UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

# Nuevos horizontes en Ecotoxicología: biomarcadores destructivos y no destructivos en codorniz (*Coturnix coturnix*) y cigüeña blanca (*Ciconia ciconia*)

**Irene de la Casa Resino**
Área de Toxicología
Departamento de Sanidad Animal

2014

UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

# NUEVOS HORIZONTES EN ECOTOXICOLOGÍA: BIOMARCADORES DESTRUCTIVOS Y NO DESTRUCTIVOS EN CODORNIZ (*Coturnix coturnix*) Y CIGÜEÑA BLANCA (*Ciconia ciconia*)

**Irene de la Casa Resino**
Área de Toxicología
Departamento de Sanidad Animal

2014

Unidad de Toxicología
Facultad de Veterinaria
Avda de la Universidad s/n
10071 Cáceres

**Marcos Pérez López y Francisco Soler Rodríguez**, Profesores Titulares de Universidad del área de Toxicología (Departamento de Sanidad Animal) de la Facultad de Veterinaria de la Universidad de Extremadura,

**INFORMAN:**

Que la presente Tesis Doctoral titulada "NUEVOS HORIZONTES EN ECOTOXICOLOGÍA: BIOMARCADORES DESTRUCTIVOS Y NO DESTRUCTIVOS EN CODORNIX (*Coturnix coturnix*) Y CIGÜEÑA BLANCA (*Ciconia ciconia*)", recogida en la presente memoria, de la que es autora la Licenciada en Veterinaria por la Universidad de Extremadura, **Dña. Irene de la Casa Resino**, ha sido realizada bajo nuestra codirección y cumple las condiciones exigidas para que su autora pueda optar al grado de **Doctor Internacional** por la **Universidad de Extremadura**, por lo que damos su aprobación para la correspondiente lectura y defensa ante el tribunal correspondiente.

Y para que conste a los efectos oportunos, firmamos el presente informe en Cáceres, a 11 de Abril de 2014.

Fdo. Marcos Pérez López

Fdo. Francisco Soler Rodríguez

MINISTERIO
DE ECONOMÍA
Y COMPETITIVIDAD

INSTITUTO NACIONAL DE INVESTIGACIÓN
Y TECNOLOGÍA AGRARIA Y
ALIMENTARIA (INIA)

SUBDIRECCIÓN GENERAL DE
INVESTIGACIÓN Y TECNOLOGÍA

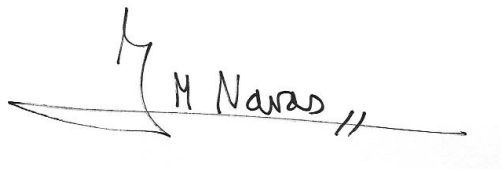
DPTO. MEDIO AMBIENTE

**José María Navas Antón,** Científico Titular de Organismos Públicos de Investigación en el Departamento de Medio Ambiente del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)**,**

**INFORMA:**

Que la presente Tesis Doctoral titulada "NUEVOS HORIZONTES EN ECOTOXICOLOGÍA" BIOMARCADORES DESTRUCTIVOS Y NO DESTRUCTIVOS EN CODORNIX (*Coturnix coturnix*) Y CIGÜEÑA BLANCA (*Ciconia ciconia*)", recogida en la presente memoria, de la que es autor la Licenciada en Veterinaria por la Universidad de Extremadura, **Dña. Irene de la Casa Resino**, ha sido realizada bajo mi codirección, junto con los Drs. Marcos Pérez López y Francisco Soler Rodríguez, y cumple las condiciones exigidas para que su autor pueda optar al grado de **Doctor Internacional** por la **Universidad de Extremadura**, por lo que doy mi aprobación para la correspondiente lectura y defensa ante el tribunal correspondiente.

Y para que conste a los efectos oportunos, firmo el presente informe en Madrid, a 21 de abril de 2014.



Fdo. José María Navas

CORREO ELECTRÓNICO:
seccmn@inia.es

Ctra. de La Coruña, Km
28040 MADRID
TEL: 91 347 39 08
FAX: 91 347 40 08

*The aim, perhaps one should say the dream, of Ecotoxicology is to understand the effect of pollutants and populations, community structure and even ecosystems*

*D.B. Peakall and L.R. Shugart (1992)*

La presente Tesis Doctoral ha sido parcialmente financiada por:

Consejería de Empleo, Empresa e Innovación del Gobierno de Extremadura (Irene de la Casa-Resino ha sido becaria PRE09001) y los fondos FEDER-UE (Ayudas a los Planes de actuación de Grupos de Investigación inscritos, referencia GRU10098).

Proyecto: RTA2009-00074-00-00. Perturbación endocrina asociada a los residuos de explotaciones ganaderas y acuícolas.
Entidad Financiadora: INIA

Proyecto: RTA2012-00053-00-00. Perturbación endocrina y efectos tóxicos asociados a la presencia de hormonas, antibióticos, micotoxinas y otras sustancias en residuos ganaderos y acuícolas
Entidad Financiadora: INIA

*Agradecimientos*

*Durante estos últimos años he pensado varias veces en el momento de escribir esta parte de la tesis, pero llegado el momento nunca pensé que me resultaría tan difícil. Espero no dejarme a nadie atrás, ya que todos de una forma u otra, habéis jugado un papel muy importante en el desarrollo de esta aventura.*

*En primer lugar mi más sincero agradecimiento a mis tres directores, ya que sin ellos este trabajo no hubiera sido posible. Al Dr. Marcos Pérez López, Marcos ¿qué decir?, gracias por enseñarme todo lo que sé sobre toxicología, por "engancharme" a la ciencia y por contagiarme ese entusiasmo y energía en todo lo que haces. Al Dr. Francisco Soler Rodríguez por su inmensa paciencia enseñándome, y por las horas de trabajo y esfuerzo que has pasado conmigo delante del cromatógrafo de gases y el HPLC. Por último, al Dr. José María Navas Antón, gracias por acogerme en el INIA como una más y por enseñarme todo lo que sé sobre alteraciones endocrinas y técnicas moleculares.*

*Al Dr. David Hernández Moreno, me faltan palabras para agradecer las miles de horas que has pasado ayudándome con esta tesis y con todo. Gracias por tener siempre una solución para todo y por tranquilizarme en los momentos de mayor estrés.*

*A las Dras. Mª Prado Míguez y Ana Oropesa, por los buenos momentos vividos en el departamento y por estar dispuestas a ayudar siempre que hacía falta.*

*A las Dras. Mª Luisa Fernández, Mª Dolores Hernando, Ana Valdehita y a todo el equipo del Departamento de Medio Ambiente del INIA, Alba, Luis, Tobias, Mona, Paul, Tania…, por todo lo que me han enseñado y los buenos momentos vividos en los laboratorios, despachos y jardines que hicieron que mis estancias allí se hicieran cortas!*

*Also thanks to Dr. Nikolaus Von Engelhardt, Dr. Vivian Goerlich-Jansson and Esther Langen. Thanks so much for the greats moments in Bielefeld.*

*A mis compañeras de departamento, Chon y Yolanda, aunque ya no estéis, empezasteis esta andadura conmigo. A los alumnos internos, Jaime, Chema, Manu, Carlos, Emilio, Miguel Ángel, José Luis, Cristóbal, Izaskun, Isra (espero que no se me olvide nadie)… porque vosotros sois los que habéis protagonizado los mejores momentos en los laboratorios, haciendo las horas más amenas. Gracias por los buenos momentos vividos y por los que vendrán.*

*A Ricardo y Cari. Cari, parte de los experimentos de la tesis llevan también tu nombre... gracias por estar a mi lado, por escucharme, por aconsejarme y por sacar siempre un ratito para lo que más nos gusta… "marujear"!*

*A mis amigos. María, simplemente me faltan las palabras…gracias por estar siempre ahí… en el laboratorio de patología infecciosa (si salimos de la facultad sin coger nada grave va a ser un milagro!) por estar en todos los momentos buenos y sobre todo en los malos… porque nadie entiende mejor las cosas cuando digo "no me pasa nada"… A mi fotógrafo preferido, el Dr. Francisco E. Martín Cano… Fran, gracias por todo, porque la parte artística de la tesis es obra tuya… y por supuesto por todo lo demás ¡que es mucho! A mi compañera de piso, cirujana personal, y sobre todo AMIGA, la Dra. Masa, por ponerle alegría y buen humor a estos últimos años de tesis y por supuesto por estar siempre al teléfono en las diversas estancias de ambas. A Lupe, porque su fuerza y buen humor siempre es arrolladora. A Antolín, por ser la prueba de que con esfuerzo y tesón todo se consigue. A Esther, estamos pasando por el mismo trance las dos a la vez, la ventaja es que lo celebraremos juntas. A Marisa, Rober, Robertín y Virginia esa GRAN familia "trekera", espero poder seguir aprendiendo y disfrutando con vosotros en futuros concursos de TREC, campeonatos de Extremadura, Andalucía, España… ¿me dejo alguno…? Y por último a JuanMa, por los buenos momentos vividos en el último año y por tener siempre esa capacidad de tranquilizarme que tan a menudo me hacía falta.*

*Por supuesto no puedo dejarme atrás a la gente que ha estado ahí desde el inicio de la carrera, Dani, Josué, Juan y Esther (mi psiquiatra particular), porque pasen los años que pasen siempre se puede contar con vosotros.*

*Y por último, a mi familia, los verdaderos responsables de que yo esté hoy donde estoy. A mi madre, la mujer con más fuerza que conozco, que se ha encargado de levantarnos cada vez que era necesario. A mi padre, porque gracias a su esfuerzo diario puedo presentar hoy mi Tesis. Y a mi hermana y mi cuñado, por darme un motivo para sonreír en los momentos más duros de los últimos dos años: LAURA.*

*A todos vosotros ¡GRACIAS!*

*A mis padres*

*A Laura*

**ÍNDICE**

# ESTRUCTURA DE LA TESIS DOCTORAL

La presente Tesis Doctoral se ha organizado siguiendo el esquema establecido de forma general para cualquier texto de índole científica. Consta en primer lugar de una ***Introducción general*** en castellano, que proporciona al lector una base teórica que facilitará la comprensión de los distintos capítulos que siguen. En ella se habla sobre los principales contaminantes objeto del presente trabajo, así como de los distintos biomarcadores utilizados para evaluar la exposición a dichos contaminantes.

A continuación se definen los ***Objetivos*** de los trabajos recopilados, que serán abordados en un total de ***6 Capítulos***, que componen el cuerpo de la tesis. Todos ellos están escritos en inglés y organizados con la estructura típica de un artículo científico. Los tres primeros capítulos son reproducciones exactas de artículos científicos ya publicados. En la ***Discusión general*** en castellano, se ofrece una visión conjunta de los resultados obtenidos en los diferentes capítulos, dándose una perspectiva integradora de su significado e implicaciones. Por último, se enumeran las principales ***Conclusiones***.

# INTRODUCCIÓN GENERAL

# 1. ECOTOXICOLOGÍA Y BIOMONITORIZACIÓN

Se entiende por biomonitorización (procedente del vocablo inglés, *biomonitoring*) el proceso de medición cuali o cuantitativo de los agentes químicos, sus metabolitos o sus productos de reacción en distintos tipos de muestras biológicas (sangre, orina, tejido graso y pelo, por ejemplo) de las poblaciones que se desea monitorizar (Needham *et al*., 2007). En este sentido la biomonitorización es uno de los caminos más directos para la estimación de las concentraciones de los más diversos compuestos químicos en el organismo vivo y en el ecosistema en su conjunto, pudiendo ser utilizada para:

- Determinar los niveles de contaminación de la población a estudio y correlacionarlos con los del ecosistema.
- Determinar los patrones de evolución que van a presentar estos contaminantes en el futuro.
- Identificar la aparición de nuevos contaminantes dentro de un cierto ecosistema.
- Permitir el desarrollo de actividades de priorización de lucha ambiental, a partir de los resultados obtenidos.
- Establecer, en resumen, si las medidas de control de los agentes químicos están siendo efectivas.

Gracias a esta herramienta, es posible recoger y analizar gran cantidad de información sobre los efectos de los agentes químicos en los seres vivos a lo largo de su existencia. Esto permite, así mismo, hacer comparaciones entre distintos estados fisiológicos, pues se dispone de datos del organismo a lo largo del tiempo, de modo que, a su vez, la información obtenida puede servir para predecir futuros cambios medioambientales que lleguen a ser importantes para el conjunto del ecosistema (Moreno, 2003).

## 1.1 Biomarcadores

En este punto parece importante definir el concepto de biomarcador y bioindicador. Se entiende por **biomarcador** aquel cambio biológico que se produce en respuesta a los contaminantes químicos en el medio ambiente y que puede ser medido en el organismo o en sus productos (heces, orina, sangre, etc.) (Van Gestel y Van Brummelen, 1996).

Según el Consejo Nacional de Investigación de EE.UU. (Henderson *et al*., 1987) y la Organización Mundial de la Salud (WHO/IPCS, 1993), los biomarcadores pueden dividirse en 3 clases:

- Biomarcadores de Exposición: abarcan la detección y medición de una sustancia exógena, su metabolito o el producto de una interacción entre un xenobiótico y

algunas moléculas o célula diana, que se mide en algún compartimento dentro de un organismo.

- Biomarcadores de Efecto: incluyen las medidas bioquímicas, fisiológicas o cualquier tipo de alteración en los tejidos o fluidos corporales de un organismo que puede ser reconocida o asociada a un posible perjuicio para la salud o enfermedad.
- Biomarcadores de Susceptibilidad: indican la capacidad inherente o adquirida de un organismo para responder a los cambios producidos por la exposición a un xenobiótico o a una sustancia específica, incluyendo cambios genéticos o en los receptores que alteran la susceptibilidad de un organismo a una determinada exposición.

Generalmente, la respuesta de los biomarcadores a los contaminantes se considera una reacción intermedia entre la exposición al xenobiótico y los efectos clínicos evidentes en el individuo. Cuando esta respuesta compensatoria se activa, la probabilidad de supervivencia del organismo se ve ya menguada, y va a depender de la capacidad del mismo a adaptarse a los cambios ambientales (van der Oost *et al*., 2003). La exposición a un xenobiótico va a producir una cascada de respuestas biológicas, y cada una de ellas va a servir, en teoría, como biomarcador. Una de las mayores utilidades de estos es que van a dar información de los efectos biológicos de los contaminantes, más allá de una mera cuantificación de los niveles ambientales (McCarthy *et al*., 1991).

Un biomarcador ideal debe presentar las siguientes características (Gil y Pla, 2001):

- La toma de muestras y el análisis debe ser simple y fiable.
- Específico para un tipo particular de exposición.
- Reflejar los efectos subclínicos y los cambios reversibles.
- Debe permitir intervenir sobre los efectos detectados o incrementar los esfuerzos preventivos.
- El uso del biomarcador debe ser éticamente aceptable.

Una inadecuada interpretación de las respuestas de los biomarcadores puede dar lugar a falsas conclusiones sobre los efectos de los contaminantes en los seres vivos o la calidad del medio ambiente. Por eso, se debe tener en cuenta que ciertas respuestas establecidas para una especie, pueden no ser útiles para otra. Además, los datos obtenidos en los estudios de laboratorio pueden ser difíciles de trasladar a los estudios de campo. Teniendo esto en cuenta, siempre que sea posible, los resultados laboratoriales de los

biomarcadores deben ser validados con una investigación de campo, aportando así, con ambos estudios, una información más real de los niveles de exposición y de la salud del medio ambiente (van der Oost *et al*., 2003).

### 1.1.1 Biomarcadores de exposición

Como se ha indicado anteriormente, estos biomarcadores comprenden la medida de la dosis interna de un compuesto químico o sus metabolitos en algún compartimento del organismo. Los niveles internos de un compuesto también indican la cantidad almacenada del mismo en algún compartimento del organismo, lo que es muy útil para evaluar procesos de acumulación de los compuestos químicos. Así, en el caso de los policlorobifenilos (PCBs), los niveles sanguíneos suelen ser indicativos de los niveles acumulados en los principales tejidos de deposición (tejidos grasos normalmente). No obstante, al evaluar la validez de un biomarcador de exposición es necesario tener en cuenta dos cosas: la capacidad analítica y la toxicocinética del mismo. Para una óptima capacidad analítica es necesaria la estandarización, pero los requerimientos específicos varían considerablemente entre las distintas especies tóxicas. Por otro lado, los distintos cambios fisiológicos del organismo, como la reproducción y la senescencia, también afectan a la toxicocinética de un xenobiótico (Gil y Pla, 2001).

### 1.1.2 Biomarcadores de efecto

#### *1.1.2.1 Biomarcadores de biotransformación*

La biotransformación o metabolismo puede ser definido como una conversión catalizada por enzimas de un compuesto químico en una forma más soluble en agua, la cual puede ser excretada por el organismo más fácilmente que el compuesto originario (Lech y Vodicnik, 1985). En la transformación de estos compuestos xenobióticos, intervienen determinadas enzimas que tienen un pequeño rango de sustratos específicos en comparación con las que intervienen en el metabolismo de compuestos endógenos (Van der Oost *et al*., 1996). La ruta de detoxificación que puede seguir un compuesto químico exógeno se encuentra subdividida en dos grandes fases: reacciones de biotransformación de fase I y de fase II.

La fase I consiste en una alteración no sintética (oxidación, reducción o hidrólisis) de la molécula originaria, que puede concluir en una reducción de la toxicidad o en una bioactivación del compuesto. Los metabolitos resultantes de las reacciones de biotransformación de fase I son conjugados en las reacciones de fase II, generando

normalmente compuestos menos tóxicos que el inicial (Nebbia, 2001). Bock (2003) también hace referencia a las reacciones de biotransformación de fase III que se llevan a cabo con enzimas como las peptidasas, hidrolasas, y β-liasas, y que se encargan del catabolismo de metabolitos conjugados para formar productos fácilmente excretables.

Dentro del grupo que conforman las enzimas de biotransformación de fase I se encuentra el sistema monooxigenasa citocromo P450 (CYP), que incluye a una superfamilia de hemoproteínas estructural y funcionalmente afines, siendo reconocidas múltiples familias y subfamilias con una gran gama de sustratos específicos (Nelson, 1999). La síntesis del grupo hemo que estas proteínas llevan en su estructura se realiza a través de una ruta metabólica con 8 pasos comúnmente denominada "metabolismo de porfirinas". Las porfirinas se producen y acumulan en los tejidos eritropoyéticos, el hígado y el riñón y son excretadas por la orina o las heces (Lim *et al*., 1984). Los niveles de porfirinas pueden verse alterados por un amplio espectro de contaminantes tales como metales y compuestos orgánicos persistentes (COPs) produciendo cambios en sus patrones de producción, acumulación o excreción (Casini *et al*., 2002). Además, los procesos de detoxificación de estos compuestos (que pueden, contrariamente al efecto buscado, generar una mayor toxicidad, por ejemplo a través de los metabolitos producidos) están mediados por su unión al receptor de hidrocarburos aromáticos (AhR). En aves se han detectado al menos dos formas de AhR, AhR1 y AhR2, siendo la primera la más transcripcionalmente activa (Yasui *et al*., 2007). Cuando un xenobiótico se une al AhR activa la transcripción de una serie de genes, entre los que se encuentra el CYP450 1A (CYP1A) (Fernández-Salguero *et al.*, 1996; Billiard *et al.*, 2006).

El CYP juega un papel esencial en la detoxificación de determinados contaminantes, incrementando su expresión en presencia de los mismos. De esta forma, la medida de los niveles de diferentes actividades enzimáticas individuales dependientes de los CYP constituye un importante indicador de la exposición a contaminantes ambientales (Miller *et al*., 2004). La actividad 7-etoxi-resorrufina-*O*-deetilasa (EROD), dependiente del CYP1A, ha sido ampliamente utilizada por diferentes autores como biomarcador de exposición a diversos contaminantes (Nebbia, 2001; Gravato y Santos, 2002; Hernández-Moreno *et al*., 2008).

Como se ha indicado anteriormente, una vez metabolizados los xenobióticos por las reacciones de fase I, estos son conjugados con sustratos endógenos por las enzimas de fase II, tales como las glutatión S-transferasas (GSTs), las uridin-difosfo-glucuronosiltransferasas (UDPGTs) y las sulfotransferasas (Pérez-López *et al*., 2002) cuya principal función es convertir a estos compuestos en sustancias más solubles en

agua y más fácilmente excretables mediante la adición de grupos polares a la molécula (Van der Oost *et al*., 2003).

Las GSTs son una amplia familia de enzimas detoxificadoras, implicadas en el proceso de conjugación de una enorme variedad de metabolitos electrofílicos, endógenos o no, con el glutatión (Pérez-López *et al*., 2002). Este es un tripéptido endógeno constituido por los aminoácidos ácido glutámico, cisteína y glicina, cuya forma reducida (GSH) se encuentra presente a altas concentraciones en la mayor parte de las células animales y vegetales (Reed, 1990). En general las reacciones catalizadas por las enzimas GSTs tienden a la obtención de productos biológicamente menos reactivos que los inicialmente metabolizados. No obstante, en ocasiones conllevan la aparición de compuestos más tóxicos que aquéllos de partida, ya sea porque la reacción desarrollada rápidamente origina un metabolito no conjugado (que requiere una posterior destoxificación), o porque el proceso que tiene lugar es reversible, permitiendo entonces la regeneración del compuesto de partida inicial (Pérez-López *et al*., 2002). En este sentido las GSTs son una familia de enzimas sumamente importantes en la prevención de peroxidación lipídica, estando demostrado que se produce una inducción de las GSTs con ciertos xenobióticos tales como hidrocarburos aromáticos policíclicos (HAPs), PCBs y compuestos fenobarbitales (Cunha *et al*., 2005).

La respuesta de las enzimas de fase II a los contaminantes ambientales es generalmente menos pronunciada que la observada en las de fase I (George, 1994), sin embargo, incluso estas menores variaciones pueden entrañar graves riesgos para los organismos (Van der Oost *et al*., 1996, Schreiber *et al*., 2006) pudiendo servir como potenciales biomarcadores de contaminación.

### *1.1.2.2 Biomarcadores de peroxidación lipídica*

La peroxidación lipídica es probablemente uno de los procesos más estudiados en referencia al daño tisular inducido por los radicales libres (moléculas que tienen un electrón desapareado en su órbita externa). Dado que estas moléculas son extremadamente inestables y tienen poder potencial para dañar las células del organismo, existen enzimas y moléculas de bajo peso molecular que poseen acción antioxidante con capacidad para proteger frente a los efectos adversos generados por las reacciones de dichos radicales libres (Machlin y Bendich, 1987; Zelikoff *et al*., 1996). El anión superóxido ($O_2^-$) y el peróxido de hidrogeno ($H_2O_2$) están fisiológicamente implicados en las reacciones químicas de varias enzimas y son empleados por multitud de células fagocíticas para eliminar bacterias (Halliwell, 1987). Sin embargo, el desequilibrio entre

la producción de especies reactivas de oxígeno (EROs) y su eliminación puede provocar el inicio de ciertas reacciones oxidativas en cadena y de la peroxidación lipídica.

Han sido ampliamente estudiados los compuestos químicos y enzimas que tienen dicha capacidad antioxidante (Figura 1). Entre ellos se encuentra: la enzima superóxido dismutasa (SOD), que convierte el anión superóxido ($O^{2-}$) en peróxido de hidrógeno ($H_2O_2$), que posteriormente debe ser detoxificado; la catalasa (CAT), que elimina el $H_2O_2$, transformándolo en oxígeno ($O_2$) y agua; el glutatión (GSH), que actúa como un antioxidante que protege a las células de las lesiones oxidativas de los radicales libres, interviniendo como sustrato de enzimas de defensa (glutatión peroxidasa, transferasa, reductasa), destoxificando aldehídos y peróxidos tóxicos y neutralizando EROs; la glutatión peroxidasa (GPx), que como se ha indicado anteriormente, cataliza la oxidación del GSH a su forma oxidada (GSSG), y la glutatión reductasa (GR), que tiene un papel muy importante en el reciclaje del GSH (Anderson, 1998).

Uno de los indicadores más utilizados para la determinación de peroxidación lipídica es el malondialdehído (MDA). El MDA es una molécula de bajo peso molecular que se forma por la descomposición primaria y secundaria de los productos de peroxidación lipídica (Janero, 1990). La existencia del MDA en el organismo conlleva potenciales efectos negativos, ya que se une a la guanina del ADN, afectando a la función de la mitocondria (y por tanto a la formación de ATP) y reduciendo la capacidad metabólica de las células. Como sustancia multi-funcional, el MDA también crea enlaces cruzados con valiosos aminoácidos (histidina, arginina, tirosina, metionina, lisina, prolina), reduciendo el valor nutritivo de la proteína al formarse polímeros metabólicamente inactivos (Halamíčková *et al*., 2003).

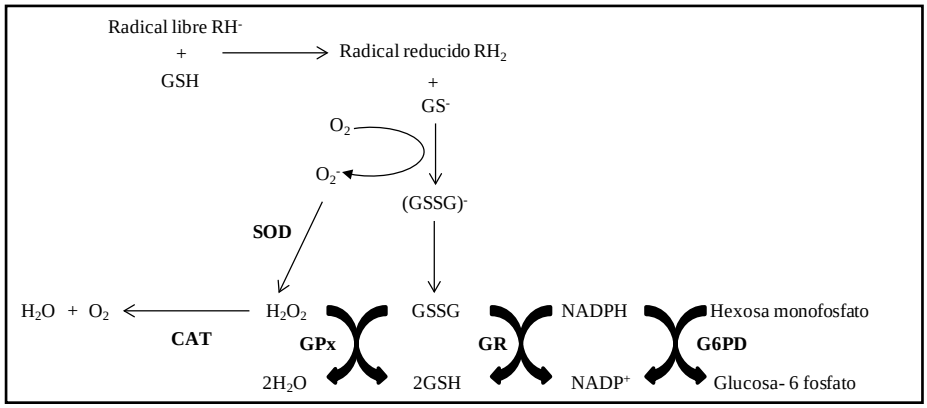



**Figura 1.** Representación simplificada del mecanismo de acción del glutatión y de las principales enzimas antioxidantes. Adaptada de Martínez (2010).

### *1.1.2.3 Biomarcadores de alteración endocrina*

La Organización Mundial de la Salud define a los alteradores endocrinos (AE)[1] como "sustancias exógenas que alteran la función del sistema endocrino y consecuentemente causan efectos adversos sobre la salud de un organismo intacto, de su progenie, o de sus poblaciones" (WHO/IPCS, 2002).

Según Navas y Segner (1998), los AE se pueden clasificar en distintos grupos atendiendo a sus mecanismos de acción:

- AE que mimetizan la acción de las hormonas, causando varios tipos de efectos, por ejemplo:
    - Efecto estrogénico: originado por sustancias que activan el receptor de estrógenos (ER) induciendo la expresión de genes dependientes de estrógenos. En el caso de los peces y las aves uno de estos genes es el de la vitelogenina (VTG).
    - Efecto androgénico: producido por sustancias que imitan la acción de los andrógenos en sus células diana, uniéndose al receptor de andrógenos y activándolo.
    - Efecto tiroideo: causado por sustancias que imitan la acción de las hormonas tiroideas.
- AE que antagonizan la acción de las hormonas esteroideas, causando distintos efectos, como:
    - Efecto antiestrogénico: ejercido por sustancias que se unen al ER bloqueándolo, de modo que los compuestos estrogénicos no pueden llegar a unirse a dicho receptor y no lo activan. No es un mecanismo frecuente en tóxicos ambientales. Existen otras sustancias antiestrogénicas que actúan uniéndose y activando el AhR que interacciona a su vez con el receptor de estrógenos bloqueando su actividad.
    - Efecto antiandrogénico: motivado por xenobióticos que se unen al receptor de andrógenos impidiendo que los andrógenos naturales lo activen.
    - Efecto antitiroideo: por el bloqueo directo de los receptores implicados en la acción de esas hormonas.

[1] La traducción literal de la denominación inglesa, *Endocrine Disruptor* (ED), al español no está oficialmente aceptada, por lo cual los autores han optado por la forma más correcta *Alterador Endocrino* (AE).

- AE que no actúan directamente sobre los receptores hormonales sino que alteran otros puntos de la ruta de acción de las hormonas, como su síntesis o su transporte. Por ejemplo, algunas sustancias pueden causar una inhibición competitiva de la transtiretina, proteína implicada en el transporte de las hormonas tiroideas.

La expresión de los ERs y TRs, así como los niveles de hormonas circulantes y los niveles de VTG han sido tradicionalmente usados como biomarcadores de la presencia de AE (Jiménez *et al.*, 2007; Sayed *et al.*, 2012).

### 1.2 Bioindicadores

Los **bioindicadores** son organismos vivos que, por sus características ecológicas, presentan una elevada sensibilidad a los cambios ambientales y reaccionan ante ellos como si fueran estímulos específicos (Capó, 2002). Cualquier especie o grupo de especies cuyas funciones, población o estado permitan evaluar la calidad ambiental de un ecosistema pueden ser utilizadas como bioindicadores. No obstante, debe tenerse en cuenta que las especies usadas han de reflejar los niveles de contaminación del medio ambiente y variar de acuerdo a ellos (Cotín, 2012). Por lo tanto parece obvio determinar sobre qué especie viva y representativa del ecosistema será más adecuado realizar dicha determinación ecotoxicológica (Casini *et al*., 2003). Si bien en los ecosistemas acuáticos existen numerosos estudios previos en este sentido, para los ecosistemas terrestres, y más concretamente para los mediterráneos, se observa una clara escasez de trabajos al respecto. Es aquí donde resalta, desde el punto de vista ecotoxicológico, el valor de las aves.

Las aves ocupan una gran variedad de medios, encontrándose distribuidas por muy diversos ambientes. En general, la mayoría de las especies de aves están bien estudiadas en cuanto a su ecología y comportamiento, son fáciles de identificar y su clasificación y sistemática está bien establecida. Dependiendo de la especie van a poseer diversos patrones de comportamiento respecto a sus movimientos, desde establecerse toda su vida en la misma zona geográfica a realizar grandes migraciones a través del globo terrestre, pasando por numerosas estrategias intermedias. Además, muchas especies de aves, debido a su longevidad (lo que las hace susceptibles a fenómenos de bioacumulación) y su elevada posición en la cadena trófica se verán expuestas a una gran variedad de factores contaminantes (Cotín, 2012). Teniendo en cuenta lo anterior, es muy probable que cuando se plantea un estudio de biomonitorización, exista una elevada probabilidad de que alguna especie de ave cumpla con los requisitos deseados.

Dentro de las aves, es de destacar el valor de los pollos a la hora de realizar un programa de biomonitorización. Numerosos estudios de campo han utilizado estos animales como bioindicadores (Baos *et al*., 2006a,b; Alvárez *et al*., 2013) ya que poseen varias ventajas con respecto a los adultos. En primer lugar, la toma de muestras es más eficaz y menos costosa que en el caso de los adultos. Los pollos suelen ser alimentados con recursos cercanos al nido por lo que son un fiel reflejo del estado ambiental de la zona de anidamiento. Además, hay especies de aves que tienden a reutilizar los nidos, lo que permite realizar estudios a lo largo de los años (Gómez Ramírez *et al*., 2012).

### 1.2.1 La codorniz como bioindicador de contaminación ambiental

La codorniz (*Coturnix coturnix*) es una especie de ave migratoria que está presente de forma general por toda la Península Ibérica, las Islas Baleares y las Islas Canarias, normalmente en altitudes no superiores a los mil cuatrocientos metros. Se observa en España durante la primavera y el verano, aunque también existe una población sedentaria que permanece en nuestro país durante todo el año. Es un ave de tamaño pequeño y fácilmente manejable en el animalario, lo que la hace susceptible de ser utilizada para estudios de laboratorio.

### 1.2.2 La cigüeña blanca como bioindicador de contaminación ambiental

La cigüeña blanca (*Ciconia ciconia*) es un ave muy representativa de los ecosistemas tanto extremeño como nacional, ampliamente distribuida por la Península Ibérica, siendo Extremadura la región donde alcanza sus mayores densidades de población dentro de Europa. En los últimos años se está produciendo un incremento progresivo de su población en toda la Península, favorecido en algunos casos porque las colonias existentes encuentran más recursos alimenticios durante todo el año (particularmente en los vertederos) lo que hace que tanto adultos como jóvenes no emprendan su migración a África y permanezcan en sus lugares de anidamiento. El pollo de cigüeña es alimentado hasta su migración con los recursos de la zona, lo que lo convierte en potenciales indicadores de la carga de diversos contaminantes persistentes en el área de anidamiento. Además, sus nidos están bien inventariados y controlados y es fácil el muestreo en los mismos antes de que los pollos alcancen la suficiente capacidad de vuelo. Todas estas consideraciones hacen que *a priori* la cigüeña blanca pueda ser considerada como un ave susceptible de ser utilizada para la monitorización del estado del medio ambiente.

**Figura 2.** Imágenes de codorniz (izquierda) y cigüeña blanca (derecha).

## 2. CONTAMINANTES AMBIENTALES OBJETO DE ESTUDIO

### 2.1 Clorotriazinas

Las clorotriazinas tales como la atrazina (ATZ), la simazina (SIM), la propazina (PRZ) y la prometrina (PRT) han sido herbicidas ampliamente utilizados para el control de malas hierbas en cultivos a lo largo de los años. No obstante, se encuentran dentro de los contaminantes que deben ser más seriamente monitorizados debido a su toxicidad y persistencia en el medio ambiente, así como por sus claros efectos negativos sobre el mismo y sobre la salud humana y animal. Además, las clorotriazinas han sido consideradas como AE debido a sus efectos sobre el sistema endocrino y la función reproductora de distintos organismos.

Uno de los representantes más importantes de la familia es la ATZ, que ejerce su efecto mediante la inducción de la enzima aromatasa (CYP450 19) (Sanderson *et al.*, 2001; Hayes *et al.*, 2002; Spanò *et al.*, 2004). Dicha enzima es el paso límite para la conversión del andrógeno testosterona (T) al estrógeno 17β -estradiol (E2). No obstante, otros estudios no han observado efectos de este herbicida sobre la actividad aromatasa (Coady *et al.*, 2005; Hinfray *et al.*, 2006) o incluso han detectado una inhibición (Benachour *et*

*al*., 2007). Además, la ATZ también puede afectar al metabolismo hepático alterando las enzimas de biotransformación de fase I y fase II que regulan la homeostasis de los esteroides sexuales. Así, la exposición a xenobióticos como la ATZ puede provocar una competición con los compuestos endógenos por el sitio activo de la enzima que los metaboliza, suponiendo, por lo tanto, una alteración en el normal equilibrio de los esteroides (Förlin y Haux, 1985).

Numerosos estudios han remarcado el efecto de la ATZ sobre el desarrollo de muchos seres vivos. De esta forma, se ha observado retraso en el inicio de la pubertad en ratas (Ashby *et al*., 2002), alteración de la organogénesis en pez cebra (Wiegand *et al*., 2001) y reducción del tamaño de la rana arbórea gris en la metamorfosis (Diana *et al*., 2000). Además, en anfibios, los estudios de diversos autores demuestran que la ATZ produce una completa feminización y castración química en los machos a bajas dosis (Hayes *et al*., 2002, 2003, 2010). Sin embargo, más escasos son los estudios en aves, aunque este herbicida parece tener una baja toxicidad aguda en las mismas, exhibiendo una $DL_{50}$ > 5000 mg/kg (U.S. EPA, 2006). Wilhelms *et al*. (2006a) en sus estudios en hembras inmaduras de codorniz no observaron alteraciones en el peso corporal, en el consumo de alimento, en la mortalidad o en los niveles de corticosterona, cuando los animales fueron expuestos a concentraciones de ATZ de 0,001 a 1000 µg/g en la dieta. Además tampoco observaron un incremento en el peso del ovario o del hígado, tejidos que son sensibles a los niveles de estrógenos circulantes. Por otra parte, en los estudios realizados por estos mismos autores en machos y embriones, tampoco se observaron alteraciones reproductivas o en el crecimiento, aunque se detectó una disminución del peso al nacimiento a dosis *in ovo* de 504 µg/kg (Wilhelms *et al.*, 2005, 2006b).

Debido a los efectos de este herbicida sobre los distintos organismos la Unión Europea (UE) decidió su prohibición en 2004 (Decisión 2004/248/CE) aunque sigue siendo uno de los herbicidas más ampliamente utilizados en EE.UU y América Latina.

### 2.2 Metales

Los metales son elementos químicos ubicuos que han acompañado al ser humano desde la antigüedad. Muchos de ellos son esenciales a determinadas concentraciones y, aunque no son sintetizados por el hombre, este tiene un papel fundamental en el aumento de los niveles de estos elementos químicos en el medio ambiente.

El cadmio (Cd), el plomo (Pb) y el mercurio (Hg) son unos de los metales más peligrosos desde un punto de vista ambiental y toxicológico, tanto para humanos como para animales (García-Fernández *et al*., 1996). Por otra parte, el zinc (Zn), el hierro (Fe) y el selenio (Se) son metales esenciales con una función concreta en el organismo a

determinadas concentraciones, mientras que a concentraciones elevadas pueden tener un efecto tóxico (Merian, 1991). Además, Cd, Pb, Hg, Zn, Se y As se encuentran dentro de la lista de los 126 contaminantes prioritarios de la Agencia de Protección Ambiental de Estados Unidos (U.S. EPA, 2013).

