between fatty tissues and liver for δ-HCH ($r=-0.391$, $p<0.01$), DDE ($r=-0.288$, $p<0.05$) and heptachlor epoxide ($r=-0.484$, $p<0.01$).

Regarding correlations between concentrations in brain and liver, a significant negative correlation was found for lindane ($r=-0.359$, $p<0.01$). More polar organochlorines such as lindane, tend to accumulate in tissues containing, proportionately, higher concentrations of phospholipids, such as brain (Kawai *et al.*, 1988), consequently, a lindane mobilization to the brain is a possible interpretation for the decrease in liver.

Finally, regarding correlations between fatty tissues, significant positive correlations were found for most compounds between abdominal and subcutaneous fat ($r=0.399-0.722$, $p<0.01$). Both fatty tissues have similar behaviour, with no significant

differences in OC accumulation ($p>0.692$ for all OC groups). However, the OC levels were highest in abdominal fat. This fact is logical since during periods of distress (such as migration), subcutaneous fat stores are metabolized firstly and these compounds are mobilized and distributed throughout the bloodstream to highly metabolically active organs, *e.g.,* the liver (Hela *et al.*, 2006).

Study of the OC groups.

Firstly, in all analyzed tissues, ΣDrins had the highest concentrations, followed by ∑DDT, ∑Endosulfan, ∑HCH and ∑Heptachlor. ΣDrins concentrations were the highest due to endrin aldehyde, which reached the highest levels of all pesticides and for all analyzed tissues. Several studies (María-Mojica *et al.*, 2000) also described high levels of this compound. ΣDDT concentrations followed the order of DDT>DDD>DDE in fat tissues. Like other authors (Borgå *et al.*, 2007; Buckman *et al.*, 2004), in our study the highest concentrations in liver were reached by DDE, meanwhile in brain tissues, this compound showed the lowest concentrations. The p,p'-DDT is metabolized in the liver, mainly to p,p'-DDE and p,p'-DDD (Gold and Brunk, 1982). Therefore, higher hepatic DDE concentrations could indicate the ability to convert DDT into DDE (Tanabe *et al.*, 1998). Fatty tissue ratios (p,p'DDE/p,p'DDT) of less than 1 indicate exposure to non-degraded DDT, and thus it was present in the environment despite its prohibition.

Regarding ΣEndosulfan group, endosulfan sulfate is the most common compound found in all tissues and reached the highest concentrations. In other studies it was also frequently detected and in high concentrations in blood samples (Martínez-López *et al.*, 2009). In regard to hexachlorocyclohexanes, β-HCH and γ-HCH (lindane) isomers were the most frequently detected. The β-HCH isomer was also the most frequently detected in previous studies in forest raptors (Martínez-López *et al.*, 2009). The β-HCH isomer reached the highest levels in all tissues analyzed except in brain samples, where the highest concentrations were reached for lindane. This isomer (β-HCH) has a greater stability against enzymatic degradation than the other isomers, and it has been demonstrated as having the lowest degradation ratio of the HCH group (WHO, 1992), which would explain its presence at higher concentrations. Kawai *et al.* (1988) found that the more polar organochlorines such as lindane, tend to accumulate in tissues like the brain, which contains high concentrations of phospholipids in proportion.

Sex, age, diet and corporal condition.

In our study, no significant differences between sexes were found, except for ∑Drins in brain, with the highest levels in females ($p=0.0449$, ∑Drins in brain of female=208.57 ng/g, ∑Drins in brain of male=41.61 ng/g), being the sex the best explanatory variable in the model of ∑Drins in brain. This could be due to the fact that all Razorbills analyzed were collected after the breeding season, thus the transference of these compounds from mother to egg is not possible.

Significant differences were observed between young and old birds in fatty tissues, with higher residue concentrations in old birds. Several studies have found a positive relationship between age and OCs in internal tissues (Borgå *et al.*, 2001; Donaldson *et al.*, 1997; María-Mojica *et al.*, 2000; Vorkamp *et al.*, 2004). The highest levels in the fat tissues of old birds may reflect a longer period of exposure in these individuals and a low elimination rate of these compounds. However, this trend was not observed for brain and liver samples, where no significant differences were found between age groups for most compounds.

Organochlorine concentrations in seabirds depend, among other factors, on trophic level and feeding habits (Borgå *et al.*, 2007; Buckman *et al.*, 2004). In studies with birds of different feeding habits it has been observed that the highest OC levels were found in piscivorous birds, followed by insectivores, omnivores and herbivores (Kunisue *et al.*, 2002). The Alcidae diet, including that of Razorbill, consisting mainly of fish (80-90% by volume) and their migratory status are factors that increase OC exposure. In the present study, Razorbill tissues showed higher liver ΣDDT concentrations (586.09 ng/g) than those of other Alcides collected by Borgå *et al.* (2001) and Buckman *et al.* (2004) in other areas (Baffin Bay and Barents Sea). These results can be only explained by the habitat, suggesting that the Mediterranean area presents higher OC levels than higher latitudes. The use of organochlorine compounds has been more intense in the Mediterranean area than at higher latitudes. Besides, the Mediterranean is a closed sea surrounded by highly industrialized countries, which constitutes a high-risk marine environment due to contamination by toxic compounds (Kuetting, 1994).

Furthermore, body condition is also a determining factor in OC levels (Bustnes *et al.*, 2008; Kenntner *et al.*, 2003; Malcolm *et al.*, 2003). In our case, no significant differences were found comparing OC concentrations and body condition. This is probably due to most of Razorbills having good body condition, so population was fairly

homogeneous and there were insufficient malnourished birds to observe such differences in our study.

Effect assessment.

Maximum residue levels for the OCs found in this study were all below the limits known to cause adverse effects in birds, such as egg breakage, brain effects, behavioural abnormalities, reproductive behaviour and mortality (Call *et al.*, 1976; Haegele and Hudson, 1977; Pratt, 1972; Sharma *et al.*, 1976; Stickel *et al.*, 1969, 1970).

During periods of distress, such as migration or breeding, the stored body fat is metabolized and the lipophilic OC are mobilized and distributed through the blood stream to highly active organs, i.e., the liver (Meador *et al.*, 2002). At worst, if we assumed that birds of this study mobilize all their fat reserves due to a stressful situation such as those mentioned above, 100% of the pesticides accumulated in fat would be released into the bloodstream. In a study on mallards dosed with 20 ppm of endrin during a large dietary experiment, Heinz and Johnson (1979) found a half-life for endrin of 3 days with 90% of residues eliminated at 33 days post-dosing. Therefore, it could be assumed that a mere 20% (approximately) of these compounds would be available for accumulation in organs. Applying this percentage to the sum of ∑Drins of both the abdominal and subcutaneous fat samples of the present study, we calculated a theoretical blood concentration of ΣDrines of approximately 2,300 ng/g, which is lower than those concentrations referred to as indicative of damage by most authors. However, we must keep in mind that OC acumulated in fat tissues would not be released at once into the bloodstream but would be gradually released over time during the stressful period. Moreover, we have evaluated the risk for the worst-case scenario, with the sum of aldrin, dieldrin, endrin and endrin aldehyde, such that if they are compared individually, the risk would be lesser. Therefore, according to the prediction made with data from other studies, the probability of risk associated to fat mobilization is low.

Chapter IV. Razorbill (Alca torda) feathers as an alternative tool for evaluating exposure to organochlorine pesticides.

OC levels in feathers.

All of the organochlorine pesticides examined were detected in Razorbill feathers. ΣDDT had the highest geometric mean concentrations (67.4 ng/g), followed by ΣHCH

(62.9 ng/g), ΣHeptachlor (61.7 ng/g), ΣEndosulfan (19.7 ng/g) and ΣDrins (10.2 ng/g). The compounds that reached the highest geometric mean levels were DDE, heptachlor epoxide, lindane and heptachlor, respectively. As in research by Dauwe *et al.* (2005) into persistent organic pollutants in great tit feathers (*Parus major*), the ΣDDT-group concentrations followed the order of DDE>DDD>DDT. The substance p,p'-DDT is metabolized by the liver, mainly to p,p'-DDE, for which p,p'-DDD is an intermediate (Gold and Brunk, 1982). Therefore, higher DDE concentrations in feathers could be explained by the ability of the organism to convert DDT into DDE (Tanabe *et al.*, 1998), as well as direct DDE inputs from the environment and prey and/or DDE transfer to feathers.

The hexachlorocyclohexane isomer γ-HCH (lindane) has a short half-life in the environment compared with other OC, and is metabolized and excreted by organisms relatively rapidly (Blus *et al.*, 1985). Therefore, the higher levels of γ-HCH in feathers probably reflect an exposure to lindane during feather growth or a release into the bloodstream by fat mobilization during feather growth. Moreover, this result shows the capacity of feathers as an excretion route for this compound. Behrooz *et al.* (2009a,b) also found that lindane was the most predominant HCH isomer in feathers, which they felt was due to recent exposure of birds to γ-HCH.

In general, levels found in Razorbill feathers are much higher than those found in the feathers of both aquatic and terrestrial bird species for ΣHCH and ΣDDT (Behrooz *et al.*, 2009a,b; Dauwe *et al.*, 2005; Jaspers *et al.*, 2006, 2007a,b, 2008, 2009). The Razorbill diet, consisting mainly of fish (80–90% volume), and their migratory status are factors that increase OC exposure. In studies of birds with different feeding habits, it has been observed that the highest OC levels were found in piscivorous birds (Kunisue *et al.*, 2002). Migratory habits must also be considered when evaluating the exposure and distribution of contaminants in the body. In this sense, Razorbills wintering in the west Mediterranean Sea may be exposed to high OC levels. The use of OC has been more intense in the Mediterranean area than in other areas. Besides, the Mediterranean is a closed sea surrounded by highly industrialized countries, which constitutes a high-risk marine environment due to contamination by toxic compounds (Kuetting, 1994).

However, interpreting feather concentrations is complex, mainly due to the molt strategy. Specifically, Razorbills have a complete post-nuptial molt (also called prebasic molt) in August-September or October, prior to migration, involving all contour

and flight feathers (Bédard, 1985). Primary and secondary feathers are replaced synchronously or nearly so, resulting in a period of flightlessness (Thompson *et al.*, 1998). In February-May there is a pre-nuptial molt (also called prealternate molt) that includes most, if not all feathers of the head and throat but none of the back, rump, or wings (Lavers *et al.*, 2009).

In periods of distress, such as migration, breeding and molt, the stored body fat is metabolized and the lipophilic OC are mobilized and distributed throughout the blood stream (Perkins and Barclay, 1997). As the blood OC concentrations are transported into the growing feathers during molt, the OC concentrations detected in primary feathers were deposited in their Northern nesting location during the post-nuptial molt, and thus, feather concentrations would indicate both OC exposure from their Northern ranges and OC incorporated during the previous winter on the Mediterranean coast albeit not yet excreted. It is feasible to assume that not all the organochlorine body burden would be mobilized during the migration. Moreover, a percentage of the OC mobilized will not be excreted and would be available for accumulation again. In this sense, Cole *et al.* (1970) and Lay *et al.* (1982) studying the elimination of 14C following intraperitoneal or intravenous injection of 14C-dieldrin in male rats, observed that between 70% and 80% of the total injected dose was excreted within 2 weeks post-dosing, and therefore, 20-30% approximately would be accumulated again.

Nonetheless, there is little data available on the relationship between organochlorine pesticides in feathers and their effects. Levels found in the present study are related to maximum concentrations in liver from (Espín *et al.*, 2010b) (Chapter III) of 1654.54 ng/g for ∑HCH, 1171.72 ng/g for ∑Heptachlor, 1965.76 ng/g for ∑Endrin, 3365.13 ng/g for ∑Endosulfan and 4176.76 ng/g for ∑DDT. These levels of exposure rarely result in adverse reproductive effects, behavioral abnormalities, or other signs of OC poisoning in birds (Pratt, 1972; Sharma *et al.*, 1976). Therefore, considering that this species undergoes a complete molt annually, we can assume there is no risk of adverse effects associated with the OC feather levels found in the present study.

Age and sex.

The best explanatory variable for most OC compounds was age ($p < 0.048$). Significant differences for some compounds were observed between young and old birds in feathers, with the highest residue concentrations residing in old birds. (Espín *et al.*, 2010b) (Chapter III) also found the highest levels in fatty tissues of old birds, which

may reflect a longer period of exposure in these individuals. Significant positive correlations were found between age and the levels of some compounds in feathers (r=0.36-0.78, p=0.000-0.009) for lindane, heptachlor epoxide, endosulfan II, DDE, DDD and δ-HCH. However, heptachlor, aldrin and DDT showed higher levels in young rather than old bird feathers, with significant negative correlations between age and levels of aldrin (r = -0.48, p = 0.000) and DDT (r = -0.43, p = 0.002). Precisely, heptachlor and DDT are compounds with a high bioconcentration factor (log Kb = 3.83 and 6.11, respectively), thus they have a higher affinity for fatty tissues, and are therefore excreted with more difficulty than other compounds.

On the other hand, several studies have concluded that females have lower OC concentrations than males in blood and internal tissues, probably due to the transference of these compounds from mother to egg (Bustnes *et al.*, 2008). In the present study, sex as an explanatory variable in the model could not explain OC compound concentrations, whether alone or in combination with age. A likely explanation for this is that the Razorbills analyzed were collected after the breeding season.

Chapter V. Razorbills (Alca torda) as bioindicators of mercury pollution in the southwestern Mediterranean.

Mercury levels in Razorbill and diet habits.

In general, mercury levels in liver and muscle (2.85 and 1.54 mg/kg dry weight, respectively) were higher than those found in alcids from other areas such as Barents Sea, Svalbard or Greenland, except for Hg concentrations in Razorbills from Portugal with higher levels, and for Hg concentrations in alcids from Aleutian archipelago of Alaska and Galicia with similar concentrations (Pérez-López *et al.*, 2006; Ribeiro *et al.*, 2009; Ricca *et al.*, 2008). No information on Hg levels in the brain of alcids has been previously published, however, Hg levels in brain (1.48 mg/kg dw) were similar to concentrations in goosander (*Mergus merganser*) wintering in Poland (1.3 mg/kg dw) (Kalisińska *et al.*, 2010). Regarding the Hg levels in feathers, concentrations found in the present study were also higher than those described in other alcids (2.66 and 1.30 mg/kg dw in vane and shaft respectively).

The higher mercury concentrations in Razorbills from this study compared with levels in piscivore Alcidae species from higher latitudes is probably due to differences in their dietary habits as a consequence of migratory processes during the winter.

Although data on the winter eating habits of Razorbills are scarce, it is well known that the prey preferred by this species have a body length between 50 and 140 mm and always below 250 mm (Bradstreet and Brown, 1985). Some fish species included in their diet during the breeding season, such as sardines (*Sardina pilchardus*), are common in the Mediterranean Sea. In this sense, certain studies have detected Hg levels between 0.077 and 0.830 mg/kg dw in a pooling fish of similar size (Arcos *et al.*, 2002). In contrast, Hg levels in prey from Svalbard, Barents Sea and Iceland were 0.010 mg/kg ww in muscle, 0.049 mg/kg dw in muscle, and 0.030-0.049 mg/kg ww in flesh, respectively (Jæger *et al.*, 2009; Joiris *et al.*, 1995; Matís, 2008). Bacci (1989) explained the elevated Hg levels in Mediterranean biota in terms of the higher Hg methylation in these waters due to the elevated water temperature (Mediterranean deep waters are approximately 10°C warmer than the Atlantic waters at the same depth). Therefore, wintering habitats may be an important factor in the contaminant body burden.

Some studies have found that levels in large predators such as seabirds (alcids included) are ten times higher than levels in medium-sized fish, the latter being components of the former's diet (Atwell *et al.*, 1998; Jæger *et al.*, 2009). Using this argument and the fact that Razorbills in the present study had mean mercury concentrations of 1.54 mg/kg dw in muscle, thus we could estimate Hg concentrations in their Mediterranean prey as being ten times lower (approximately 0.154 mg/kg dw). This is consistent with the observations of Arcos *et al.* (2002) who recorded mercury levels of 0.140-0.156 mg/kg dw (whole fish) in three different fish species from the Mediterranean Sea with lengths between 74-196 mm, thus being potential prey of Razorbills as mentioned above.

Body condition, sex and age.