A pesar de que muchos de ellos (como por ejemplo Cd, Pb, Hg y Zn) están presentes de forma natural en el medio ambiente, gran parte de las emisiones provienen de actividades antrópicas. Desde un punto de vista ambiental, el Cd es un elemento relativamente raro en la litosfera. Por afinidad química, se le encuentra junto al Zn, en proporción muy variable. Las principales fuentes de contaminación son: la minero-metalurgia de metales no ferrosos, la metalurgia del hierro y acero, la fabricación de fertilizantes fosfatados, la incineración de residuos de madera o carbón, y la combustión de aceite y gasolina. Debido a sus múltiples usos y a la larga vida media de las moléculas de las que forma parte es previsible que se vaya acumulando progresivamente en el medio ambiente (Wayland y Scheuhammer, 2011).

En el caso del Pb, el principal origen del mismo en el medio ambiente a altas concentraciones es nuevamente antropogénico. Una de las fuentes más importantes de Pb durante años ha sido el uso de gasolinas con Pb como antidetonante pero estos combustibles están prohibidos en España desde 1998 (Directiva 98/70/CE; Real Decreto 785/2001). La ingestión de perdigones de caza es una de las fuentes más importantes de Pb para las aves (Kendall *et al*., 1996). 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). Además, los aledaños de las minas, los vertederos y las plantas industriales constituyen una de las fuentes adicionales de este metal (Henny *et al.*, 1991, 1994; García-Fernandez *et al.*, 1995). Los lodos procedentes de plantas de tratamiento que en muchos casos se añaden como abono a los suelos agrícolas (Pain, 1995), así como las pinturas plomadas (Finkelstein *et al*., 2003) pueden suponer una fuente adicional de Pb.

El Hg, considerado como un contaminante ambiental, posee un origen antropogénico como fungicida, herbicida y conservante de semillas en agricultura. Entre los usos industriales constituyen una fuente de Hg las papeleras, la industria electroquímica, pinturas y pilas, la industria de los catalizadores, la combustión de carbones a partir de minería, la incineración de basuras, el consumo de combustibles fósiles, los lodos de depuradoras y los vertidos industriales (Nriagu y Pacyna, 1988). Además, también tiene un origen ambiental a partir de erupciones volcánicas, partículas volátiles y flujos procedentes del medio marino (Nriagu, 1989).

Por su parte entre las principales fuentes de As se encuentran los pesticidas y agentes conservantes de la madera, la fundición de metales no férreos, la fabricación de aleaciones y semiconductores, la combustión del carbón así como las erupciones volcánicas (Moreno, 2003).

Los restantes metales considerados en la presente tesis se consideran esenciales, y están presentes de forma natural en la mayoría de los organismos.

### 2.2.1 Factores que afectan a la toxicidad de los metales en aves

Es importante remarcar que al igual que para muchos otros contaminantes, en el caso de los metales los efectos tóxicos no van a depender únicamente de su concentración en los tejidos, sino que se van a ver influenciados por factores tales como:

- Especie: según diversos estudios algunas especies son más sensibles a los metales que otras. Así, las especies altriciales son mucho más sensibles a los niveles de Pb que las precociales (Hoffman *et al*., 1985). Además, distintas especies de buitres son capaces de sobrevivir a concentraciones de Pb más elevadas que otras aves rapaces (Redig *et al*., 1991; Carpenter *et al*., 2003; García-Fernández *et al*., 2005).
- Edad: este factor va a ser muy importante en los niveles de metales pesados acumulados en el organismo. Estudios realizados en aves demuestran que los niveles de Cd y Pb aumentan con la edad, mientras que los de Hg y Se disminuyen (Gochfeld *et al*., 1996). Además los individuos jóvenes presentan mayor sensibilidad a los efectos de Pb y Cd que los adultos (Scheuhammer, 1987).
- Sexo: la acumulación de metales se va a ver influenciada por el sexo del animal y el tipo de metal. Así, en el caso del Pb se han observado mayores concentraciones en las hembras que en los machos, hecho atribuido al incremento de la absorción de Pb intestinal durante la época de puesta debido a la necesidad de calcio para la formación del huevo (Finley y Dieter, 1978; Pattee, 1984; Pain y Amiardtriquet, 1993). Sin embargo, son los machos los que acumulan mayores niveles de Cd y Zn. Esta diferencia parece estar relacionada, en el caso del Zn, con la síntesis de metalotioneinas, que también es mayor en los machos (Debacker *et al*., 2001; Barjaktarovic *et al*., 2002).
- Alimentación: la concentración de metales pesados va a estar determinada en muchos casos por los hábitos alimenticios, los cuales determinan a veces algunas

de las diferencias encontradas entre especies (Pain y Amiardtriquet, 1993; García-Fernández *et al*., 1995).

- Hábitat: en función del lugar donde habiten las aves, estas se van a ver más o menos expuestas a metales pesados. Por ejemplo, las aves que habitan zonas de caza tendrán mayor probabilidad de sufrir intoxicaciones por el consumo de perdigones de Pb. Además, sus predadores también incrementan la posibilidad de consumir una presa con perdigones en sus tejidos (García-Fernández *et al*., 2005). La proximidad a las fuentes de producción de estos metales (refinerías, minerías…) incrementa la susceptibilidad de intoxicación por dichos metales (Blanco *et al*., 2003).
- Condiciones climáticas: se ha observado que ciertas aves expuestas a Pb a baja temperatura presentaron mayor sensibilidad y mortalidad que aves similares expuestas a este metal pero en ambientes templados (Kendall y Scanlon, 1984).
- Otros factores: tales como la condición corporal del animal, la forma química en la que el contaminante es almacenado, así como el nivel y la duración de la exposición (Pain, 1996).

### 2.2.2 Efectos tóxicos de los metales en aves

En el caso del Cd son escasos los estudios que reflejan una toxicidad aguda del metal, aunque la exposición crónica en la dieta puede producir daño intestinal y reducción de la captación de nutrientes, daño renal y en el metabolismo de la vitamina D, defectos en el esqueleto, alteraciones en la osmorregulación y en el metabolismo energético, alteraciones en la reproducción y en el sistema endocrino, así como anemia y alteraciones en el comportamiento (Wayland y Scheuhammer, 2011).

En relación al Pb, los efectos subletales afectan a los sistemas nervioso, renal y circulatorio, resultando en cambios fisiológicos, bioquímicos y de comportamiento en las aves (Scheuhammer, 1987). El metabolismo de las vitaminas se puede ver afectado (Baksi y Kenny, 1978) y en algunos animales pueden aparecer cegueras (Pattee *et al*., 1981). Además, la intoxicación por Pb deprime la actividad de ciertas enzimas como la ácido δ -aminolevulínico deshidratasa (ALAD), fundamental para la producción de hemoglobina, reduciendo los niveles de esta proteína y el hematocrito a lo largo del tiempo (Redig *et al*., 1991; Grasman y Scanlon, 1995). La intoxicación por Pb produce también reducción del rendimiento reproductivo en aves, apareciendo adelgazamiento de la cáscara del huevo (Grandjean, 1976), disminución de la producción de huevos (Edens y Garlich, 1983), degeneración testicular (Kendall *et al*., 1981; Veit *et al*., 1983) y reducción de esperma en los túbulos seminíferos (Kendall *et al*., 1981).

Una vez que el Hg se introduce en el organismo se deposita en varios tejidos, uniéndose a grupos sulfhidrilos, interfiriendo con distintos sistemas enzimáticos al igual que ocurre en el caso del Cd (Debacker *et al*., 2001). La exposición crónica al Hg tiene efecto tóxico fundamentalmente en el sistema nervioso central (habiéndose descrito pérdida de funcionalidad motora, incoordinación, apatía, temblores, cambios en el comportamiento, eretismo y depresión severa) y en el riñón (proteinuria, enzimuria y necrosis del túbulo proximal) (Heinz y Locke, 1976; Spalding *et al*., 2000). Así mismo una exposición crónica al Hg puede causar importantes efectos en la reproducción como atresia gonadal, reducción de la fertilidad de los huevos, disminución del éxito reproductivo, disminución en la producción de huevos, huevos más ligeros, más pequeños, alteración de la incubación, y del crecimiento de la pluma (Hill y Soares, 1984; Burger, 1994). Además, como consecuencia de una exposición crónica al Hg también se ha descrito hipertrofia de la glándula tiroides, taquicardia, gingivitis, así como alteraciones hematológicas, hormonales e histológicas. Por otra parte, la intoxicación aguda por inhalación de concentraciones elevadas de Hg elemental puede provocar bronquitis corrosiva y neumonitis aguda, que puede causar la muerte. La ingestión de la forma inorgánica produce ulceraciones corrosivas del sistema digestivo, acompañada de hemorragias y necrosis del tubo digestivo (Moreno, 2003).

Es posible encontrar compuestos muy diferentes de As, tanto orgánicos como inorgánicos. En general, los compuestos inorgánicos son más tóxicos que los orgánicos y las formas trivalentes más tóxicas que las pentavalentes. La acción tóxica del As se atribuye a su afinidad por el azufre, uniéndose y desactivando enzimas que contienen grupos –SH y requieren ácido lipoico como coenzima. Uno de sus efectos más importantes se asocia a la alteración de la fosforilación oxidativa, desacoplándola (Eisler, 2004). La exposición crónica a compuestos inorgánicos conduce a efectos neurotóxicos tanto en el sistema nervioso central como en el periférico. Son comunes las lesiones cutáneas tales como cambios de pigmentación (melanosis) (Tseng, 1977) y las lesiones en órganos internos pertenecientes a los aparatos y sistemas respiratorio, digestivo, circulatorio o renal (Capó, 1998). En aves se han descrito una serie de síntomas como son: descoordinación muscular, debilidad, lentitud de movimientos, convulsiones, incapacidad para mantenerse erguidas, parálisis parciales e incluso alteraciones teratogénicas (Puzanová, 1980).

Con respecto a los restantes metales, aunque esenciales, también se ha descrito toxicidad a altas dosis. De esta forma, un exceso de Se produce hepatotoxicidad y teratogenicidad. Además, altos niveles de selenometalotioneina en aves han producido una disminución de los nacimientos y deformidades en los embriones (Spallholz y Hoffman, 2002). En el caso del Zn, la exposición a niveles tóxicos en aves se produce principalmente por la

presencia de alambres galvanizados y clips para la construcción de recintos (Ritchie *et al*., 1994). Los síntomas producidos por una intoxicación por Zn en aves incluyen poliuria, polidipsia, problemas gastrointestinales, pérdida de peso, temblores, anemia, cianosis e hiperglicemia. Los síntomas sistémicos están asociados a la hipoproteinemia que produce daño en el riñón, en el sistema gastrointestinal y en el páncreas. La pancreatitis es la lesión más característica, siendo evidenciada tanto en aves silvestres como de cautividad (Beyer *et al*., 2004). En el caso del Fe un exceso del mismo puede producir incremento de EROs a través de la reacción de Fenton (Fowler *et al*., 2011).

### 2.2.3 Interpretación de la concentración de metales en sangre de aves

A la hora de realizar un estudio de biomonitorización es importante, no sólo la elección de la especie y la edad del individuo como se ha indicado anteriormente, sino también el tipo de muestra en el que se va a llevar a cabo dicha biomonitorización. Los metales pesados tienden a acumularse en tejidos concretos. Así, el Pb tiende a acumularse en hueso, el Cd en riñón, el Hg en riñón e hígado, y el Zn, el Cu y el Fe en hígado. En cambio, la sangre representa una exposición a corto tiempo, es decir, refleja la dieta frecuente (García-Fernández *et al*., 1996, 2005). La interpretación de los niveles de metales en aves se centrará en aquellos estudiados en la presente Tesis doctoral.

Con respecto al Pb, Pain (1996) propuso como niveles basales en sangre de aves acuáticas 200 µg/l. A partir de ese límite y hasta 500 µg/l se considera intoxicación subclínica. Los niveles correspondientes a una intoxicación clínica se encontrarían entre 500-1000 µg/l mientras que se hablaría de una intoxicación clínica severa a partir de niveles superiores a 1000 µg/l.

Es importante conocer los niveles umbral de este metal en sangre, ya que se puede relacionar más fácilmente con los efectos sobre la salud de las aves, y además estos valores sanguíneos presentan una variación 30 veces menor entre especies que los niveles determinados en hueso o como exposición externa (mg Pb/kg/día) (Pain, 1996; Buekers *et al*., 2009).

El Cd se acumula normalmente en hígado y riñón (92%) y sólo un 0.5 % del total se detecta en sangre (García-Fernández *et al.*, 1996). Son escasos los estudios que establecen niveles de Cd en sangre y no se ha establecido un rango umbral (Martínez-López *et al.*, 2005), aunque algunos autores han observado alteraciones histológicas en hígado y riñón a partir de concentraciones sanguíneas de 17 µg/l en faisanes (Świergosz y Kowalska, 2000). Además debe tenerse en cuenta que los niveles sanguíneos detectados en sangre de pollos suelen reflejar niveles de exposición recientes, mientras

que los niveles de adultos reflejan bioacumulación a lo largo del tiempo (García-Fernández *et al*., 1996; Spahn y Sherry, 1999).

En relación al Hg, concentraciones sanguíneas inferiores a 1000 µg/l en aves acuáticas no han producido ningún efecto negativo reproductivo o de comportamiento, razón por la cual se considera como valor umbral en estas aves. En cambio, a niveles entre 1000-3000 µg/l se han observado alteraciones fisiológicas, del comportamiento y reproductivas (Evers *et al.*, 2008; Alvárez *et al.*, 2013). Niveles superiores a 3000 µg/l son considerados de alto riesgo para la salud de las aves, observándose cambios comportamentales y reducción en la supervivencia y en el número de pollos nacidos (Alvárez *et al*., 2013).

Con respecto al As, la información sobre las concentraciones tóxicas es limitada. Burger y Gochfeld (1997) detectaron valores sanguíneos de 18 µg/l en gaviotas de Franklin, procedentes de áreas con baja contaminación, estableciéndose este valor como referencia en sangre de aves (Benito *et al*., 1999).

En relación a los restantes metales y metaloides esenciales, la información sobre sus concentraciones tóxicas en muchos casos es escasa. Se consideran niveles basales de Se en sangre de aves no marinas aquellos que se encuentran entre 100-400 µg/l (Ohlendrof y Heinz, 2011). Con respecto al Zn, se consideran valores normales en sangre de pollos los situados entre 1450-3400 µg/l, considerándose fisiológicos en psitácidas niveles inferiores a 2000 µg/l (Puschner *et al*., 1999). En el caso del Fe son escasos los estudios sobre su toxicidad en aves. En cigüeña negra (*Ciconia nigra*) en libertad y en águila calva (*Haliaeetus leucocephalus*) se han determinado valores sanguíneos fisiológicos de 1933 ± 1136 mg/l (Lanzarot *et al*., 2005) y 1490 µg/l (Bowerman *et al*., 2000) respectivamente. Por otra parte, Ritchie *et al*. (1994) establecen como niveles normales en tucanes valores <3500 µg/l y en guacamayo de 790-1350 µg/l.

### 2.3 Contaminantes orgánicos persistentes

Los compuestos orgánicos persistentes han sido identificados como algunos de los mayores contaminantes medioambientales (tanto acuáticos como terrestres), resultado de la actividad humana (Herrera *et al*., 1996; Van Wyk *et al*., 2001). Dentro de este grupo se incluyen los PCBs y los plaguicidas organoclorados (OCPs). La mayoría de ellos tienen restringido su uso o está prohibida su producción a lo largo del mundo (Ryan *et al.*, 2013; Addison *et al.*, 2014).

Los PCBs constituyen una familia de compuestos químicos representada por 209 congéneres que se diferencian en función del número y la posición del cloro en la

estructura bifenilo, formada por dos anillos de benceno (Figura 3). El lugar que ocupen esos átomos de cloro, así como, la posición de los anillos, son críticos en la determinación de la toxicidad de estos compuestos. Así, en relación estructural a la toxicidad, los PCB se dividen en dos categorías distintas: coplanares y no coplanares. Los coplanares tienen una estructura bastante rígida, con los dos anillos en el mismo plano. Esto le da a la molécula una estructura similar a las dibenzo-*p*-dioxinas y dibenzofuranos, y actúan como agonistas del AhR en los organismos. Por otra parte, los PCB no coplanares, con átomos de cloro en las posiciones orto, presentan una toxicidad más reducida y no relacionada con la activación del AhR (O'Hara y Rice, 1996). Los PCBs suelen entrar en el medio ambiente como mezclas, como por ejemplo Aroclor en Estados Unidos, Clophen en Alemania y Kanechlor en Japón (Barron *et al*., 1995). Los efectos tóxicos de estas mezclas en las aves incluyen retrasos en el crecimiento, disminución de la atención paterna y efectos neurológicos, entre otros (Dahlgren *et al*., 1972; Peakall y Peakall, 1973).

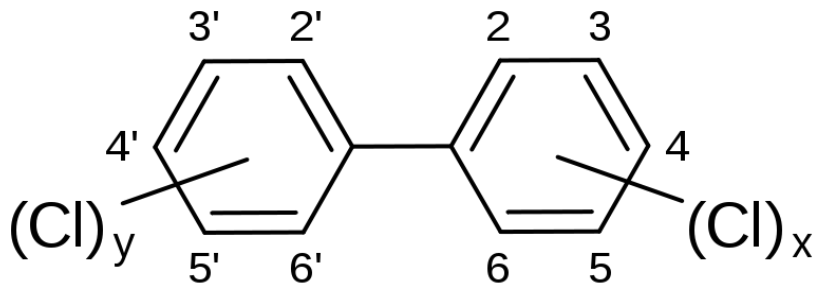



**Figura 3.** Estructura química básica de los PCBs. Los números 2-6 y 2'-6' representan posibles posiciones del átomo de cloro (Cl) dentro de cada anillo bencénico.

Los OCs conforman un grupo de compuestos cuya estructura química se corresponde con la de hidrocarburos clorados con pesos moleculares entre 291 y 545. Los principales componentes de este grupo son el diclorodifeniltricloroetano (DDT) y sus metabolitos diclorodifenildicloroetileno (DDE) y diclorodifenildicloroetano (DDD), aldrín, dieldrín, heptacloro, clordano, endrín y los distintos isómeros del hexaclorociclohexano (α -HCH, β-HCH, Δ-HCH y γ-HCH). Una de las características comunes de estos compuestos es su baja solubilidad en agua y su elevada solubilidad en lípidos y solventes orgánicos, lo que les hace susceptibles de acumularse en tejidos grasos. Además presentan una baja presión de vapor, una alta estabilidad química y una elevada resistencia a la degradación por microorganismos. La vida media de estos compuestos en el organismo va a depender del tipo de compuesto, las condiciones del animal, la edad y el sexo (Blus *et al*., 1996).

La mayor fuente de contaminación de estos compuestos es su aplicación para controlar plagas agrícolas en cultivos, masas forestales, suelos o aguas estancadas o para luchar contra plagas causantes de problemas de salud en el ser humano (Blus *et al*., 1996; O'Hara y Rice, 1996). La principal vía de entrada de estos compuestos al organismo de las aves es la oral, por consumo de alimentos, y la pulmonar, por contaminación atmosférica. Una vez ingeridos, la absorción intestinal está determinada por los constituyentes de la dieta (cantidad de fibra y grasa), así como por la ingesta total de comida (Heath y Vandekar, 1964). Debido a su persistencia en el medio ambiente y su liposolubilidad, se acumulan principalmente en el tejido adiposo y se biomagnifican a lo largo de la cadena alimentaria. Son eliminados por la bilis o por las heces, mientras que los metabolitos se eliminan principalmente por orina si presentan una alta polaridad. En el caso de las aves, durante la época de puesta eliminan estos compuestos a través de la yema de huevo debido a la elevada afinidad de estos por los lípidos que forman parte del mismo (Ross *et al*., 2008; Van den Steen *et al*., 2009).

Las aves se han utilizado tradicionalmente como bioindicadores de contaminación por COPs (Mundy *et al.*, 1982; Stickel *et al.*, 1984; Tanabe *et al.*, 1998; Gómez-Ramírez *et al.*, 2012). Numerosos estudios han reflejado los efectos de estos contaminantes en las mismas, incluyendo acciones tan variadas como adelgazamiento y debilitación de la cáscara del huevo (Lundholm, 1997), feminización de los machos y comportamiento sexual anormal (Fry y Toone, 1981; Iwaniuk *et al*., 2006), inhibición de la puesta de huevos y disminución del tamaño de la nidada (Larson *et al*., 1996), disminución en el éxito de eclosión (King y Krynitsky, 1986), incremento en la frecuencia de deformidades en los embriones (Fry y Toone, 1981; Larson *et al*., 1996) así como reducción del tamaño del cerebro y neurotoxicidad (Iwaniuk *et al*., 2006). Sin embargo, son más escasos los estudios que utilizan la sangre para monitorizar estas sustancias en las aves. Residuos sanguíneos de diferentes OCPs se han asociado a alteraciones del comportamiento, reducción del éxito reproductivo y la supervivencia de los pollos (Bustnes *et al.*, 2001, 2003, 2005; Verreault *et al.*, 2004). La tabla 1 muestra una pequeña relación de los estudios realizados en sangre de aves en los que se han medido contaminantes clorados.

A la hora de elegir una especie de ave como bioindicadora de la contaminación por estos pesticidas, es muy importante conocer las rutas de migración de la misma (si las hubiera), ya que estas se pueden ver expuestas a ellos en las zonas de migración. Así, Henny *et al*. (1990) en la bahía de Commencement (Washington) encontraron un incremento en la concentración de PCBs, DDE y clordano en achichilique occidental (*Aechmophorus occidentalis*) después del invierno. Además, en Rusia, se ha observado un incremento de los niveles de OCPs en aves migratorias después de pasar el invierno

en el sur de Asia (Kunisue *et al*., 2002) y en EE.UU. altos niveles de DDE en aves se han asociado a los lugares de migración en América Latina (Mora, 1997).

Diversos trabajos han asociado las concentraciones de OCs con efectos tóxicos en aves. Así, Enderson *et al*. (1982) estableció que la concentración más baja de DDE en la dieta que produce adelgazamiento de la cáscara y disminución de la producción de huevos en halcón peregrino (*Falco peregrinus*) era de 1 µg/g. Estudios posteriores, realizados por Deweese *et al*. (1986), establecieron como niveles críticos de DDE 3 µg/g basándose en un estudio experimental en aves rapaces. A esta concentración se observaron serios problemas reproductivos, e incluso mortalidad de los adultos. En relación al dieldrín, la LOAEL en patos es de 7 µg/g en hígado y 2,5 µg/g en cerebro, mientras que la NOAEL es inferior a 1 µg/g en ambos órganos (Nebeker *et al*., 1992). Niveles de 8 µg/g, y de 5 µg/g en cerebro se han establecido como de riesgo mortal para el heptacloro y clordano respectivamente (Wiemeyer, 1996). Con respecto al hexaclorobenceno (HCB), no se ha determinado una dosis letal aunque diversos estudios experimentales en codorniz han observado mortalidad en los animales expuestos a 100 µg/g en la dieta (Vos *et al*., 1971).

**Tabla 1.** Investigaciones recogidas en la literatura científica sobre pesticidas clorados en sangre de aves. PCBs: policlorobifenilos; DDTs: diclorodifeniltricloroetano y sus metabolitos; HCH: hexaclorociclohexano; DDE: diclorodifenildicloroetano; HCB: hexaclorobenceno; PBDE: polibromodifeniléter; PFC: perfluorocarbono.

| Especie | n | edad | Pesticidas detectados | Bibliografía |
|---|---|---|---|---|
| Cigüeña blanca (*Ciconia ciconia*) | 200 | pollos | PCBs, DDTs | Blazquez *et al*., 2006 |
| Cigüeña blanca (*Ciconia ciconia*) | 17 | pollos | PCBs, DDTs | Saez *et al*., 2008 |
| Cigüeña blanca (*Ciconia ciconia*) | 7 y 5 | pollos y adultos | PCBs, DDTs | Saez *et al*., 2009 |
| Gavión hiperbóreo (*Larus hyperboreus*) | 22 | adultos | HCHs, clordanos, DDTs, HCB, PCBs | Henriksen *et al*., 1998 |
| Gavión hiperbóreo (*Larus hyperboreus*) | 31 | adultos | HCHs, DDE, oxiclordano, PCBs | Bustnes *et al*., 2001 |
| Gavión hiperbóreo (*Larus hyperboreus*) | 111 | adultos | HCHs, HCB, DDE, oxiclordano, PCBs | Bustnes *et al*., 2003 |
| Gavión hiperbóreo (*Larus hyperboreus*) | 27 | adultos | HCB, oxiclordano, DDE, PCBs | Bustnes *et al*., 2005 |
| Gavión hiperboreo (*Larus hyperboreus*) | 49 | adultos | PCBs, clordanos, heptacloro epóxido. | Ross *et al*., 2008 |
| Gavilán americano (*Accipiter striatus*) | | adultos | DDE, mirex, oxiclordano, heptacloro epóxido, dieldrín, HCB, PCBs | Elliot y Shutt, 1993 |
| Pigargo europeo (*Haliaeetus albicilla*) | 14 | pollos | PCBs, DDTs, HCB, HCHs, clordanos | Eulaers *et al*., 2011 |
| Alimoche común (*Neophron percnopterus*) | 27 | adultos | PCBs, DDTs | Gomara *et al*., 2004 |
| Cernícalo americano (*Falco sparverius*) | 20 | adultos | DDE | Henny y Meeker, 1981 |
| Águila calzada (*Hieraaetus pennatus*) | 62 | pollos | HCHs, α-endosulfan, β-endosulfan, endosulfan sulfato | Martínez-López *et al*., 2009 |
| Cernícalo americano (*Falco sparverius*), turpial gorjeador (*Sturnella neglecta*) y cenzontle (*Mimus polyglottos*) | 4, 2 y 2 | adultos | HCHs, heptacloro, heptacloro epoxido, aldrín, *p,p'*-DDE, endosulfan, endosulfan sulfato | Rivera-Rodríguez *et al*., 2007 |
| Pardela cenicienta (*Calonectris diomedea, Calonectris borealis* y *Calonectris edwardsii*), pardela chica (*Puffinus baroli* y *Puffinus boydi*) y pardela pichoneta (*Puffinus yelkouan* y *Puffinus mauretanicus*) | 68, 17 y 13 | adultos | PCBs, DDTs | Roscales *et al*., 2011 |
| Azor común (*Accipiter gentilis*), águila real (*Aquila chrysaetos*) y pigargo europeo (*Haliaeetus albicilla*) | 16, 2 y 5 | pollos | PCBs, *4,4'*-DDE, HCB, clordano, PBDEs, PFCs, β-HCH, heptacloro epóxido, HCB | Sonne *et al*., 2010 |
| Azor común (*Accipiter gentilis*), águila real (*Aquila chrysaetos*) y pigargo europeo (*Haliaeetus albicilla*) | 56, 12 y 36 | pollos | PCBs, *4,4'*-DDE, HCB, clordano, PBDEs, PFCs. | Sonne *et al*., 2012 |
| Buitre dorsiblanco africano (*Pseudogyps africanus*), buitre de El Cabo (*Gyps coprotheres*) y buitre orejudo (*Torgos tracheliotos*) | 60 | pollos y adultos | HCHs, heptacloro epóxido, α-clordano, β-clordano, aldrín, DDE, endosulfan, endosulfan sulfato | van Wyk *et al*., 2001 |

## REFERENCIAS

Addison, R., Muir, D., Ikonomou, M., Harwood, L., Smith, T., Alikamik, J. (2014). Temporal trends in "legacy" organochlorine contaminants in blubber of ringed seals (*Phoca hispida*) from Ulukhaktok, NT, Canada between 1972 and 2010. Science of the Total Environment 466**,** 564-576.

Alvárez, C.R., Moreno, M.J., Alonso, L.L., Gómara, B., Bernardo, F.G., Martín-Doimeadios, R.R., González, M. (2013). Mercury, methylmercury, and selenium in blood of bird species from Doñana National Park (Southwestern Spain) after a mining accident. Environmental Science and Pollution Research 20**,** 5361-5372.

Anderson, M.E. (1998). Glutathione: an overview of biosynthesis and modulation. Chemico-Biological Interactions 111**,** 1-14.

Ashby, J., Tinwell, H., Stevens, J., Pastoor, T., Breckenridge, C. (2002). The effects of atrazine on the sexual maturation of female rats. Regulatory Toxicology and Pharmacology 35**,** 468-473.

Baksi, S., Kenny, A. (1978). Effect of lead ingestion on vitamin D3 metabolism in Japanese quail. Research Communications in Chemical Pathology and Pharmacology 21**,** 375-378.

Baos, R., Blas, J., Bortolotti, G.R., Marchant, T.A., Hiraldo, F. (2006a). Adrenocortical response to stress and thyroid hormone status in free-living nestling White storks (*Ciconia ciconia*) exposed to heavy metal and arsenic contamination. Environmental Health Perspectives 114**,** 1497-1501.

Baos, R., Forero, M.G., Gómez, G., Gónzalez, M.J., Hiraldo, F., Jiménez, B., Jovani, R., Tella, J.L. (2006b). Relationships between T-cell mediated immnune response and Pb, Zn. Cu, Cd, and as concetration in blood of nestling White storks (*Ciconia ciconia*) and Black kites (*Milvus migrans*) from Doñana (Southwestern Spain) after the Aznalcollar toxic spill. Environmental Toxicology and Chemistry 25**,** 1153-1159.

Barjaktarovic, L., Elliott, J., Scheuhammer, A. (2002). Metal and metallothionein concentrations in scoter (*Melanitta* spp.) from the Pacific Northwest of Canada, 1989–1994. Archives of Environmental Contamination and Toxicology 43**,** 0486-0491.

Barron, M., Galbraith, H., Beltman, D. (1995). Comparative reproductive and developmental toxicology of PCBs in birds. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 112**,** 1-14.

Benachour, N., Moslemi, S., Sipahutar, H., Seralini, G.E. (2007). Cytotoxic effects and aromatase inhibition by xenobiotic endocrine disrupters alone and in combination. Toxicology and Applied Pharmacology 222**,** 129-140.

Benito, V., Devesa, V., Muñoz, O., Suñer, M.A., Montoro, R., Baos, R., Hiraldo, F., Ferrer, M., Fernández, M., González, M.J. (1999). Trace elements in blood collected from birds

feeding in the area around Doñana National Park affected by the toxic spill from the Aznalcóllar mine. Science of the Total Environment 242**,** 309-323.

Beyer, W., Dalgarn, J., Dudding, S., French, J., Mateo, R., Miesner, J., Sileo, L., Spann, J. (2004). Zinc and lead poisoning in wild birds in the Tri-State Mining District (Oklahoma, Kansas, and Missouri). Archives of Environmental Contamination and Toxicology 48**,** 108-117.

Billiard, S.M., Timme-Laragy, A.R., Wassenberg, D.M., Cockman, C., Di Giulio, R.T. (2006). The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish. Toxicological Sciences 92**,** 526-536.

Blanco, G., Frías, O., Jiménez, B., Gómez, G. (2003). Factors influencing variability and potential uptake routes of heavy metals in black kites exposed to emissions from a solid-waste incinerator. Environmental Toxicology and Chemistry 22**,** 2711-2718.

Blázquez, E., Aguirre, J., Martínez-Haro, M., Mateo, R., Jiménez, B. (2006). The use of White stork (*Ciconia ciconia*) nestlings in a biomonitoring programme for organochlorines through the region of Madrid (Spain). Organohalogen Compounds 68**,** 2081-2084.

Blus, L., Wiemeyer, S.N., Henny, C.J. (1996). Organochlorine pesticides. En*:* Fairbrother, A., Locke, L.N., Hoff, G.L. (eds.). Noninfectious diseases of wildlife (2nd edition). United States, Iowa State University Press.

Bock, K.W. (2003). Vertebrate UDP-glucuronosyltransferases: functional and evolutionary aspects. Biochemical Pharmacology 66**,** 691-696.

Bowerman, W.W., Stickle, J.E., Sikarskie, J.G., Giesy, J.P. (2000). Hematology and serum chemistries of nestling bald eagles (*Haliaeetus leucocephalus*) in the lower peninsula of MI, USA. Chemosphere 41**,** 1575-1579.

Buekers, J., Steen Redeker, E., Smolders, E. (2009). Lead toxicity to wildlife: Derivation of a critical blood concentration for wildlife monitoring based on literature data. Science of the Total Environment 407**,** 3431-3438.

Burger, J. (1994). Heavy metals in avian eggshells: another excretion method. Journal of Toxicology and Environmental Health, Part A 41**,** 207-220.

Burger, J., Gochfeld, M. (1997). Age differences in metals in the blood of herring (*Larus argentatus*) and Franklin's (*Larus pipixcan*) gulls. Archives of Environmental Contamination and Toxicology 33**,** 436-440.

Bustnes, J.O., Erikstad, K.E., Skaare, J.U., Bakken, V., Mehlum, F. (2003). Ecological effects of organochlorine pollutants in the Arctic: a study of the glaucous gull. Ecological Applications 13**,** 504-515.

Bustnes, J.O., Miland, Ø., Fjeld, M., Erikstad, K.E., Skaare, J.U. (2005). Relationships between ecological variables and four organochlorine pollutants in an artic glaucous gull (*Larus hyperboreus*) population. Environmental Pollution 136**,** 175-185.

Bustnes, J.O., Skaare, J.U., Erikstad, K.E., Bakken, V., Mehlum, F. (2001). Whole blood concentrations of organochlorines as a dose metric for studies of the glaucous gull (*Larus hyperboreus*). Environmental Toxicology and Chemistry 20**,** 1046-1052.

Capó, M. (1998). Incidencia ecotoxicológica de los metales pesados en poblaciones humanas. Madrid, Ciencia Veterinarias.

Capó, M. (2002). Principios de Ecotoxicología. Diagnóstico, tratamiento y gestión del medio ambiente. Madrid, McGraw-Hill Profesional.

Carpenter, J.W., Pattee, O.H., Fritts, S.H., Rattner, B.A., Wiemeyer, S.N., Royle, J.A., Smith, M.R. (2003). Experimental lead poisoning in turkey vultures (*Cathartes aura*). Journal of Wildlife Diseases 39**,** 96-104.

Casini, S., Fossi, M.C., Cavallaro, K., Marsili, L., Lorenzani, J. (2002). The use of porphyrins as a non-destructive biomarker of exposure to contaminants in two sea lion populations. Marine Environmental Research 54**,** 769-773.

Casini, S., Fossi, M.C., Leonzio, C., Renzoni, A. (2003). Review: porphyrins as biomarkers for hazard assessment of bird populations: destructive and non-destructive use. Ecotoxicology 12**,** 297-305.

Coady, K.K., Murphy, M.B., Villeneuve, D.L., Hecker, M., Jones, P.D., Carr, J.A., Solomon, K.R., Smith, E.E., Van Der Kraak, G., Kendall, R.J. (2005). Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in *Xenopus laevis*. Ecotoxicology and Environmental Safety 62**,** 160-173.

Cotín, J. (2012). Birds as bioindicators of pollution in aquatic and terrestrial environments. Tesis Doctoral, Universitat de Barcelona.

Cunha, I., García, L.M., Guilhermino, L. (2005). Sea-urchin (*Paracentrotus lividus*) glutathione S-transferases and cholinesterase activities as biomarkers of environmental contamination. Journal of Environmental Monitoring 7**,** 288-294.

Dahlgren, R.B., Linder, R.L., Carlson, C. (1972). Polychlorinated biphenyls: their effects on penned pheasants. Environmental Health Perspectives 1**,** 89-101.

Debacker, V., Schiettecatte, L.S., Jauniaux, T., Bouquegneau, J.M. (2001). Influence of age, sex and body condition on zinc, copper, cadmium and metallothioneins in common guillemots (*Uria aalge*) stranded at the Belgian coast. Marine Environmental Research 52**,** 427-444.

Decisión 2004/248/CE. Decisión de la Comisión de 10 de marzo de 2004 relativa a la no inclusión de la atrazina en el anexo I de la Directiva 91/414/CEE del Consejo y a la

retirada de las autorizaciones de los productos fitosanitarios que contengan esta sustancia activa. Diario Oficial de la Unión Europea nº 78, de 16 de marzo de 2004, pp 53-55.

Deweese, L.R., McEwen, L.C., Hensler, G.L., Petersen, B.E. (1986). Organochlorine contaminants in passeriformes and other avian prey of the Peregrine falcon in the Western United States. Environmental Toxicology and Chemistry 5**,** 675-693.

Diana, S.G., Resetarits, W.J., Schaeffer, D.J., Beckmen, K.B., Beasley, V.R. (2000). Effects of atrazine on amphibian growth and survival in artificial aquatic communities. Environmental Toxicology and Chemistry 19**,** 2961-2967.