Body condition was generally good in the Razorbills in the present study and no effect of nutritional status on tissue mercury concentrations was detectable. There was also no significant difference in Hg accumulation between males and females, probably because Razorbills were collected after the breeding season. Moreover, the amount of Hg eliminated into eggs is usually small compared to the amount transferred into feathers during the moult (Furness, 1993). There were significant differences between age groups in Hg kidney, muscle, brain and feather concentrations ($p < 0.022$) with residues being greater in older birds. This could occur if adult individuals eat larger, more contaminated prey and/or may simply reflect an accumulation of Hg in their

tissues over a longer period of time (Kojadinovic *et al.*, 2007). Although feathers act as an excretion route for about 90% of the mercury (Burger, 1993), a certain percentage of the body burden is not evacuated into the plumage (Lewis and Furness, 1991), and this can lead to an increase of mercury with age in the internal tissues of some species.

Exposure assessment.

The distribution pattern of mercury was liver>feather-vane>kidney>muscle>brain>feather-shaft. The chronic daily exposure to Hg implies a balance between compartment concentrations. This may explain the distribution pattern observed and the strong correlations in Hg levels between internal tissues ($r > 0.80$, $p < 0.001$).

The Hg kidney:liver ratio has been proposed as a means of distinguishing chronic MeHg from inorganic Hg exposure (Scheuhammer, 1987); kidney:liver ratios much greater than 1 reflecting exposure to inorganic Hg, whereas ratio closer to 1 (and <2) being characteristic of exposure to MeHg. In the present study, the mean kidney:liver ratio in the Razorbills was 0.78 and so probably reflected exposure to MeHg via dietary intake. This is consistent with the fact that almost all (close to 100%) of the total Hg detected in the muscle of several fish species is MeHg (Scheuhammer, 1987).

The total mercury concentrations in this study were within ranges cited as background levels in wild birds (1-10 mg/kg ww in liver; Fimreite, 1974), with a maximum concentration of 1.7 mg/kg ww in liver. These results seem to indicate that the exposure to mercury in this species is low.

Effect assessment.

The kidney levels of Hg in the present study (2.23 mg/kg dw) were 25 times lower than those reported as causing reproductive alterations and brain lesions in American black ducks (*Anas rubripes*) (16.0 mg/kg ww in kidney) (Finley and Stendell, 1978). In addition, the brain levels were 37 to 50 times lower than those described as causing behavioral changes in pigeons (12-16 mg/kg ww in brain) (Evans *et al.*, 1982). Therefore, it is likely that renal and brain Hg concentrations found in the present study were low and insufficient to cause adverse effects in these birds. Despite this, Hg concentrations in liver (2.85 mg/kg dw, 0.89 ppm ww) were close to the critical level associated with high embryo/duckling mortality and brain lesions described by (Zillioux *et al.*, 1993) (1-2 mg/kg ww in liver). Nonetheless, it should be noted that the total Hg concentration would probably not be the best indicator of toxic effects, and more

importance should be given to organic mercury as it seems to be considerably more toxic than inorganic Hg to animals at high trophic levels (Wolfe *et al.*, 1998).

The role of feathers in the mercury distribution pattern.

If we consider the whole feather instead of the separate Hg levels in vane and shaft, feather Hg concentrations were higher than levels for internal tissues in adult birds. In this sense, Burger (1993) considered that > 90% of a bird's total mercury body burden may be sequestered into feathers during the moult, where Hg binds with feather keratin in the form of methylmercury (Thompson and Furness, 1989a, 1989b).

The mean concentration in vanes was twice as high as for shafts, this difference being statistically significant ($p<0.001$). Several hypotheses could be used to explain these differences: i) the influence of external contamination, in spite of the exhaustive washing process carried out prior to the analytical determination; ii) influence of the chemical structure of the vanes and shafts and their Hg-binding capacity, as suggested by Dauwe *et al.* (2003); and/or iii) Hg accumulates in the barbs after passing through the shaft (Jaspers *et al.*, 2007b).

The higher correlation coefficients between internal tissues and shafts than those between internal tissues and vanes may corroborate the hypothesis that vanes may accumulate some external Hg (adsorbed to feather waxes) that is not removed by the washing method performed. Therefore, despite vanes having higher Hg levels than shafts, the latter may be a better indicator of internal tissue levels. Therefore, we can use feather shafts, as a model of a non-invasive sample for predicting accumulated Hg levels in target organs (brain and/or kidney). The prediction equations for Hg concentrations, obtained via GLMs, are described below for both dry and wet weight (Eq. 1 and Eq. 2). Sex and age were not significant when included as factors. Finally, all models were statistically significant ($p<0.001$).

Brain Hg (μg/Kg dry weight) = 1050+0.34*Shaft Hg (μg/Kg) (Eq. 1)

Kidney Hg (μg/Kg dry weight) = 1567+0.51*Shaft Hg (μg/Kg) (Eq. 2)

Assuming that concentrations in feathers reflect circulating concentrations in the body at the time of their formation, collection time may have an influence on the correlations between internal tissues and feathers. Razorbills usually arrive to the Spanish Mediterranean coastline in October, where they remain until April (Bédard, 1985). Since the Razorbills were found on the Mediterranean coastline in February

2007, the Hg concentrations found in internal tissues most likely correspond to the concentrations accumulated by the birds up until that date, and would reflect the most recent exposure during the previous overwintering. However, the levels in feathers reflect the blood concentrations during feather growth, after the breeding moult (July to September 2006) in breeding grounds prior to migration to Spain (Pyle, 2009). Therefore, feather Hg concentrations are related to those in internal tissues, but for the year prior to collection (from October 2005 to September 2006). Accordingly, Hg concentrations in feathers and internal tissues do not reflect the same period of Hg exposure.

Chapter VI. Factors influencing mercury concentrations in nestling Eagle Owls (Bubo bubo).

Mercury levels in blood and feathers of Nestling Eagle Owls.

Mean mercury concentrations in blood of nestling Eagle owls were 36.09 µg/l. There is no data available about mercury concentrations in blood of Eagle owls. However, levels found were much lower than those reported for fish-eating raptors (Jagoe *et al.*, 2002; Langner *et al.*, 2012). Mean Hg concentrations in back feathers of nestling Eagle owls in the present study (328.88 µg/Kg) were slightly higher than those reported in owls by other authors (Ortego *et al.*, 2006; Solonen and Lodenius, 1990; Zolfaghari *et al.*, 2007). These results suggest that Hg levels detected in Eagle owl chicks from Southeastern Spain can be considered low.

Moreover, Hg levels found in Eagle owls were below those described to cause toxic effects. Only three chicks had Hg concentrations in feathers between 3 and 5 mg/Kg, close to the critical level described by NAS (1978) (5 mg/kg in feathers) associated with reproductive impairment. Nevertheless, for the overall population it is unlikely that Hg pollution can negatively affect the breeding performance.

On the other hand, feathers represent an important route of Hg elimination in birds (Kenow *et al.*, 2007), thus it seems logical that feathers have significant higher mercury concentrations than blood ($p<0.001$). Feathers were still developing when they were sampled. Therefore, as expected, we could find a positive correlation between Hg concentrations in blood of chicks and levels found in their back feathers ($r = 0.339$, $p = 0.001$, and $n = 229$). Hence, a unique growing back feather of Eagle owl could be enough to estimate Hg concentrations in blood of this terrestrial bird species. We provide the equation estimated by simple linear regression calculated considering Hg

concentrations in blood and feathers of 229 nestling Eagle owls from southestern Spain: Log Hg in blood (μg/L, ww) = -0.255 + 0.617 * Log Hg in feathers (μg/Kg).

Mercury concentrations in Eagle Owls according to studied area. Are they affected by their diet habits or by the contamination sources?

When studying spatial variations, we found significant differences in Eagle owl blood Hg concentrations ($p < 0.001$, n=623) between Northern and Southern areas, with Hg levels of 33.77 ± 148.90 μg/L in the North and 41.46 ± 128.26 μg/L in the South. León *et al.* (2008) observed that in Northern areas the rabbit is very abundant and therefore, it is the main prey of Eagle owls (71% of the diet). By contrast, in the Southern area the rabbit comprises only 35% of the diet, with rats (23%), pigeons (14%), partridges (5.26%), hedgehogs (5.26%) and yellow-legged gulls (3.16%) as other preys (León *et al.*, 2008). In the present study, we found a significant positive correlation between Hg levels in blood of Eagle owl and muscle of rabbits in the Northern area (r= 0.489, $p < 0.001$) where the availability of rabbit is higher (León *et al.*, 2008), but no significant correlation was found in the south, which firm up the diet composition described by León *et al.* (2008).