Directiva 98/70/CE. Directiva 98/70/CE del Parlamento Europeo y del Consejo de 13 de octubre de 1998 relativa a la calidad de la gasolina y el gasóleo y por la que se modifica la Directiva 93/12/CEE del Consejo. Diario Oficial de la Unión Europea nº 350, de 28 de diciembre de 1998, pp 58-68.

Edens, F., Garlich, J. (1983). Lead-induced egg production decrease in leghorn and Japanese quail hens. Poultry Science 62**,** 1757-1763.

Eisler, R. (2004). Arsenic hazards to humans, plants, and animals from gold mining. Reviews of Environmental Contamination and Toxicology 180**,** 133-165.

Elliott, J.E., Shutt, L. (1993). Monitoring organochlorines in blood of sharp-shinned hawks (*Accipiter striatus*) migrating through the great lakes. Environmental Toxicology and Chemistry 12**,** 241-250.

Enderson, J.H., Craig, G.R., Burnham, W.A., Berger, D.D. (1982). Eggshell thinning and organochlorine residues in Rocky Mountain peregrines, *Falco peregrinus*, and their prey. Canadian Field-Naturalist 96**,** 255-264.

Eulaers, I., Covaci, A., Hofman, J., Nygård, T., Halley, D.J., Pinxten, R., Eens, M., Jaspers, V.L. (2011). A comparison of non-destructive sampling strategies to assess the exposure of white-tailed eagle nestlings (*Haliaeetus albicilla*) to persistent organic pollutants. Science of the Total Environment 410**,** 258-265.

Evers, D.C., Savoy, L.J., DeSorbo, C.R., Yates, D.E., Hanson, W., Taylor, K.M., Siegel, L.S., Cooley Jr, J.H., Bank, M.S., Major, A. (2008). Adverse effects from environmental mercury loads on breeding common loons. Ecotoxicology 17**,** 69-81.

Fernandez-Salguero, P.M., Hilbert, D.M., Rudikoff, S., Ward, J.M., Gonzalez, F.J. (1996). Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced toxicity. Toxicology and Applied Pharmacology 140**,** 173-179.

Finkelstein, M.E., Gwiazda, R.H., Smith, D.R. (2003). Lead poisoning of seabirds: Environmental risks from leaded paint at a decommissioned military base. Environmental Science and Technology 37**,** 3256-3260.

Finley, M.T., Dieter, M.P. (1978). Influence of laying on lead accumulation in bone of mallard ducks. Journal of Toxicology and Environmental Health, Part A 4**,** 123-129.

Förlin, L., Haux, C. (1985). Increased excretion in the bile of 17β-[3H]estradiol-derived radioactivity in rainbow trout treated with β-naphthoflavone. Aquatic Toxicology 6**,** 197-208.

Fowler, B.A., Nordberg, G.F., Nordberg, M., Friberg, L. (2011). Handbook on the toxicology of metals. USA, Academic Press.

Fry, D.M., Toone, C.K. (1981). DDT-induced feminization of gull embryos. Science 213**,** 922-924.

García-Fernández, A., Martínez-López, E., Romero, D., María-Mojica, P., Godino, A., Jiménez, P. (2005). High levels of blood lead in griffon vultures (*Gyps fulvus*) from Cazorla Natural Park (Southern Spain). Environmental Toxicology 20**,** 459-463.

García-Fernández, A., Romero, D., Martínez-López, E., Navas, I., Pulido, M., María-Mojica, P. (2005). Environmental lead exposure in the European Kestrel (*Falco tinnunculus*) from Southeastern Spain: the influence of leaded gasoline regulations. Bulletin of Environmental Contamination and Toxicology 74**,** 314-319.

García-Fernández, A., Sanchez-García, J., Gómez-Zapata, M., Luna, A. (1996). Distribution of cadmium in blood and tissues of wild birds. Archives of Environmental Contamination and Toxicology 30**,** 252-258.

García-Fernandez, A.J., Sanchez-Garcia, J.A., Jimenez-Montalban, P., Luna, A. (1995). Lead and cadmium in wild birds in Southeastern Spain. Environmental Toxicology and Chemistry 14**,** 2049-2058.

George, S.G. (1994). Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. Aquatic Toxicology 4**,** 37-85.

Gil, F., Pla, A. (2001). Biomarkers as biological indicators of xenobiotic exposure. Journal of Applied Toxicology 21**,** 245-255.

Gochfeld, M., Belant, J.L., Shukla, T., Benson, T., Burger, J. (1996). Heavy metals in laughing gulls: gender, age and tissue differences. Environmental Toxicology and Chemistry 15**,** 2275-2283.

Gómara, B., Ramos, L., Gangoso, L., Donázar, J., González, M. (2004). Levels of polychlorinated biphenyls and organochlorine pesticides in serum samples of Egyptian Vulture (*Neophron percnopterus*) from Spain. Chemosphere 55**,** 577-583.

Gómez-Ramírez, P., Martínez-López, E., García-Fernández, A., Zweers, A., van den Brink, N. (2012). Organohalogen exposure in a Eurasian Eagle owl (*Bubo bubo*) population from Southeastern Spain: Temporal–spatial trends and risk assessment. Chemosphere 88**,** 903-911.

Grandjean, P. (1976). Possible effect of lead on egg-shell thickness in kestrels 1874–1974. Bulletin of Environmental Contamination and Toxicology 16**,** 101-106.

Grasman, K., Scanlon, P. (1995). Effects of acute lead ingestion and diet on antibody and T-cell-mediated immunity in Japanese quail. Archives of Environmental Contamination and Toxicology 28**,** 161-167.

Gravato, C., Santos, M. (2002). *In vitro* liver EROD activity inhibition by aromatic hydrocarbon-receptor agonists. Fresenius Environmental Bulletin 11**,** 342-346.

Halamíčková, A., Vorlová, L., Smutná, M., Svobodová, Z., Buchtová, H. (2003). Comparison of malondialdehyde concentrations in different muscle areas of tench *Tinca tinca* L. Fish Physiology and Biochemistry 29**,** 305-312.

Halliwell, B. (1987). Oxidants and human disease: some new concepts. The FASEB Journal 1**,** 358-364.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A. (2002). Herbicides: feminization of male frogs in the wild. Nature 419**,** 895-896.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A. (2003). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environmental Health Perspectives 111**,** 568-575.

Hayes, T.B., Khoury, V., Narayan, A., Nazir, M., Park, A., Brown, T., Adame, L., Chan, E., Buchholz, D., Stueve, T. (2010). Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proceedings of the National Academy of Sciences 107**,** 4612-4617.

Heath, D., Vandekar, M. (1964). Toxicity and metabolism of dieldrin in rats. British Journal of Industrial Medicine 21**,** 269-279.

Heinz G.H., Locke, L.N. (1976). Brain lesions in mallard ducklings from parents fed methylmercury. Avian Diseases 20**,** 9-17.

Henderson, R., Hobbie, J., Landrigan, P., Mattisoti, D., Perera, F., Pfttaer, E., Silbergeld, E., Wogan, G. (1987). Biological markers in environmental health research. Environmental Health Perspectives 7**,** 3-9.

Henny, C.J., Blus L.J., Hoffman, D.J., Grove, R.A. (1994). Lead in hawks, falcons and owls downstream from a mining site on the Coeur d'Alene River, Idaho. Environmental Monitoring and Assesments 29, 267-288.

Henny, C.J., Blus, L.J., Grove, R.A. (1990). Western grebe, *Aechmophorus occidentalis*, wintering biology and contaminant accumulation in Commencement Bay, Puget Sound, Washington (USA). Canadian Field-Naturalist 104, 460-472.

Henny, C.J., Blus, L.J., Hoffman, D.J., Grove, R.A., Hatfield, J.S. (1991). Lead accumulation and osprey production near a mining site on the Coeur d'Alene River, Idaho. Archives of Environmental Contamination and Toxicology 21**,** 415-424.

Henny, C.J., Meeker, D.L. (1981). An evaluation of blood plasma for monitoring DDE in birds of prey. Environmental Pollution Series A, Ecological and Biological 25**,** 291-304.

Henriksen, E.O., Gabrielsen, G.W., Utne Skaare, J. (1998). Validation of the use of blood samples to assess tissue concentrations of organochlorines in glaucous gulls, *Larus hyperboreus*. Chemosphere 37**,** 2627-2643.

Hernández-Moreno, D., Soler-Rodríguez, F., Míguez-Santiyán, M.P., Pérez-López, M. (2008). Hepatic monooxygenase (CYP1A and CYP3A) and UDPGT enzymatic activities as biomarkers for long-term carbofuran exposure in tench (*Tinca tinca* L). Journal of Environmental Science and Health Part B 43**,** 395-404.

Herrera, A., Arino, A., Conchello, P., Lazaro, R., Bayarri, S., Perez-Arquillue, C., Garrido, M., Jodral, M., Pozo, R. (1996). Estimates of mean daily intakes of persistent organochlorine pesticides from Spanish fatty foodstuffs. Bulletin of Environmental Contamination and Toxicology 56**,** 173-177.

Hill, E.F., Soares, J.H. (1984). Subchronic mercury exposure in coturnix and a method of hazard evaluation. Environmental Toxicology and Chemistry 3**,** 489-502.

Hinfray, N., Porcher, J.-M., Brion, F. (2006). Inhibition of rainbow trout (*Oncorhynchus mykiss*) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 144**,** 252-262.

Hoffman, D.J., Franson, J.C., Pattee, O.H., Bunck, C.M., Anderson, A. (1985). Survival, growth, and accumulation of ingested lead in nestling American kestrels (*Falco sparverius*). Archives of Environmental Contamination and Toxicology 14**,** 89-94.

Iwaniuk, A.N., Koperski, D.T., Cheng, K.M., Elliott, J.E., Smith, L.K., Wilson, L.K., Wylie, D.R. (2006). The effects of environmental exposure to DDT on the brain of a songbird: changes in structures associated with mating and song. Behavioural Brain Research 173**,** 1-10.

Janero, D.R. (1990). Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radical Biology and Medicine 9**,** 515-540.

Jiménez, B., Mori, G., Concejero, M.A., Merino, R., Casini, S., Fossi, M.C. (2007). Vitellogenin and zona radiata proteins as biomarkers of endocrine disruption in peregrine falcon (*Falco peregrinus*). Chemosphere 67**,** S375-S378.

Kendall, R., Scanlon, P. (1984). The toxicology of lead shot ingestion in ringed turtle doves under conditions of cold exposure. Journal of Environmental Pathology, Toxicology and

Oncology : Official Organ of the International Society for Environmental Toxicology and Cancer 5**,** 183-192.

Kendall, R.J., Lacker, T.E., Bunck, C., Daniel, B., Driver, C., Grue, C.E., Leighton, F., Stansley, W., Watanabe, P.G., Whitworth, M. (1996). An ecological risk assessment of lead shot exposure in non-waterfowl avian species: Upland game birds and raptors. Environmental Toxicology and Chemistry 15**,** 4-20.

Kendall, R.J., Veit, H.P., Scanlon, P.F. (1981). Histological effects and lead concentrations in tissues of adult male Ringed turtle doves that ingested lead shot. Journal of Toxicology and Environmental Health Part A 8**,** 649-658.

King, K.A., Krynitsky, A.J. (1986). Population trends, reproductive success, and organochlorine chemical contaminants in waterbirds nesting in Galveston Bay, Texas. Archives of Environmental Contamination and Toxicology 15**,** 367-376.

Kunisue, T., Minh, T.B., Fukuda, K. Watanabe, M., Tanabe, S., Titenko, A.M. (2002). Seasonal variation of persistent organochlorine accumulation in birds from Lake Baikal, Russia, and the role of the south Asian region as a source of pollution for wintering migrants. Environmental Science and Technology 1, 1396-1404.

Lanzarot, M.P., Barahona, M.V., Andres, M.I.S., Fernández-García, M., Rodríguez, C. (2005). Hematologic, protein electrophoresis, biochemistry, and cholinesterase values of free-living black stork nestlings (*Ciconia nigra*). Journal of Wildlife Diseases 41**,** 379-386.

Larson, J.M., Karasov, W.H., Sileo, L., Stromborg, K.L., Hanbidge, B.A., Giesy, J.P., Jones, P.D., Tillitt, D.E., Verbrugge, D.A. (1996). Reproductive success, developmental anomalies, and environmental contaminants in double-crested cormorants (*Phalacrocorax auritus*). Environmental Toxicology and Chemistry 15**,** 553-559.

Lech, J.J., Vodicnik, M.J. (1985). Biotransformation. En*:* Rand, G.M., Petrocelli, S.R. (eds.). Fundamentals of aquatic toxicology; methods and applications. Nueva York, Hemisphere Publishing Corporation.

Lim, C., Rideout, J., Peters, T. (1984). High-performance liquid chromatography of dicarboxylic porphyrins and metalloporphyrins: retention behaviour and biomedical applications. Journal of Chromatography A 317**,** 333-341.

Lundholm, C.D. (1997). DDE-induced eggshell thinning in birds: effects of *p,p'*-DDE on the calcium and prostaglandin metabolism of the eggshell gland. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 118**,** 113-128.

Machlin, L.J., Bendich, A. (1987). Free radical tissue damage: protective role of antioxidant nutrients. The FASEB Journal 1**,** 441-445.

Martínez, M. (2010). Contaminación por perdigones de plomo en humedales y evaluación de los aportes de grit como medida para reducir el plumbismo en aves acuáticas. Tesis doctoral, Universidad de Castilla la Mancha.

Martínez-López, E., María-Mojica, P., Martinez, J.E., Calvo, J.F., Romero, D., García-Fernández, A.J. (2005). Cadmium in feathers of adults and blood of nestlings of three raptor species from a nonpolluted Mediterranean forest, southeastern Spain. Bulletin of Environmental Contamination and Toxicology 74**,** 477-484.

Martínez-López, E., Romero, D., María-Mojica, P., Martínez, J.E., Calvo, J.F., García-Fernández, A.J. (2009). Changes in blood pesticide levels in booted eagle (*Hieraaetus pennatus*) associated with agricultural land practices. Ecotoxicology and Environmental Safety 72**,** 45-50.

McCarthy, J.F., Halbrook, R.S., Shugart, L.R. (1991). Conceptual strategy for design, implementation, and validation of a biomarker-based biomonitoring capability. Oak Ridge National Lab., TN (USA).

Merian, E. (1991). Metals and their compounds in the environment: occurrence, analysis and biological relevance, VCH Verlagsgesellschaft mbH.

Miller, K., Addison, R., Bandiera, S. (2004). Hepatic CYP1A levels and EROD activity in English sole: biomonitoring of marine contaminants in Vancouver Harbour. Marine Environmental Research 57**,** 37-54.

Mora, M.A. (1997). Transboundary pollution: persistent organochlorine pesticides in migrant birds of the Southwestern United States and Mexico. Environmental Toxicology and Chemistry 16**,** 3-11.

Moreno, M.D. (2003). Toxicología ambiental. Evaluación del riesgo para la salud humana. Madrid, McGraw-Hill.

Mundy, P.J., Grant, K.I., Tannock, J., Wessels, C.L. (1982). Pesticide residues and eggshell thickness of griffon vulture eggs in Southern Africa. The Journal of Wildlife Management 46**,** 769-773.

Navas, J.M., Segner, H. (1998). Antiestrogenic activity of anthropogenic and natural chemicals. Environmental Science and Pollution Research 5**,** 75-82.

Nebbia, C. (2001). Biotransformation enzymes as determinants of xenobiotic toxicity in domestic animals. The Veterinary Journal 161**,** 238-252.

Nebeker, A.V., Griffis, W.L., Stutzman, T.W., Schuytema, G.S., Carey, L.A., Scherer, S.M. (1992). Effects of aqueous and dietary exposure of dieldrin on survival, growth and bioconcentration in mallard ducklings. Environmental Toxicology and Chemistry 11**,** 687-699.

Needham, L.L., Calafat, A.M., Barr, D.B. (2007). Uses and issues of biomonitoring. International Journal of Hygiene and Environmental Health 210**,** 229-238.

Nelson, D.R. (1999). Cytochrome P450 and the individuality of species. Archives of Biochemistry and Biophysics 369**,** 1-10.

Nriagu, J.O. (1989). A global assessment of natural sources of atmospheric trace metals. Nature 338**,** 47-49.

Nriagu, J.O., Pacyna, J.M. (1988). Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature 333**,** 134-139.

O'Hara, T.M., Rice, C.D. (1996). Polychlorinated biphenyls. En*:* Fairbrother, A., Locke, L.N. Hoff, G.L. (eds.). Noninfectious diseases of wildlife (2nd edition). USA, Iowa State University Press.

Ohlendrof, H.M., Heinz, G.H. (2011). Selenium in birds. En*:* Beyer, W.N., Meador, P.M. (eds.) Environmental contaminants in biota (2nd edition). Nueva York, Taylor and Francis.

Pain, D., Amiardtriquet, C. (1993). Lead poisoning of raptors in France and elsewhere. Ecotoxicology and Environmental Safety 25**,** 183-192.

Pain, D.J. (1995). Lead in the environment. En*:* Hoffman, D.J., Rattner, B.A., Burton, G.A.J. Cairns, J.J. (eds.). Handbook of Ecotoxicology. USA, CRC Press.

Pain, D.J. (1996). Lead in waterfowl. En*:* Beyer, W.N., Heinz, G.H., Redmon-Norwood, A.W. (eds.). Environmental contaminants in wildlife. USA, CRC Press.

Pattee, O.H. (1984). Eggshell thickness and reproduction in American kestrels exposed to chronic dietary lead. Archives of Environmental Contamination and Toxicology 13**,** 29-34.

Pattee, O.H., Wiemeyer, S.N., Mulhern, B.M., Sileo, L., Carpenter, J.W. (1981). Experimental lead-shot poisoning in bald eagles. The Journal of Wildlife Management 45**,** 806-810.

Peakall, D.B., Peakall, M.L. (1973). Effect of a polychlorinated biphenyl on the reproduction of artificially and naturally incubated dove eggs. Journal of Applied Ecology 10**,** 863-868.

Pérez-López, M., Nóvoa-Valiñas, M.C., Melgar-Riol, M.J. (2002). Glutathione S-transferase cytosolic isoforms as biomarkers of polychlorinated biphenyl (Arochlor-1254) experimental contamination in rainbow trout. Toxicology Letters 136**,** 97-106.

Puschner, B., Judy, L.S., Galey, F.D. (1999). Normal and toxic zinc concentrations in serum/plasma and liver of psittacines with respect to genus differences. Journal of Veterinary Diagnostic Investigation 11**,** 522-527.

Puzanová, L. (1980). Teratogenic effect of different toxic elements on young chick embryos. Folia Morphologica 28**,** 354-356.

Real Decreto 581/2001. Real Decreto 581/2001, de 1 de junio, por el que en determinadas zonas húmedas se prohíbe la tenencia y el uso de municiones que contengan plomo para el ejercicio de la caza y el tiro deportivo. Boletín Oficial del Estado nº 143, de 15 de junio de 2001, pp 21284-21285.

Real Decreto 785/2001. Real Decreto 785/2001, de 6 de julio, por el que se adelanta la prohibición de comercialización de las gasolinas con plomo y se establecen las

especificaciones de las gasolinas que sustituirán a aquéllas. Boletín Oficial del Estado nº 162, de 7 de julio de 2001, pp 24774-24776.

Redig, P.T., Lawler, E.M., Schwartz, S., Dunnette, J.L., Stephenson, B., Duke, G.E. (1991). Effects of chronic exposure to sublethal concentrations of lead acetate on heme synthesis and immune function in red-tailed hawks. Archives of Environmental Contamination and Toxicology 21**,** 72-77.

Reed, D.J. (1990). Glutathione: toxicological implications. Annual Review of Pharmacology and Toxicology 30**,** 603-631.

Ritchie, B.W., Harrison, G.J., Harrison, L.R., Zantop, D.W. (1994). Avian medicine: principles and application. Florida, Wingers Publishing.

Rivera-Rodríguez, L.B., Rodríguez-Estrella, R., Ellington, J.J., Evans, J.J. (2007). Quantification of low levels of organochlorine pesticides using small volumes ($\leq 100\,\mu l$) of plasma of wild birds through gas chromatography negative chemical ionization mass spectrometry. Environmental Pollution 148**,** 654-662.

Roscales, J.L., González-Solís, J., Muñoz-Arnanz, J., Jiménez, B. (2011). Geographic and trophic patterns of OCs in pelagic seabirds from the NE Atlantic and the Mediterranean: A multi-species/multi-locality approach. Chemosphere 85**,** 432-440.

Ross, M.S., Verreault, J., Letcher, R.J., Gabrielsen, G.W., Wong, C.S. (2008). Chiral organochlorine contaminants in blood and eggs of glaucous gulls (*Larus hyperboreus*) from the Norwegian Arctic. Environmental Science and Technology 42**,** 7181-7186.

Ryan, M., Stern, G., Kidd, K., Croft, M., Gewurtz, S., Diamond, M., Kinnear, L., Roach, P. (2013). Biotic interactions in temporal trends (1992-2010) of organochlorine contaminants in the aquatic food web of Lake Laberge, Yukon Territory. Science of the Total Environment 443**,** 80-92.

Sáez, M., Aguirre, J.I., Blázquez, E., Jiménez, B. (2008). Organochlorines in White stork (*Ciconia ciconia*): a comparison of levels in eggs and nestlings. Organohalogen Compounds 70**,** 1133-1136.

Sáez, M., Aguirre, J.I., Jiménez, B. (2009). Organochlorines in plasma of nestlings and adults White stork (*Ciconia ciconia*) from Madrid, Spain. Organohalogen Compounds 71**,** 2915-2918.

Sanderson, J.T., Letcher, R.J., Heneweer, M., Giesy, J., Van den Berg, M. (2001). Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environmental Health Perspectives 109**,** 1027-1031.

Sayed, A.E.-D.H., Mahmoud, U.M., Mekkawy, I.A. (2012). Reproductive biomarkers to identify endocrine disruption in Clarias gariepinus exposed to 4-nonylphenol. Ecotoxicology and Environmental Safety 78**,** 310-319.

Scheuhammer, A. (1987). The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: a review. Environmental Pollution 46**,** 263-295.

Schreiber, E.A., Otter, R.R., van den Hurk, P. (2006). A biomarker approach to measure biological effects of contaminant exposure in largemouth bass from Lake Conestee, South Carolina, USA. Environmental Toxicology and Chemistry 25**,** 1926-1932.

Sonne, C., Bustnes, J.O., Herzke, D., Jaspers, V.L., Covaci, A., Eulaers, I., Halley, D.J., Moum, T., Ballesteros, M., Eens, M. (2012). Blood plasma clinical-chemical parameters as biomarker endpoints for organohalogen contaminant exposure in Norwegian raptor nestlings. Ecotoxicology and Environmental Safety 80**,** 76-83.

Sonne, C., Bustnes, J.O., Herzke, D., Jaspers, V.L., Covaci, A., Halley, D.J., Moum, T., Eulaers, I., Eens, M., Ims, R.A. (2010). Relationships between organohalogen contaminants and blood plasma clinical-chemical parameters in chicks of three raptor species from Northern Norway. Ecotoxicology and Environmental Safety 73**,** 7-17.

Spahn, S.A., Sherry, T.W. (1999). Cadmium and lead exposure associated with reduced growth rates, poorer fledging success of Little Blue Heron chicks (*Egretta caerulea*) in South Louisiana wetlands. Archives of Environmental Contamination and Toxicology 37**,** 377-384.

Spalding, M.G., Frederick, P.C., McGill, H.C., Bouton, S.N., Richey, L.J., Schumacher, I.M., Blackmore, C.G., Harrison, J. (2000). Histologic, neurologic, and immunologic effects of methylmercury in captive great egrets. Journal of Wildlife Diseases 36**,** 423-435.

Spallholz, J.E., Hoffman, D.J. (2002). Selenium toxicity: cause and effects in aquatic birds. Aquatic Toxicology 57**,** 27-37.

Spanò, L., Tyler, C.R., Aerle, R.V., Devos, P., Mandiki, S., Silvestre, F., Thomé, J.-P., Kestemont, P. (2004). Effects of atrazine on sex steroid dynamics, plasma vitellogenin concentration and gonad development in adult goldfish (*Carassius auratus*). Aquatic Toxicology 66**,** 369-379.

Stickel, W., Stickel, L., Dyrland, R., Hughes, D. (1984). DDE in birds: Lethal residues and loss rates. Archives of Environmental Contamination and Toxicology 13**,** 1-6.

Świergosz, R., Kowalska, A. (2000). Cadmium accumulation and its effects in growing pheasants *Phasianus colchicus* (L.). Environmental Toxicology and Chemistry 19**,** 2742-2750.

Tanabe, S., Senthilkumar, K., Kannan, K., Subramanian, A. (1998). Accumulation features of polychlorinated biphenyls and organochlorine pesticides in resident and migratory birds from South India. Archives of Environmental Contamination and Toxicology 34**,** 387-397.

Tseng, W.-P. (1977). Effects and dose-response relationships of skin cancer and blackfoot disease with arsenic. Environmental Health Perspectives 19**,** 109-119.

U.S. EPA. United States Environmental Protection Agency (2013). Finalization of Atrazine IRED, and Completion of Tolerance Reassessment and Reregistration Eligibility Process (April 6, 2006). Science Chapter for Atrazine: Environmental Fate and Effects. Office of Prevention, Pesticides and Toxic Substances,Washington, DC.

U.S. EPA. United States Environmental Protection Agency (2013). Priority pollutants. http://water.epa.gov/scitech/methods/cwa/pollutants.cfm. Consulta 08-04-2014.

Van den Steen, E., Jaspers, V.L., Covaci, A., Neels, H., Eens, M., Pinxten, R. (2009). Maternal transfer of organochlorines and brominated flame retardants in blue tits (*Cyanistes caeruleus*). Environment International 35**,** 69-75.

Van der Oost, R., Beyer, J., Vermeulen, N.P. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13**,** 57-149.

Van der Oost, R., Goksøyr, A., Celander, M., Heida, H., Vermeulen, N.P. (1996). Biomonitoring of aquatic pollution with feral eel (*Anguilla anguilla*) II. Biomarkers: pollution-induced biochemical responses. Aquatic Toxicology 36**,** 189-222.

Van Gestel, C., Van Brummelen, T. (1996). Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. Ecotoxicology 5**,** 217-225.

Van Wyk, E., Bouwman, H., van der Bank, H., Verdoorn, G.H., Hofmann, D. (2001). Persistent organochlorine pesticides detected in blood and tissue samples of vultures from different localities in South Africa. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 129**,** 243-264.

Veit, H.P., Kendall, R.J., Scanlon, P.F. (1983). The effect of lead shot ingestion on the testes of adult Ringed turtle doves (*Streptopelia risoria*). Avian Diseases 27**,** 442-452.

Verreault, J., Skaare, J.U., Jenssen, B.M., Gabrielsen, G.W. (2004). Effects of organochlorine contaminants on thyroid hormone levels in Arctic breeding glaucous gulls, *Larus hyperboreus*. Environmental Health Perspectives 112**,** 532-537.

Vos, J., Van der Maas, H., Musch, A., Ram, E. (1971). Toxicity of hexachlorobenzene in Japanese quail with special reference to porphyria, liver damage, reproduction, and tissue residues. Toxicology and Applied Pharmacology 18**,** 944-957.

W.H.O./I.P.C.S. (1993). Biomarkers and risk assessment: concepts and principles. International programme on Chemical Safety, World Health Organization. Ginebra, Suiza.

W.H.O./I.P.C.S. (2002). IPCS global assessment of the state-of-the-science of endocrine disruptors. International Programme on Chemical Safety, World Health Organization. Ginebra, Suiza.

Wayland, M., Scheuhammer, A.M. (2011). Cadmium in birds. En*:* Beyer, W.N., Meador, P.M. (eds.). Environmental Contaminants in Biota (2nd edition). Nueva York, Taylor and Francis.

Wiegand, C., Krause, E., Steinberg, C., Pflugmacher, S. (2001). Toxicokinetics of atrazine in embryos of the Zebrafish (*Danio rerio*). Ecotoxicology and Environmental Safety 49**,** 199-205.

Wiemeyer, S.N. (1996). Other organochlorine pesticides in birds. En*:* Beyer, W.N., Heinz, G.H., Redmon-Norwood, A.W. (eds.). Environmental Contaminants in Wildlife. Florida, CRC Press.

Wilhelms, K.W., Cutler, S.A., Proudman, J.A., Anderson, L.L., Scanes, C.G. (2005). Atrazine and the hypothalamo-pituitary-gonadal axis in sexually maturing precocial birds: studies in male Japanese quail. Toxicological Sciences 86**,** 152-160.

Wilhelms, K.W., Cutler, S.A., Proudman, J.A., Carsia, R.V., Anderson, L.L., Scanes, C.G. (2006a). Lack of effects of atrazine on estrogen-responsive organs and circulating hormone concentrations in sexually immature female Japanese quail (*Coturnix coturnix japonica*). Chemosphere 65, 674-681.

Wilhelms, K.W., Fitzpatrick, K.F., Scanes, C.G., Anderson, L.L. (2006b). *In ovo* exposure to a triazine herbicide: effects of atrazine on circulating reproductive hormones and gonadal histology in young Japanese quail. Archives of Environmental Contamination and Toxicology 51, 117-122.

Yasui, T., Kim, E. Y., Iwata, H., Franks, D. G., Karchner, S. I., Hahn, M. E., Tanabe, S. (2007). Functional characterization and evolutionary history of two aryl hydrocarbon receptor isoforms (AhR1 and AhR2) from avian species. Toxicological Sciences 99, 101-117.

Zelikoff, J.T., Wang, W., Islam, N., Twerdok, L.E., Curry, M., Beaman, J., Flescher, E. (1996). Assays of reactive oxygen intermediates and antioxidant enzymes: potential biomarkers for predicting the effects of environmental pollution. Techniques in Aquatic Toxicology 1**,** 287-306.

# OBJETIVOS

El objetivo general de la presente Tesis Doctoral es la evaluación de la exposición a clorotriazinas, metales pesados y compuestos clorados en aves, así como la determinación de una batería de biomarcadores de la exposición a estos contaminantes.

De esta forma, la Tesis está dividida en dos partes. La primera parte está centrada en los efectos producidos por las clorotriazinas en codorniz, prestando especial atención a los biomarcadores de alteración endocrina.

La segunda parte de la Tesis se centra en un estudio de biomonitorización de metales y compuestos clorados en sangre de pollos de cigüeña blanca.

Para cada una de las partes, y sus capítulos correspondientes, se han determinados los siguientes objetivos específicos:

**Parte 1. Efecto de los herbicidas triazínicos en ensayos *in vitro* en cultivos celulares e *in vivo* en codorniz (*Coturnix coturnix*)**

*Capítulo I. Las clorotriazinas no activan el receptor de hidrocarburos aromáticos, el receptor de estrógenos y el receptor de hormona tiroidea en ensayos in vitro (de la Casa-Resino et al., 2014).*

Realizar una serie de ensayos *in vitro* para evaluar si la atrazina (ATZ), la simazina (SIM), la prometrina (PRT) y la propazina (PRZ), así como los metabolitos de la atrazina, desetil-triazina (DEA) y deisopropil-triazina (DIA), causan transactivación del receptor de hidrocarburos aromáticos (AhR), del receptor de estrógenos (ER) o del receptor de hormona tiroidea (TR).

*Capítulo II. Alteración endocrina causada por la administración oral de atrazina en codorniz (Coturnix coturnix) (de la Casa-Resino et al., 2012).*

Determinar los posibles efectos de la atrazina, tras su administración intermitente a dosis controladas, en los niveles hormonales que controlan la reproducción en codorniz. A su vez, evaluar los efectos de este herbicida a nivel transcripcional y a nivel enzimático sobre el CYP1A.

*Capítulo III. Biomarcadores no destructivos en codornices (Coturnix coturnix) expuestas al herbicida atrazina (de la Casa-Resino et al., 2013).*

Estudiar una batería de biomarcadores no destructivos de la exposición a ATZ en codorniz. Evaluar el efecto del herbicida sobre los niveles de porfirinas fecales. A su vez, estudiar el efecto del mismo sobre los niveles de antioxidantes endógenos, glutatión reducido (GSH) y las actividades enzimáticas glutatión reductasa (GR) y glutatión S-transferesa (GST), así como sobre las membranas celulares, en función de los niveles de malondialdehído (MDA).

**Parte 2. Estudio de biomarcadores de estrés oxidativo, metales y contaminantes orgánicos persistentes en sangre de pollos de cigüeña blanca (*Ciconia ciconia*)**

*Capítulo IV. La cría cerca de una planta de tratamiento de residuos sólidos urbanos puede influir en los niveles sanguíneos de metales (Cd, Pb, Hg, Fe, Zn) y metaloides (Se, As) en los pollos de cigüeña blanca (Ciconia ciconia).*

Evaluar la exposición a metales (Cd, Pb, Hg, Fe, y Zn) y metaloides (Se y As) en pollos de cigüeña blanca procedentes de tres colonias diferentes en Extremadura, representativas de tres influencias antropogénicas diferentes: dehesa, planta de tratamiento de residuos sólidos urbanos (PTRSU) y agricultura intensiva. Estudiar cómo influye el ambiente donde los progenitores se alimentan en los niveles sanguíneos de estos elementos inorgánicos en las crías.

*Capítulo V. Biomarcadores de estrés oxidativo asociados a la contaminación por metales en sangre de cigüeña blanca (Ciconia ciconia) en España.*

Evaluar la exposición a metales (Pb, As y Hg) en sangre de pollos de cigüeña blanca procedentes de dos ambientes diferentes en Extremadura. Estudiar los efectos de la exposición a dichos metales sobre los niveles de biomarcadores de estrés oxidativo, incluyendo el glutatión reducido (GSH), la actividad enzimática glutatión S-transferasa (GST) y el malondialdehído (MDA).

*Capítulo VI. Contaminantes clorados en sangre de pollos de cigüeña blanca (Ciconia ciconia) procedentes de diferentes colonias en España.*

Evaluar la exposición a compuestos orgánicos persistentes, bifenilos policlorados (PCBs) y pesticidas organoclorados (OCPs) en pollos de cigüeña blanca procedentes de tres

colonias diferentes en Extremadura, representativas de tres influencias antropogénicas diferentes: dehesa, planta de tratamiento de residuos sólidos urbanos (PTRSU) y agricultura intensiva.. Estudiar cómo influye el ambiente donde los progenitores se alimentan en los niveles sanguíneos de estos contaminantes en las crías.

# CAPÍTULO I

## Las clorotriazinas no activan el receptor de hidrocarburos aromáticos, el receptor de estrógenos y el receptor de hormona tiroidea en ensayos *in vitro*.

*de la Casa-Resino, I., Navas, J.M., Fernández-Cruz, M.L. (2014). Chlorotriazines do not activate the aryl hydrocarbon receptor, the estrogen receptor and thyroid receptor in in vitro assays. Alternative to Laboratory Animals 42:1-6.*

**RESUMEN:** Las clorotriazinas atrazina (ATZ), prometrina, propazina y simazina son herbicidas ampliamente utilizados. Sin embargo, su aplicación ha sido causa de amplio debate debido a sus efectos como disruptores endocrinos en distintos organismos. Los estudios llevados a cabo para elucidar los efectos de estos pesticidas a nivel hormonal y celular son controvertidos. En el presente estudio, se ha evaluado la habilidad de estas clorotriazinas y de dos metabolitos de la ATZ, desetil-s-clorotriazina y deisopropil-s-clorotriazina para producir una respuesta mediada a través del receptor de estrógenos (Er), el receptor de hidrocarburos aromáticos (Ahr) y el receptor de hormonas tiroideas (Tr) en distintos modelos celulares *in vitro*. Se realizaron ensayos para evaluar la activación transcripcional del Er y el Tr. La inducción de la actividad etoxiresorrufina-O-deetilasa (EROD) en las células RTG-2 se utilizó como indicador de la activación del AhR. No se obtuvo respuesta en ninguno de los ensayos realizados con ninguna de las seis clorotriazinas analizadas. Estos resultados indican que no es probable que dichas clorotriazinas causen su efecto endocrino a través de estos receptores.

**Palabras clave**: receptor de hidrocarburos aromáticos, clorotriazinas, disrupción endocrina, receptor de estrógenos, receptor tiroideo.

**ABSTRACT:** Atrazine, prometryn, propazine and simazine are chlorotriazines that are commonly employed as herbicides. However, their use is a major cause of concern, due to their reported endocrine disrupting effects in different taxa. Data from studies on the molecular and cellular processes underlying the hormonal action of these substances are contradictory. In the present article, the ability of these chlorotriazinesand the atrazine metabolites, desethyl-s-chlorotriazine and desisopropyl-s-chlorotriazine, to trigger responses mediated by the oestrogen receptor (Er), aryl hydrocarbon receptor (Ahr) and thyroid receptor (Tr), was studied by using in vitro approaches. Transcriptional activation assays were applied to observe the activation of Er and Tr. The induction of ethoxyresorufin-O-deethylase (EROD) activity in the RTG-2 cell line served as an indicator of Ahr activation. No responses were found in any of the assays, with any of the six chlorotriazines tested. Our observations indicate that the chlorotriazines tested are unlikely to cause their endocrine effects via these receptors.

**Keywords:** aryl hydrocarbon receptor, atrazine, chlorotriazines, endocrine disruption, oestrogen receptor, thyroid receptor.

## 1. INTRODUCTION

Chlorotriazines (Figure 1), such as atrazine (ATZ), prometryn (PRT), propazine (PRZ) and simazine (SIM), are widely-used herbicides that have been excluded in the EU from the registration process for use on crops. However, they are still registered in the USA, and ATZ is the most frequently used herbicide in the country. These chlorotriazines can provoke alterations to the endocrine function of organisms, and are therefore considered to be endocrine disrupters (EDs). Hayes et al. (2010) have reported oestrogenic effects in the African clawed frog (*Xenopus laevis*), such as feminisation and chemical castration, following exposure to ATZ. In addition, ATZ provoked changes in the reproductive organs of the Japanese quail (*Coturnix coturnix japonica*; Wilhelms *et al.*, 2006), and altered the plasma levels of 17β-oestradiol (E2) and vitellogenin in the common quail (*Coturnix coturnix coturnix*) at environmentally relevant concentrations (de la Casa-Resino *et al.*, 2012). Although ATZ and SIM do not structurally resemble steroid hormones, it has been suggested that these chlorotriazines alter oestrogen receptor (Er) activity and expression in rats, by acting as Er agonists or antagonists (Eldridge *et al.*, 1994). However, contradictory results have been obtained in a number of studies on the interaction of ATZ, SIM and/or PRT with the Er (Tennant *et al.*, 1994; Connor *et al.*, 1996; Tran *et al.*, 1996; Graumann *et al.*, 1999). In contrast to oestrogenic substances, certain other chemicals are able to elicit anti-oestrogenic effects (i.e. they block the action of oestrogens in target cells or tissues). Some of these chemicals mediate their anti-oestrogenic activity through the aryl hydrocarbon receptor (Ahr). In mammals, ATZ is initially metabolised in the liver by cytochrome P450 (CYP) 1A (CYP1A; Hanioka *et al.*, 1998). CYP1A expression is dependent on Ahr activation. The possible effect of ATZ on the Ahr is the subject of intense debate. Thyroid hormones play a key role in regulating development and maintaining the homeostasis of physiological functions in organisms. No alterations in the plasma levels of triiodothyronine, thyroxine or thyroid stimulating hormone were observed in rats exposed to ATZ (Laws *et al.*, 2000; Stoker *et al.*, 2002). However, no information is available with regard to the effects of other chlorotriazines on thyroid function. Given these contradictory results and the lack of information on any chlorotriazines other than ATZ, the present study employed an array of *in vitro* bioassays to assess whether ATZ, SIM, PRZ, PRT and the ATZ metabolites, desethyl-s-chloro-triazine (DEA) and desisopropyl-s-chlorotriazine (DIA; Figure 1), might cause the transactivation of the Ahr, the Er or the thyroid receptor (Tr).

## 2. MATERIAL AND METHODS

### 2.1 Chemicals

ATZ (6-chloro-4-N-ethyl-2-N-propan-2-yl-1,3,5-triazine- 2,4-diamine), SIM (6-chloro-N,N´-diethyl-1,3,5-triazine-2,4-diamine), PRZ (6-chloro-2-N,4-N-di[propan-2-yl]-1,3,5-triazine-2,4-diamine) and PRT (6-methylsulfanyl-2-N,4-N,di[propan-2-yl]-1,3,5-triazine-2,4-diamine), β-naphthoflavone, E2 and 5-triiodo-L-thyronine (T3), were purchased from Sigma-Aldrich (Madrid, Spain). Stock solutions of these compounds were prepared in dimethyl sulphoxide (DMSO≥ 99.9% purity; Sigma -Aldrich). The stock solutions were diluted in culture medium at a maximum solvent concentration of 0.3% (v/v), which was shown to have no effect on the assays. Ethoxyresorufin, resorufin, nicotinamide adenine dinucleotide phosphate reduced (NADPH), bovine serum albumin (BSA) and fluorescamine were from Sigma-Aldrich (Madrid, Spain). L-Glutamine (200 mM), Ultra-Glutamine, fetal bovine and horse serums (FBS and FHS, respectively), penicillin–streptomycin (10,000 U/ml), hygromycin B, geneticin, non-essential aminoacids 100× (NEAA), Eagle's Minimum Essential Medium (EMEM) and Dulbecco's Minimal Essential Medium (DMEM), were purchased from Lonza (Barcelona, Spain).

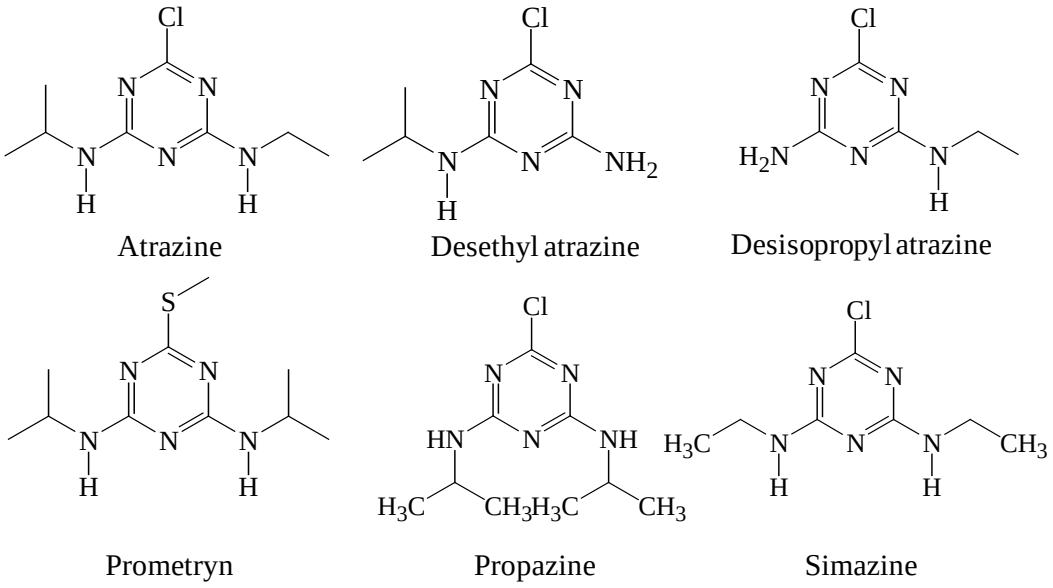



**Figure 1.** Molecular structures of the chlorotriazines used in this study.

### 2.2 Cell lines culture and exposure

Three cell lines were used for the exposure assays. The fibroblast-like RTG-2 cell line, isolated from rainbow trout (*Oncorhynchus mykiss*) gonads, was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in 96-well plates (Costar, VWR, Spain), at a density of 2.5 × 104 cells/well, at 20ºC and with 5% v/v $CO_2$. The medium used was EMEM supplemented with 1% penicillin/streptomycin, 10% FBS, 1% L-glutamine and 1% NEAA. Cells of the HERLUC cell line (HEK-293 [human embryonic kidney] cell line, stably expressing the sea bass oestrogen receptor sbERα, and cotransfected with a construct containing the luciferase gene under the control of oestrogen-responsive elements), generated as described in Quesada et al. (2012), were cultured in 96-well plates at a density of 25 × 104 cells/well in a 5% $CO_2$ humidified atmosphere at 37°C. The culture medium for this cell line was DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 2% UltraGlutamine. The PC-DR-LUC cell line, derived from PC-12 (rat adrenal pheochromocytoma) cells stably expressing the avian thyroid receptor avTRα1, and transfected with a gene encoding luciferase as reporter gene, was a kind gift from Dr. Juan Bernal (CSIC, Madrid, Spain). The cells were cultured in 96-well plates at a density of 25 × 104 cells/well, in 5% $CO_2$ and at 37°C. The medium for PC-DR-LUC cells consisted of DMEM with 4.5 g/L glucose, 15% serum (10% FHS + 5% FCS), 1% penicillin-streptomycin, 1mM UltraGlutamine, 0.8 mg/ml geneticin and 0.8 mg/ml hygromycin B. In all cases, the cells were exposed for 24 hours to serially doubling dilutions of the six compounds under investigation. The exposure concentrations ranged from 0.78 to 100 μM. Control cells received the maximal vehicle (DMSO) concentration that was used with the exposed cells (0.3% v/v). β-Naphthoflavone (0.25 μM), E2 (1 μM) and T3 (1 nM) were included in each assay as positive controls, since these compounds are potent inducers of the Ahr, Er and Tr, respectively. Three independent experiments were performed for each compound in the different cell lines. For each experiment, each concentration of the test or control chemicals was applied in triplicate.

### 2.3 Ethoxyresorrufin-O-deethylase (EROD) activity

The possible activation of the Ahr, which elicits a signaling cascade resulting in CYP1A induction, was assessed in RTG-2 cells through measurements of EROD activity, a standard assay for detecting CYP1A activity. EROD activity and protein content measurements were performed as described in Fernández-Cruz *et al*. (2011). After 24 hours of treatment, the medium was removed, the cells were washed with phosphate buffered saline at pH 7.5 (PBS), and the entire plates were flash-frozen in liquid

nitrogen. The plates were stored at -80ºC for approximately 1 hour before EROD activity and protein content were determined. Briefly, to measure EROD activity, 75 µl of a 2.5 µM ethoxiresorufin solution was added to each well and the reaction triggered with the addition of 12.5 µl of a NADPH solution (1.2 mg/ml in PBS). Fluorescence measurements, taken at the start (time 0) and at 10-minute intervals for 30 minutes, were performed in a Tecan Genios microplate reader (Tecan Group Ltd, Männedorf, Switzerland) at 230 nm excitation and 590 nm emission wavelengths. The amount of resorufin generated was calculated by interpolating the fluorescence readouts in a resorufin standard curve. Protein content was determined by adding 75 µl of a fluorescamine solution (0.15 mg fluorescamine/ml acetonitrile) and measuring fluorescence at 360 nm excitation and 590 nm emission wavelengths. A BSA curve was used as standard to measure the protein content.

### 2.4 Er and Tr activation

To study the activation of the Er and the Tr, HERLUC and PC-DR-LUC cell lines were used, respectively. The measurements of transcriptional activation based on luminescence readouts for these two cell lines were performed according to the protocol of Quesada *et al*. (2012). Luciferase activity was measured by using a luciferase reporter gene assay kit (Biodetection Systems, Amsterdam, The Netherlands) according to the manufacturer's instructions, with minor modifications. Briefly, 90 µl or 120 µl of PBS (for the HER-LUC or the PCDR-LUC cell the lysis buffer were added to each well of the multiwell plate. After 15 minutes, 80 µl (Hlines, respectively) and 30 µl of ER-LUC) or 50 µl (PC-DR-LUC) of the luciferase reagent was added and bioluminescence was measured in the culture plates with a luminometer (MicroBeta Trilux; Perkin Elmer, Waltham, MA, USA).

### 2.5 Statistical Analysis

Statistical analysis was performed with Sigma Plot, version 12.0 (Jandel Scientific, San Rafael, CA, USA). Significant differences among treatments, with respect to the control, were determined by one-way repeated measures analysis of variance (RM ANOVA, $P<0.05$) followed by the Dunnett's test. Previously, the normality (Shapiro-Wilk test, $P<0.05$) and variance ($P<0.05$) of the distribution were tested.

## 3. RESULTS AND DISCUSSION

Although the expected results were obtained with the positive controls in each the in vitro assays (Table 1), no significant responses were observed in these assays with any of the six chlorotriazines and metabolites tested (i.e. ATZ, PRT, PRZ, SIM, DIA and DEA; data not shown). This indicates that none of these compounds exert endocrine disrupting effects observed *in vivo* through their interaction with the Ahr, Er or Tr. However, the results reported in the literature with regard to the activation of the Er by chlorotriazines are contradictory. Tennant *et al*. (1994) found that the interaction of ATZ with Er was weak, and appeared only at extremely high concentrations. Tran *et al*. (1996) reported that ATZ and SIM inhibited an oestrogen-induced response in yeast cells stably transfected with the Er and a reporter gene. However, these data could not be replicated by Graumann *et al*. (1999), who reported no inhibitory effects of ATZ on E2-mediated transactivation in yeast. Moreover, Connor *et al*. (1996) reported no oestrogenic or anti-oestrogenic activity of ATZ and SIM in the MCF-7 cell line. Recent *in vitro* studies have also concluded that ATZ, SIM, PRT and their metabolites do not interact with the Er (Oh *et al.*, 2003; Roberge *et al.*, 2004; Kojima *et al.*, 2010). None of the chlorotriazines tested in the present study -i.e. ATZ, SIM, PRZ and PRT, and the ATZ metabolites, DEA and DIA- were able to activate the Er. These results confirm previous observations, suggesting a lack of activity of chlorotriazines on the Er. This led us to conclude that chlorotriazines are unlikely to provoke alterations in the oestrogen system through direct interaction with the Er, as had previously been reported by a number of authors. A variety of substances, such as aromatase inhibitors, retinoids, peroxisomal proliferators, progestins, vitamin D3 analogues, and halogenated aromatic hydrocarbons, can also modulate oestrogenic responses via mechanisms that do not involve the Er. Recently, based on results obtained with fish and mammalian cells, it was hypothesised that ATZ might be able to cause an increase in the expression of the CYP19A1 gene (Suzawa and Ingraham, 2008), which encodes aromatase, i.e. the enzyme that converts testosterone to E2. This is a target gene of the nuclear receptor NR5A steroidogenic factor (SF-1). Suzawa and Ingraham (2008) proposed that ATZ leads to a stimulatory effect on this receptor that was not mediated by direct interaction with the receptor, but by other mechanisms such as receptor phosphorylation. It has been suggested that chlorotriazines may alter hepatic metabolism, by influencing the expression and activity of detoxifying activities, such as those of CYP1A, although contradictory results have been obtained with the rainbow trout and the common carp. Salaberria *et al*. (2009) reported a decrease in basal CYP1A gene expression after intraperitoneal injection of ATZ in rainbow trout, while Chang *et al*. (2005) found an increase after exposure of the common carp to ATZ. A study of chlorotriazine metabolism in rat liver microsomes found a significant

correlation between EROD activity and the formation of ATZ metabolites, indicating that EROD might play a role in ATZ metabolism, and therefore might possibly modulate ATZ-mediated toxic effects (Hanioka *et al*., 1998). However, other studies found that ATZ did not alter hepatic CYP1A activity in rainbow trout (Egaas *et al*., 1993), nor EROD activity in birds (de la Casa-Resino *et al*., 2012) and ranid frogs when exposed in the wild (Murphy *et al*., 2006). Since this CYP is induced through activation of the Ahr, it is essential to clarify the possible interaction of ATZ and other chlorotriazines with this receptor. In this study, none of the chorotriazines tested were able to induce EROD activity, indicating that the Ahr is not activated by any of these compounds. The results obtained in the present study suggest that the induction of EROD activity observed by other researchers is not mediated by a direct interaction of chlorotriazines with the Ahr. Other mechanisms, such as activation of the receptor by intermediate metabolites or receptor phosphorylation, could also be acting in this case, although this needs further investigation. There are few reports that evaluated the effects of ATZ on thyroid function. In two articles, this compound did not alter thyroid function in rats (Rooney *et al.*, 2003; Son *et al.*, 2003). However, high doses of ATZ (618 mg/kg/day for 6 days) caused a dose-dependent decrease in serum T3 levels in adult female Wistar rats (Kornilovskaya *et al.*, 1996). Stoker *et al*. (2002) observed the opposite effect: an increase in total T3 when Wistar rats were exposed to 200 mg/kg of ATZ. This effect was not seen when animals were exposed to the three chlorinated metabolites, i.e. DEA, DIA and diaminochlorotriazine. In an attempt to shed light on these contradictory findings, the present work investigated a possible interaction between chlorotriazines and the Tr by using a transactivation assay. We have demonstrated that the chlorotriazines studied did not have an effect on the Tr, which suggests that the observations reported by other authors could be caused by effects of ATZ on a different element in the sequence of events that regulate the production and release of thyroid hormones.

**Table 1.** The *in vitro* assays used to test the receptor-mediated effects of four chlorotriazines and two atrazine metabolites

| **Compound** | **Receptor/*in vitro* assay used to test the response** | | |
|---|---|---|---|
| | Oestrogen receptor (Er) | Thyroid receptor (Tr) | Aryl hydrocarbon receptor (Ahr) |
| Atrazine (ATZ)<br>Propazine (PRZ)<br>Simazine (SIM)<br>Prometryn (PRT)<br>Desethyl-s-chlorotriazine (DEA)<br>Desisopropyl-s-chlorotriazine (DIA) | Transcriptional activation assay with the HER-LUC cell line | Transcriptional activation assay with the PC-DR-LUC cell line | Ethoxyresorufin-O-deethylase (EROD) activity assay with the RTG-2 cell line |

## 4. CONCLUSIONS

Although oestrogenic, anti-oestrogenic and thyrogenic effects of chlorotriazines, (especially ATZ) have been described, the results presented here, together with those reported in the literature, clearly support the view that chlorotriazines and their primary metabolites do not exert their effects through the Er, Ahr or Tr. This study contributes to the further understanding of the mechanism of action of these compounds, and highlights the need for further studies to determine the point of action of these substances on the cascade of events that regulate the production, release and function of these hormones.

## ACKNOLEDGEMENTS

Irene de la Casa-Resino was supported by fellowship PRE09001 from Consejería de Empleo, Empresa e Innovación (Gobierno de Extremadura, Spain). The authors acknowledge the support of INIA project RTA2012-00053-00-00 and of the Marie Curie Initial Training Network Environmental ChemOinformatics (ECO; Grant Agreement No. 238701).

## REFERENCES

Chang, L.W, Toth, G.P, Gordon, D.A, Graham, D.W, Meier, J.R, Knapp, C.W, deNoyelles, F.J, Jr., Campbell, S. Lattier, D.L. (2005). Responses of molecular indicators of exposure in mesocosms: common carp (*Cyprinus carpio*) exposed to the herbicides alachlor and atrazine. Environmental Toxicology and Chemistry 24, 190-197.

Connor, K., Howell, J., Chen, I., Liu, H., Berhane, K., Sciarretta, C., Safe, S., Zacharewski, T. (1996). Failure of chloro-S-triazine-derived compounds to induce estrogen receptor-mediated responses *in vivo* and *in vitro*. Fundamental and Applied Toxicology 30, 93-101.

De la Casa-Resino, I., Valdehita, A., Soler, F., Navas, J.M., Perez-Lopez, M. (2012). Endocrine disruption caused by oral administration of atrazine in European quail (*Coturnix coturnix coturnix*). Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 156, 159-165.

Egaas, E., Skaare, J.U., Svendsen, N.O., Sandvik, M., Falls, J.G., Dauterman,W.C., Collier, T.K., Netland, J. (1993). A comparative study of effects of atrazine on xenobiotic metabolizing enzymes in fish and insect, and of the in vitro phase II atrazine metabolism in some fish, insects, mammals and one plant species. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 106, 141-149.

Eldridge, J.C., Fleenor-Heyser, D.G., Extrom, P.C., Wetzel, L.T., Breckenridge, C.B., Gillis, J.H., Luempert, L.G., Stevens, J.T. (1994). Short-term effects of chlorotriazines on estrus in female Sprague-Dawley and Fischer 344 rats. Journal of Toxicology and Environmental Health 43, 155-167.

Fernandez-Cruz, M.L., Valdehita, A., Alonso, M., Mann, E., Herradon, B., Navas, J.M. (2011). Biological and chemical studies on aryl hydrocarbon receptor induction by the p53 inhibitor pifithrin-alpha and its condensation product pifithrin-beta. Life Sciences 88, 774-783.

Graumann, K., Breithofer, A., Jungbauer, A. (1999). Monitoring of estrogen mimics by a recombinant yeast assay: synergy between natural and synthetic compounds? Science of the Total Environment 225, 69-79.

Hanioka, N., Jinno, H., Tanaka-Kagawa, T., Nishimura, T., Ando, M. (1998). Changes in rat liver cytochrome P450 enzymes by atrazine and simazine treatment. Xenobiotica 28, 683-698.

Hayes, T.B., Khoury, V., Narayan, A., Nazir, M., Park, A., Brown, T., Adame, L., Chan, E., Buchholz, D., Stueve, T. (2010). Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proceedings of the National Academy of Sciences 107, 4612-4617.

Kojima, H., Takeuchi, S., Nagai, T. (2010). Endocrine-disrupting potential of pesticides via nuclear receptors and aryl hydrocarbon receptor. Journal of Health Science 56, 374-386.

Kornilovskaya, I.N., Gorelaya, M.V., Usenko, V.S., Gerbilsky, L.V., Berezin, V.A. (1996). Histological studies of atrazine toxicity on the thyroid gland in rats. Biomedical and Environmental Sciences 9, 60-66.

Laws, S.C., Ferrell, J.M., Stoker, T.E., Schmid, J., Cooper, R.L. (2000). The effects of atrazine on female wistar rats: an evaluation of the protocol for assessing pubertal development and thyroid function. Toxicological Sciences 58, 366-376.

Murphy, M.B., Hecker, M., Coady, K.K., Tompsett, A.R., Jones, P.D., Newsted, J.L., Wong, H.L., du Preez, L.H., Solomon, K.R., Carr, J.A., Smith, E.E., Kendall, R.J., Van Der Kraak, G., Giesy, J.P. (2006). Sediment TCDD-EQs and EROD and MROD activities in ranid frogs from agricultural and nonagricultural sites in Michigan (USA). Archives of Environmental Contamination and Toxicology 51, 467-477.

Oh, S.M., Shim, S.H., Chung, K.H. (2003). Antiestrogenic action of atrazine and its major metabolites *in vitro*. Journal of Health Science 49, 65-71.

Quesada-García, A., Valdehita, A., Fernández-Cruz, M.L., Leal, E., Sánchez, E., Martín-Belinchón, M., Cerdá-Reverter, J.M., Navas, J.M. (2012). Assessment of estrogenic and thyrogenic activities in fish feeds. Aquaculture 338-341, 172-180.

Roberge, M., Hakk, H., Larsen, G. (2004). Atrazine is a competitive inhibitor of phosphodiesterase but does not affect the estrogen receptor. Toxicology Letters 154, 61-68.

Rooney, A.A., Matulka, R.A., Luebke, R.W. (2003). Developmental atrazine exposure suppresses immune function in male, but not female Sprague-Dawley rats. Toxicological Sciences 76, 366-375.

Salaberria, I., Hansen, B.H., Asensio, V., Olsvik, P.A., Andersen, R.A., Jenssen, B.M. (2009). Effects of atrazine on hepatic metabolism and endocrine homeostasis in rainbow trout (*Oncorhynchus mykiss*). Toxicology and Applied Pharmacology 234, 98-106.

Son, H.Y., Nishikawa, A., Okazaki, K., Lee, K.Y., Imazawa, T., Hirose, M. (2003). Lack of modifying effects of atrazine and/or tamoxifen on thyroid carcinogenesis in rats pretreated with N-bis(2-hydroxypropyl)nitrosamine (DHPN). Food Chemical Toxicology 41, 1811-1816.

Stoker, T.E., Guidici, D.L., Laws, S.C., Cooper, R.L. (2002). The effects of atrazine metabolites on puberty and thyroid function in the male Wistar rat. Toxicological Sciences 67, 198-206.

Suzawa, M., Ingraham, H.A. (2008). The herbicide atrazine activates endocrine gene networks via non-steroidal NR5A nuclear receptors in fish and mammalian cells. Plos One 3, 294 e2117.

Tennant, M.K., Hill, D.S., Eldridge, J.C., Wetzel, L.T., Breckenridge, C.B., Stevens, J.T. (1994). Chloro-s-triazine antagonism of estrogen action: limited interaction with estrogen receptor binding. Journal of Toxicology and Environmental Health 43, 197-211.

Tran, D.Q., Kow, K.Y., McLachlan, J.A., Arnold, S.F. (1996). The inhibition of estrogen receptor-mediated responses by chloro-S-triazine-derived compounds is dependent on 250 estradiol concentration in yeast. Biochemical and Biophysical Research Communications 227, 140-146.

Wilhelms, K.W., Fitzpatrick, K.F., Scanes, C.G., Anderson, L.L. (2006). In ovo exposure to a triazine herbicide: effects of atrazine on circulating reproductive hormones and gonadal histology in young Japanese quail. Archives of Environmental Contamination and Toxicology 51, 117-122.

# CAPÍTULO II

---

**Alteración endocrina causada por la administración oral de atrazina en codorniz (*Coturnix coturnix coturnix*).**

*de la Casa-Resino. I., Valdehita, A., Soler, F., Navas, J.M., Pérez-López, M. (2012). Endocrine disruption caused by oral administration of atrazine in European quail (Coturnix coturnix coturnix). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 156: 159-165*

**RESUMEN:** El herbicida atrazina (ATZ) ha sido ampliamente utilizado y numerosos estudios han demostrado su efecto tóxico en la reproducción de ratas, peces, y anfibios, presentando una $DL_{50}$ en aves de 5000 mg/kg. En el presente estudio, se procedió a la administración de dos dosis únicas de ATZ a codornices (*Coturnix coturnix coturnix*) durante los días 0, 5 y 10 de experimento, llevándose a cabo el muestreo durante los días 15, 30 y 45. La ATZ incrementó significativamente la expresión del receptor de estrógenos α (ERα) a ambas dosis en el día 30 de muestreo. Se observó también una inducción importante en los niveles plasmáticos de β17 -estradiol (E2). La ATZ a la concentración de 100 mg/kg incrementó los niveles de vitelogenina (VTG) plasmática, aunque este efecto no estuvo relacionado con los niveles de expresión hepáticos de la proteína. A su vez, la ATZ no tuvo ningún efecto sobre la expresión hepática del citocromo P450 1A (CYP1A) o en su actividad enzimática asociada etoxiresorufina-O-deetilasa (EROD). La exposición a ATZ provocó un claro efecto estrogénico en hembras de codorniz adultas, aunque serán necesarios más estudios para establecer el efecto en el desarrollo sexual o en la reproducción de estas aves en ambos sexos en libertad.

**Palabras clave:** Atrazina, CYP1A4, estradiol, etoxiresorufina-O-deetilasa, codorniz vitelogenina.

**ABSTRACT:** The widely used herbicide atrazine (ATZ) has been reported to exhibit reproductive toxicity in rats, fish and amphibians, with an avian LD50 of 5000 mg/kg. In the present work, ATZ was administered as a single oral dose of 25 or 100 mg/kg to female European quail (Coturnix coturnix coturnix) at days 0, 5 and 10 of the experiment being the animals sampled at days 15, 30 and 45. ATZ significantly increased the expression of hepatic estrogen receptor α (ERα) at both doses at day 30. An important increase was also observed in plasma 17β-estradiol (E2) concentrations. ATZ at 100 mg/kg increased the circulating concentration of vitellogenin (Vtg), but this effect was not related with an increase in hepatic Vtg mRNA levels. ATZ had no effect on the hepatic expression of both cytochrome P450 1A4 (CYP1A4) or the related biotransformation activity ethoxyresorufin-O-deethylase (EROD). These results led to the conclusion that ATZ provokes an estrogenic effect in sexually mature females of European quail. Further studies are necessary to establish the effect on sexual development or reproduction of female and male birds in the wild.

**Keywords:** Atrazine, CYP1A4, Estradiol, Ethoxyresorufin-O-deethylase, European quail, Vitellogenin.

## 1. INTRODUCTION

In the last decades, increasing attention has been paid to evaluating the adverse effects of endocrine disrupting chemicals (EDCs). These are defined as exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, its progeny, or (sub) populations" (WHO/IPCS, 2002). These chemicals can mimic the action of sex steroid hormones, estrogens and androgens, by binding to hormone receptors or influencing cell signaling pathways. In addition, they may alter production and breakdown of natural hormones or modify levels and function of hormone receptors (Soto *et al.,* 1995).

Atrazine (6-chloro-N-ethyl-N′-isopropyl-1,3,5-triazine-2,4-diamine) (ATZ) is a chloro-s-triazine herbicide that inhibits photosynthesis in plants (Shimabukuro, 1967). Although it has been excluded from a registration process of pesticides in the European Union, it is still one of the most widely used herbicides in the world and may be applied before and after planting to control broadleaf and grassy weeds (U.S. EPA, 2006; APVMA, 2010). ATZ annual sales in the U.S. are approximately 33–36 million kg (Kiely *et al.,* 2004). This herbicide exhibits low acute toxicity to birds with a dietary $LD_{50}$>5000 mg/kg in Japanese quail (*Coturnix coturnix japonica*) (U.S. EPA, 2006). The current ecological risk assessment for ATZ established by the U.S. EPA reports a dietary no-observed-adverse-effect-concentration (NOAEC) of 225 mg/kg and a lowest-observed-adverse-effect-concentration (LOAEC) of 675 mg/kg. At this concentration, a reduction in egg production and embryo viability together with an increase in defective eggs were observed in bobWhite quail (*Colinus virginianus*) and mallard duck (*Anas platyrhynchos*) (U.S. EPA, 2006).

ATZ has been considered as EDC due to alterations caused on endocrine activities in various taxa. In amphibians, several studies have reported that ATZ induces complete feminization and chemical castration in frogs at low doses (Hayes *et al.,* 2002, 2006, 2010). Also, it has been demonstrated that ATZ has estrogenic effects in rainbow trout (*Oncorhynchus mykiss*) in vivo (Salaberria *et al.,* 2009) and on reproductive output in fathead minnow (*Pimephales promelas*) (Tillitt *et al.,* 2010). In rat males, ATZ caused persistent estrous, reduced the testosterone (T) levels (Cooper *et al.,* 1996, 1999, 2000; McMullin *et al.,* 2004) and increased the circulating concentration of 17β-estradiol (E2) (Stoker *et al.,* 2000). In birds, the number of studies about the effectof ATZ on adult animals is scarce so that there is little information about the effects of ATZ on avian reproduction. Wilhelms *et al.,* (2005, 2006a, b) conducted studies to evaluate the effects of ATZ *in ovo* and on immature male and female quails. T and E2 plasma levels were slightly increased when immature bird males were exposed to 1000 mg/kg and 10 mg/kg

of ATZ, respectively. However, no remarkable effects were detected on the mortality of immature female Japanese quails, neither on the weights of liver, ovary or oviduct nor on plasma concentration of luteinizing hormone (LH).

Several mechanisms of action have been proposed to explain the disruptive action of ATZ on endocrine systems although, in general, alterations in the synthesis and/or metabolism of estrogens have been reported, for instance in fish (Thibaut and Porte, 2004). Also in amphibians it has been hypothesized that ATZ endocrine effects are caused by an increase in the expression and/or activity of aromatase, the enzyme that converts androgens to estrogens (Hayes *et al.*, 2002). In accordance with these reports, Salaberria *et al.*, (2009) suggested that in rainbow trout (*O. mykiss*) ATZ caused a rise in blood E2, which triggered an increase in the production of vitellogenin (Vtg). Considering the pivotal role that these prototypical feminine hormones play in key processes as growth, development and sexual differentiation in animals any alteration in the normal functioning of estrogens can lead to important deleterious effects on organisms. The action of these hormones is mediated by specific nuclear estrogen receptors (ER). In the particular case of quails two isoforms of ER have been described: ERα and ERβ being ERα the most abundant isoform in the liver (Mattsson *et al.*, 2008).

Vitellogenesis is one of the key processes regulated by estrogens in oviparous vertebrates (Arukwe and Goksøyr, 2003). Vtg is a major precursor of egg-yolk proteins, which provide energy reserves for embryonic development in eggs (Nagler *et al.*, 1987; Marin and Matozzo, 2004). Vtg is normally produced in the liver of egg-laying mature females in response to endogenous estrogens (Wahli *et al.*, 1981; Denslow *et al.*, 1999), however its production can be induced by xenoestrogens in adult males as well as in juveniles of both sexes (Lazier, 1978; Wiskocil *et al.*, 1980; Elbrecht *et al.*, 1984; Mattsson *et al.*, 2011). Considering the high plasma concentrations reached by Vtg after appropriate stimulation this protein has been widely used as a very sensitive and easy to analyze biomarker of estrogenic effects.

The effects of a chemical in an organism will be influenced by the metabolic pathways activated by this chemical. Several studies have reported that cytochrome P4501A (CYP1A) plays an important role in the catabolism of ATZ (Adams *et al.*, 1990; Hanioka *et al.*, 1999; Chang *et al.*, 2005). In mammals, ATZ is metabolized in the liver by cytochrome P450 oxidation to dealkylated, chlorinated metabolites (Bakke *et al.*, 1971; McMullin *et al.*, 2003). CYP1A is induced after ligand-activation of the aryl hydrocarbon receptor (AhR), in various tissues in vertebrates (Whitlock, 1999; Jonsson *et al.*, 2003). Typical AhR inducers are planar, polycyclic and aromatic compounds (Waller and McKinney, 1995) including polyaromatic hydrocarbons (PAHs), dioxins and

polychlorinated biphenyls (PCBs) (Shimada *et al.,* 2001). However, there is increasing evidence that AhR can also be activated by chemicals showing structural features different from the typical planar AhR ligands including for instance pesticides or pharmaceuticals (Denison and Nagy, 2003; Navas *et al.,* 2004; Boronat *et al.,* 2007; Fernández-Cruz *et al.,* 2011). In birds the CYP1A4 isoformexhibits catalytic specificity for aryl hydrocarbon hydroxylase and ethoxyresorufin O-deethylase (EROD) activities and the measurement of EROD together with the estimation of CYP1A4 mRNA levels are practical methods for quantifying the induction of this cytochrome in birds (Jonsson *et al.,* 2003; Herve *et al.,* 2010).

It must also be taken into account that the activated AhR can interact with the action of ER through a variety of mechanisms provoking estrogenic or, more frequently, antiestrogenic processes (Ohtake *et al.,* 2003; Navas and Segner, 2008). For instance, in cultured fish hepatocytes the exposure to typical AhR inducers such as PAHs or PCBs lead to a reduction of Vtg production previously induced by 17β-estradiol (E2). And this effect was parallel to an induction of AhR dependent responses, as the increase of EROD activity and of CYP1A protein levels (Anderson *et al.,* 1996; Smeets *et al.,* 1999; Navas and Segner, 2000).

The European quail (*Coturnix coturnix coturnix*) is a model species for studying avian endocrinology and is often used to test the endocrine-disrupting potential of chemicals *in vivo* (Mattsson *et al.,* 2008). As before indicated, in other bird species ATZ leads to an increase of defective eggs (U.S. EPA, 2006) and in a variety of species it has been described as an endocrine disruptor. Taking all this into account, the main aim of the present work was to determine the possible effects of intermittent administration of single doses of ATZ under realistic conditions on the hormonal control of reproduction of European quail. Since the activation of detoxification pathways caused by ATZ can also influence possible hormonal alterations, the induction of CYP1A4 was also estimated at the transcriptional and enzymatic levels.

## 2. MATERIAL AND METHODS

### 2.1 Chemicals

Atrazine, ethylenediaminetetraacetic acid (EDTA), MgCl2, Tris–HCl, NaCl, bovine serum albumin (BSA), glycerol, sodium dodecyl sulphate (SDS), Tween-20, dithiothreitol (DTT), bromophenol blue, and protease inhibitors (aprotinin, leupeptin and pestatin A) were purchased from Sigma-Aldrich (Germany). Acrylamide–bisacrylamide, polyvinylidene fluoride (PVDF) sheets and SYBR Green were from Bio-Rad (CA, US).

Mouse anti-bird Vtg monoclonal antibody was from Biosense Laboratories (Bergen, Norway).

### 2.2 Animals and treatment

A total of 45 adult female European quails (*C. coturnix coturnix*), free from apparent clinical ailment, were obtained from a local quail farm. The birds were placed in cages under constant temperature, humidity and lighting conditions (12 h light/dark cycle). Feed and water were offered *ad libitum* for the duration of the experiment. One of the limitations found for designing the experimental groups was the lack of information relative to similar previous works carried out in vivo with birds. It was decided to use at least two very different doses of ATZ and at least three time points for sampling. For reducing at a maximal the number of used animals it was decided to allocate only five quails in each experimental unit (for each group and sampling point). This design would allow detecting any effect of ATZ on the treated birds rendering the sufficient power in the statistical tests for detecting significant differences. After two weeks of acclimatization, birds were divided into three groups of 15 animals (C, D1 and D2). Birds of group C received only the vehicle solvent (corn oil) and served as control. ATZ, dissolved in corn oil, was orally administered (through crop tubing) at days 0, 5 and 10 of the experiment at a dose of 25 mg/kg body mass, group D1, or, 100 mg/kg body mass, group D2. The birds were monitored daily for clinical signs of discomfort. At days 15, 30 and 45 of the experiment, 5 animals of each group were sacrificed in a $CO_2$ chamber. The blood was collected from the heart of each bird, and centrifuged at 3000 rpm for 10 min in order to obtain the plasma. Internal organs were examined for possible macroscopic damage. Livers were removed immediately and stored at −80 °C.