Omnivorous species such as rats, hedgehogs and gulls could be classified as secondary consumers (Lourenço *et al.*, 2011) and are expected to have higher Hg levels than primary consumers such as rabbits, partridges and pigeons. Following this reasoning, it seems logical that Eagle owls in the Southern area, with a higher proportion of secondary consumers (31.58%) and even mesopredators in their diet, are expected to have higher Hg concentrations in their tissues.

However, local contamination sources may also contribute to the highest concentrations found in Eagle owl from the south. In the Southern area there is a subarea corresponding to an ancient mine site. If Eagle owls from this mine area (n=80) are excluded, no significant differences are found between areas for Hg concentrations in blood of Eagle owls, with Hg levels of 33.77 ± 148.90 μg/L in the North (n=435) and 29.93 ± 61.44 μg/L in the South (n=108). However, mean mercury levels in blood of chicks from the ancient mine site (57.03 ± 182.76 μg/L, n=80) were significantly higher ($p < 0.001$) than the mean concentration found in chicks from the rest of the sampled population (33.01 ± 136.02 μg/L, n=543). Similarly, no location-related differences were found for Hg concentrations in muscle of European rabbit, but when rabbits from the ancient mine site are excluded, mean Hg levels in muscle of rabbit were significantly lower (12.46 ± 12.38 μg/Kg ww, $p = 0.020$) than those found in

rabbits from the mine area (24.98 ± 12.76 µg/Kg ww). Therefore, it seems that the diet composition is not the explanation of the higher Hg concentrations in Eagle owls from the Southern area in comparison with the Northern area.

The southeast of the Iberian Peninsula has traditionally suffered a great extraction of their mineral resources, and high amounts of wastes have remained within mining areas. These materials are strongly enriched in heavy metals (Faz Cano *et al.*, 2001). Moreover, many factories manufacturing chemical products were constructed 50 years ago near the city of Cartagena, in the Southern area. As a consequence, soils of this area have also been affected by industrial wastes (Faz Cano *et al.*, 2001). In this sense, a study conducted in the mid-1990s shows that the highest concentrations of metals were found in blood of wild birds (Eagle owl included) from Cartagena in comparison with three different locations in Murcia Region (García-Fernández *et al.*, 1995). Moreover, Eagle owl chicks from the mining area “Sierra Minera Cartagena-La Unión” or their surroundings had higher lead concentrations than the rest of the population in the period from 2003 to 2007 (Gómez-Ramírez *et al.*, 2011).

Furthermore, precipitation along the Southern area (annual precipitation 318 mm) is slightly higher than in the Northern area (annual precipitation 292 mm) (SIGA, 2012), which may contribute to a higher Hg removal from the atmosphere and local wet deposition. Therefore, although the studied area is not considered Hg polluted, mining influence and the industrial complex seems to be the main cause of the higher mercury levels in Eagle owls and their main prey, the European rabbit, from this particular subzone. These results support the fact that spatial differences in Hg concentrations in Eagle owls appear to be mostly related to local contamination, and probably diet composition plays a role of less extent.

Mercury concentrations in Eagle Owls according to year.

Significant differences in blood mercury concentrations between years were found in Eagle owls ($p < 0.001$, $n = 623$). However, Hg concentrations found in muscle of European rabbit were very similar between years, with no significant differences between them. When faced with rabbit scarcity this generalist predator will diversify its diet to include other preys and predators (Lourenço *et al.*, 2011). This fact could affect to the differences found between years in Hg blood concentrations. However, meteorological conditions may play a major role in these results.

Rainfalls in Murcia Region were variable during the seven years of the study (CREM, 2013), and they may contribute to a higher Hg removal from the atmosphere and local wet deposition. Moreover, stormwater runoff also washes off surfaces that may contain mercury and contributes to its transport. The two years with higher mercury levels in blood of Eagle owls were 2007 and 2009, precisely two of the years with higher rainfalls (397 and 402 L/m2, respectively) (CREM, 2013). The lack of differences in rabbit Hg levels between years seems to conflict with this explanation. However, there is small number of samples and only samples of 3 years were available to analyze, making it difficult to observe a trend.

Positive correlations between Hg concentrations in blood of nestling Eagle owls and in muscle of their prey were found in samples collected in 2009 ($r= 0.380$, $p = 0.029$), 2011 ($r= 0.614$, $p = 0.001$) and 2012 ($r= 0.556$, $p = 0.020$). Moreover, Hg levels in blood of Eagle owls and in muscle of rabbit were very similar in samples collected in 2011 and 2012. These results suggest that blood mercury is greatly influenced by Hg ingested through the consumption of rabbit.

When Generalized Linear Models were performed, we found that both year and area combined also have a significant effect on mercury concentrations in blood of nestling Eagle owls ($p<0.001$, $n=623$). In this sense, local contamination seems to be the main cause of differences between areas, and rainfalls could have a major role in the differences found in blood Hg concentrations of Eagle owls between years.

PART 2. Induction of oxidative stress in metal exposed wild birds.

Chapter VII. Effects of heavy metals exposure on oxidative stress biomarkers in Eurasian Eagle owl (Bubo bubo).

Metals concentrations in blood.

Mean lead levels in blood of Eagle owl (mean= 3.3 ± 5.2 µg/dl) were lower than those found by García-Fernández *et al.* (1995) (mean=8.3 ± 6.7 µg/dl) and García-Fernández *et al.* (1997) (mean=7.6 µg/dl, median=6.5 ± 2) in Eagle owls from Murcia Region (Southeastern Spain), and similar to those found by Gómez-Ramírez *et al.* (2011) (3.7 ± 4.2 µg/dl) in nestling Eagle owls from the same studied region. Studies in Southeast Spain (Murcia) show lead exposure has decreased in the last 10 years (García-Fernández *et al.*, 2008b). With the information reviewed by García-Fernández *et al.* (2008), lead concentrations found by Gómez-Ramírez *et al.* (2011) and levels found in the present study, we can conclude that there is a clear decrease in lead

exposure in the last 20 years in Eagle owls from Murcia Region. Some actions such as the closure of mines in 1991 and the ban on leaded petrol in 2001 most probably have an influence in the decrease in accumulated lead tissue concentrations in Eagle owls.

In general, concentrations were similar to those found in raptor species from non-polluted areas (Henny *et al.*, 1994; Martínez-López *et al.*, 2004). Only four individuals presented Pb concentrations higher than 20 µg/dl, which is the minimum blood level for considering physiological effects in Falconiformes according to Franson (1996). However, more recent studies have found that Pb concentrations below 15 µg/dl in blood are enough to cause sublethal effects, such as inhibition of aminolevulinic acid dehydratase (ALAD) activity in raptors in the field (Gómez-Ramírez *et al.*, 2011; Martínez-López *et al.*, 2004).

Significant differences were found in Pb concentrations between areas, with the highest levels in Eagle owls from the Southern area (1.2 ± 1.1 and 5.4 ± 6.7 µg/dl in Northern and Southern area respectively). Local contamination sources have probably contributed to the highest concentrations we found in Eagle owl from the south. In the Southern area an important industrial zone were constructed 50 years ago near the city of Cartagena (García-Fernández *et al.*, 1995). Moreover, in this subarea there are some nests located close to an ancient mine site called "Sierra Minera Cartagena-La Unión". In fact, when only individuals from the ancient mine site were selected, we obtained mean Pb concentrations of 7.6 µg/dl (n=29), significantly higher ($p<0.001$) than the levels found in the rest of the population (mean Pb levels=2.1 µg/dl, n=112) and close to those described 15 years ago by García-Fernández *et al.* (1997). The low concentrations found in the Northern area were expected, since nests are located far from potential sources for heavy metals.

Regarding Hg concentrations, there is few data available about Hg levels in blood of Eagle owls (Chapter VI). However, concentrations found (2.3 µg/dl) were much lower than those reported for fish-eating raptors (Jagoe *et al.*, 2002; Langner *et al.*, 2012). GLMs showed that the best explanatory variables were area and age. As explained in Chapter VI, although the region is not considered Hg polluted, area under mining influence and the industrial zone seems to be the main cause of the higher mercury levels in Eagle owls from the area under mining influence than levels in owls from the rest of the study region. Besides, higher Hg levels in adult individuals may occur if they eat larger, more contaminated prey and/or may simply reflect an accumulation of mercury in their tissues over a longer period of time (Kojadinovic *et al.*, 2007).