### 2.3 RNA extraction and single step quantitative real-time RT-PCR (RTqPCR) of Vtg, ERα and CYP1A4

Total RNA was extracted from approximately 0.1 mg European quail liver, using TRI® reagent (Sigma-Aldrich GmbH), according to the manufacturer's instructions. One-step Retrotranscription and real time Polymerase Chain Reaction (RT-qPCR) analysis was carried out with 200 ng RNA using an iScript RT-PCR kit with SYBR Green according to the manufacturer's instructions. The thermal cycling conditions were 50 °C for 10 min, followed by 40 cycles of 95 °C for 5 min, 95 °C for 10 s and 58 °C for 30 s. All PCR reactions were performed on a LineGene 9600 system BIOER Technology (Hangzhou, China) with specific primers previously reported in the literature (Table 1). Negative

controls with water instead of RNA or without reverse transcriptase were run in parallel to exclude any kind of contamination. The melting curves of the qPCR products confirmed that no unspecific sequences were amplified. RT-qPCR results were expressed as Ct values, where Ct was defined as the threshold cycle number at which the product is first detected by fluorescence. Expression of β-actin was constant among the different bird cohorts. Thus, β-actin was found to be an appropriate housekeeping gene for normalization in this study. Relative quantification was measured using the comparative Ct method also referred to as the $2^{-\Delta\Delta Ct}$. It represents the amount of target normalized to the endogenous control (β-actin) and relative to the mean value from the set of birds considered as control, where $\Delta\Delta Ct=(Ct_{target}-Ct_{ß\text{-}actin})_{Atrazine}-(Ct_{target}-Ct_{ß\text{-}actin})_{Control}$. The fold change in relative expression was then determined by calculating $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). This method was chosen because the efficiency values of PCR for the target genes and the β-actin were around 2.00. All the RT-qPCR analyses were performed in two independent experiments.

**Table 1.** Oligonucleotide primers used in RT-qPCR analysis for CYP1A4, Vtg, ER and β-actin gene expression in liver of European quail.

| mRNA | Primers | Sequence (5'…… 3') | Product size (bp) | GenBank accession no. |
|---|---|---|---|---|
| CYP1A4 | sense | GGATGTCAATACCCGTTTCG | 109 | **GQ906939** |
| | antisense | CTGCCCAATCAATGAGTCTG | | |
| Vtg | sense | GAAAACCCTGAGCAACGGATAG | 80 | **AF199490** |
| | antisense | TGGAACATCATCATGGAAATCTTG | | |
| ER | sense | CTTGCAGACAGAGAATTAGTGCACA | 68 | **AF442965** |
| | antisense | GTTAAATCCACAAATCCTGGAACTC | | |
| β-actin | sense | AAATTGTGCGTGACATCAAGGA | 76 | **AF199488** |
| | antisense | GAGGCAGCTGTGGCCATCT | | |

## 2.4 Vtg purification and Western blot

Plasma (250 μL) was added to 1 ml of a 20 mM EDTA solution (pH 7.7). After mixing, 80 μL of 0.5 M $MgCl_2$ was added, and the content of the tube was gently mixed by inversion. The resulting precipitate was collected by centrifugation at 2500 g for 15 min. The supernatant liquid was discarded, and the pellet was dissolved in 150 μL of 1 M NaCl, 50 mM Tris–HCl buffer at pH 7.5. Vigorous mixing was avoided to minimize Vtg denaturation. The tube was centrifuged for 30 min at 2500 g to remove any insoluble material, the supernatant fluid was decanted into another tube, and Vtg was precipitated

by the addition of distilled water (Wiley *et al.*, 1979). Purified plasma protein content was measured by the Lowry Protein Assay® (Bio-Rad), using BSA as standard. 60 μg of total protein from plasma purified Vtg was solubilized in 50 mM Tris–HCl buffer (pH 6.8) containing 10% (v/v) glycerol, 3% (w/v) SDS, and 0.01% bromophenol blue. Proteins were resolved on a 7.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF sheets. The PVDF sheets were soaked in methanol. Excess protein-binding sites were saturated with PBS containing 0.1% Tween-20 and 5% non-fat dried milk. Blotted membranes were incubated for 2 h with mouse monoclonal anti-bird Vtg antibody (1:1000). Excess of primary antibody was removed by washing three times with PBST (PBS containing 0.1% Tween-20) and then incubated for 1 h with secondary anti-mouse antibody (1:4000) from G-Biosciences (MO, USA). After extensive washing with PBS, the bands were visualized by means femto-Chromo™ HRP (G-Biosciences) designed for chromogenic visualization using Horseradish peroxidase (HRP). The bands were analyzed semiquantitatively, using the imaging system Gel Doc™ XR from Bio-Rad.

### 2.5 ELISA of Vtg

To quantify the plasma Vtg levels, an enzyme linked inmunosorbent assay (ELISA) kit® for Japanese Quail Vitellogenin (Trans Genic Inc. Japan) was used. Plasma protein content was previously measured by the Lowry Protein Assay from Bio-Rad, using bovine serum albumin as standard. 150 μg of total protein content was used to carry out the ELISA kit according to the manufacturer's instructions.

### 2.6 E2 assay

To identify ATZ-induced changes in circulating concentrations of reproductive hormones, plasma concentrations of E2 were determined using the E2 enzyme-linked immunosorbent assay (ELISA) sensitive kit® (sensitivity of 1.4 pg/ml) obtained from DEMEDITEC Diagnostics (Germany). Plasma protein content was measured by the Lowry Protein Assay from Bio-Rad, using bovine serum albumin as a standard. In this case, 340 μg of total protein content was used to perform ELISA according to the manufacturer's instructions.

### 2.7 EROD activity

EROD activity was measured in liver microsomes. Liver samples (0.1 g) were homogenized in a Potter–Elvehjem tissue homogenizer in 1 ml of buffer containing 50 mM Tris–HCl, 0.25 M sucrose, 2 mM EDTA, 150 mM KCl, 1 mM dithiothreitol (DTT) and protease inhibitors (aprotinin, leupeptin and pestatin A). The homogenate was centrifuged at 8000 g for 20 min at 4 °C and the resulting supernatant was further ultracentrifuged at 100000 g for 60 min at 4 °C. The microsome pellet obtained from ultracentrifugation was suspended in 100 μL of the same buffer at 4 °C. EROD assay was conducted according to the method of Burke and Mayer (1974) with slight modifications. Microsomal fraction (5 μL) was incubated for 30 min in a 96-well plate with 7-ethoxyresorufin (5 μM), and NADPH (1.2 mg/ml). The fluorescence was read at minute 10, 20 and 30 at an excitation wavelength of 530 nm and at emission wavelength of 590 nm with a TECAN Genios spectrofluorometer (TECAN, Switzerland). The concentration of microsomal protein was determined as described above.

### 2.8 Statistics

All data were analyzed using statistical software Prism 5 for Windows version 5.03 (GraphPad software, Inc., La Jolla, CA, USA). Results were expressed as mean±S.E.M. Taking into account that the n value of each experimental group was 5, statistical analyses were performed using a non-parametric Kruskal-Wallis test. Differences among groups ($P$<0.05) were determined with Dunn's test.

## 3. RESULTS

ATZ administered via the diet at concentrations 25 mg/kg or 100 mg/kg had no effect on mortality or feed intake in European quail. All animals were free from apparent clinical ailment during the experiment and the macroscopic examination of the internal organs did not show any damage.

### 3.1 Effect of ATZ on ERα gene expression and plasma E2 levels

No differences among groups in ERα mRNA levels were found at days 15 and 45 (Fig. 1A). However, at day 30 a significant ($P$<0.05) induction was observed in groups D1 and D2 (receiving 25 and 100 mg/kg, respectively) with respect to control group C (Fig. 1A). E2 plasma levels did not show statistically significant differences among groups at any of the sampling times (Fig. 1B). However, again at day 30 an increase in the mean values

was observed in both treatment groups (D1 and D2), being stronger in the group receiving the highest dose of ATZ (D2). This result is in accordance with the significant increase observed in the expression of the ERα in the same groups and at the same time point.

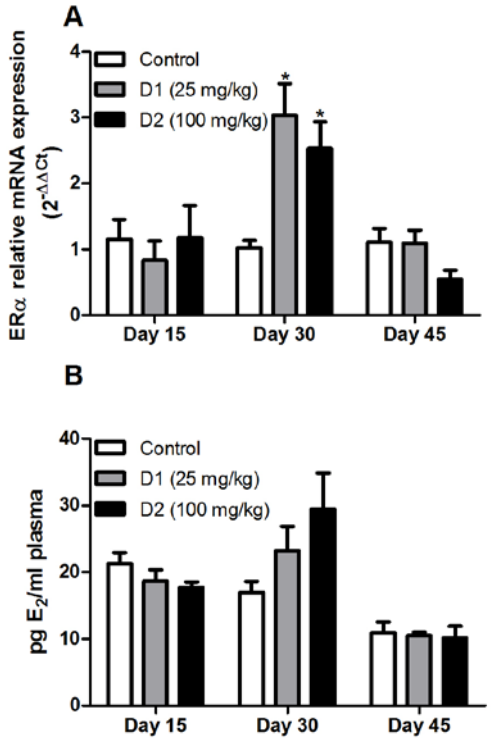



**Figure 1.** Effect of atrazine on ERα hepatic mRNA and E2 plasma levels. ERα mRNA expression was determined by RT-qPCR in European quail livers (A). The results show the mRNA ratio defined as fold change in gene expression. Plasma levels of E2 determined by ELISA (B). Columns represent mean±SEM (n=5). Asterisks reflect significant differences between groups at day 30 (*$P$<0.05).

### 3.2 Effect of ATZ on Vtg mRNA levels and plasma Vtg concentrations

Results obtained for Vtg mRNA levels did not show any significant difference ($P$>0.05) neither among groups nor at any of the sampling times (data not shown). This lack of differences was probably associated with a high variability in the Vtg mRNA among individuals. Vtg protein concentrations were semiquantitatively compared among groups at all the sampling times by means of Western blot. Results are presented in Fig. 2A. No apparent differences among groups were found at days 15 and 45. However, a

statistically significant ($P$<0.05) induction was found at day 30 in group D2 (100 mg/kg) where Vtg plasma levels were about three times higher than those of the control group. In order to confirm and quantify the increase of Vtg detected at day 30 by means of Western blot plasma Vtg concentrations in the different groups were quantified at day 30 by means of ELISA. Fig. 2B shows the ELISA results which confirm the data obtained by Western blot in the case of group D2 (100 mg/kg), where an important induction (although no significant $P$>0.05) of Vtg concentration was detected. However, the D1 group results did not correspond with the Western blot results, showing an increase of Vtg plasma concentration that reached even higher values than in group D2.

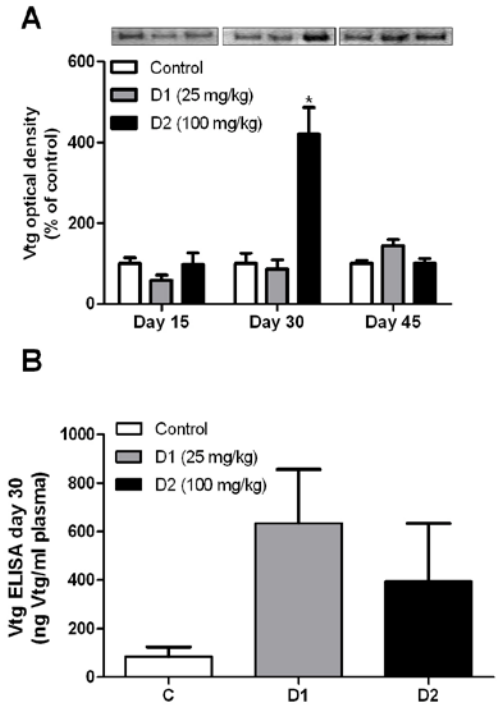



**Figure 2.** Effect of atrazine on Vtg plasma levels. Relative Vtg expression determined by means of Western blot (A). Immunodetection using antibody against Vtg was performed followed by densitometry of the corresponding bands. A representative experiment is shown in the upper panels. Plasma Vtg concentration in samples from day 30 was quantified by means of ELISA (B). The columns represent mean±SEM (n=5). Asterisks reflect significant differences between groups at day 30 (*$P$<0.05).

### 3.3 Effect of ATZ on CYP1A4 expression and EROD activity

Finally, to evaluate any interaction of ATZ with AhR pathways that could be modulating the observed endocrine effects, the levels of CYP1A4 mRNA and of its associated enzymatic activity, EROD, were studied. For CYP1A4 mRNA levels (Fig. 3A) no statistically significant differences ($P$>0.05) were observed among groups at any of the sampling times. It must be signaled however, that CYP1A4 mRNA detected in group D1 exhibited an increase in the mean values with time, and that in group D2 this increase was only observed at day 30, decreasing to values similar to those of controls at day 45. These tendencies did not find any correspondence with the observed EROD activity that was approximately the same for all groups at all the sampling times (Fig. 3B).

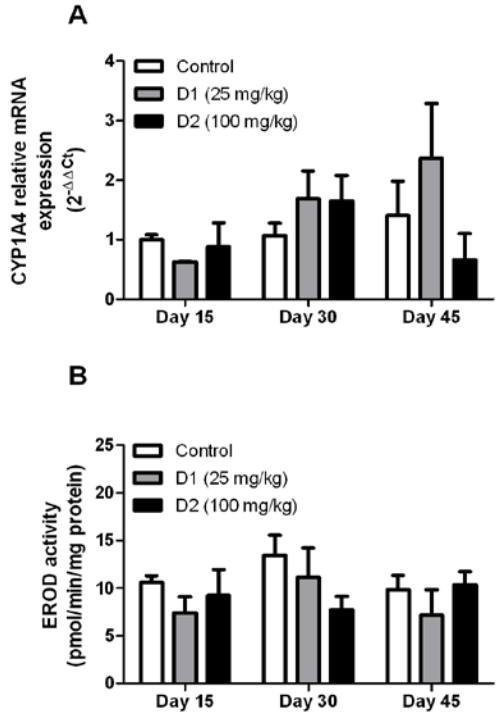



**Figure 3.** Effect of atrazine on the hepatic CYP1A4 mRNA expression (A) determined by RT-qPCR in the liver of European quail. The results show the mRNA ratio defined as fold change in gene expression. 7-ethoxyresorufin-O-deethylase (EROD) activity in European quail livers from three samplings (days 15, 30 and 45). Columns represent mean±SEM (n=5).

## 4. DISCUSSION

The experiments performed in the present work were designed to evaluate the effects of ATZ as an endocrine disruptor in sexuallymature female birds after intermittent oral administration. ATZ is primarily applied in the U.S. on corn, sugarcane and sorghum crops normally in spring (KY Department of Agriculture, 2007) so that animals in these temperate regions could be exposed to this chemical at the time of sexual maturation and start of reproductive activity. Although a continuous exposure to the toxicant through ingestion of contaminated corns or vegetables is possible in birds, we have considered that an intermittent exposure was more realistic taking into account possible variability in the accessibility to this contaminated food. In this kind of studies it is possible that disruptive effects appear late after treatment due to the time needed to modify the compensation mechanisms that maintain the homeostasis of organisms. To cover these long term effects, possible alterations in the experimental animals were monitored 15, 30 and 45 days after the beginning of the treatment (and 5, 20 and 25 days after administration of the third and last dose of ATZ). The concentrations used, 25 mg/kg (D1) and 100 mg/kg (D2), were chosen based on the study performed by Hussain *et al.,* (2011), where only the 100 mg/kg dose provoked a decrease in the food intake and alterations in some hematological parameters. These two doses are lower than those considered as NOAEC or LOAEC, 225 mg/kg and 675 mg/kg, respectively, by U.S. EPA (2006). Moreover, ATZ concentrations can reach even 960 mg/kg post-application and exceed the avian reproductive NOAEC for up to 35 days post-application (U.S. EPA, 2006). Taking all this into account the used doses could be representative of a real exposure situation in the wild. In addition, ATZ was not administered in a continuous way, but only three times with five days interval. All this makes the conditions used in the present work closer to realistic field situations. Oral administration of ATZ following the described protocol provoked for both doses used an increase in the ERα expression ($P<0.05$) and in the circulating E2 plasma levels 30 days after the beginning of the exposure, and this induction disappeared 15 days later. This is in contrast with the results obtained by Wilhelms *et al.,* (2006a,b) in Japanese quail. These authors did not find any increase in the weight of estrogen sensitive organs as oviduct, liver or ovary after oral administration of ATZ at concentrations of approximately 109 mg/kg/day for 14 days (Wilhelms *et al.,* 2006a). ATZ did not cause neither any change on the levels of LH. The authors stated that these results were indicative of the lack of overt toxicity or estrogenicity of ATZ in quail at the used dietary concentrations. Similarly, injections of ATZ in Japanese quail eggs at concentrations reaching 504 μg/kg did not provoke alterations in E2 or T plasma concentrations measured in birds 14 days after hatching (Wilhelms *et al.,* 2006b). Our results, however, clearly show that ATZ provokes an

increase of E2 plasma levels that is associated with typical estrogenic effects as the increase in ER mRNA levels in liver or the increase of Vtg plasma concentrations. It has been described that ATZ administered to male Japanese quail for two weeks at a concentration of 10 mg/kg induced a 3.6 fold increase in plasma concentrations of E2 with respect to controls (Wilhelms *et al.,* 2005) although this effect was not observed in birds fed with higher ATZ doses (100 mg/kg and 1000 mg/kg). Divergences in results related with the induction of E2 plasma levels or with estrogenic effects after ATZ exposure appear also in fish related literature. For instance, in rainbow trout intraperitoneal injection of ATZ at a dose of 2 or 200 μg/kg did not provoke significant effects on levels of E2 or T as measured six days later (Salaberria *et al.,* 2009). However, an induction of Vtg was observed in the same experiment, which led the authors to suggest that ATZ could have induced a pulse of endogenous E2 responsible for the Vtg production by stimulation of aromatase activity. The authors also argue that this transient increase of E2 levels could have not been detected in their 6 days experiment because after a single E2 injection, E2 homeostasis is reinstated after only 16 to 24 h (Korte *et al.*, 2000). In fathead minnow exposed to ATZ concentrations of 0.5 μg/L to 50 μg/L in a flow through system for 30 days, a decrease in fecundity was observed in females due to a reduction in spawning events related with alterations in the maturation of oocytes but no statistical difference in T or E2 plasma levels in males or females with respect to the controlswere observed (Tillitt *et al.,* 2010). However, in goldfish (*Carassius auratus*) a suppression of plasma androgens (11-ketotestosterone and T) and an induction of E2 were observed after 21 days exposure to 1000 μg/L ATZ (Spano *et al.,* 2004). Divergences in the effects of ATZ have also been reported in mammals. In rats, exposure to 1 mg/L ATZ for 4 months in drinking water had no effects in the expression of the ERα in bone narrow cells in males or females (Cimino-Reale *et al.,* 2008). In contrast, recent studies observed that exposure to the herbicide increased the plasma levels of E2 in male rats after a daily dose of 200 mg/kg for 15 days (Victor-Costa *et al.,* 2010).

The increase in plasma levels of E2 observed in this and in other cited works could be related to the stimulation of the aromatase activity, which transforms the androgen T to E2. This mechanism has been suggested to explain the feminization observed in American leopard frog (*Rana pipiens*) after ATZ exposure (Sanderson *et al.,* 2000; Hayes *et al.,* 2002, 2003). Moreover, a decrease of T was observed in exposed frogs (Hayes *et al.,* 2002). Corroborating this hypothesis Hayes *et al.,* (2010) also found a decrease in plasma T levels in male African clawed frogs (*Xenopus laevis*) exposed to ATZ 2.5 ppb in ethanol) under controlled laboratory conditions. In female zebrafish ATZ significantly induced aromatase expression (Suzawa and Ingraham, 2008). Aromatase expression is controlled by the induction of the ER (Sanderson *et al.,* 2000; Hayes *et al.,*

2002), and although ATZ appears not to be a direct ER agonist (Connor *et al.,* 1996; Roberge *et al.,* 2004; Suzawa and Ingraham, 2008) other mechanisms different than ER activation by ATZ could explain the induction of aromatase activity. For instance, in the H295R human adrenocortical carcinoma cell line, exposure to ATZ led to an elevation of aromatase activity parallel to an increase of the concentration of cyclic adenosine monophosphate (cAMP) (Sanderson *et al.,* 2000, 2001). The phosphodiesterase (PDE) family of enzymes hydrolyzes cAMP to 5-AMP. By means of fluorescence polarization Roberge *et al.,* (2004) evidenced that ATZ was able to interact with PDE. This interaction would inhibit PDE activity resulting in elevated levels of cAMP, which may result in elevated aromatase mRNA (Sanderson *et al.,* 2000; Mehats *et al.,* 2002). In zebrafish Suzawa and Ingraham (2008) proposed a complex mechanism involving NR5A receptor activation, as well as receptor phosphorylation, amplification of cAMP, and PI3K signaling. Whether these mechanisms are acting also in the case of quail should be determined in future studies, but is out of the scope of this article. In the present work, the induction of E2 production observed in female European quails after ingestion of ATZ was not immediate and could only be observed after 30 days of the start of exposure and 20 days after the third and last administration of ATZ. All this suggests that ATZ could act through a cascade of mechanisms that need some time to be effective what would be in agreement with a process involving alterations in the pathways regulating the action of aromatase and/or other enzymes instead of a direct action on ER.

Despite the fact that we did not find any significant effect in the expression of liver Vtg mRNA, probably due to data variability, a significant induction of Vtg protein levels was observed by means of Western blot at day 30 in D2 exposed animals. This induction was also detected by means of ELISA but in this case for both treated groups, D1 and D2, at day 30, although no statistically significant differences were observed with respect to controls. Taking into account that in oviparous vertebrates Vtg production is under the control of the ER which is activated by E2 (Matozzo *et al.,* 2008), the observed increase of Vtg levels at day 30 is in accordance with the significant induction observed in the expression of ERα and of E2 plasma levels at the same time point. The lack of correspondence between Western blot and ELISA results in the D1 group could be due to a loss of Vtg during the purification process needed for Western blot analysis. This purification involves a laborious and tedious multiple step method that could have led to some losses in the sample.

In order to determine any possible modulatory effect of catabolic activities on ATZ action, the induction of CYP1A4 by ATZ was also studied. No significant changes in the CYP1A4 mRNA expression or in the CYP1A4 dependent EROD activity was observed at any dose or day of treatment compared to control. CYP1A4 expression is regulated by

AhR. To our knowledge no interactions of ATZ with the AhR have been reported previously. In addition, our own experiments performed in vitro with fish cell lines expressing AhR and CYP1A, and with mammalian cell lines permanently transfected with reporter genes under the control of the AhR, failed to show any induction of AhR by ATZ and other triazine derivatives (unpublished results). All this is in agreement with the lack of effect of ATZ on CYP1A4 observed in the present study. Our data are also in accordance with those obtained in rats by Islam *et al.,* (2002) who did not find any effect of ATZ neither on the expression of CYP1A1 nor on EROD activity. However, ATZ has been reported to induce the activity of CYP1A in common carp (*Cyprinus carpio*) (Chang *et al.,* 2005) and zebrafish (Dong *et al.,* 2009). In contrast, in rainbow trout a dose dependent liver inhibition of the CYP1A expression after a single ATZ injection has been observed (Salaberria *et al.,* 2009). In this case, the reduction in CYP1A expression was attributed to the observed increase of E2 plasma levels that would provoke an activation of ER that, on his hand, is able to inhibit AhR dependent processes as, for instance, CYP1A expression (Navas and Segner, 2000, 2001; Salaberria *et al.,* 2009). Little is known about the effects of ATZ in the expression of the CYP1A4 and on EROD activity in birds. Several studies have reported that ATZ is metabolized by phase I reactions mediated by CYP450 (Adams *et al.,* 1990; Hanioka *et al.,* 1999; Chang *et al.,* 2005). In the present work, differences between controls and treated animals in the liver expression of CYP1A4 and EROD levels (more important at day 30) seem to indicate a possible effect of ATZ at this level. Thus, further studies are required to elucidate this phenomenon.

## 5. CONCLUSION

In summary, the administration of three single doses of ATZ with five day intervals at concentrations that could be considered as environmentally relevant and lower than the NOAEC caused an increase of E2 plasma levels in mature female quails 20 days (day 30 of the experiment) after the last administration. This increase was not detected five days (day 15 of the experiment) after the last administration and E2 plasma levels reached again control values 35 days after the last administration of ATZ (day 45 of the experiment). This increase in E2 plasma levels was parallel to a raise in the expression of ERα expression that concomitantly led to an increase of Vtg plasma levels. These results indicate that ATZ could be causing negative effects on the endocrine system that could have consequences on wild bird populations. However, these results, in particular considering the limited number of quails used, should be considered as preliminary. Further studies should be performed in order to explore the mechanisms underlying the

endocrine activity of ATZ and to establish possible long term effects at the population level.

## ACKNOWLEDGEMENTS

Irene de la Casa-Resino was supported by fellowship PRE09001 from Consejería de Empleo, Empresa e Innovación (Gobierno de Extremadura, Spain). The INIA team acknowledges the support of INIA project RTA2009-00074-00-00 in all the work relatedwith endocrine disruption. The experimental design has been approved by the Ethical Committee of the University of Extremadura and all the work has been carried out in accordance with the ethical requirements of the current legislation (EU Directive 2010/63/EU for animal experiments).

## REFERENCES

Adams, N.H., Levi, P.E., Hodgson, E. (1990). In vitro studies of the metabolism of atrazine, simazine, and terbutryn in several vertebrate species. Journal of Agricultural and Food Chemistry 38, 1411-1417.

Anderson, M.J., Miller, M.R., Hinton, D.E. (1996). In vitro modulation of 17 beta-estradiol induced vitellogenin synthesis: effects of cytochrome P450 1A1 inducing compounds on rainbow trout (*Oncorhynchus mykiss*) liver cells. Aquatic Toxicology 34, 327-350.

Arukwe, A., Goksøyr, A. (2003). Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comparative Hepatology 2, 4.

Astraulian Pesticides and Veterinary Mecicines Authority (APVMA), 2010. Atrazine toxicity: analysis of potential modes of action. http://www.apvma.gov.au.

Bakke, J.E., Robbins, J.D., Feil, V.J. (1971). Metabolism of 2-methoxy-4-ethylamino-6-secbutylamino-s-triazine by the dairy cow and the goat. Journal of Agricultural and Food Chemistry 19, 462-466.

Boronat, S., Casado, S., Navas, J.M., Piña, B. (2007). Modulation of aryl hydrocarbon receptor transactivation by carbaryl, a nonconventional ligand. Federation of European Biochemical Societies Journal 274, 4347-4359.

Burke, M.D., Mayer, R.T. (1974). Ethoxyresorufin: direct fluorimetric assay of a microsomal Odealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metabolism and Disposition 2, 583-588.

Chang, L.W., Toth, G.P., Gordon, D.A., Graham, D.W., Meier, J.R., Knapp, C.W., deNoyelles, F.J., Campbell, S., Lattier, D.L. (2005). Responses of molecular indicators of exposure in

mesocosms: common carp (*Cyprinus carpio*) exposed to the herbicides alachlor and atrazine. Environmental Toxicolgy and Chemistry 24, 190-197.

Cimino-Reale, G., Ferrario, D., Casati, B., Brustio, R., Diodovich, C., Collotta, A., Vahter, M., Gribaldo, L. (2008). Combined in utero and juvenile exposure of mice to arsenate and atrazine in drinking water modulates gene expression and clonogenicity of myeloid progenitors. Toxicology Letters 180, 59-66.

Connor, K., Howell, J., Chen, I., Liu, H., Berhane, K., Sciarretta, C., Safe, S., Zacharewski, T. (1996). Failure of chloro-S-triazine-derived compounds to induce estrogen receptormediated responses *in vivo* and *in vitro*. Fundamental and Applied Toxicology 30, 93-101.

Cooper, R.L., Stoker, T.E., Goldman, J.M., Parrish, M.B., Tyrey, L. (1996). Effect of atrazine on ovarian function in the rat. Reproductive Toxicology 10, 257-264.

Cooper, R.L., Goldman, J.M., Stoker, T.E. (1999). Neuroendocrine and reproductive effects of contemporary-use pesticides. Toxicology and Industrial Health 15, 26-36.

Cooper, R.L., Stoker, T.E., Tyrey, L., Goldman, J.M., McElroy, W.K. (2000). Atrazine disrupts the hypothalamic control of pituitary-ovarian function. Toxicological Sciences 53, 297-307.

Denison, M.S., Nagy, S.R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annual Review of Pharmacology and Toxicology 43, 309-334.

Denslow, N.D., Chow, M.C., Kroll, K.J., Green, L. (1999). Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicology 8, 385-398.

Dong, X., Zhu, L., Wang, J., Wang, J., Xie, H., Hou, X., Jia, W. (2009). Effects of atrazine on cytochrome P450 enzymes of zebrafish (*Danio rerio*). Chemosphere 77, 404-412.

Elbrecht, A., Lazier, C.B., Protter, A.A., Williams, D.L. (1984). Independent developmental programs for two estrogen-regulated genes. Science 225, 639-641.

Fernández-Cruz, M.L., Valdehita, A., Alonso, M., Mann, E., Herradón, B., Navas, J.M. (2011). Biological and chemical studies on aryl hydrocarbon receptor induction by the p53 inhibitor pifithrin-α and its condensation product pifithrin-β. Life Sciences 88, 774-783.

Hanioka, N., Jinno, H., Tanaka-Kagawa, T., Nishimura, T., Ando, M. (1999). *In vitro* metabolism of chlorotriazines: characterization of simazine, atrazine, and propazine metabolism using liver microsomes from rats treated with various cytochrome P450 inducers. Toxicology and Applied Pharmacology 156, 195-205.

Hayes, T.B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A.A., Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low

ecologically relevant doses. Proceedings of the National Academy of Sciences U.S.A. 99, 5476-5480.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A. (2003). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environmental Health Perspectives 111, 568-575.

Hayes, T.B., Stuart, A.A., Mendoza, M., Collins, A., Noriega, N., Vonk, A., Johnston, G., Liu, R., Kpodzo, D. (2006). Characterization of atrazine-induced gonadal malformations in African clawed frogs (*Xenopus laevis*) and comparisons with effects of an androgen antagonist (cyproterone acetate) and exogenous estrogen (17beta-estradiol): support for the demasculinization/feminization hypothesis. Environmental Health Perspectives 114, 134-141.

Hayes, T.B., Khoury, V., Narayan, A., Nazir, M., Park, A., Brown, T., Adame, L., Chan, E., Buchholz, D., Stueve, T., Gallipeau, S. (2010). Atrazine induces complete feminizationand chemical castration in male African clawed frogs (*Xenopus laevis*). Proceedings of the National Academy of Sciences U.S.A. 107, 4612-4617.

Herve, J.C., Crump, D., Jones, S.P., Mundy, L.J., Giesy, J.P., Zwiernik, M.J., Bursian, S.J., Jones, P.D., Wiseman, S.B., Wan, Y., Kennedy, S.W. (2010). Cytochrome P4501A induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. Toxicology Sciences 113, 380-391.

Hussain, R., Mahmood, F., Khan, M.Z., Khan, A., Muhammad, F. (2011). Pathological and genotoxic effects of atrazine in male Japanese quail (*Coturnix japonica*). Ecotoxicology 20, 1-8.

Islam, M.O., Hara, M., Miyake, J. (2002). Inductionof P-glycoprotein, glutathione-S-transferase and cytochrome P450 in rat liver by atrazine. Environmental Toxicology and Pharmacology 12, 1-6.

Jonsson, M., Abrahamson, A., Brunstrom, B., Brandt, I., Ingebrigtsen, K., Jorgensen, E.H. (2003). EROD activity in gill filaments of anadromous and marine fish as a biomarker of dioxin-like pollutants. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 136, 235-243.

Kentucky Department of Agriculture (2007). Guidelines for atrazine use and application for groundwater and surface water protection. Kentucky Department of Agriculture, Kentucky.

Kiely, T., Donaldson, D., Grube, A. (2004). Pesticide industry sales and usage -2000 and 2001 market estimates. U.S. Environmental Protection Agency, Office EPA-733- R-04-001, p. 33.

Korte, J.J., Kahl, M.D., Jensen, K.M., Pasha, M.S., Parks, L.G., LeBlanc, G.A., Ankley, G.T. (2000). Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA

and protein expression after estradiol treatment. Environmental Toxicology and Chemistry 19, 972–981.

Lazier, C.B. (1978). Ontogeny of the vitellogenic response to estradiol and of the soluble nuclear oestrogen receptor in embryonic-chick liver. Biochemical Journal 174, 143–152.

Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402-408.

Marin, M.G., Matozzo, V. (2004). Vitellogenin induction as a biomarker of exposure to estrogenic compounds in aquatic environments. Marine Pollution Bulletin 48, 835-839.

Matozzo, V., Gagne, F., Marin, M.G., Ricciardi, F., Blaise, C. (2008). Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review. Environment International 34, 531-545.

Mattsson, A., Olsson, J.A., Brunstrom, B. (2008). Selective estrogen receptor alpha activation disrupts sex organ differentiation and induces expression of vitellogenin II and very low-density apolipoprotein II in Japanese quail embryos. Reproduction 136, 175-186.

Mattsson, A., Olsson, J.A., Brunstrom, B. (2011). Activation of estrogen receptor alpha disrupts differentiation of the reproductive organs in chicken embryos. General and Comparative Endocrinology 172, 251-259.

McMullin, T.S., Brzezicki, J.M., Cranmer, B.K., Tessari, J.D., Andersen, M.E. (2003). Pharmacokinetic modeling of disposition and time-course studies with [14C]atrazine. Journal of Toxicology and Environmental Health, Part A 66, 941-964.

McMullin, T.S., Andersen, M.E., Nagahara, A., Lund, T.D., Pak, T., Handa, R.J., Hanneman, W.H. (2004). Evidence that atrazine and diaminochlorotriazine inhibit the estrogen/progesterone induced surge of luteinizing hormone in female Sprague–Dawley rats without changing estrogen receptor action. Toxicological Sciences 79, 278-286.

Mehats, C., Andersen, C.B., Filopanti, M., Jin, S.L., Conti, M. (2002). Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. Trends in Endocrinology and Metabolism13, 29-35.

Nagler, J.J., Ruby, S., Idler, D.R., So, Y.P. (1987). Serum phosphoprotein phosphorus and calcium levels as reproductive indicators of vitellogenin in highly vitellogenic mature female and estradiol-injected immature rainbow trout (*Salmo gairdneri*). Canadian Journal of Zoology 65, 2421-2425.

Navas, J.M., Segner, H. (2000). Modulation of 7-ethoxyresorufin-O-deethylase (EROD) activity by estradiol and octylphenol. Marine Environmental Research 50, 157-162.

Navas, J.M., Segner, H. (2001). Estrogen-mediated suppression of cytochrome P4501A (CYP1A) expression in rainbow trout hepatocytes: role of estrogen receptor. Chemico-Biological Interactions138, 285-298.

Navas, J.M., Segner, H. (2008). *In vitro* screening of the antiestrogenic activity of chemicals. Expert Opinion on Drug Metabolism and Toxicology 4, 605-617.

Navas, J.M., Chana, A., Herradón, B., Segner, H. (2004). Induction of cytochrome P4501A (CYP1A) by clotrimazole, a non-planar aromatic compound. Computational studies on structural features of clotrimazole and related imidazole derivatives. Life Sciences 76, 699-714.

Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y., Kato, S. (2003). Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature 423, 545-550.

Roberge, M., Hakk, H., Larsen, G. (2004). Atrazine is a competitive inhibitor of phosphodiesterase but does not affect the estrogen receptor. Toxicology Letters 154, 61-68.

Salaberria, I., Hansen, B.H., Asensio, V., Olsvik, P.A., Andersen, R.A., Jenssen, B.M. (2009). Effects of atrazine on hepatic metabolism and endocrine homeostasis in rainbow trout (*Oncorhynchus mykiss*). Toxicology and Applied Pharmacology 234, 98-106.

Sanderson, J.T., Seinen, W., Giesy, J.P., van den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicology Sciences 54, 121-127.

Sanderson, J.T., Letcher, R.J., Heneweer, M., Giesy, J.P., van den Berg, M. (2001). Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environmental Health Perspectives 109, 1027-1031.

Shimabukuro, R.H. (1967). Atrazine metabolism and herbicidal selectivity. Plant Physiology 42, 1269-1276.

Shimada, T., Oda, Y., Gillam, E.M., Guengerich, F.P., Inoue, K. (2001). Metabolic activation of polycyclic aromatic hydrocarbons and other procarcinogens by cytochromes P450 1A1 and P450 1B1 allelic variants and other human cytochromes P450 in *Salmonella typhimurium* NM2009. Drug Metabolism and Disposition 29, 1176-1182.

Smeets, J.M.W., van Holsteijn, I., Giesy, J.P., van den Berg, M. (1999). The anti-estrogenicity of Ah receptor agonists in carp (*Cyprinus carpio*) hepatocytes. Toxicology Sciences 52, 178-188.

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Serrano, F.O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental Health Perspectives 103, 113-122.

Spano, L., Tyler, C.R., van Aerle, R., Devos, P., Mandiki, S.N., Silvestre, F., Thome, J.P., Kestemont, P., 2004. Effects of atrazine on sex steroid dynamics, plasma vitellogenin

concentration and gonad development in adult goldfish (*Carassius auratus*). Aquatic Toxicoligy 66, 369-379.

Stoker, T.E., Laws, S.C., Guidici, D.L., Cooper, R.L. (2000). The effect of atrazine on puberty in male Wistar rats: an evaluation in the protocol for the assessment of pubertal development and thyroid function. Toxicology Sciences 58, 50-59.

Suzawa, M., Ingraham, H.A. (2008). The herbicide atrazine activates endocrine gene networks via non-steroidal NR5A nuclear receptors in fish and mammalian cells. PLoS One 3, e2117.

Thibaut, R., Porte, C. (2004). Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. The Journal of Steroid Biochemistry and Molecular Biology 92, 485-494.

Tillitt, D.E., Papoulias, D.M., Whyte, J.J., Richter, C.A. (2010). Atrazine reduces reproduction in fathead minnow (*Pimephales promelas*). Aquatic Toxicology 99, 149-159.

United States Environmental Protection Agency (U.S. EPA) (2006). Finalization of Atrazine IRED, and Completion of Tolerance Reassessment and Reregistration Eligibility Process. Science Chapter for Atrazine: Environmental Fate and Effects. Office of Prevention, Pesticides and Toxic Substances,Washington, DC.

Victor-Costa, A.B., Bandeira, S.M.C., Oliveira, A.G., Mahecha, G.A.B., Oliveira, C.A. (2010). Changes in testicular morphology and steroidogenesis in adult rats exposed to atrazine. Reproductive Toxicology 29, 323-331.

Wahli, W., Dawid, I.B., Ryffel, G.U., Weber, R. (1981). Vitellogenesis and the vitellogenin gene family. Science 212, 298-304.

Waller, C.L., McKinney, J.D. (1995). Three-dimensional quantitative structure activity relationships of dioxins and dioxin-like compounds: model validation and Ah receptor characterization. Chemical Research in Toxicology 8, 847-858.

Whitlock, J.P. (1999). Induction of cytochrome P4501A1. Annual Review of Pharmacology and Toxicology 39, 103-125.

WHO/IPCS (World Health Organization/International Petroleum Chemical Society) (2002). In: Damstra, T., Barlow, S., Bergman, A., Kavlock, R., Van Der Kraak, G. (Eds.), Global assessment of the state-of-the-science of endocrine disruptors. WHO/IPCS/EDC/02.2. World Healh Organization, Geneva, Switzerland.

Wiley, H.S., Opresko, L., Wallace, R.A. (1979). New methods for the purification of vertebrate vitellogenin. Analytical Biochemistry 97, 145-152.

Wilhelms, K.W., Cutler, S.A., Proudman, J.A., Anderson, L.L., Scanes, C.G. (2005). Atrazine and the hypothalamo-pituitary-gonadal axis in sexually maturing precocial birds: studies in male Japanese quail. Toxicological Sciences 86, 152-160.

Wilhelms, K.W., Cutler, S.A., Proudman, J.A., Carsia, R.V., Anderson, L.L., Scanes, C.G. (2006a). Lack of effects of atrazine on estrogen-responsive organs and circulating hormone concentrations in sexually immature female Japanese quail (*Coturnix coturnix japonica*). Chemosphere 65, 674-681.

Wilhelms, K.W., Fitzpatrick, K.F., Scanes, C.G., Anderson, L.L. (2006b). *In ovo* exposure to a triazine herbicide: effects of atrazine on circulating reproductive hormones and gonadal histology in young Japanese quail. Archives of Environmental Contamination and Toxicology 51, 117-122.

Wiskocil, R., Bensky, P., Dower, W., Goldberger, R.F., Gordon, J.I., Deeley, R.G. (1980). Coordinate regulation of two estrogen-dependent genes in avian liver. Proceedings of the National Academy of Sciences U.S.A 77, 4474-4478.

# CAPÍTULO III

---

**Biomarcadores no destructivos en codornices (*Coturnix coturnix coturnix*) expuestas al herbicida atrazina.**

*de la Casa-Resino, I., Hernández-Moreno, D., Navas, J. M., Soler, F., Pérez-López, M. (2013). Non-destructive multibiomarker approach in European quail (Coturnix coturnix coturnix) exposed to the herbicide atrazine. Archives of Environmental Contamination and Toxicology, 65: 567-574.*

**RESUMEN:** En el presente estudio se ha evaluado el efecto de la administración oral de atrazina (ATZ) (25 y 100 mg/kg durante los días 0, 5 y 10 de experimento) en cuatro biomarcadores no destructivos: porfirinas fecales y niveles sanguíneos de glutatión S-transferasa, glutatión reductasa, glutatión reducido y malondialdehído (MDA) en codorniz europea (*Coturnix coturnix coturnix*). Las principales porfirinas detectadas en heces fueron la uroporfirina I (UPI), y coproporfirinas I y III (CPI y III). La dosis más baja de ATZ causó un incremento significativo ($P$<0,05) en UPI y CPIII en el día 5 del experimento. Además, la dosis más alta de atrazina causó una inducción de CPI y una disminución en los niveles de MDA el día 30 de experimento.

**Palabras clave:** Atrazina, codorniz, glutatión S-transferasa (GST), glutatión reductasa, glutatión reducido (GSH), malondialdehído (MDA), porfirinas.

**ABSTRACT:** The effect of orally administered atrazine (ATZ) (25 or 100 mg/kg, at days 0, 5 and 10 of the experiment) was studied in European quail (*Coturnix coturnix coturnix*) on four non destructive biomarkers: fecal porphyrins, blood glutathione-S-transferase (GST), glutathione reductase (GR), reduced glutathione (GSH) and malondialdehyde (MDA). Uroporphyrin I (UPI) and coproporphyrins I and III (CPI, CPIII) were the main porphyrins detected in feces. The lowest dose provoked a significant ($P$<0.05) increase in UPI and CPIII at day 5 and the highest dose an induction of CPI and a significant (P<0.05) decrease in MDA levels at day 30.

**Keywords:** Atrazine, European quail, glutathione S-transferase (GST), glutathione reductase, reduced glutathione (GSH), malondialdehyde (MDA), porphyrins.

## 1. INTRODUCTION

Many ecosystems are contaminated with industrial, domestic and agricultural chemicals, such as herbicides and insecticides, which show an ubiquitous presence at the regional and even global levels (Jin *et al.,* 2010). During the last 40 years, atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, ATZ) has been one of the most extensively used herbicides in the world, although it has been excluded from a registration process of pesticides in the European Union (Commission Decision 2004/248/EC) it is still used in USA (U.S.EPA 2006). It is primarily applied to increase the yield of food crops such as soy, maize and sugarcane, and is one of the most commonly used pesticides in the United States (Jin *et al.,* 2010). ATZ has been considered as an endocrine disruptor due to alterations caused on hormone regulated systems in various taxa (Hayes *et al.,* 2011; Salaberria *et al.,* 2009; McMullin *et al.,* 2004; de la Casa-Resino *et al.,* 2012). However, little is known about its effects on different metabolic pathways in adult birds. The current ecological risk assessment for ATZ in avian species established by the U.S. EPA reports a dietary non-observed-adverse-effect-concentration (NOAEC) of 225 mg/kg and a lowest-observed-adverse-effect-concentration (LOAEC) of 675 mg/kg (U.S.EPA 2006). When causing its toxic effects ATZ can provoke a variety of alterations in different detoxification and metabolic pathways that can be detected through variations in levels of different substances or enzyme activities. The detection of these variations for instance in feces allow the use of non destructive biomarkers for observing such toxic effects.

In the detoxification of organic xenobiotics cytochromes P450 (cyp) play a key role by catalyzing the oxidation of organic substances in phase I biotransformation reactions. Cyps are hemoproteins with an heme group in their structure (Stine and Brown, 2006) which allow them to carry out electron transport. The heme group synthesis occurs in all body cells, but is especially productive in erythropoietic cells in bone marrow, where nearly all of the heme is used for hemoglobin, and in the liver, where the heme is primarily needed by cyps (Daniell *et al.,* 1997). Heme synthesis is produced by a metabolic pathway that involves eight enzyme-controlled steps, and is often referred to as porphyrin metabolism. Porphyrins and porphyrinogens are cyclic tetrapyrroles that are intermediates for heme biosynthesis. They are produced and accumulated in trace amounts in erithropoietic tissues, the liver and kidneys and are excreted via urine or feces (Lim *et al.,* 1984). It is well known that the heme biosynthesis may be altered by a broad spectrum of environmental contaminants such as polychlorinated biphenyls (PCBs), dioxins, organochlorine and organophosphorous pesticides or polyaromatic hydrocarbons (PAHs), leading to changes in the porphyrins accumulation or excretion profiles (Marks, 1985; Nichol *et al.,* 1982; Frydrych *et al.,* 2006; Casini *et al.,* 2002;

Hernández-Zavala *et al.,* 1999). These changes are also related with the ability of these substances to provoke the induction of those cyps involved in the transformation of the molecules for detoxification (Lämsä *et al.,* 2012). For instance, dioxins and related compounds, such as some PCBs, furanes or PAHs, all of them exhibiting polycyclic and polyaromatic molecules with two or more rings in the same plane (planar molecules) are able to induce the cyp1A (Navas and Segner, 1998; Safe *et al.,* 1991) which on its hand play a key role in the initial oxidation of these substances at the start of the detoxification process. In birds, it has been reported that some xenobiotics such as mercury, hexachlorobenzene (HCB) or polychlorinated biphenyls (PCBs), for example, are able to modify porphyrin levels in feces of Japanese quail (Carpenter *et al.,* 1985; Fossi *et al.,* 1996; Leonzio *et al.,* 1996) and seabirds of the Chilean coast (Casini *et al.,* 2001). However, at present, there is a substantial lack of information about the effect of ATZ on porphyrin levels in birds.

Moreover, ATZ may be responsible for induction of oxidative stress, by interfering with different endpoints and reactive oxygen species (ROS) production in some species. Free radicals are highly reactive and toxic compounds derived from oxidative stress. All the cellular components can be damaged by free radicals but lipids with unsaturated double bonds are especially susceptible (Cross *et al.,* 1987; Pryor, 1982; Sevanian and Hochstein, 1985). Malondialdehyde (MDA) is a main oxidation product of peroxidized polyunsaturated fatty acids and increased MDA level is an important index of lipid peroxidation. To prevent oxidation-induced damage, organisms must have effective antioxidant systems. Some components of these systems involve reduced glutathione (GSH) and certain antioxidant enzymes including free radical scavenging enzymes such as, glutathione peroxidase (GPx) or catalese (CAT) and enzymes as glutathion reductase (GR) withs an important role in GSH recycling (Banerjee *et al.,* 1999; Elia *et al.,* 2002). Other associated enzymes are the glutathione S-transferase (GST) which is involved in the general detoxification process of ATZ through conjugation with GSH (Egaas *et al.,* 1993).

Therefore, the objective of the present study was to investigate if ATZ produce any alteration in the profile of different biomarkers. For that, the levels of fecal porphyrins were quantified to evaluate a possible affectation of the heme group by ATZ, related with phase I metabolism. In addition, blood GST and GR activity as well as the GSH levels were studied to determine any possible effect of ATZ on the induction of oxidative stress. Moreover, the effect of the herbicide on membranes was examined based on the measurement of lipid peroxidation in terms of MDA. Finally, and as a second goal, it was also assessed the possible use of any of these biomarkers in European quail (*Coturnix coturnix coturnix*) as indicators of the presence of ATZ in the environment.

## 2. MATERIAL AND METHODS

### 2.1 Chemicals

Porphyrin standards containing the following type I isomers of octa- (uroporphyrin I, UPI), hepta- (7-CP), hexa- (6-CP), penta- (5-CP), tetra- (coproporphyrin I, CPI) carboxylic porphyrins and coproporphyrin III (CPIII) were obtained from Frontier Scientific Europe (Lancashire, UK). HCl (37%) and acetonitrile (ACN) HPLC grade were from Scharlab (Barcelona, Spain). The herbicide ATZ (98.9%) and all other reagents were of analytical grade and purchased from Sigma-Aldrich (Madrid, Spain).

### 2.2 Animals and treatment

The experimental design has been approved by the Ethical Committee of the University of Extremadura and all the work has been carried out in accordance with the ethical requirements of the current legislation (Directive 2010/63/EU). 45 adult female European quail, free from apparent clinical ailment, were obtained from a local quail farm. The birds (100-150 g) were placed in cages under constant temperature, humidity and lighting conditions (12-h light/dark cycle). Feed and water were offered *ad libitum* during the experiment. After 2 weeks of acclimatization, birds were divided into three equal groups (C, D1 and D2 with 15 animals each one). The birds from group C did not receive ATZ, only the vehicle, corn oil and were considered as control. Regarding the other groups, ATZ dissolved in corn oil was orally administered (through crop tubing) at day 0, 5 and 10 of the experiment as follow: group D1, 25 mg/kg body weight, and group D2, 100 mg/kg body weight. The concentrations were chosen based on the study performed by Hussain *et al.*, (2011), where only the 100 mg/kg dose provoked a decrease in food intake and alterations in some hematological parameters. Moreover, these two doses are lower than those considered as NOAEC or LOAEC, 225 mg/kg and 675 mg/kg, respectively, by U.S. EPA (2006). The birds were monitored daily for clinical signs. At days 0, 5, 10, 15, 30 and 45 of the experiment, feces were collected from the cages of each working group and homogenized before analysis. At days 15, 30 and 45 of the experiment, 5 animals of each group were sacrificed in $CO_2$ chamber. For full performance, the obtained tissues were used in a variety of analyses not restricted to the study presented here. See for instance de la Casa-Resino *et al.*, (2012). The blood was collected from the heart of each bird, and centrifuged at 3000 rpm for 10 min in order to obtain the plasma and erythrocyte pellet. The pellet was homogenized in 0.1 M phosphate buffer ($KH_2PO_4/K_2HPO_4$) pH 7.4 and used to determine GSH and MDA levels. 2 ml of the pellet homogenate was centrifuged at 13,200 g for 20 min to obtain the post-mitochondrial fraction (Gravato *et al.*, 2005). The pellet was discarded and the

supernatant was used to determine the protein content (Bradford, 1976) and the GST activity.

### 2.3 Porphyrin determination

Porphyrins were extracted from fecal homogenates and isolated using a modification of the procedures described by Mateo *et al.,* (2004). In brief, 0.1 g of quail excreta were vortexed in Eppendorf® tubes for 15 s with 0.25 ml of HCl 3 N, 0.3 ml of ACN, and 0.3 ml of distilled water. Samples were centrifuged for 10 min at 16,100 x g in an AvantiTM 30 centrifuge (Beckman Coulter, CA). Each homogenate was assayed in triplicate. Supernatants were filtered using 45 µm PTFE filters (Teknokroma, Barcelona, Spain) and transferred to glass vials for HPLC analysis. The samples were analyzed by and HPLC technique modified from Hernández-Moreno *et al.,* (2012). An LC-20 PROMINENCE HPLC system (Shimadzu, Japan) with autosampler, and fluorescence detector (RF-10Axl) was used. Separation was achieved using a Supelco LC 18 (Supelco, MO) column (25 cm x 4.6 mm x 5 µm particle size). All the chromatographic conditions and quantifications were controlled using LabSolutions software (Shimadzu Corporation, Kyoto, Japan). The initial mobile phase was ammonium acetate (1 M, pH 7.3)/ACN (83:13, v/v), and separation was obtained using a gradient with ACN increasing from 13 to 80 % in 30 min. Afterwards the original ammonium acetate /ACN rate was applied for 15 more minutes. The initial flow rate was 1 ml/min, changing to 1.5 ml/min between minutes 17 and 30. The total runtime was 45 min. The fluorescence detector was set at 400 nm excitation and 622 nm emission.

A porphyrin standard containing carboxylic porphyrins and coproporphyrin III was prepared by dissolving the standards in 3 N HCl. The quantification of samples was conducted using calibration curves constructed with standard solutions (0-500 nM). Extraction efficiencies were evaluated by processing aliquots of porphyrin standards and comparing them with samples spiked with aliquots of porphyrin standards through the extraction procedure. Recovery of the extraction procedure was calculated comparing standard solutions with samples and samples spiked with porphyrins.

### 2.4 Blood glutathione S-transferase (GST) and glutathione reductase (GR) activities, reduced glutathione (GSH) and lipid peroxidation

Blood GST activity was determined using the method of Cohen *et al.,* (1964) modified by Habig *et al.,* (1974) with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction was measured spectrophotometrically at 340 nm each 20 seconds during 5

minutes. Blood GR activity was determined using the method of Cribb *et al.*, (1989) with 5,5'-dithiobis(2-nitrobenzoic acid) as substrate.

GSH levels were measured using a fluoromethric method (Hissin and Hilf, 1976) at 350 nm excitation and 425 nm emission. Accumulation of MDA was used as a calibrator of the lipid peroxidation and evaluated spectrophotometrically at 550 nm by measuring the presence of thiobarbituric acid reactive substances (TBARS), according to the method proposed by Recknagel *et al.*, (1982) using a microplate reader.

### 2.5 Statistical analysis

Data were analyzed using statistical software Prism 5 version 5.03 for Windows (GraphPad software, Inc., CA). Data were tested for normality (test of Kolmogorov–Smirnov and Shapiro–Wilk) and homoscedasticity (Levene's test). Since data did not show a normal distribution and the variances were not homogeneous the statistical analyses were performed using a non-parametric Kruskal-Wallis test (Zar 1984). To compare each treatment (dose) with its own control the Dunn´s test was applied ($P<0.05$). Moreover, a Spearman test was used to determine correlations between GST, GSH and MDA levels in the three sampling days (15, 30 and 45).

## 3. RESULTS

ATZ administered through crop tubing at concentrations 25 mg/kg or 100 mg/kg had no effect on mortality or feed intake (*ad livitum*) in European quail. All animals were free from apparent clinical ailment during the whole experiment.

### 3.1 Effect of ATZ on fecal porphyrins levels

The chromatographic profile of the 6 porphyrins with a run time of 45 minutes is presented in Figure 1a. Figure 1b shows the porphyrin profile found in a control feces sample. Only three main peaks were found in all samples, corresponding to UPI, CPI and CPIII. Recoveries obtained for quail feces spiked with porphyrins were within 94-100% with coefficients of variation less than 30 % for UPI and CPI and less than 11 % for CPIII.

Figure 2 shows the variation in porphyrin content (UPI, CPI and CPIII respectively) in excreta of quails after ATZ administration. The result are expressed as % of the control in order to avoid the variability due to the physiological status of the animals, those raw

control values being 2.95; 4.67; 6.48; 4.15; 5.16 (UI), 8.50; 27.07; 29.21; 17.63; 39.45 (CI) and 59.98; 108.19; 114.54; 112.39; 181.57 (CIII) for day 5, 10, 15, 30 and 45 respectively. CPIII appeared with the highest levels, followed by CPI and UPI.

An important increase respect to control in UPI levels at the beginning of the experiment (days 5 and 10 of the experiment) at the lowest dose, 25 mg/kg, was found. This increase had statistical significance ($P$=0.027) at day 5. At days 15 and 45 of the experiment the D1 group results were slightly lower than those of C, without statistical relevance. Regarding CPIII the results were similar to UPI. An important increase in CPIII levels in group D1 was found at days 5 and 10 ($P$=0.048 and $P$=0.027 respectively), and later stabilized. No remarkable differences were found in D2 respect to C.

In the case of CPI, although a general trend of enhancing was observed in group D1 at days 5, 10 and 30 of the experiment, it had not statistical significance. In contrast, an induction (respect to control) in D2 group at the end of the experiment (day 30) was found ($P$=0.027).

### 3.2 Effect of ATZ on blood GST and GR enzymatic activity, GSH and MDA levels

No significant ($P$>0.05) differences in GST activity among groups were observed at any of the sampling times (Fig. 3a). Nevertheless, it is worth noting that an important trend to enhancing was found in the levels of GST on day 45 at both exposed and control animals.

With respect to the levels of GR (Fig. 3d), it was observed a trend in exposed animals with respect to controls on days 30 and 45, showing higher levels of GR enzymatic activity the exposed animals. Nevertheless, no significative ($P$>0.05) differences were found at any of the sampling time.

GSH blood levels showed a similar pattern than GST, at day 30 of the experiment; moreover, no remarkable differences were found between dose and control groups at any sampling time (fig. 3c). In contrast, MDA levels decreased with respect to the control in ATZ treated birds 20 days after the last administration (i.e. day 30 of the experiment, Fig. 3b) reaching in group D2 (high dose, 100 mg/kg) significantly lower ($P$=0.022) levels than in controls. Similarly to GSH results, no significant differences were found in MDA levels at days 5 and 35 of the experiment. A significative negative correlation between GSH and MDA in the three (15, 30 and 45) sampling days was observed when both C and exposed groups were considered (correlation coefficient -0.871;-0.593; -0.767 and $P$=0.00002; $P$=0.020 and $P$=0.01 respectively).

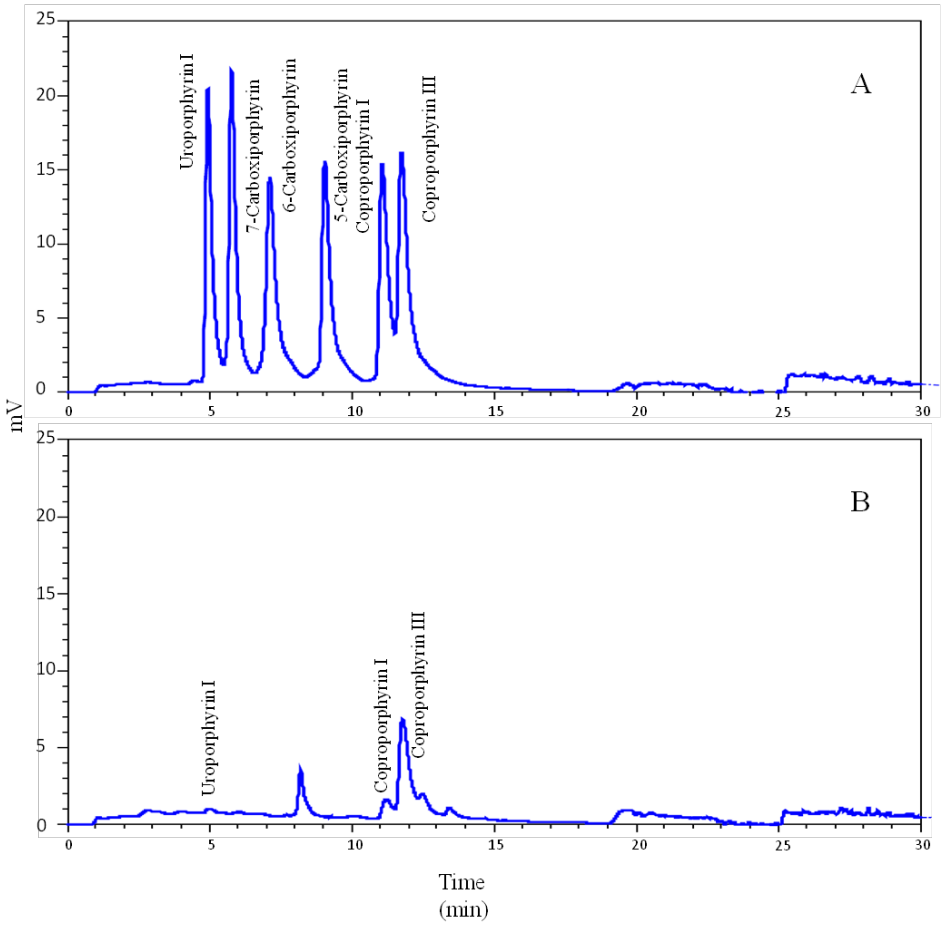



**Figure 1.** HPLC profile (25 µl) of porphyrin acid standards (500 nM of each one, a) and a sample of feces (control, b).

## 4. DISCUSSION

The experiments performed in the present work were designed to assess the effects of ATZ on different non-destructive biomarkers in order to evaluate their usefulness as tools for recognizing exposure of birds to ATZ. This herbicide is mainly applied in the U.S. on corn, sugarcane and sorghum crops normally in spring (KY Department of Agriculture, 2007). Although a continuous exposure to the toxicant through ingestion of contaminated corns or vegetables is possible in birds, we considered that an intermittent exposure was more realistic taking into account possible variability in the accessibility to this contaminated food. Usually, effects appear late after exposure due to the time needed to modify the compensation mechanisms that maintain the homeostasis of organisms. To cover these long term effects, possible alterations in the experimental animals were

monitored 5, 10, 15, 30 and 45 days after the beginning of the treatment. In addition, ATZ was not administered in a continuous way, but only three times with five days interval. All this makes the conditions used in the present work closer to realistic field situations.

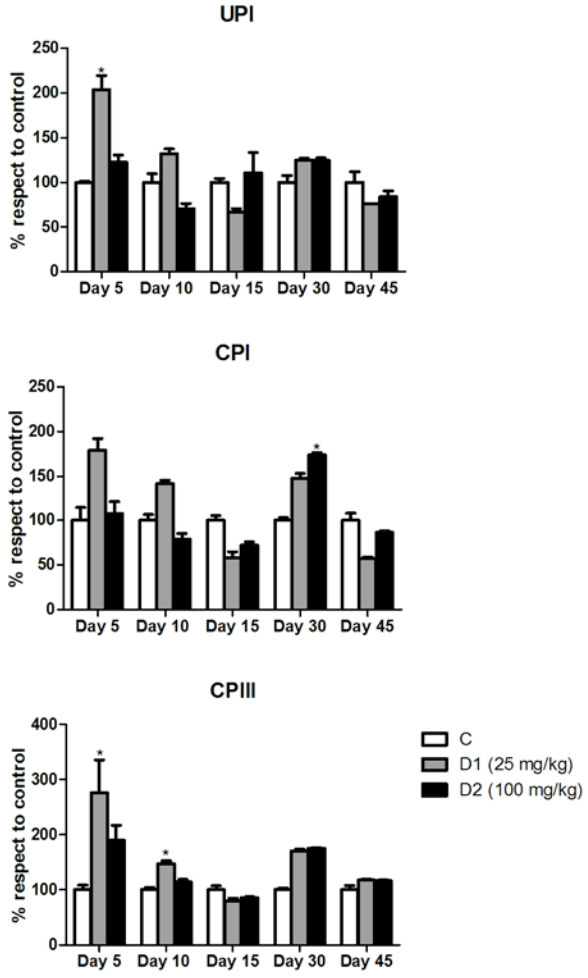



**Figure 2.** UPI, CPI, and CPIII levels in feces of European quail exposed to ATZ, i.e., groups D1 (25 mg/kg bw) and D2 (100 mg/kg bw). * Statistically significant difference compared with the control ($P$<0.05).

The importance of porphyrin profile determination in the assessment of the toxic response to exposure to porphyrinogenic compounds has been highlighted. Sometimes the total porphyrins may remain constant even when subtle changes to the porphyrin profile can be found (Kennedy *et al.*, 1986). The porphyrin extraction method used in

this study showed good recoveries for all the porphyrins studied. This could be accomplished with a double extraction with an ACN:HCl 3 N mixture that permitted the solubilization of the less polar porphyrins from biological samples, followed by centrifugation without further purification steps (Mateo *et al.*, 2004, 2006). Results showed that the main porphyrins isolated in feces were UPI, CPI and CPIII, with a clear predominance of CPIII. The porphyrins formed towards the end of the metabolic pathway, as coproporphyrins, are normally found in feces due to their hydrophobicity. In addition, the hepatobiliary tract is the main route of elimination for the latter compounds, but small amounts of uroporphyrin and heptacarboxyl porphyrin may appear in fecal excreta (Zaider and Bickers, 1998). The findings presented in this paper are characterized by elevated UPI and CPIII 5 days after the first administration of ATZ (at the beginning of the experiment), and the increase of CPI 20 days after the last administration of ATZ, in both cases at low doses (25 mg/kg bw). Similar results were found in pigeons (*Columba livia*) exposed to air pollution. In that study, the porphyrin profile showed a predominance of both coproporphyrins and UPI, as observed in the present study. Moreover, an increase in the mean of total porphyrins were found in animals exposed to normal air pollution compared with pigeons exposed to clean air (Sicolo *et al.*, 2009). Increased porphyrin levels were observed in Japanese quail (*C. coturnix japonica*) exposed to different xenobiotics. Carpenter *et al.*, (1985) found an increase in the total liver porphyrin content in quails exposed to 500 mg HCB/kg/day for 10 days. This increase was 10 fold higher than control group after 5 days of exposure. Other researchers have induced porphyrias in the Japanese quail by administering much lower doses for longer periods, such as the study performed by Elliot *et al.*, (1997) where an increase of hepatic 4-carboxyporphyrin was found in quails exposed to Arochlor 1254 (7 mg/kg bw/day) for 4 weeks. These results agree with those of other studies with mammals. For instance, in male and female rabbits exposed to two different doses of diazinon (25 and 125 µg/kg bw), only UPI and CPI were found when feces were analyzed by HPLC. However, UPI levels in animals exposed to diazinon were clearly decreased compared to control rabbits. Males showed a clear inhibitory effect in both doses that was statistically significant at 20 and 30 days after exposure ($P<0.05$). The same was found with female rabbits, in this case with statistically significant differences ($P<0.05$) among the three sampling times (days 10, 20 and 30 of the experiment). As happened in the present study, the most important effect (pesticide-associated porphyrin inhibitory effect) was specially observed in animals exposed to the lowest dose (Hernandez-Moreno *et al.*, 2012). In kidney tissue of rats exposed to hexabromobenzene (HBB) at dose 15, 75 and 375 mg/kg bw an increase in the UPI levels when animals were exposed to the lowest dose (15 mg/kg bw) of HBB on day 7 was observed (Frydrych, 2006). In liver, however, the increase in the porphyrin levels takes place

during the whole experiment, not only at the beginning (Frydrych, 2006). Heme synthesis in erythroid cells differs from that in hepatocytes; it is linked to tissue differentiation and the half-life of the same end-product is quite different (Fujita *et al.*, 1991). Regulation in liver is exquisitely sensitive to fluctuations in intracellular heme levels because it needs to respond rapidly to the requirements for synthesis. In contrast, in the bone marrow a quick response it is not necessary (Abraham, 1991). The presence of ATZ in the avian organism could trigger an increase in the heme synthesis necessary for the CYP. The CYP hepatic activity did not show any differences between control and dosed groups as previously reported (de la Casa-Resino *et al.*, 2012). This CYP activity was assayed at day 15 of the experiment after the last ATZ administration, whereas the most significant porphyrin differences were found previously, at days 5 and 10. The results obtained in the present study seem to indicate a clear porphyrinogenic action of ATZ at low doses. However further studies are necessary to clarify these point and to establish the causality of these findings.

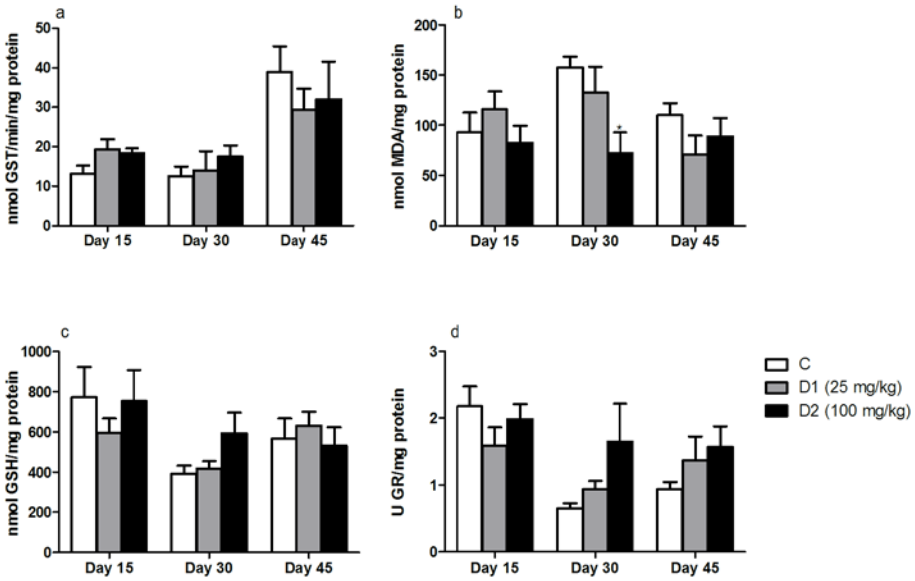



**Figure 3.** Activities of GST (a) and GR (d), as well as MDA (b) and GSH (c) levels, in blood of female European quail exposed to ATZ, i.e., groups D1 (25 mg/kg bw) and D2 (100 mg/kg bw). Values are presented as a mean ± SEM (n = 5). *Statistically significant difference compared with the control ($P$<0.05).

Our data constitute the first report about oxidative stress in blood of birds treated orally with ATZ. In blood, normal erythrocyte function depends on the intactness of cell membrane which is the target for many toxic factors including pesticides (Banerjee *et al.*,

1999). We hypothesize that lipid peroxidation (evaluated as MDA levels) and GSH are modified in blood 20 days after the last administration (day 30 of the experiment). This appears to be an adaptive response to oxidative stress. Oxidative stress has also been reported in the hepatic and adrenal tissues of fish sampled in areas impacted by agrochemicals such as simazine, atrazine, and deethyl-atrazine (Dorval *et al.,* 2005). The capacity of pesticides to induce oxidative stress in different organs of mammals and invertebrates is also known (Bagchi *et al.,* 1995; Salaberria *et al.,* 2009; Abarikwu *et al.,* 2010). In our study, the dose-dependent increase in the activity of GST at day 30 might be partly related to the availability increased of the substrate, GSH, although it was not statistically significative in any case. Moreover, the changes obtained in MDA concentration allow us to conclude that the erythrocyte antioxidant defense system still effectively protects from the action of ATZ-induced free radicals.

## 5. CONCLUSION

In conclusion, porphyrins levels in bird excreta may be used as indicators of exposure to ATZ, thus providing a non-destructive method useful for monitoring wildlife exposure to ATZ and to different kind of chemical pollutants.

The experimental data obtained with birds, designed as a long-term experiment under controlled laboratory conditions can be considered as a useful reference for comparisons with biomarkers response of organisms living in polluted environments.

## ACKNOWLEDGEMENT

Irene de la Casa-Resino was supported by fellowship PRE09001 from Consejería de Empleo, Empresa e Innovación (Gobierno de Extremadura, Spain). Authors wish to thank the "Ministerio de Educación y Ciencia" from Spain, which financially supported this work (CTM2007-60041). The INIA team acknowledges the support of INIA project RTA2009-00074-00-00 in all the work related with endocrine disruption.

## REFERENCES

Abarikwu, S.O., Adesiyan, A.C., Oyeloja, T.O., Oyeyemi, M.O., Farombi, E.O. (2010). Changes in sperm characteristics and induction of oxidative stress in the testis and epididymis of experimental rats by a herbicide, atrazine. Archives of Environmental Contamination and Toxicology 58, 874-882.

Abraham, N.G. (1991). Molecular regulation-biological role of heme in hematopoiesis. Blood Reviews 5, 19-28.

Bagchi, D., Bagchi, M., Hassoun, E.A., Stohs, S.J. (1995). *In vitro* and *in vivo* generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. Toxicology 104, 129-140.

Banerjee, B.D., Seth, V., Bhattacharya, A., Pasha, S.T., Chakraborty, A.K. (1999). Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. Toxicology Letters 107, 33-47.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Carpenter, H.M., Williams, D.E., Buhler, D.R. (1985). Hexachlorobenzene-induced porphyria in Japanese quail: changes in microsomal enzymes. Journal of Toxicology and Environmental Health, Part A 15, 431-444.

Casini, S., Fossi, M.C., Cavallaro, K., Marsili, L., Lorenzani, J. (2002). The use of porphyrins as a non-destructive biomarker of exposure to contaminants in two sea lion populations. Marine Environmental Research 54, 769-773.

Casini, S., Fossi, M.C., Gavilan, J.F., Barra, R., Parra, O., Leonzio, C., Focardi, S. (2001). Porphyrin levels in excreta of sea birds of th chilenan coasts as nondestructive biomarker of exposure ton environmental pollutants. Archives of Environmental Contamination and Toxicology 41, 65-72.

Cohen, A.J., Smith, J.N., Turbert, H. (1964). Comparative detoxication. 10. The enzymic conjugation of chloro compounds with glutathione in locusts and other insects. Biochemical Journal 90, 457-464.

Cribb, A.E., Leeder, J.S., Spielberg, S.P. (1989). Use of a microplate reader in an assay of glutathione reductase using 5,5'-dithiobis(2-nitrobenzoic acid). Analitical Biochemistry 183, 195-196.