Cadmium was detected above the detection limit only in the 26% of the samples. Cd concentrations (0.07 µg/dl) were similar to those found by García-Fernández *et al.* (1995) and Gómez-Ramírez (2011) (0.1 µg/dl), and were within the range considered as low exposure levels in birds (0.01 to 0.28 µg/dl) (García-Fernández *et al.*, 1996). Non significant differences in Cd concentrations were found according to area and age. Finally, concentrations of the essential metals Zn and Cu (Zn=311 and Cu=10.6 µg/dl) were within the range of physiologic levels in several health bird species (163-495 µg/dl for Zn and 13-120 µg/dl for Cu) including different raptor species (García-Fernández *et al.*, 2005b).

Oxidative stress in Eagle owl.

- *Oxidative stress biomarkers in Eagle owl.*

Studies published so far shown that metals can induce oxidative stress, but the response is very variable depending on the concentration of exposed metals, duration of exposure and species studied (Hoffman *et al.*, 2000a; Ji *et al.*, 2006; Mateo and Hoffman, 2001). In the present study significant location-related differences were found only for GST activity, with lower activity in the Southern area relative to Northern area (GST= 11.2 and 9.3 nmol/min/mg protein in Northern and Southern area respectively). When GLMs were performed, area was a variable with significant effect on the model of GST. GST is used to catalyse the conjugation of GSH with cytotoxic aldehydes produced during lipid peroxidation (Halliwell and Gutteridge, 1999) and GSH conjugation with pollutants, and some GST isozymes posess non-Se-dependent GPx activity (Prohaska and Ganther, 1977). An experimental study with Mallards (*Anas platyrhynchos*) has shown a reduction in GST activity in lead-treated individuals (Mateo *et al.*, 2003). In this sense, it is possible that the higher concentrations of Pb and Hg in owls from the Southern area in comparison to the Northern area could induce a GST depletion in this population. No differences were found in TBARS concentrations in red blood cells of Eagle owl between areas (0.05 µmol/g). The lack of differences in oxidative damage to membrane lipids (TBARS) suggests that the antioxidant capacity of both populations is able to deal with oxidant species and mantain TBARS levels in the same amount.

- *Effect of metal concentrations in oxidative stress biomarkers.*

Despite the low levels of metals, several oxidative stress biomarkers correlated with contaminant concentrations.

When only Eagle owls from the Northern area were selected, it was found positive relationships between CAT activity and Hg concentrations (r=0.343, F=9.23, p=0.003), SOD activity and Hg concentrations (r=0.313, F=7.485, p=0.008), and GST activity and Cd levels (r=0.374, F=11.254, p=0.001). These results suggest a possible protective response by an increase of antioxidant enzymes activities in individuals from the Northern area under exposure of Cd and Hg. In this sense, Hussain *et al.* (1999) reported increased CAT activity in mice exposed to mercury, what was explained as a possible compensatory mechanism to scavenge ROS levels produced as a result of Hg accumulation. Moreover, mercury stimulates the activity of copper-zincSOD (Gurer and Ercal, 2000), probably as a protective effect. Besides, rats treated with cadmium had an increase of 17% in liver GST activity (Jurczuk *et al.*, 2006). As suggested by Jurczuk *et al.* (2006), GST may be induced by this metal, since GST catalyzes the conjugation of Cd with GSH.

Glutathione peroxidase (GPx)

GPx activity was inversely correlated with cadmium concentrations (r=-0.22, p=0.008), and it was also found a marginally inverse correlation between GPx activity and lead concentrations (r=-0.15, p=0.07). When GLMs were performed, the best model for GPx activity was constructed only with Cd (X2=7.17, p=0.007). The GPx activity and cadmium concentration inverse relationship was still true and even stronger when only the individuals from the Southern area were selected (r=-0.508, F=23.29, p=<0.001). Cadmium concentrations ≥ 0.3 µg/dl in blood produced an inhibition of 32% in GPx activity in red blood cells of Eagle owls. GPx activity also showed a 47% decrease in liver of starlings (*Sturnus vulgaris*) treated with Cd, due to inhibition of selenium-dependent fraction of the enzyme (Congiu *et al.*, 2000). GPx enzyme reduces peroxides in cells, such as the transformation of H_2O_2 to H_2O by oxidizing GSH (Koivula and Eeva, 2010), and requires selenium as a cofactor (ExPASy, 2012), thus the formation of Cd-Se complex as a protective effect of Se against Cd toxicity could be the reason of the negative correlation found between GPx activity and Cd levels in the present study. Antagonistic effect between lead and selenium have also been described (Schrauzer, 1987), resulting in reduced selenium uptake that may affect GPx activity. Several authors have found an inhibition of GPx activity in Pb exposed birds (Chapter VIII; Mateo *et al.*, 2003; Somashekaraiah *et al.*, 1992).

Catalase (CAT)

CAT activity was inversely related with concentrations of the single metals Cd (r=-0.36, p=<0.001), Pb (r=-0.24, p=0.004) and Cu (r=-0.24, p=0.004). The best explanatory variables when GLMs were constructed were Cd and Pb (X^2=23.12, p<0.001). CAT enzyme catalyzed H_2O_2 to H_2O and molecular oxygen (Koivula and Eeva, 2010). CAT activity has been inhibited following both in vivo and in vitro exposure to Cd in rats and fish species (Koizumi and Li, 1992; Palace *et al.*, 1993; Pruell and Engelhardt, 1980; Roméo *et al.*, 2000). Palace *et al.* (1993) suggested a direct structural alteration of the enzyme and depression of CAT synthesis by cadmium. In the present study, cadmium levels ≥ 0.3 µg/dl in blood produced an inhibition of 25% in CAT activity in red blood cells of Eagle owls. Moreover, CAT enzyme has heme as the prosthetic group (ExPASy, 2012), and it is known that lead reduces the absorption of iron in the gastrointestinal tract and inhibits the heme biosynthesis (Gurer and Ercal, 2000). Several authors have found inhibition of CAT activity in lead-exposed animals (Sandhir and Gill, 1995; Sandhir *et al.*, 1994).

Total glutathione (tGSH)

Regarding tGSH levels, no relationship was found with any single metal and no significant models were constructed with the studied variables. However, linear regression analysis was performed selecting only the individuals living in the ancient mine site (n= 29) in order to elucidate if the significant higher levels of lead in this subarea (7.64 µg/dl) in comparison with the rest of the population could have an effect on tGSH concentrations. In this sense, it was found a significant negative relationship between blood Pb concentrations and tGSH levels in red blood cells of Eagle owls from the mining area (r=-0.392, p=0.039). A depletion of 16% in tGSH levels were associated with lead concentrations ≥ 15 µg/dl in Eagle owls from the ancient mine site. Several studies have found a reduction in GSH concentrations in lead-exposed birds (Mateo *et al.*, 2003; Somashekaraiah *et al.*, 1992), which may be explained by GSH role in the excretion of this metal through lead binding to GSH because of its affinity for sulfhydryl groups (Sharma *et al.*, 2011).

Thiobarbituric acid-reactive substances (TBARS)

TBARS levels were positively related with Hg concentrations (r=0.23, p=0.007). The explanatory variables in the best model were age and Hg concentrations (X2=11.44, p=0.003). When only Eagle owls from the Southern area were selected, the

relationship between TBARS and Hg levels was stronger (r=0.434, F=15.096, p=<0.001), and a new relationship between TBARS levels and Pb concentrations was found (r=0.259, F=4.687, p=0.034). An increase in lipid peroxidation after lead (Hoffman *et al.*, 2000a, 2000b; Mateo and Hoffman, 2001; Mateo *et al.*, 2003; Somashekaraiah *et al.*, 1992) and mercury exposure (Hoffman *et al.*, 2005; Huang *et al.*, 1996) has been found in birds and rats in several studies. In Chapter VIII it was also found a correlation between Pb and Hg concentrations and TBARS levels in Griffon vultures. The correlations found are indicative of an effect of mercury and lead on lipid peroxidation, particularly in the Southern area. Lead concentrations ≥ 10 μg/dl produced a TBARS induction of 28%, and mercury concentrations ≥10 μg/dl resulted in a TBARS induction of 107% in Eagle owls from the Southern area. In this sense, several mechanisms may be responsible for lipid peroxidation by these metals. Both Hg and Pb may induce generation of ROS, associated with lipid peroxidation in membranes (Lund *et al.*, 1991; Monteiro *et al.*, 1989; Ribarov and Bochev, 1982; Verity *et al.*, 1975). Moreover, as explained above, both metals can alter levels of GSH (Flora *et al.*, 2008) and the activity of antioxidant enzymes (Gstraunthaler *et al.*, 1983; Sandhir and Gill, 1995; Schrauzer, 1987; Zalups and Lash, 1996), which interfere in the protection against lipid peroxidation.