Cross, C.E., Halliwell, B., Borish, E.T., Pryor, W.A., Ames, B.N., Saul, R.L., McCord, J.M., Harman, D. (1987). Oxygen radicals and human disease. Annals of Internal Medicine 107, 526-545.

Daniell, W.E., Stockbridge, H.L., Labbe, R.F., Woods, J.S., Anderson, K.E., Bissell, D.M., Bloomer, J.R., Ellefson, R.D., Moore, M.R., Pierach, C.A., Schreiber, W.E., Tefferi, A., Franklin, G.M. (1997). Environmental chemical exposures and disturbances of heme synthesis. Environmental Health Perspectives 105, 37-53.

de la Casa-Resino, I., Valdehita, A., Soler, F., Navas, J.M., Pérez-López, M. (2012). Endocrine disruption caused by oral administration of atrazine in European quail (*Coturnix coturnix*

*coturnix*). Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology 156, 159-165.

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union 2010, 273, 33-79.

Dorval, J., Leblond, V., Deblois, C., Hontela, A. (2005). Oxidative stress and endocrine endpoints in White sucker (*Catostomus commersoni*) from a river impacted by agricultural chemicals. Environmental Toxicology and Chemistry 24, 1273-1280.

Egaas, E., Skaare, J.U., Svendsen, N.O., Sandvik, M., Falls, J.G., Dauterman, W.C., Collier, T.K., Netland, J. (1993). A comparative study of effects of atrazine on xenobiotic metabolizing enzymes in fish and insect, and of the *in vitro* phase II atrazine metabolism in some fish, insects, mammals and one plant species. Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology 106, 141-149.

Elia, A.C., Waller, W.T., Norton, S.J. (2002). Biochemical responses of bluegill sunfish (*Lepomis macrochirus*, Rafinesque) to atrazine induced oxidative stress. Bulletin of Environmental Contamination and Toxicology 68, 809-816.

Elliott, J.E., Kennedy, S.W., Lorenzen, A. (1997). Comparative toxicity of polychlorinated biphenyls to Japanese quail (*Coturnix c. japonica*) and American kestrels (*Falco sparverius*). Journal of Toxicology and Environmental Health, Part A 51, 57-75.

Fossi, M.C., Casini, S., Marsili, L. (1996). Porphyrins in excreta: a nondestructive biomarker for the hazard assessment of birds contaminated with PCBs. Chemosphere 33, 29-42.

Frydrych, B., Szymańska, J.A., Świderska-Comer, A. (2006). Concentration of porphyrins in rat liver and kidneys after repeated administration of hexabromobenzene and tetrabromobisphenol-A. Polish Journal of Environmental Studies 15, 543-548.

Fujita, H., Yamamoto, M., Yamagami, T., Hayashi, N., Sassa, S. (1991). Erythroleukemia differentiation. Distinctive responses of the erythroid-specific and the nonspecific delta-aminolevulinate synthase mRNA. The Journal of Biological Chemistry 266, 17494-17502.

Gravato, C., Oliveira, M., Santos, M.A. (2005). Oxidative stress and genotoxic responses to resin acids in mediterranean mussels. Ecotoxicology and Environmental Safety 61, 221-229.

Habig, W.H., Pabst, M.J., Jakoby, W.B. (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. The Journal of Biological Chemistry 249, 7130-7139.

Hayes, T.B., Khoury, V., Narayan, A., Nazir, M., Park, A., Brown, T., Adame, L., Chan, E., Buchholz, D., Stueve, T., Gallipeau, S. (2011). Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proceedings of the National Academy of Sciences U.S.A. 107, 4612-4617.

Hernandez-Moreno, D., Perez-Lopez, M., Miguez, M.P., Soler, F., Jimenez, B. (2012). Porphyrin levels in excreta of rabbit as non-destructive biomarkers of diazinon exposure. Environmental Toxicology and Pharmacology 34, 466-472.

Hernández-Zavala, A., Del Razo, L.M., García-Vargas, G.G., Aguilar, C., Borja, V.H., Albores, A., Cebrián, M.E. (1999). Altered activity of heme biosynthesis pathway enzymes in individuals chronically exposed to arsenic in Mexico. Archives of Toxicology 73, 90-95.

Hissin, P.J., Hilf, R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analitical Biochemistry 74, 214-226.

Hussain, R., Mahmood, F., Khan, M.Z., Khan, A., Muhammad, F. (2011). Pathological and genotoxic effects of atrazine in male Japanese quail (*Coturnix japonica*). Ecotoxicology 20, 1-8.

Jin, Y., Chen, R., Liu, W., Fu, Z. (2010). Effect of endocrine disrupting chemicals on the transcription of genes related to the innate immune system in the early developmental stage of zebrafish (*Danio rerio*). Fish and Shellfish Immunology 28, 854-861.

Kennedy, S.W., Wigfield, D.C., Fox, G.A. (1986). Tissue porphyrin pattern determination by high-speed high-performance liquid chromatography. Analitical Biochemistry 157, 1-7.

Kentucky Department of Agriculture (2007). Guidelines for atrazine use and application for groundwater and surface water protection. Kentucky Department of Agriculture, Kentucky.

Lämsä, V., Levonen, A.L., Sormunen, R., Yamamoto, M., Hakkola, J. (2012). Heme and heme biosynthesis intermediates induce heme oxygenase-1 and cytochrome P450 2A5, enzymes with putative sequential roles in heme and bilirubin metabolism: different requirement for transcription factor nuclear factor erythroid- derived 2-like 2. Toxicological Sciences 130, 132-144.

Leonzio, C., Fossi, M.C., Casini, S. (1996). Porphyrins as biomarkers of methylmercury and PCB exposure in experimental quail. Bulletin of Environmental Contamination and Toxicology 56, 244-250.

Lim, C.K., Rideout, J.M., Peters, T.J. (1984). High-performance liquid chromatography of dicarboxylic porphyrins and metalloporphyrins: retention behaviour and biomedical applications. Journal of Chromatography 317, 333-341.

Marks, G.S. (1985). Exposure to toxic agents: the heme biosynthetic pathway and hemoproteins as indicator. Critical Reviews in Toxicology 15, 151-179.

Mateo, R., Castells, G., Green, A.J., Godoy, C., Cristofol, C. (2004). Determination of porphyrins and biliverdin in bile and excreta of birds by a single liquid chromatography-ultraviolet detection analysis. Journal of Chromatography B 810, 305-311.

Mateo, R., Taggart, M.A., Green, A.J., Cristofol, C., Ramis, A., Lefranc, H., Figuerola, J., Meharg, A.A. (2006). Altered porphyrin excretion and histopathology of greylag geese (*Anser anser*) exposed to soil contaminated with lead and arsenic in the Guadalquivir Marshes, southwestern Spain. Environmental Toxicology and Chemistry 25, 203-212.

McMullin, T.S., Andersen, M.E., Nagahara, A., Lund, T.D., Pak, T., Handa, R.J., Hanneman, W.H. (2004). Evidence that atrazine and diaminochlorotriazine inhibit the estrogen/progesterone induced surge of luteinizing hormone in female Sprague-Dawley rats without changing estrogen receptor action. Toxicological Sciences 79, 278-286.

Navas, J.M., Segner, H. (1998). Antiestrogenic activity of anthropogenic and natural chemicals. Environmental Science and Pollution Research 5, 75-82.

Nichol, A.W., Elsbury, S., Elder, G.H., Jackson, A.H., Rao, K.R.N. (1982). Separation of impurities in diazinon preparations and their effect on porphyrin biosynthesis in tissue culture. Biochemical Pharmacology 31, 1033-1038.

Recknagel, R.O., Glende, E.A., Walker, R.L., Lowery, K. (1982). Lipid peroxidation biochemistry measurement and significance in liver cell injury. In: Plaa, G.L., Hewitt, W.R. (Eds) Toxicology of the liver. Raven Press, New York, pp 213-241.

Safe, S., Astroff, B., Harris, M., Zacahrewski, T., Dickerson, R., Romkes, M., Biegel, L. (1991). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and related compounds as antioestrogens: characterization and mechanism of action. Pharmacology and Toxicology 69, 400-409.

Salaberria, I., Hansen, B.H., Asensio, V., Olsvik, PA., Andersen, R.A., Jenssen, B.M. (2009). Effects of atrazine on hepatic metabolism and endocrine homeostasis in rainbow trout (*Oncorhynchus mykiss*). Toxicology and Applied Pharmacology 234, 98-106.

Sevanian, A., Hochstein, P. (1985). Mechanisms and consequences of lipid peroxidation in biological systems. Annual Review of Nutrition 5, 365-390.

Sicolo, M., Tringali, M., Orsi, F., Santagostino, A. (2009). Porphyrin pattern and methemoglobin levels in *Columba livia* applied to assess toxicological risk by air pollution in urban areas. Archives of Environmental Contamination and Toxicology 57. 732-740.

Stine, K.E., Brown, T.M. (2006). Principles of toxicology. 2nd edition. Taylor and Francis, New York.

U.S. EPA (2006). Finalization of Atrazine IRED, and Completion of Tolerance Reassessment and Reregistration Eligibility Process (April 6, 2006). Science Chapter for Atrazine: Environmental Fate and Effects. U.S. EPA, Washington DC.

Zaider, E., Bickers, D.R. (1998). Clinical laboratory methods for diagnosis of the porphyrias. Clinics in Dermatology 16, 277-293.

Zar, J.H. (1984). Biostatistical analysis. 2nd edn. Prentice Hall, Inc., New Jersey.

# Capítulo IV

**La cria cerca de una planta de tratamiento de residuos sólidos urbanos puede influir en los niveles sanguíneos de metales (Cd, Pb, Hg, Fe, Zn) y metaloides (Se, As) en los pollos de cigüeña blanca (*Ciconia ciconia*).**

*de la Casa-Resino, I., Hernández-Moreno, D., Castellano A., Pérez-López, M., Soler, F. Breeding near a landfill may influence blood metals (Cd, Pb, Hg, Fe, Zn) and metalloids (Se, As) in White Stork (Ciconia ciconia) nestlings. Submitted to Ecotoxicology.*

**RESUMEN:** Se han determinado los niveles de cadmio, mercurio, selenio, hierro, zinc y arsénico en muestras de sangre de 59 pollos de cigüeña blanca procedentes de 3 colonias situadas en tres ambientes diferentes en el oeste de España. La colonia de referencia estaba situada en "Llanos de Cáceres y Sierra de Fuentes", una zona de especial protección para aves (ZEPA). La segunda colonia estaba situada cerca (4,9 km) de una planta de tratamiento de residuos sólidos urbanos (PTRSU) y la tercera colonia en un área de agricultura intesiva con una PTRSU (1,5 km). El análisis de las muestras de sangre se llevó a cabo mediante ICP-MS. En todos los casos, las mayores concentraciones se cuantificaron para los dos metales esenciales, zinc y hierro, seguidos por plomo>selenio>mercurio>arsénico>cadmio. En cuanto a los metales tóxicos, las mayores concentraciones se encontraron para el plomo (23,27-146,4 µg/l), aunque en todos los casos las concentraciones detectadas estuvieron por debajo de aquellas consideradas como causantes de efectos subclínicos. Los niveles de metales detectados en la sangre de los pollos no estuvieron relacionados con los niveles detectados en los suelos cercanos a la colonia previamente publicados, lo que parece indicar que las PTRSU son las principales fuentes de metales en las crías de cigüeña blanca. Estos resultados muestran que los niveles de metales en los pollos de cigüeña blanca puenden estar influenciados por el uso de las PTRSU como zonas de alimentación por los progenitores. No obstante, son necesarios más estudios para determinar el contenido en metales en el alimento de los padres asi como la influencia de la distancia a las PTRSU y así poder establecer las causas de los resultados obtenidos.

**Palabras clave:** cigüeña blanca, metales, sangre, contaminación, PTRSU.

**ABSTRACT:** Cadmium, lead, mercury, selenium, iron, zinc and arsenic levels were measured in blood samples from 59 free-ranging White stork nestlings from colonies located in 3 different environmental conditions in Western Spain. The reference colony was situated in "Llanos de Cáceres y Sierra de Fuentes", an Area of Special Interest for Bird Protection. A second colony was located close to (4.9 km) an urban landfill and a third one was close to both an intensive agricultural area and an urban landfill (1.5 km). Blood samples were diluted and elemental analysis was performed using ICP-MS. In all cases, the essential metals zinc and iron were found at the highest mean concentrations followed by lead>selenium>mercury>arsenic>cadmium. Regarding toxic metals, the highest concentrations were found for lead (ranging from 23.27 to146.4 µg/L) although in all cases the concentrations were lower than those considered to cause subclinical effects. The metals levels detected in the chick's blood were not related to the previously reported levels in the soil next to the colonies, which may indicate that landfills are the

main source of metals in White stork nestlings. The present data showed that metal levels in White stork chicks may be influenced by the use of landfills as feeding areas by the parents. However, more studies on the metal content in the feed of White stork and the influence of the distance to the landfill are necessary to establish the causality of these findings.

**Keywords:** White stork, metals, blood, pollution, landfill.

## 1. INTRODUCTION

Metals and metalloids are natural components in the environment and many of them are essential micronutrients for organisms. In general, they could be concentrated through food webs, and thus the species situated on the top accumulate high levels of metals (Hernández *et al.,* 1999; Koivula and Eeva, 2010). Inputs of metals to the environment as a result of anthropogenic activities are difficult to measure due to the wide natural inputs from the erosion of rocks, wind-blowing dusts, volcanic activity and forest fires. Moreover, many metals are essential to live organisms (i.e. Fe, Se and Cu), but they can become toxic at high concentrations. However, some metals and metalloids such as Pb, Hg, Ni or As are generally not required for metabolic activity and are toxic to living organisms at low concentrations (Merian, 1991).

Avian species, through their trophic relationships, represent ideal indicators for assessing environmental pollution by metals (Baos *et al.,* 2006a,b; Alvarez *et al.,* 2013; Binkowski *et al.,* 2013). The White storks (*Ciconia ciconia*), a large (2.2–4.4 Kg), long-lived wading bird that breeds from North Africa to Northern Europe. At the latitude of the Iberian Peninsula, they have a prolonged breeding season (from February to July), with chicks hatching after 33–34 days of incubation and depending on both adults for food and shelter during approximately 75 days before fledging (Cramp and Simmons, 1980). Developing organisms has a potentially elevated susceptibility to pollution compared to adults and White stork chicks are usually used as bioindicators of environmental contamination by metals (Benito *et al.,* 1999; Hernández *et al.,* 1999; Smits *et al.,* 2005; Baos *et al.,* 2006a,b, 2012; Kamiński *et al.,* 2009; Goutner *et al.,* 2011; Cabo *et al.,* 2012; Tkachenco *et al.,* 2012; Álvarez *et al.,* 2013) and other contaminants (Blazquez *et al.,* 2006).

White stork is mentioned in EU Birds Directive (Directive 2009/147/EC) because there has been a major decline of their population through the twentieth century. Several factors have been put forward to explain this decrease, including alterations of the breeding habitat, changes in European climate, food resources, head power lines and pesticides between others (Dallinga and Schoenmakers, 1987). However, in South European countries (France, Spain and Portugal) this trend seems to be reversed. This difference could be due to local reintroduction programs and the availability of food at rubbish dumps, but the more extensive change is still not completely understood (Tortosa *et al.,* 2002). White stork is a colonial species that feeds mainly on wildlife preys; however, landfills have become in the latter years an important source of nutrients, and for that reason an increasing percentage of the population is sedentary (Blanco 1996; Kruszyk and Ciach 2010; Massemin-Challet *et al.,* 2006; Peris 2003; Tortosa *et al.,*

2002). The food provided by landfills has a positive influence in the breeding success in this species (Tortosa *et al.,* 2002). However, it can be the way of exposure to other pollutants (Clarkson *et al.,* 1983) which can influence negatively the future reproduction success. Landfills have been reported as areas that may increase the levels of metals in the soil around them (Earle *et al.,* 1999; Hernández *et al.,* 1998; Matejczyk *et al.,* 2011; Rushton 2003). In White stork nestlings several toxic effects (leg deformities, altered adrenocortical stress response or even genotoxic effects) have been associated with metals contamination after a mining spill in Southwestern Spain (Baos *et al.,* 2006a,b; Smits *et al.,* 2005). Developmental exposure to these chemicals, even at low levels, may be particularly detrimental, with potential long-term effects on reproduction and ultimately individual fitness (Baos *et al.,* 2012). Moreover, White stork adults migrate long distances but the prefledging young are fed entirely on food resources obtained locally by their parents and are appropriate as sentinels of contaminants within a local environment (Blázquez *et al.,* 2006).

The present study aimed to provide data on the incidence of persistent toxic substances in White stork nestlings from three different colonies of Extremadura (West of Spain), representative of three different environmental influences (grassland, urban landfill and agricultural area near to an urban landfill). Specifically, the presence of selected metals (Cd, Pb, Hg, Fe, and Zn) and metalloids (Se and As) was assessed in blood of 59 chicks to study how the local environment where their parents were feeding could influence their exposure to these compounds.

## 2. MATERIAL AND METHODS

### 2.1 Reagents

Solvents and reagents used were of analytical grade or high purity grade and purchased from Panreac (Moncada i Reixac, Spain) and Merck (Darmstad, Germany). Milli-Q water was used to prepare solutions and dilutions.

The internal standard used for elemental analysis by ICP-MS was a solution of Yttrium, Rhenium, Rhodium and Tellurium (10 mg/L) purchased from Perkin Elmer, Inc. (Shelton, USA).

### 2.2 Study area

Fieldwork was conducted in three colonies located in Cáceres province (Extremadura region, Spain) during spring 2011 covering a gradient of potential contamination (Figure

1). The reference colony (colony A) was located in “Los Llanos de Cáceres y Sierra de Fuentes” (39º 28’ 17.53’’ N, 6º 10’ 35.00’’ W), a natural area far from apparent sources of pollution, considered a Special Protection Area for Birds under the Directive 2009/147/CE and forming an integral part of the NATURA 2000 ecological network. It is a pseudo steppe zone crossed by 4 rivers (Tajo, Almonte, Tamuja and Salor) that contains an important number of protected steppe birds like Bustard (*Otis tarda*), Little Bustard (*Tetrax tetrax*) and Lesser Kestrel (*Falco naumanni*). It is characterized by rainfed crops, mainly cereals but some legumes too, that are used for feeding livestock, sheep and cattle, in an extensive farming.

The second colony (colony B) was located in a grassland area similar to the colony A but with more trees (Holm oak, *Quercus ilex*) (39º 18’ 37.13’’ N, 6º 29’ 47.53’’ W), at a distance of 13.4 km far from Cáceres town (95668 inhabitants, 2012) and close (4.9 km) to a landfill that handled 61826 tons of solid waste in 2011 (Domínguez *et al.*, 2011) where the White storks frequently go to feed during their breeding period (Medina *et al.*, 1998). An important road (“national” road) was 15 m far from the nearest nest in this colony. The third colony (colony C) was located between the towns of Navalmoral de la Mata (17401 inhabitants, 2012) and Talayuela (9269 inhabitants, 2012) (39º 18’ 37.13’’N, 6º 29’ 47.53’’ W). It was situated in a grassland area with Quercus ilex trees used for livestock and next to an important intensive agricultural area crossed by the Tietar river which is characterized for large tobacco, pepper, tomato, asparagus and corn crops. Close to colony C (1.5 km) there was a landfill that handled 39446 tons of solid waste in 2011 (Domínguez *et al.*, 2011). Both landfills receive urban waste from the cities and towns nearby and were built according the Directive 1999/31/CE. They consist on a pre-constructed ‘cell’ lined with an impermeable layer (man-made or natural) and with controls to minimize emissions to water, land or air. The distance between colonies A and B was 26.2 km and between colonies A and C was 71.7 km. The distance between colonies B and C was 97 km. All nests were placed on trees.

### 2.3 Field procedure and blood sampling

During the spring of 2011, with the regional government permission (Gobierno de Extremadura CN10/0306), White stork nests were selected and sampled in each colony depending on its accessibility (A, n = 11; B, n = 8; C, n = 6). The sampled nests were dispersed into the colony. The number of animals sampled was as big as possible according to the accessibility to the nest, the number of nests in the colony, the weight (2.3-3 kg), the developmental state of nestling (3-4 weeks) and the climate on sampling days (avoiding the hottest time of the day). Both standardized capture and handling

method were performed in order to produce the minimal stress to the animals. All nestlings from each colony were taken down from their nests, gently restrained by hand during blood collection, physical examination, weighing and measuring.

Blood samples (about 5 ml) were taken from the tarsal vein with heparinized 0.8x25 mm needle and 5 ml syringe. The whole blood samples were refrigerated until arriving to the laboratory, where they were stored at -80ºC in 1.5 ml plastic tubes previously washed with $HNO_3$ 2%.

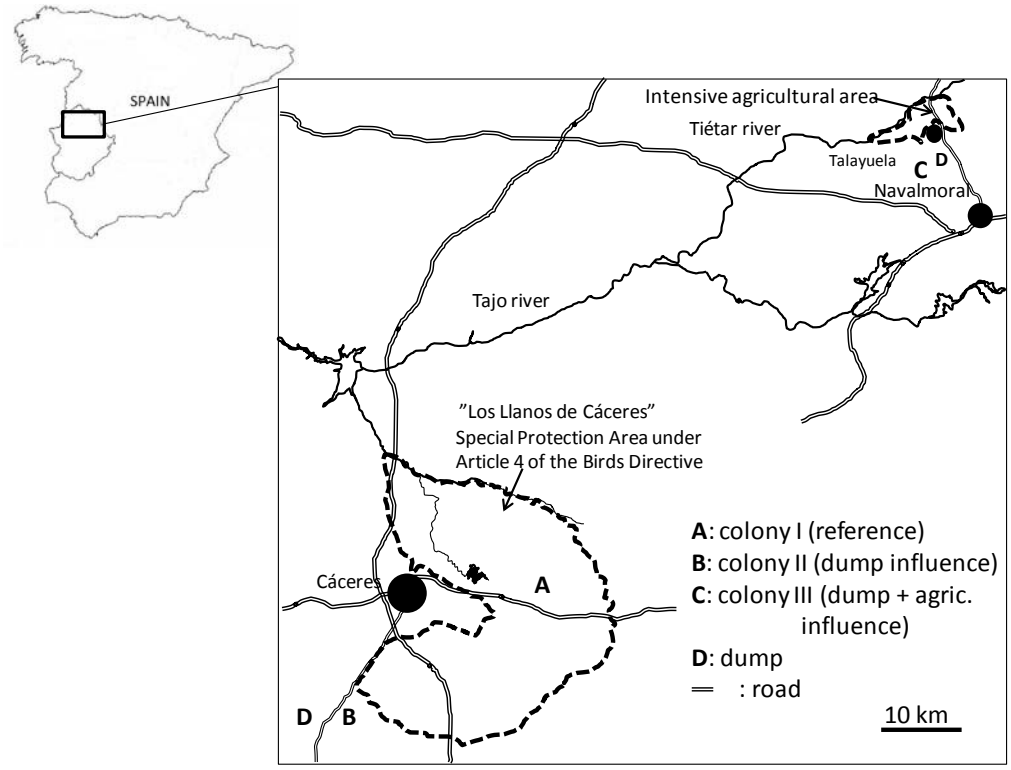



**Figure 1.** Geographical location of the study area (Cáceres province). A: reference colony; B: close to a landfill, and C: close to a landfill and an agricultural area. The landfills (D) are also indicated.

## 2.4 Determination of metals and metalloids

Analyses were done in the Elemental and Molecular Analysis Laboratory of the Research Support Service (SAIUEX, accredited by ISO 9001:2008) from the University of Extremadura. Blood samples were thawed and gently shaken using an orbital shaker during 20 s for homogenization. An aliquot of 200 µl of blood was taken and then 50 µl of isopropanol and 25 µl of the internal standard solution were added. This mix was

filled up to 5 ml with an aqueous solution containing NH4OH (0.7 mM), Triton X-100 (0.07 % v/v) and EDTA (0.01 mM) and finally vortexed. A Platform collision cell inductively coupled plasma mass spectrometer ICP-MS NexION 300D equipped with and S10 automatic autosampler (PerkinElmer, Inc., Shelton, CT) was used for element determination. For an optimal nebulization of the sample a Peltier-cooled (2 ºC) cyclonic chamber and a low-flow (0.25 ml/min) Meinhard concentric nebulizer was employed to generate homogeneous sample aerosols. ICP-MS is generally used for the determination of metals because it provides sufficiently low detection limits and allows the simultaneous determination of several metals. Every working day the ICP-MS was optimized to obtain the highest values of intensity indicated by the ratios: CeO/Ce < 2.5%, Ce++/Ce < 3% and background (220) < 1 cps. The Limits of Detection (LODs) of the elements referred to blood were 0.25 µg/L for cadmium (Cd), 0.21 µg/L for lead (Pb), 3.01 µg/L for mercury (Hg), 1.55 µg/L for selenium (Se), 13.45 µg/L for zinc (Zn) and 0.5 µg/L for arsenic (As). Calibrating solutions were daily prepared from a 10 mg/L Multielement Calibration Standard 3 (PerkinElmer, Inc., Shelton, CT) and assayed as the samples. A whole blood certified reference sample (Seronorm® Trace Elements Whole Blood) was used for elemental accuracy. The values obtained for all these elements were consistent with the certified reference values. Recoveries obtained were between 92% for Hg and 107% for Se and coefficients of variation for replicate samples (n=5) were lower than 6.5 %. Metal levels were not adjusted for percent recovery.

### 2.5 Statistical analysis

Data were grouped by colony in two ways. Firstly, the nestling was used as the sampling unit. Secondly, the nest was used as the sampling unit since in several nests more than one nestling was present. In this case the metal mean value of the chicks was assigned as the value of the nest. Data were analyzed using statistical software Prism 5 version 5.03 for Windows (GraphPad software, Inc., CA). Data were tested for normality (test of Kolmogorov–Smirnov and Shapiro–Wilk) and homoscedasticity (Levene's test). Since data did not show a normal distribution and the variances were not homogeneous the statistical analyses were performed using a non-parametric Kruskal-Wallis test (Zar, 1984). Differences among colonies were determined with the Dunn´s test ($P$<0.05). Moreover, a Spearman test was used to determine correlations between metals.

## 3. RESULTS AND DISCUSSION

Table 1 shows the levels of metals (Cd, Pb, Hg, Fe and Zn) and metalloids (Se and As) in White stork chicks and nests from colonies living close to an urban landfill (colony B) and to an agriculture area and an urban landfill (colony C) compared with a reference zone (colony A). There were no changes in the statistical analysis using the nests or nestling as sampling unit. For that reason, all the results and discussion are referred to the nestling as sampling unit (Figure 1). When working with wildlife, usually one major difficulty is the establishment of an absolutely controlled condition, and in most of the cases the best option may be the use of a reference population for comparison. The widespread distribution of many pollutants makes difficult, if not impossible, to find a true control population (Norris, 2000). The stork colony considered as reference was in a natural area far from apparent sources of contamination. Since breeding storks generally forage near their nesting sites (Cramp and Simmons, 1980) those animals from colony A were very unlikely to have foraged in potentially contaminated areas, supporting their validity as a reference colony.

Numerous studies have demonstrated the utility of chicks in biomonitoring studies of environmental pollution (Baos *et al.,* 2006a,b; Goutner *et al.,* 2011; Tkachenko and Kurhaluk, 2012). Metal concentrations in blood of nestling can be derived from local exposure to food and water as well as from the egg content. However, the metal content derivative from eggs (which comes from the mother) has been reported to be minimal compared with that from the nestling exposure since hatching to fledging. Therefore, it is considered that metal levels in chick blood reflect concentrations of metals recently absorbed from local exposure and are very useful in biomonitoring studies (Blanco *et al.,* 2003; Blázquez *et al.,* 2006; Burger and Gochfeld, 1997; García-Fernández *et al.,* 1996).

When considering all the analyzed elements, Zn and Fe were quantified at the highest concentrations, according to their relevance as essential elements with a wide range of physiological functions, followed by Pb>Se>Hg>As>Cd.

Cd is accumulated in liver and kidney (92%) and only 0.5 % of the total burden is detected in blood, which is frequently used in monitoring works using live birds (García-Fernández *et al.,* 1996; Nordberg *et al.,* 1985). In our study all Cd concentrations in blood were < LOD (0.25 µg/L) except in the case of 5 animals from colony C, where detectable cadmium concentrations were found. Therefore, Cd levels are not drawn in Figure 2. These Cd levels were lower than those obtained by García-Fernández *et al.,* (1996) in several wild bird species including White stork (*Ciconia ciconia*). These authors found blood Cd concentrations higher than 1 µg/L in 64 % of the birds, with higher levels in adults than in immature. Thus, blood Cd concentrations in chicks normally reflect recent exposure from areas surrounding the colony while adults blood

levels reflect bioaccumulation rates along the years (García-Fernández *et al.,* 1996; Spahn and Sherry, 1999). Taking this into account, the results of Cd obtained in the present study may reflect low exposure levels, mainly coming from food and atmosphere.

Significant differences in blood Pb levels were found between the reference area (colony A) and the other two areas under human influence ($P<0.001$) (Fig 2a). The highest levels were found in colony C (146.40 ± 140.20 µg/L) and colony B (107.10 ± 170.09 µg/L), both places in close proximity to a landfill. The lowest levels were found in the reference colony A (23.27 ± 25.14), although it was very close to an important road (sampled nests were at a distance between 10 and 200 m). This fact may suggest that gasoline is not a source for Pb in the environment nowadays, as García-Fernández *et al.,* (2005) have previously demonstrated in European kestrel. Similar results were found in the study of Kamiński *et al.,* (2009), where the highest levels of Pb were found in White stork from polluted areas.

It has been considered that background blood Pb concentration in birds is <200 µg/L (Franson and Pain, 2011). Moreover, Scheuhammer (1987) established that values ≤ 150 µg/L in blood indicate an absence of abnormal Pb exposure, and levels around 200 µg/L are indicative of slightly elevated Pb exposure. Other authors considered that a blood Pb concentration higher than 200 µg/L is indicative of sublethal exposure (Samuel *et al.,* 1992). Results obtained in our study showed mean values lower than 200 µg/L, although three animals from colony B (from the same nest) and two animals from colony C (from different nests) exceeded this limit (251.2, 318.9, 780.5 and 502.7, 200.7 µg/L, respectively). Pb levels detected in birds from colony A indicate that they were exposed to low environmental contamination. However, Pb exposure seems to be more important when feeding in landfill which may cause subacute intoxication in some birds. These results support the well-known close relationship between human activities and Pb exposure (Blanco *et al.,* 2003; Bikowski *et al.,* 2013). It has been indicated that compost obtained from municipal solid waste (MSW) contains more heavy metals than the background concentrations present in soil. Moreover, Zn and Pb are numerically the elements present in the largest amounts in MSW-compost, being Pb the most limiting element for using mechanically-segregated compost in private gardens (Smith, 2009).

**Table 1.** Mean, median, minimum and maximum concentrations (µg/L) for each element studied in blood of White stork nestlings from different environments. [a]statistical differences ($P$<0.05) respect to A. [b]statistical differences ($P$<0.01) respect to A. [c]statistical differences ($P$<0.001) respect to A. [d]statistical differences ($P$<0,05) respect to B. *Data refered to the 5 animals in which were detected cadmiun levels.

| Element | Colony nestling (n) | Mean ± SD | Median | Minimun | Maximum | Colony nest (n) | Mean ± SD | Median | Minimun | Maximun |
|---|---|---|---|---|---|---|---|---|---|---|
| Cd | A (27) | < 0.25 | < 0.25 | < 0.25 | < 0.25 | A (11) | < 0.25 | < 0.25 | < 0.25 | < 0.25 |
| | B (22) | < 0.25 | < 0.25 | < 0.25 | < 0.25 | B (8) | < 0.25 | < 0.25 | < 0.25 | < 0.25 |
| | C* (10) | 0.49 ± 0.06 | 0.49 | 0.40 | 0.58 | C* (6) | 0.47 ± 0.10 | 0.47 | 0.40 | 0.54 |
| Pb | A (27) | 23.27 ± 25.14 | 17.10 | 5.52 | 119.5 | A (11) | 21.25 ± 14.11 | 18.00 | 8.50 | 53.03 |
| | B (22) | 107.1 ± 170.9[c] | 46.70 | 27.80 | 780.5 | B (8) | 100.2 ± 142.3[a] | 48.60 | 28.00 | 450.2 |
| | C (10) | 146.4 ± 140.2[c] | 100.3 | 53.7 | 502.7 | C (6) | 170.4 ± 96.35[c] | 136.2 | 75.30 | 292.0 |
| Hg | A (27) | 8.89 ± 5.70 | 7.58 | 3.47 | 31.20 | A (11) | 9.08 ± 4.83 | 246.7 | 195.7 | 375.9 |
| | B (22) | 24.36 ± 19.46[b] | 15.70 | 6.24 | 62.10 | B (8) | 24.01 ± 16.36[a] | 378.6 | 321.1 | 447.4 |
| | C (10) | 53.03 ± 35.26[c] | 51.50 | 16.00 | 118.3 | C (6) | 85.20 ± 102.9[c] | 341.6 | 292 | 365.4 |
| Se | A (27) | 82.67 ± 29.61 | 78.80 | 43.20 | 181.8 | A (11) | 79.81 ± 21.37 | 73.80 | 53.60 | 123.4 |
| | B (22) | 142.6 ± 34.32[c] | 142.4 | 92.80 | 213.9 | B (8) | 144.8 ± 33.43[a] | 140.7 | 97.10 | 206.9 |
| | C (10) | 199.5 ± 49.87[c] | 185.2 | 135.2 | 278.0 | C (6) | 213.5 ± 46.30[c] | 211.5 | 153.8 | 292.0 |
| Zn | A (27) | 2078 ± 522.5 | 1903 | 1321 | 3515 | A (11) | 2074 ± 493.0 | 1857 | 1716 | 3223 |
| | B (22) | 2312 ± 295 | 2259 | 1845 | 2724 | B (8) | 2295 ± 192.5 | 2391 | 2043 | 2493 |
| | C (10) | 2814 ± 239.6[c,d] | 2766 | 2541 | 3244 | C (6) | 2447 ± 1073[a] | 2797 | 292.0 | 3244 |
| Fe | A (27) | 269.6 ± 67.54 | 283.9 | 107.5 | 396.7 | A (11) | 265.9 ± 61.34 | 246.7 | 195.7 | 375.9 |
| | B (22) | 386.9 ± 50.21[c] | 369.0 | 329.0 | 499.0 | B (8) | 382.6 ± 43.87[b] | 378.6 | 321.1 | 447.4 |
| | C (10) | 338.4 ± 22.55 | 339.3 | 292.0 | 366.9 | C (6) | 338.1 ± 27.87 | 341.6 | 292.0 | 365.4 |
| As | A (27) | 2.35 ± 1.93 | 1.51 | 0.91 | 10.60 | A (11) | 2.05 ± 0.96 | 1.60 | 1.10 | 3.61 |
| | B (22) | 22.48 ± 31.82[c] | 11.10 | 2.91 | 108.1 | B (8) | 20.91 ± 30.92[b] | 10.70 | 3.70 | 96.70 |
| | C (10) | 23.04 ± 6324[c] | 24.90 | 10.80 | 31.90 | C (6) | 66.35 ± 110.7[c] | 23.80 | 10.80 | 292.0 |

Pb concentrations obtained in this study are similar, but slightly higher in colonies B and C, to those obtained by Baos *et al.,* (2006b) in White stork chicks from Puebla del Rio (Sevilla, Spain), at 1 km from the area affected by the Aznalcóllar mine spill occurred in 1998. However, Baos *et al.,* (2006a) found blood Pb levels of 90.7 ± 51 µg/L in White stork chicks from a reference colony also located in Cáceres province (the exact place is not indicated) in a natural area far from urban environments and other apparent sources of pollution, being these levels intermediate when compared to the range of those obtained in the present study (lower than those obtained in colonies B and C, but higher than those obtained in the reference colony A). Cabo *et al.,* (2012) found blood Pb levels in the range 105-222.6 µg/L in White stork chicks from different areas of Madrid province (Spain), with the higher levels found in the Southeast area characterized by the presence of industries and several rubbish dumps. Our data are not very different to those found in other bird species in different locations from Spain. In Black kite (*Milvus migrans*) nestlings sampled at different distances from a solid waste incinerator, blood Pb levels of 7.21- 221.40 µg/L have been reported (Blanco *et al.,* 2003), detecting a significant negative correlation related to the distance to the solid waste incinerator.