- *Relationship between oxidative stress biomarkers and size of the brood.*

Koivula *et al.* (2011) found a positive relationship between GST activity and the size of the brood. The higher GST activity in large broods in Great tit (*Parus major*) was explained by an increase within the brood competition for food and space may cause increased oxidative stress among nestlings in larger broods (Koivula *et al.*. 2011). However, in the present study we found negative relationships between the number of nestlings in each nest and GPx activity (r=-0.316, p<0.001, n=133) or CAT activity (r=-0.201, p=0.021, n=133), showing lower enzyme activities in larger broods. Several studies have found increased oxidative stress in nestlings from larger broods (Costantini *et al.*, 2006, 2010), and nestling Common starlings raised in experimentally enlarged broods had lower total antioxidant capacity (Bourgeon *et al.*, 2011).

Chapter VIII. Effects of heavy metals exposure on oxidative stress biomarkers in Griffon Vulture (Gyps fulvus).

Metals concentrations in blood.

Mean blood Pb concentrations in Griffon vultures from Alcoy (15.32 µg/dl) were similar to (Donázar *et al.*, 2002; Shlosberg *et al.*, 2012) or higher than those found in vultures by other authors (Gangoso *et al.*, 2009; Hernández and Margalida, 2009). Regarding mean blood Pb concentrations in vultures from Cinctorres (41.44 µg/dl), they were significant higher than those found in Alcoy in the present study and similar to those found in Griffon vultures from Southern Spain outside hunting season and from Murcia, Southeastern Spain (García-Fernández *et al.*, 1995, 2005a). However, lead concentrations in both populations were lower than those found in Griffon vultures from Cazorla Natural Park during hunting season (García-Fernández *et al.*, 2008b).

Since sampling in the present study was done in September-November at the beginning of the hunting season in Valencian Community (Order 1, 2011), and concentrations detected were much lower than those found in Griffon vultures from Cazorla Natural Park during hunting season (García-Fernández *et al.*, 2008b), it is possible that lead levels in Griffon vultures from Alcoy and Cinctorres (median=12.37 and 16.26 µg/dl, respectively) are the normal or background levels in vultures feeding on porcine origin carcasses during the whole year.

However, three individuals from Cinctorres had high lead concentrations (83, 290 and 362 µg/dl). The blood levels of lead found in these vultures probably indicate recent exposure to large amounts of this metal (García-Fernández *et al.*, 1995). Some studies suggested that the ingestion of lead in metallic form is able to provoke these blood Pb concentrations (García-Fernández *et al.*, 2008b; Hoffman *et al.*, 1981). Several authors have demonstrated that lead ammunition can produce hundreds of small fragments contaminating animal carcasses and discarded viscera that serve as food sources for scavengers (Hunt *et al.*, 2006; Knopper *et al.*, 2006). In Spain, the use of lead shot has been banned only in wetlands included on Ramsar's list because of the risk to waterfowl (Royal Decree 581, 2001). However, lead ammunition is still used in big- and small-game hunting. In Valencian Community big-game hunting is allowed and regulated for Iberian wild goat (*Capra pyrenaica*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), wild boar (*Sus scrofa*), mouflon (*Ovis aries*) and barbary sheep (*Ammotragus lervia*) (Order 1, 2011). There are also many species allowed for small-game hunting such as partridge (*Alectoris rufa*),

European rabbit (*Oryctolagus cuniculus*), red fox (*Vulpes vulpes*) and several pigeon species (*Columba sp.*) (Order 1, 2011). Although the diet of Griffon vultures from this study is mainly based on porcine origin carcasses provided in feeding stations, they can feed from wild species dead in the field (Donázar, 1993). Therefore, the ingestion of game meat with bullet fragments in carcasses or lead shot embedded in their flesh is probable. This may be the major cause of the blood lead concentrations found in the three individuals from Cinctorres with high blood lead concentrations.

Twenty-seven vultures from Alcoy and 18 vultures from Cinctorres had lead levels below 20 µg/dl, mentioned by Franson (1996) as the minimum blood lead level necessary in Falconiformes for considering physiological effects. Besides, 9 individuals from Alcoy and 8 from Cinctorres had Pb concentrations between 20 and 50 µg/dl, considered as threshold value for physiological effects (Franson, 1996). Finally, 4 individuals from Cinctorres had Pb levels higher than 50 µg/dl, with 2 vultures (290.48 and 362.13 µg/dl) with concentrations higher than those considered by Franson (1996) as threshold value in individuals with probable clinical symptoms (100 µg/dl). Although Franson (1996) established 20 µg/dl as the blood Pb level for considering physiological effects, more recent studies have found that lead concentrations below 15 µg/dl in blood can cause sublethal effects such as inhibition of aminolevulinic acid dehydratase (ALAD) activity in raptors and waterbirds in the field (Gómez-Ramírez *et al.*, 2011; Martínez-Haro *et al.*, 2011; Martínez-López *et al.*, 2004). Therefore, according to the concentrations found in the present study, some individuals could be susceptible to suffer physiological effects due to Pb concentration.

Regarding Hg, due to the methylation and bioaccumulation of methylmercury in the aquatic systems, few papers have studied the concentrations of this metal in blood of terrestrial birds of prey (Chapter VI; Shlosberg *et al.*, 2012). Hg concentrations in blood of Griffon vultures in the present study were similiar to those found in nestlings Eagle owls (*Bubo bubo*) from southeast of Spain (Chapter VI), and seem to be very low to cause any adverse effects on vultures (2.27 µg/dl in Alcoy and 1.72 µg/dl in Cinctorres). In fact, levels of mercury in blood considered as no observed adverse effect level in adult Common loon (*Gavia immer*) are two orders of magnitude larger (1 µg/ml) (Evers *et al.*, 2004). As regards to Cd concentrations in blood of Griffon vultures in the present study (0.018 µg/dl in Alcoy and 0.025 µg/dl in Cinctorres), levels were lower than those found in vultures by other authors (Donázar *et al.*, 2002; García-Fernández *et al.*, 1995). The levels producing sub-lethal effects are unknown for Cd, but concentrations were below those considered as normal in non-polluted zones (0.1

µg/dl, (García-Fernández *et al.*, 1995). Finally, Zn and Cu concentrations in blood of Griffon vultures in the present study (Zn=332 and 347 µg/dl, Cu=20.4 and 26.8 µg/dl, in Alcoy and Cinctorres respectively) are in the range of Cu and Zn levels in healthy birds (García-Fernández *et al.*, 2005b) and seem to be low to cause any adverse effects on vultures.

Oxidative stress biomarkers.

- *Oxidative stress biomarkers in Griffon vultures from two areas of Spain.*

Griffon vultures from Cinctorres displayed significantly higher CAT and GST activities, and higher concentrations of total GSH and TBARS in red blood cells compared to vultures from Alcoy (CAT=0.55 and 1.18 µmol/min/mg protein, GST=7.26 and 9.79 nmol/min/mg protein, total GSH=4.56 and 5.42 µmol/g, TBARS=0.034 and 0.050 µmol/g, in Alcoy and Cinctorres respectively). However, no significant differences in red blood cells GPx and SOD activities were found between vulture populations (GPx=415.6 and 483.1 U/g protein, SOD=929.9 and 827.7 U/g protein, in Alcoy and Cinctorres respectively). The general trend observed in the present study is an increase in enzymatic and non-enzymatic antioxidant mechanisms in Griffon vultures from Cinctorres. This result may be interpreted as a protective response against the higher TBARS levels, since these mechanisms may contribute together to the scavenging of ROS and alleviating oxidative damage. Besides, as explained above, lead concentrations were significantly higher in blood of Griffon vultures from Cinctorres than in vultures from Alcoy. This may be related with the highest TBARS levels found in vultures from Cinctorres, since it is known that Pb may induce generation of ROS, which is associated with lipid peroxidation in erythrocytic membranes (Gurer and Ercal, 2000).

- *Effect of metal concentrations in oxidative stress biomarkers.*

Almost all the relationships found between metal concentrations and oxidative stress biomarker responses were found in vultures from Alcoy, or when all samples were pooled together. However, in Griffon vultures from Cinctorres only two significant correlation was found (CAT-Zn r=0.38, p=0.04 and LogGPx-LogPb r=-0.40, p=0.003).

Glutathione peroxidase (GPx)

In vultures from Alcoy, GPx activity was directly correlated with blood Cd (r=0.37, p=0.026) and Cu concentrations (r=0.37, p=0.026). GPx enzyme uses H_2O_2 as

substrate, and Cd exposure has been shown to increase H2O2 levels in rat pituitary membrane (Pillai *et al.*, 2002). Concentrations of Cd ≥ 0.05 µg/dl in blood were able to produce an induction of 33.3% in GPx activity. GPx was inversely related with blood Pb concentrations (r=-0.40, p=0.003) in vultures from Cinctorres. When both populations were pooled together, GPx was inversely correlated with Pb concentrations (r=-0.31, p=0.013), and the best-fitting generalized linear model for GPx activity only included an effect of Pb (X^2=6.37, p=0.012). GPx enzyme requires selenium as a cofactor (ExPASy, 2012), and Schrauzer (1987) indicated antagonistic effects between lead and selenium, resulting in reduced selenium uptake that may affect GPx activity. Experimental studies with lead-treated birds have found an inhibition of this enzyme (Mateo *et al.*, 2003; Somashekaraiah *et al.*, 1992). In the present study, concentrations of lead ≥ 15 µg/dl in blood were able to produce an inhibition of 12.5% in GPx activity, and lead levels ≥ 25 µg/dl produced an inhibition of 16.7% in GPx activity in Griffon vultures.

Superoxide dismutase (SOD)

Both Hg (r=0.41, p=0.012) and Cu (r=0.40, p=0.016) levels as single metals show a significant positive relationship with SOD activity in Griffon vultures from Alcoy. When GLMs were performed both Hg and Cu became included in the top-ranked model (X^2=12.7, p=0.002), and SOD activity seemed to depend mostly on Hg concentrations when all individuals were pooled together (X^2=7.16, p=0.007). Results clearly show that Hg has an effect on this enzyme activity despite the low levels found in this study, and mercury concentrations in blood ≥ 3 µg/dl can produce an induction of 10% in SOD activity. In this sense, changes in SOD enzyme are dependent on exposure time and level of Hg (Ji *et al.*, 2006), thus low-dose Hg exposure would result in increased levels of SOD as a protective response of the redox-defense system (Elia *et al.*, 2003). Moreover, the effect of Cu in SOD activity is logical since copper is a cofactor of this enzyme (ExPASy, 2012).

Catalase (CAT)

CAT activity was positively correlated with Cd (r=0.34, p=0.045) and Cu (r=0.40, p=0.015) concentrations in blood of vultures. In this sense, Cd concentrations ≥ 0.05 µg/dl in blood were able to induce CAT activity in 44%. As explained above, Cd exposure has been shown to increase H_2O_2 levels in rat pituitary membrane (Pillai *et al.*, 2002), and CAT catalyzes H_2O_2 to H_2O and oxygen (Koivula and Eeva, 2010). Moreover, it was found an almost significant negative relationship between Pb and

CAT activity in vultures from Alcoy (r=-0.32, p=0.055). When all individuals were pooled together, the top-ranked model for CAT activity included an effect of area and Pb concentrations (X^2=55.96, p=<0.001). Catalase enzyme has heme as the prosthetic group (ExPASy, 2012), and lead is known to reduce the absorption of iron in the gastrointestinal tract and to inhibit the heme biosynthesis (Gurer and Ercal, 2000). Several authors have found catalase activity inhibition in lead-exposed animals (Sandhir and Gill, 1995; Sandhir *et al.*, 1994). Concentrations of lead ≥ 15 µg/dl in blood were able to produce an inhibition of 11.3% in CAT activity, while lead levels ≥ 20 µg/dl resulted in a CAT inhibition of 15.8%.

Thiobarbituric acid-reactive substances (TBARS)

TBARS concentrations were significantly positively correlated with Pb (r=0.35, p=0.004) and Zn (r=0.31, p=0.012) levels when all individuals were pooled together. The best-fitting model for TBARS concentrations was constructed with Pb, Zn and Hg concentrations as covariates in vultures from Alcoy (X^2=23.22, p=<0.001), and the same variables including area when all individuals where pooled together. Lead concentrations ≥ 15 µg/dl produced a TBARS induction of 10.7%, while lead levels ≥ 30 µg/dl produced an induction of 13.4% in red blood cells of Griffon vultures. In Eagle owl, lead concentrations ≥ 10 µg/dl produced a TBARS induction of 28% (Chapter VII), suggesting that Griffon vulture is more resistant to sublethal effects of lead than other species. In this sense, García-Fernández *et al.* (2008) found high lead concentrations in Griffon vultures (750-1100 µg/dl) with no observable effects, suggesting that this species may be more tolerant to lead exposure.

Lead-induced lipid peroxidation has been associated with several mechanisms (Mateo and Hoffman, 2001). In this sense, lead can produce ROS that attack membranes by its interaction with haemoglobin and by its capacity of ALAD inactivation and the consequent accumulation of the pro-oxidant aminolevulinic acid in erythrocytes. Moreover, lead inhibits GSH because the binding of this molecule with lead or aldehydic products of lipid peroxidation, and it can also inhibit antioxidant enzymes involved in the protection of the cell such as GPx, SOD or CAT. These effects on GSH and antioxidant enzymes reduce the protection of membranes to ROS attack and lipid peroxidation. Lead may also alter membrane integrity and fatty acid composition increasing susceptibility of membranes to oxidative attack (Gurer and Ercal, 2000). As discussed above, in the present study lead concentrations have inverse relationships with GPx and CAT activity, two important scavengers of H_2O_2.

These correlations together with the positive effect of Pb concentrations on TBARS levels suggest that the inhibition of antioxidant enzymes can play a role in the increase of lipid peroxidation.

Regarding the positive correlation between Zn and TBARS, may be related to a protective effect by an increased amount of this essential metal. Several authors have proposed that one function of zinc is the maintenance of membrane structure and function (Bettger and O'Dell, 1981). In this sense, dietary Zn deficiency increased the susceptibility to lipid peroxidation in rats (Sullivan *et al.*, 1980).

CONCLUSIONS

PART 1. Biomonitoring of persistent environmental pollutants in wild birds: feathers as a biomonitoring tool.

Chapter I. Feathers as a biomonitoring tool of polyhalogenated compounds: a review.

Since Chapter I is a review, we have prepared some recommendations, key uncertainties and conclusions.

- Further research should focus on the structure of the feather, its binding affinities for PHCs, and the stability of these compounds in feathers.

- The deposition rate (pollutant load that enters feathers daily) is proposed as a unit of measurement, which allows any part of a feather to be validly compared to a different part of the same or other feathers. In this regard, further studies are required in order to provide the growth rate of feathers in different wild bird species.

- Further studies with newly grown feathers and blood samples are required in order to clarify the relationship between feather and internal tissue contaminant concentrations. It is also necessary to eliminate the bias of external contamination by preen oil not removed by washing techniques and avoid the bias of the time elapsed between the last molt period and the time of sampling.

- Several studies have found that external contamination does occur in feathers, and preen oil is probably the main source of external contamination. Further experimental studies are needed to determine methods for discriminating between the internal and external contamination of feathers by organic pollutants.

- The use of external contamination markers such as certain metals with a low absorption rate and therefore with low endogenous deposition in feathers may be useful for discriminating between internal and external contamination. Further research must be carried out on this matter in order to look for organic external contamination markers.

- Barb material is structurally too complex to be washed effectively using the techniques proven so far. Rachis may be a better indicator of internal organic pollutant deposition in feathers than barbs. However, in developing or very recently molted feathers, surface external contamination would be expected to be minimal and thus barbs may remain valuable.

- All feather types could possibly be used, but the ideal feather depends on the molting pattern of the species and the end point of the monitoring study. Further studies are needed on the suitability of each type of feather.

- We recommend monitoring male feathers during the breeding season in order to eliminate the potential bias of off-loading contaminants into eggs. However, depending on the end point this recommendation may not be appropriate, i.e. studies on the influence of egg laying or studies on differences in exposure according to sex.

- The importance of fat mobilization for migration, egg laying or other factors in the relative levels of feather and internal organ tissue contamination partly depends on the time when samples are collected. There is a need to collect further quantitative data as to what degree this time may influence correlations between feather and other tissue contaminant levels.

Chapter II. Development of an analytical method for extracting organochlorine pesticides from feathers.

It was selected the technique with a greater recovery for the organochlorine pestices. Mean recoveries in spiked samples ranged from 46.13% to 146.05%.

Organochlorine levels were significantly higher in unwashed samples than in washed ones for some compounds, in both vane and shaft, suggesting a possible interference by external contamination from atmospheric deposition or oil secreted by the preen gland. Moreover, higher levels of certain compounds in unwashed barbs than in unwashed shafts can be explained by the higher probability of organic pollutant deposition in barbs than in shafts.

Chapter III. Assessment of organochlorine pesticide exposure in a wintering population of Razorbills (Alca torda) from the southwestern Mediterranean.

This is the first study of organochlorine pesticides in *Alca torda* species. The OC levels in tissues were higher than those of other studies on Alcides, which is probably due to the habitat in which they were found. In spite of this, these concentrations were below the limits known to cause adverse effects in birds.

Respect to the OC concentrations, no differences were found between sexes, probably due to it being outside the Razorbills' breeding season. However, age does affect the concentration of these compounds in fatty tissues.

The p,p'DDE/p,p'DDT ratio in fatty tissues was lower than 1, which indicates exposure to non-degrading DDT, despite the ban on its use. In liver, the ratio greater than 1 indicates a greater concentration of DDE, which is explained by the liver's capacity of transforming DDT to DDE.

According to the results, the species tested could be a good biomonitor of organochlorine concentrations.

Chapter IV. Razorbill (Alca torda) feathers as an alternative tool for evaluating exposure to organochlorine pesticides.

This is the first study of organochlorine pesticides in Razorbill feathers. The OC concentrations in Razorbill feathers were higher than those observed in the feathers of other bird species from Belgium or Iran, which is probably influenced by the Razorbill diet and migration status. In spite of this, levels found in the feathers of the present study are related to concentrations in internal tissues below those that provoke adverse reproductive and behavioral effects, and other signs of organochlorine pesticide poisoning in birds.

Age affects the concentration of OC pesticides in feathers. However, no differences were found between OC concentrations by gender. Feathers appear to be a promising tool for OC biomonitoring in seabirds since it is possible to quantify OC compounds.

Chapter V. Razorbills (Alca torda) as bioindicators of mercury pollution in the southwestern Mediterranean.

According to the results, the Razorbill could be a good sentinel species for Hg pollution in the Mediterranean area. The higher levels in their tissues compared to concentrations in other Alcidae species from higher latitudes are probably due to their dietary habits over the winter in that area, although the concentrations found do not seem to be associated with risks for the Razorbill's health. It is feasible to assume that Razorbills in the southwestern Mediterranean were chronically exposed to relatively low levels of MeHg via dietary intake, probably below 0.5 ppm. Results indicate that feathers are an excellent nondestructive tool for monitoring mercury levels in Razorbills, and feather shafts may be a better indicator of internal tissue levels than feather vanes. We have proposed prediction equations for brain and kidney Hg concentrations using feather shafts as noninvasive samples. However, the time between the moult and the moment of sample collection should be considered. Finally, this work provides a solid understanding of Razorbill Hg exposure both in their wintering and breeding grounds, and shows that this species could be useful for assessing marine environmental health in the Mediterranean area.

Chapter VI. Factors influencing mercury concentrations in nestling Eagle Owls (Bubo bubo).

Hg levels detected in Eagle owl chicks from Southeastern Spain can be considered low, and it is unlikely that Hg pollution can negatively affect the breeding performance. A unique growing back feather of nestling Eagle owl could be enough to estimate mercury concentrations in blood of this terrestrial bird species.

Blood Hg concentrations in Eagle owls reflect Hg levels in muscle of rabbits, which is more evident in the nest from the Northern area, where rabbits are the main prey of Eagle owl. Although the studied region is not considered Hg polluted, area under mining influence and the industrial zone seems to contribute in the higher mercury levels in Eagle owls and their main prey, the European rabbit, from the area under mining influence than levels in owls and rabbits from the rest of the study region. This result supports the fact that spatial differences in Hg concentrations in Eagle owls appear to be mostly related to local contamination, and probably diet composition plays a role of less extent. Rainfalls could be the main cause of the differences in Hg concentrations found in blood of nestling Eagle owls between years.

PART 2. Induction of oxidative stress in metal exposed wild birds.

Chapter VII. Effects of heavy metals exposure on oxidative stress biomarkers in Eurasian Eagle owl (Bubo bubo).

The present study provides information about oxidative stress in Eagle owls exposed to different levels of several metals. Although individuals from Southern area (around an ancient mine site) have significant higher Pb and Hg concentrations, significant lower GST and GPx activities, and marginal higher tGSH levels; the lack of differences in oxidative damage to membrane lipids (TBARS) suggests that the antioxidant capacity of both populations is able to deal with oxidant species and maintain TBARS levels in the same amount. Despite the low levels of metals, several oxidative stress biomarkers were correlated with contaminant concentrations. Negative relationships between cadmium and lead levels and GPx or CAT activities were found. In the Southern area, TBARS was positively correlated with Pb and Hg, and when only Eagle owls from the mining area were selected, a negative relationship between Pb concentrations and tGSH levels was found. Finally, results suggested a possible protective response in Eagle owls from the Northern area, since the low exposure levels of Hg in Eagle owls resulted in increased activities of antioxidant enzymes CAT and SOD.

Moreover, this study provides threshold concentrations at which metals cause effects on antioxidant system in Eagle owls. Blood cadmium concentrations greater than 0.3 µg/dl produced an inhibition of 25% in CAT activity and 32% in GPx activity in red blood cells of Eagle owls. A depletion of 16% in tGSH levels were associated with lead concentrations higher than 15 µg/dl in Eagle owls from the mining area. Finally, lead concentrations upper than 10 µg/dl produced a TBARS induction of 28%, and mercury concentrations higher than 10 µg/dl resulted in a TBARS induction of 107% in Eagle owls from the Southern area.

Chapter VIII. Effects of heavy metals exposure on oxidative stress biomarkers in Griffon Vulture (Gyps fulvus).

Since sampling in the present study was done at the beginning of the hunting season, it is possible that lead levels found in Griffon vultures from Alcoy and Cinctorres were the normal or background levels in vultures feeding on porcine origin carcasses during the whole year. Then, it is possible that ingestion of game meat with

bullet fragments in carcasses or lead shot embedded in their flesh could be the cause of the high blood lead concentrations found in three Griffon vultures from Cinctorres.

The general trend observed in oxidative stress status is an increase in antioxidant mechanisms and oxidative stress products (CAT and GST activity, and tGSH and TBARS concentrations) in Griffon vultures from Cinctorres, which may be interpreted as a protective response against raised amount of ROS that may be generated by higher metal concentrations. Several metal-related effects were observed in antioxidant enzymes of Griffon vultures. Inverse relationships between lead and GPx or CAT activity were found. Besides, direct correlations between cadmium and GPx or CAT, and mercury and SOD were found. Lead had a significant effect on lipid peroxidation in Griffon vultures. The positive correlations found between some oxidative stress biomarkers prove that antioxidant defence operates as a balanced and coordinated system.

The present study provides threshold concentrations at which metals caused effects on antioxidant system in Griffon vultures. Blood cadmium concentrations greater than 0.05 µg/dl produced an induction of 33% in GPx activity and of 44% in CAT activity in red blood cells of vultures from Alcoy. Mercury concentrations in blood higher than 3 µg/dl produced an induction of 10% in SOD activity. Concentrations of lead upper than 15 µg/dl in blood were able to produce an inhibition of 12.5% in GPx activity and 11.3% in CAT activity, and a TBARS induction of 10.7% in red blood cells of Griffon vultures.

Our results suggest that antioxidant enzymes, particularly GPx, CAT and SOD, as well as lipid peroxidation as biomarker of oxidative damage, may function as useful biomarkers of metal induced effects on antioxidant system in Griffon vultures.

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