Hg levels were significantly higher in colonies B (24.36 ± 19.46 µg/L) ($P<0.01$) and C (53.03 ± 35.26 µg/L) ($P<0.001$) when compared with colony A (8.89 ± 5.70 µg/L) (Fig 2b). Inorganic mercury disappears rapidly from blood (Peakall and Lovett, 1972), but blood can be considered a suitable matrix to indicate the current mercury burden in wild birds (Kahle and Becker, 1999). Therefore, data presented in this study are indicative of recent exposure. In all cases, the concentration of mercury was below 1000 µg/L, which has been reported as the threshold of high risk for birds (Álvarez *et al.,* 2013; Evers *et al.,* 2008). In our study Hg in White stork nestling has never exceeded this limit. Moreover, levels obtained were lower than those quantified by Álvarez *et al.,* (2013) in White storks chicks from Doñana National Park (SW of Spain) after the Aznalcóllar mine spill accident (121 µg/L). In White stork nestlings from different areas in Madrid province (Spain) a range from 324.5-749.7 µg/L of total mercury has been found, with the highest concentrations found in the Southeast area characterized for the presence of an industrial zone with some rubbish dumps (Cabo *et al.,* 2012). These values are higher than those detected in the present study. However, our results are similar to those obtained by the same authors (40.8 µg/L) in White stork nestling in Aragón (Spain).

Something similar occurs with Se levels (Fig 2c). The highest values were found in colony C (199.50 ± 49.87 µg/L), followed by colony B (142.6 ± 34.32 µg/L) and the reference colony A (82.67 ± 29.61 µg/L). All of them were into the range (100-400 µg/L) considered as background levels in bird's blood (U.S. DI, 1998).

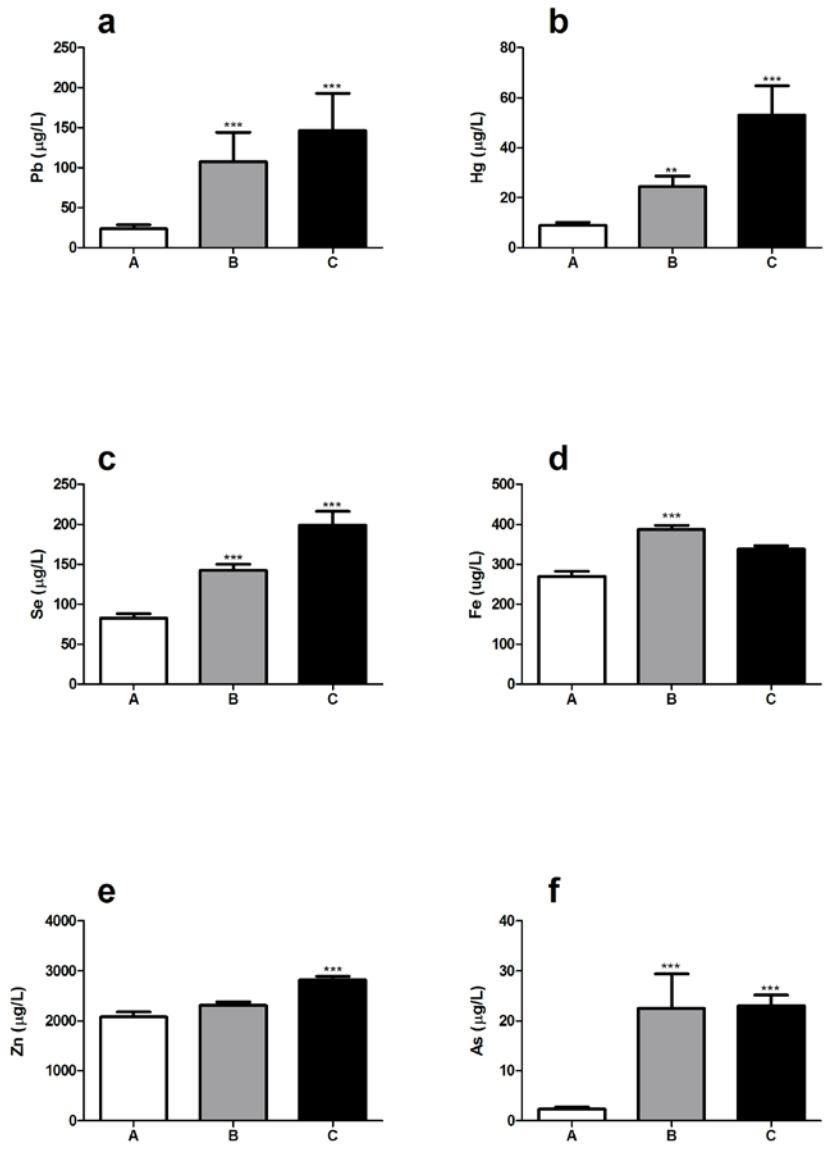



**Figure 2.** Lead (a), mercury (b), selenium (c), iron (d), zinc (e) and arsenic (f) levels in blood of White stork nestlings from three different colonies. A: reference colony; B: close to a landfill, and C: close to a landfill and an agricultural area. ***P*<0.01 and ****P*<0.001 statistical differences respect to colony A.

Fe levels were higher in colonies B (386.90 ± 50.21 µg/L) and C (338.90 ± 22.55 µg/L) than in the reference area, colony A (269.60 ± 67.54 µg/L) (Fig 2d). However, unlike the other metals, the highest levels were found in colony B being this difference statistically significant with respect to colony A. Probably, the differences between colonies may be due to differences in geochemistry between the sites, but no data on Fe in the soils are

available to support it. Fe is an essential element, which is especially important as the component of haemoglobin. Data available about the toxicity of this metal is scarce. In human blood Fe levels above 3500-5000 µg/L are considered toxic (Pagana and Pagana, 2010).

Zn levels were higher in colonies B (2312 ± 295 µg/L) and C (2814 ± 239.60 µg/L) than in colony A (2078 ± 522.50 µg/L), but the results were statistically significant only in the case of colony C ($P<0.05$) with respect to colony A (Fig 2e). These results are similar to those obtained by Baos *et al.,* (2006a) in White stork chicks from other colonies in Cáceres and Doñana National Park (South of Spain) (2800 ± 500 and 2900 ± 400 µg/L, respectively). Moreover, our results are in the range 1840-3300 µg/L which is also similar to the levels detected by Baos *et al.,* (2006b) in White stork nestlings from the Doñana National Park during the years 1999-2003 (after the Aznalcóllar toxic spill). Other authors have observed Zn mean values in blood of 7500 µg/kg ww in birds from contaminated areas (Benito *et al.,* 1999; Falandysz *et al.,* 1988). Zn concentrations were reported to vary depending on the Cd concentrations. This may be due to the induction of metallothionein synthesis by high accumulation of Cd leading to greater binding of Zn and hence increased Zn uptake for essential cellular functions.

Arsenic (As) levels were higher in colonies C (23.04 ± 6.32 µg/L) and B (22.48 ± 31.82 µg/L) than in colony A (2.34 ± 1.93 µg/L) (Fig 2f), showing statistical significance ($P<0.001$). Thus, the presence of this metalloid at high concentrations in colonies B and C may be associated to the presence of the landfill next to both colonies. Baos *et al.,* (2006b) found similar values of As in blood of stork nestlings sampled during four years at the Doñana National Park area. However, our values are lower than those obtained by Baos *et al.,* (2006a) in a White stork colony in Cáceres province (48.6 ± 46.8 µg/L) theoretically considered as "reference or not contaminated". The information about the threshold value regarding this metalloid is limited in nestling blood. Burger and Gochfeld (1997) reported an As value of 18 µg/L in young Franklin's gulls from uncontaminated areas, being considered that value as reference in a study from Benito *et al.,* (1999). Colonies B and C reflected slightly higher levels than this reference value.

Strong correlations were found between pairs of all the metals studied except for the pair Hg-Zn (Table 2). Correlation between essential and non-essential metals might indicate a possible implication of the essential metals in the detoxification of metals (Blanco *et al.,* 2003). Exposure to Pb may increase the levels of metal-binding proteins such as protoporphyrins and metallothioneins, producing significant correlations between Pb and essential metals (Elliott *et al.,* 1992; Stewart *et al.,* 1996). The correlations observed in the present study do not agree with those reported by Blanco *et al.,* (2003) as they did not

find any correlation between Pb and Zn or As. However, they reported that the relation between Pb and Zn may depend on the levels of copper which was out of the scope of this article.

**Table 2.** Correlation study between the pairs of the studied elements. [a]statistical significance *P*<0,001.

| | | Hg | Se | Zn | Fe | As |
|---|---|---|---|---|---|---|
| Pb | *r* | 0.507 | 0.643 | 0.437 | 0.493 | 0.87 |
| | *P* value | 0.000[a] | 0.000[a] | 0.000[a] | 0.000[a] | 0.000[a] |
| Hg | *r* | | 0.621 | 0.137 | 0.223 | 0.676 |
| | *P* value | | 0.000[a] | 0.313 | 0.099 | 0.000[a] |
| Se | *r* | | | 0.611 | 0.682 | 0.786 |
| | *P* value | | | 0.000[a] | 0.000[a] | 0.000[a] |
| Zn | *r* | | | | 0.615 | 0.507 |
| | *P* value | | | | 0.000[a] | 0.000[a] |
| Fe | *r* | | | | | 0.57 |
| | *P* value | | | | | 0.000[a] |

The blood concentration of the considered elements varied in accordance with the location of the colonies. In general, the highest concentrations were found in the colony C, situated near a landfill and an intensive agricultural area, followed by the colony situated near the landfill (colony B). The lowest levels were found in the reference colony (A), as expected. However, the study was not designed specifically to evaluate the impact of proximity to landfills. Since metals are ubiquitous, special care should be taken to identify the source, and in our case the presence of landfill or agricultural activity needs to be considered. It is well known that White stork population has increased during the last years in Spain, and this growth has been attributed to the extended source of food provided by rubbish dumps throughout the year (Blázquez *et al.*, 2006; Peris, 2003). However, the origin of toxic metals is diverse and other potential sources need to be considered. There is no industrial activity around the colonies considered, but the metal content in soil of the area where birds live must be taken into account. From soils, metals transfer into the food chain through plants or small prey and finally reach birds. Agriculture is a source of contamination for soil and crops with different metals and metalloids by means of the application of municipal solid waste compost, sewage irrigation, addition of manures, fertilizer and pesticide applications, etc. (Hargreaves *et al.*, 2008; Khan *et al.*, 2008, 2013).

Since the metal concentrations in ingested soil may be higher than in prey items, the soil can be an important pathway of exposure to birds as well. In order to compare the metal concentrations in blood with those in soils, data of metal concentrations (Cd, Hg, Pb and Zn) in soils of Spain reported by López-Arias and Grau-Corbí, (2005), including the areas of the present study, were used as reference. Concentrations of Cd (<LOD), Pb (6 mg/kg) and Zn (18 mg/kg) in the soil close to (1.2 km) colony C were lower than those obtained in the soil close to (7.5 km) colony A (0.10, 20 and 91 mg/kg respectively) and in the soil close to (3.5 km) colony B (<LOD, 19 and 98 mg/kg respectively). Cd was detectable only in the soil of the reference colony A, but not in its chicks. However, Cd was only detectable in chicks from colony C. Moreover, levels of Pb in soils close to colonies A and B were similar; but their concentrations in blood were statistically different. In colony C, the nearness to the landfill (1.5 km) seems to be de main source of Pb levels in these animals, since soil from that area showed the lowest metal concentrations and the animals the highest ones. Thus, regarding these metals, soil composition and agricultural activity do not seem to influence Cd, Pb and Zn concentrations in blood, and the main source of pollution to birds seems to be the landfill located close to colonies B and C. To ensure this statement, the knowledge of the frequency of landfill visits by White stork would be necessary. However, we have no data about this fact and it would be interesting to determine it in future research using telemetry data for the adult foraging ranges. Medina *et al.,* (1998) reported that 88.9 % of White stork specimens in landfills in Extremadura were birds below 4 years of age and 11.1 % were over 4.

Regarding Hg levels, López-Arias and Grau-Corbí, (2005) detected higher concentrations in the soil near to colonies B (0.02 mg/kg) and C (0.02 mg/kg) than those detected in A (<LOD), which may indicate that in the case of Hg the soil might be also a source of pollution by this metal and should be taken into account when interpreting the results.

It has been reported that soil affected by the landfills may increase its concentration in some trace elements as B, Zn, Fe, Mn, Cr, Pb, Hg or Cd (Earle *et al.,* 1999; Hernández *et al.,* 1998; Matejczyk *et al.,* 2011; Rushton 2003). Landfilling of solid wastes is still a common practice in many Mediterranean countries and may cause ecological and sanitary problems, even when at the present they are sealed. Although the health impact on human population living near landfill sites has been evaluated in some aspects as birth weights, reproductive disorders, cancer incidence or congenital malformations (Forastiere *et al.,* 2011; Johnson and DeRosa 1995; Rushton 2003), to our knowledge there are no studies about the presence of contaminants in birds feeding in a landfill site or in its surroundings. This is the first report on the relation between landfills and the

increase in blood metal content in birds due to breeding close to and feeding in these sites.

## 4. CONCLUSIONS

The present study shows the differences in blood metal and metalloid concentrations in nestlings from three different colonies of White stork under the influence of natural environment, agricultural activity, and solid waste treatment (landfills). The agricultural activity has no influence in the blood levels of Cd, Pb and Zn in White storks chicks according to the soil levels reported in the literature in these locations. Landfills seem to be the most important source of metals for White stork chicks in colonies breading next to them. However, more studies are necessary to evaluate the metal content in the White stork food, and if the soil concentration of Fe, Se and As are related to blood levels of these elements in these birds. More investigations are necessary, in order to check whether the distance from the nest to the landfill is inversely related to the metal concentrations in chicks, controlling the visits to landfills by their parents.

## ACKNOLEDGEMENTS

Irene de la Casa-Resino was supported by fellowship PRE09001 from Consejería de Empleo, Empresa e Innovación (Gobierno de Extremadura, Spain).

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## REFERENCES

Álvarez, C.R., Moreno, M.J., Alonso, L.L., Gómara, B., Bernardo, F.G., Martín-Doimeadios, R.R., González, M. (2013). Mercury, methylmercury, and selenium in blood of bird species from Doñana National Park (Southwestern Spain) after a mining accident. Environmental Science and Pollution Research 20, 5371-5372.

Baos, R., Blas, J., Bortolotti, G.R., Marchant, T.A., Hiraldo, F. (2006a). Adrenocortical response to stress and thyroid hormone status in free-living nestling White storks (*Ciconia ciconia*) exposed to heavy metal and arsenic contamination. Environmental Health Perspectives 114, 1497-1501.

Baos, R., Forero, M.G., Gómez, G., Gónzalez, M.J., Hiraldo, F., Jiménez, B., Jovani, R., Tella, J.L. (2006b). Relationships between T-cell mediated immnune response and Pb, Zn, Cu, Cd, and As concentration in blood of nestling White storks (*Ciconia ciconia*) and Black kites (*Milvus migrans*) from Doñana (Southwestern Spain) after the Aznalcóllar toxic spill. Environmental Toxicology and Chemistry 25, 1153-1159.

Baos, R., Jovani, R., Serrano, D., Tella, J.L., Hiraldo, F. (2012). Developmental exposure to a toxic spill compromises long-term reproductive performance in a wild, long-lived bird: the White stork (*Ciconia ciconia*). PLoS ONE 7:e34716.

Benito, V., Devesa, V., Muñoz, O., Suñer, M.A., Montoro, R., Baos, R., Hiraldo, F., Ferrer, M., Fernández, M., González, M.J. (1999). Trace elements in blood collected from birds feeding in the area around Doñana National Park affected by the toxic spill from the Aznalcóllar mine. Science of The Total Environment 6, 309-323.

Binkowski, L.J., Meissner, W. (2013). Levels of metals in blood samples from Mallards (*Anas platyrhynchos*) from urban areas in Poland. Environmental Pollution 178, 336-342.

Blanco, G. (1996). Population dynamics and communal roosting of White storks foraging at a Spanish refuse dump. Colonial Waterbirds 19, 273-276.

Blanco, G., Frias, O., Jimenez, B., Gomez, G. (2003). Factors influencing variability and potential uptake routes of heavy metals in Black kites exposed to emissions from a solid-waste incinerator. Environmental Toxicology and Chemistry 22, 2711-2718.

Blázquez, E., Aguirre, J., Martínez-Haro, M., Mateo, R., Jiménez, B. (2006). The use of White stork (*Ciconia ciconia*) nestlings in a biomonitoring programme for organochlorines through the region of Madrid (Spain). Organohalogen Compound 68, 2081-2085.

Burger, J., Gochfeld, M. (1997). Age differences in metals in the blood of Herring (*Larus argentatus*) and Franklin's (*Larus pipixcan*) gulls. Archives of Environmental Contamination and Toxicology 33, 436-440.

Cabo, P., Espín, S., Martínez-López, E., Roscales, J.L., Jiménez, B., García-Fernández, A.J. (2012). Metales pesados en sangre de pollos de Cigüeña blanca (*Ciconia ciconia*) de Madrid y Aragón. Revista de Toxicología 29, 72.

Clarkson, T.W., Weiss, B., Cox, C. (1983). Public health consequences of heavy metals in dump sites. Environmental Health Perspectives 48, 113-127.

Cramp, S., Simmons, K.E.L. (1980). The Birds of the Western Palearctic. Oxford University Press, Oxford.

Dallinga, J.H., Schoenmakers, S. (1987). Regional decrease in the number of White storks (*Ciconia ciconia*) in relation to food resources. Colon waterbird 10, 167-177.

Directive 1999/31/CE of the European Parliament and of the Council of 26 April 1999 on the landfill of waste. Official Journal of the European Union 182, 1-19.

Directive 2009/147/EC of the European Parliament and of the Council of 30 November 2009 on the conservation of wild birds. Official Journal of the European Union 20, 7-25

Domínguez, J.A., Moreno, F.A., González, F., Pita, M.A. (2011). Informe ambiental de Extremadura 2011. Consejería de Agricultura, Desarrollo Rural, Medio Ambiente y Energía de la Junta de Extremadura. http://extremambiente.gobex.es/. Accessed 3 October 2013.

Earle, C.D.A., Rhue, R.D., Earle, J.F.K. (1999). Mercury in a municipal solid waste landfill. Waste Management and Research 17, 305-312.

Elliott, J., Scheuhammer, A., Leighton, F., Pearce, P. (1992). Heavy metal and metallothionein concentrations in Atlantic Canadian seabirds. Archives of Environmental Contamination and Toxicology 22, 63-73.

Evers, D.C., Savoy, L.J., DeSorbo, C.R., Yates, D.E., Hanson, W., Taylor, K.M., Siegel, L.S., Cooley, J.H., Bank, M.S., Major, A., Munney, K., Mower, B.F., Vogel, H.S., Schoch, N., Pokras, M., Goodale, M.W., Fair, J. (2008). Adverse effects from environmental mercury loads on breeding Common loons. Ecotoxicology 17, 69-81.

Falandysz, J., Jakuczun, B., Mizera, T. (1988). Metals and organochlorines in four female White-Tailed eagles. Marine Pollution Bulletin 19, 521-526.

Forastiere, F., Badaloni, C., DeHoogh, D., von Kraus, M.K., Martuzzi, M., Mitis, F., Palkovicova, L., Porta, D., Presiss, P., Ranzi, A., Perucci, A.A., Briggs, D. (2011). Health impact assessment of waste management facilities in three European countries. Environmental Health 10, 53.

Franson, J.C., Pain, D.J. (2011). Lead in birds. In: Beyer, W.N., Meador, J.P. (Eds) Environmental Contaminants in Biota: Interpreting Tissue Concentrations. CRC Press Taylor and Francis Group, Boca Raton, Florida, pp 563-593.

García-Fernández, A.J., Sánchez-García, J.A., Gómez-Zapata, M., Luna, A. (1996). Distribution of cadmium in blood and tissues of wild birds. Archives of Environmental Contamination and Toxicology 30, 252-258.

García-Fernández, A.J., Romero, D., Martinez-López, E., Navas, I., Pulido, M., Maria-Mojica, P. (2005). Environmental lead exposure in the European kestrel (*Falco tinnunculus*) from southeastern Spain: the influence of leaded gasoline regulations. Bulletin of Environmental Contamination and Toxicology 74, 314-319.

Goutner, V., Becker, P., Liordos, V., Tsachalidis, E. (2011). Mercury in White stork (*Ciconia ciconia*) chick feathers from northeastern mediterranean areas in relation to age, brood size, and hatching order. Archives of Environmental Contamination and Toxicology 61, 327-336.

Hargreaves, J., Adl, M.S., Warman, P.R. (2008). A review of the use of composted municipal solid waste in agriculture. Agriculture, Ecosystems and Environment 123, 1-14.

Hernández, A.J., Adarve-Alcazar, M.J., Pastor, J. (1998). Some impacts of urban waste landfills on Mediterranean soils. Land Degradation and Development 9, 21-33.

Hernández, L.M., Gómara, G., Fernández, M., Jiménez, B., González, M.J., Baos, R., Hiraldo, F., Ferrer, M., Benito, V., Suner, M.A., Devesa, V., Munoz, O., Montoro, R. (1999). Accumulation of heavy metals and As in wetland birds in the area around Doñana National Park affected by the Aznalcóllar toxic spill. Science of the Total Environment 242, 293-308.

Johnson, B.L., DeRosa, C.T. (1995). Chemical mixtures released from hazardous waste sites: implications for health risk assessment. Toxicology 105, 145-156.

Kahle, S., Becker, P.H. (1999). Bird blood as bioindicator for mercury in the environment. Chemosphere 39, 2451-2457.

Kamiński, P., Kurhalyuk, N., Kasprzak, M., Jerzak, L., Tkachenko, H., Szady-Grad, M., Klawe, J.J., Koim, B. (2009). The impact of Element–Element interactions on antioxidant enzymatic activity in the blood of White Stork (*Ciconia ciconia*) chicks. Archives of Environmental Contamination and Toxicology 56, 325-337.

Khan, K., Lu, Y., Khan, H., Ishtiaq, M., Khan, S., Waqas, M., Wei, L., Wang, T. (2013). Heavy metals in agricultural soils and crops and their health risks in Swat District, northern Pakistan. Food and Chemical Toxicology 58, 449-458.

Khan, S., Cao, Q., Zheng, Y., Huang, Y., Zhu, Y. (2008). Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. Environmental Pollution 152, 686-692.

Koivula. M.J., Eeva, T. (2010). Metal-related oxidative stress in birds. Environmental Pollution 158, 2359-2370.

Kruszyk, R., Ciach, M. (2010). White Storks, *Ciconia ciconia*, forage on rubbish dumps in Poland: a novel behaviour in population. European Journal of Wildlife Research 56, 83-87.

López-Arias, M., Grau-Corbí, J.M. (2005). Metales pesados, materia orgánica y otros parámetros de la capa superficial de los suelos agrícolas y de pastos de la España Peninsular. II: Resultados provinciales y puntuales. INIA, Madrid.

Massemin-Challet, S., Gendner, J.P., Samtmann, S., Pichegru, L., Wulgue, A., Le Maho, Y. (2006). The effect of migration strategy and food availability on White Stork *Ciconia ciconia* breeding success. Ibis 148, 503-508.

Matejczyk, M., Plaza, G.A., Nalęcz-Jawecki, G., Ulfig, K., Markowska-Szczupak, A. (2011). Estimation of the environmental risk posed by landfills using chemical, microbiological and ecotoxicological testing of leachates. Chemosphere 82, 1017-1023.

Medina, F., Avilés, J.M., Sánchez, A. (1998). Diferencias intraespecíficas en el uso de un vertedero por parte de la Cigüeña Blanca (*Ciconia ciconia*) en el oeste de la Península Ibérica. Butlletí del Grup Català d'Anellament 15, 9-14.

Merian, E. (1991). Metals and their compounds in the environment: occurrence, analysis and biological relevance. WILEY-VCH, Weinheim.

Nordberg, G.F., Kjellstrom, T., Nordberg, M. (1985). Kinetics and metabolism. In: Freiberg, L., Elinder, G.C., Kjellstrom, T., Nordberg, G.F. (Eds). Cadmium and health: a toxicological and epidemiological appraisal. Exposure, dose and metabolism. CRC Press, Boca Raton, Florida, pp 103-178.

Norris, D.O. (2000). Endocrine disruptors of the stress axis in natural populations: How can we tell? American Zoologist 40, 93-401.

Pagana, K.D., Pagana, T.J. (2010). Mosby's Manual of Diagnostic and Laboratory Tests, 4th edn. Mosby, St. Louis.

Peakall, D.B., Lovett, R.J. (1972). Mercury: Its Occurrence and Effects in the Ecosystem. BioScience 22, 20-25.

Peris, S.J. (2003). Feeding in urban refuse dumps: ingestion of plastic objects by the White Stork (*Ciconia ciconia*). Ardeola 50, 81-84.

Rushton, L. (2003). Health hazards and waste management. British Medical Bulletin 68, 183-197.

Samuel, M.D., Bowers, E.F., Franson, J.C. (1992). Lead exposure and recovery rates of black ducks banded in Tennessee. Journal of wildlife diseases 28, 555-561.

Scheuhammer, A.M. (1987). The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: A review. Environmental Pollution 46, 263-295.

Smith, S.R. (2009). A critical review of the bioavailability and impacts of heavy metals in municipal solid waste composts compared to sewage sludge. Environment International 35, 142-156.

Smits, J.E., Bortolotti, G.R., Baos, R., Blas, J., Hiraldo, F., Xie, Q. (2005). Skeletal pathology in White storks (*Ciconia ciconia*) associated with heavy metal contamination in southwestern Spain. Toxicologic Pathology 33, 441-448.

Spahn, S.A., Sherry, T.W. (1999). Cadmium and lead exposure associated with reduced growth rates, poorer fledging success of Little Blue Heron chicks (*Egretta caerulea*) in South Louisiana Wetlands. Archives of Environmental Contamination and Toxicology 37, 377-384.

Stewart, F., Furness, R., Monteiro, L. (1996). Relationships between heavy metal and metallothionein concentrations in Lesser Black-Backed Gulls, *Larus fuscus*, and Cory's Shearwater, *Calonectris diomedea*. Archives of Environmental Contamination and Toxicology 30, 299-305.

Tkachenko, H., Kurhaluk, N. (2012). Pollution-induced oxidative stress and biochemical parameter alterations in the blood of White stork nestlings *Ciconia ciconia* from regions with different degrees of contamination in Poland. Journal of Environmental Monitoring 14, 3182-3191.

Tortosa, F., Caballero, J., Reyes-López, J. (2002). Effect of rubbish dumps on breeding success in the White Stork in southern Spain. Waterbirds 25, 39-43.

U.S. Department of the Interior (U.S. DI) (1998). Guidelines for interpretation of the biological effects of selected constituents in biota, water, and sediment. National Irrigation Water Quality Program Information Report Nº 3. USDI, Denver, CO.

Zar, J.H. (1984). Biostatistical analysis, 2nd edn. Prentice Hall, New Jersey.

# CAPÍTULO V

---

**Biomarcadores de estrés oxidativo asociados a la contaminación por metales en sangre de cigüeña blanca (*Ciconia ciconia*) en España.**

*de la Casa-Resino, I., Hernández-Moreno, D., Castellano, A., Soler Rodríguez, F., Pérez-López, M. Biomarkers of oxidative stress associated to metal pollution in blood of White stork (Ciconia ciconia) from Spain. Submitted to Environmental Monitoring and Assessment*.

**RESUMEN:** En el presente estudio se han evaluado los efectos de la contaminación por tres metales (Pb, As y Hg) en los niveles de distintos biomarcadores sanguíneos en pollos de cigüeña blanca muestreados en dos áreas diferentes de la provincia de Cáceres (España): un area de referencia (n=27), y un area cercana a una planta de tratamiento de residuos sólidos urbanos (PTRSU), donde las cigüeñas se alimentan principalmente (n=22). Las dos areas consideradas mostraron diferentes patrones de biomarcadores y contaminantes, sugiriendo diferentes niveles de contaminación ambiental. La colonia situada en la zona de referencia mostró los niveles más bajos de Pb, Hg y As (con medias de 23,04 ± 4,56, 8,39 ± 1,15 y 21,58 ± 6,68 µg/l, respectivamente) en comparación con la colonia situada cerca de la PTRSU (103,30 ± 35,77, 23,55 ± 4,13 y 2,34 ± 0,37 µg/l, respectivamente), siendo esta diferencia estadísticamente significativa ($P$<0,001). Sin embargo, y en relación a la bibliografía, los niveles de metales detectados en el presente estudio están por debajo del límite considerado como causante de efectos tóxicos en aves. En relación a los biomarcadores de estrés oxidativo, la colonia cercana a la PTRSU mostró niveles sanguíneos significativamente más altos de GSH ($P$<0,001) y GST ($P$<0.005), mientras que en los niveles de MDA (con valores medios de 0,11 nmol/mg proteína) no se obtuvieron diferencias significativas entre ambas zonas de muestreo. Estos resultados sugieren que los pollos de cigüeña blanca pueden ser considerados como centinelas para monitorización de la contaminación por metales, y los niveles de GSH y GST podrían ser útiles biomarcadores de la contaminación por estos elementos, aunque son necesarios más estudios para esclarecer las causas de estos resultados.

**Palabras clave:** biomarcadores, sangre, cigüeña blanca, metales, estrés oxidativo.

**ABSTRACT:** Blood biomarkers and levels of three different metals (Pb, As and Hg) were used to determine pollution effects in nestlings of White stork, sampled on two different areas of Caceres (Spain): a reference site (n=27), and a site affected by an important landfill, where storks go to feed (n=22). The two considered populations showed different biomarker and contaminant patterns, suggesting different levels of environmental contamination. In the population nesting in the natural protected area, lower blood concentrations of the toxic elements Pb, Hg and As (means of 23.04 ± 4.56, 8.39 ± 1.15 and 21.58 ± 6.68 µg/l) were quantified when compared to those from the site located close to the landfill (103.30 ± 35.77, 23.55 ± 4.13 and 2.34 ± 0.37 µg/l, respectively), this difference being statistically significant ($P$<0.001). However, and according to the literature, the level of contamination by metals is generally below the levels of toxicological effects for the White stork. Regarding the antioxidant biomarkers, animals close to the landfill displayed significantly higher blood activities of GSH

($P$<0.001) and GST ($P$<0.005), whereas MDA levels (mean values of 0.11 nmol/mg prot) did not significantly differed from both sampling sites. These results suggest that White stork nestlings can be considered a suitable sentinel for monitoring metal contamination and GSH and GST could be used as sensitive biomarkers for metal exposure in this species even if further studies are necessary to establish the causality of these findings.

**Keywords:** biomarkers, blood, White stork, metals, oxidative stress.

## 1. INTRODUCTION

The value of birds as bioindicators is directly associated to their use as qualitative and quantitative accumulators of a broad variety of contaminants (mainly pesticides and heavy metals), based on logistically convenient and non-destructive avian matrices such as eggs, feathers or blood, and on high biomagnification rates in dose dependent responses (Becker, 2003). Wild birds have been shown to be particularly useful as bioindicators because they are sensitive to pollutants and are important structural components of the ecosystem (Kekkonen *et al.*, 2012; Swaileh and Sansur, 2006). More specifically, the White stork, *Ciconia ciconia*, is a bioindicative species model for large-scale animal research and a good indicator of the natural environment quality (Kaluga, 2006). In the wild, storks live only in places where the environment is not severely transformed and birds are able to find rich feeding grounds assuring their survival (Tkachenko and Kurhaluk, 2012). Some of the main known threats to the breeding population of White stork include natural factors, but also anthropogenic factors, e.g. industrial activities and environmental contaminants, can reduce productivity of their populations. In fact, our previous investigation demonstrated that the concentrations of some heavy metals (e.g. Pb, As and Hg) in blood of chicks were different between individuals when comparing areas submitted to different levels of anthropogenic influence of western Spain (unpublished results).

In this sense, advances in chemical analytical techniques during the last few years make the study of pollutants in very small amounts of material possible now, such as in small blood samples which can be obtained from a free-ranging bird without harming it, a method of sampling that will gain in importance in years to come, as analytical techniques are further refined (Becker, 2003). However, environmental monitoring of those metals can be performed not only by measuring concentrations of the specific element of interest but also by means of biomarkers, which have successfully been used in environmental monitoring and assessment around the world to detect exposure to and effects of chemicals. Most of heavy metals are very reactive elements, they are toxic to living organisms when interfering with metabolism and important biochemical reactions, and they produce alterations in enzymatic activities and free radical levels (Koivula *et al.*, 2011).

Toxic metals have their specific ecotoxicological impact upon the course of the level of pro- and antioxidant activity of enzymes and on the development of lipoperoxidation processes. In fact, several studies demonstrate a relationship between metal exposure and formation of the superoxide radical, hydroxyl radical (mainly via Fenton reaction) and other reactive oxygen species (ROS), which finally produce malondialdehyde (MDA), an

end product of lipid peroxidation. Against those oxidant compounds, antioxidants are substances that have the ability to inhibit free radical generations, scavenge free radicals and reduce oxidation and damage caused by the radicals (Koivula and Eeva, 2010). The main antioxidant is glutathione (GSH), which detoxifies metabolic by-products and xenobiotics by redox reactions or by conjugation. Other antioxidant enzymes are also important, like glutathione-S-transferases (GST) which are able to inactivate and remove free radicals (Isaksson *et al.*, 2009; Leaver and George, 1998).

In recent years, a rising interest has been directed towards the antioxidant system of birds, where oxidative responses have been described in relation to fecundity, survival, plumage coloration or other ecophysiological parameters (Costantini *et al.*, 2006; Bertrand *et al.*, 2006; Geens *et al.*, 2009). However, oxidative stress related to metals has barely been studied in wild free-living birds, and in general those studies are associated to aquatic and seabirds, whereas terrestrial studies have been centered primarily on small passerines. Moreover, most of the studies have been focused on a single metal, and there is a general lack of knowledge concerning the cooperative action of different metals in wild populations, which realistically may be chronically exposed simultaneously to multiple contaminants (Koivula and Eeva, 2010).

In the present study, White stork chicks were of particular interest due to desirable properties as research objects. Young individuals are logistically easier to handle due to less mobility compared to adults and they are also expected to show less physiological and age-related intra-species variance than what is normally occurring among older individuals (Hegseth *et al.*, 2011). With those considerations, the aim of this study was to characterize antioxidant defenses (GSH, GST) and MDA in the blood of White stork chicks breeding in two different types of environments. Simultaneously, the levels of three toxic elements (Pb, As and Hg) were quantified in blood samples. The final purpose was to estimate the impact of the considered environmental contaminants on the activity and levels of the antioxidant defense of free-living White storks.

## 2. MATERIAL AND METHODS

### 2.1 Reagents

Solvents and reagents used were of analytical grade or high purity grade and purchased from Panreac (Moncada i Reixac, Spain), Merck (Darmstad, Germany) and Sigma-Aldrich (St. Louis, USA). Milli-Q water was used to prepare solutions and dilutions.

The internal standard used for elemental analysis by ICP-MS was a solution of Yttrium, Rhenium, Rhodium and Tellurium (10 mg/L) purchased from Perkin Elmer, Inc. (Shelton, USA).

### 2.2 Study area

The studies were carried out in 2011 during nestling season (spring) in free-living White stork colonies situated in Cáceres (West of Spain), covering a gradient of potential contamination (Figure 1). The reference colony (colony A) was located far from industries or apparent contaminant sources, in a natural area called "Los Llanos de Cáceres y Sierra de Fuentes", considered a Special Protection Area for Birds under the Directive 2009/147/CE and forming an integral part of the NATURA 2000 ecological network. In this sampling area, a total of 27 individuals were sampled.

The second colony, identified as B, was located in a grassland area similar to the colony A, situated 13.4 km from the city of Caceres (95668 inhabitants, 2012), and close to (4.9 km) a landfill that handled 61826 tons of solid waste in 2011 (Domínguez *et al.*, 2011) where the White storks frequently go to feed during their breeding period (Medina *et al.*, 1998). An important road was 15 m far from the nearest nest in this colony. A total of 22 chicks were sampled in this colony. The distance between colonies A and B was 26.2 km, and all nests were placed on trees.

In all cases, the behavioral observation of the chicks, as well as their physical examinations suggested that they were all healthy. The experiments were conducted in accordance with both the Guidelines of the European Union Council and the Ethical Commission of the University, and with the regional government permission (CN10/0306). All handling methods were optimized for reducing to minimal levels the stress to the animals. Each chick was retrieved from the nest and blood samples (about 5 ml) were taken from nestlings via vein-puncture of the tarsal vein with heparinized 0.8x25 mm needle and 5 ml syringe. The whole blood samples were refrigerated until arriving to the laboratory, where 3 aliquots were made. 1 ml of whole blood was stored at -80ºC in 1.5 ml plastic tubes previously washed with $HNO_3$ 2% for metal measurement. The remaining blood was centrifuged at 3000 rpm for 10 min to obtain plasma and erythrocyte pellet. The pellet was homogenized in the ratio of 1 g of pellet to 10 ml of phosphate buffer 0.1 M ($KH_2PO_4/K_2HPO_4$ [pH 7.4]) and used to determine GSH and MDA levels. The homogenate pellet was centrifuged at 13200 g for 20 min to obtain the post-mitochondrial fraction (Gravato *et al.*, 2005). The supernatant was used to determine protein content (Bradford, 1976) and GST activity.

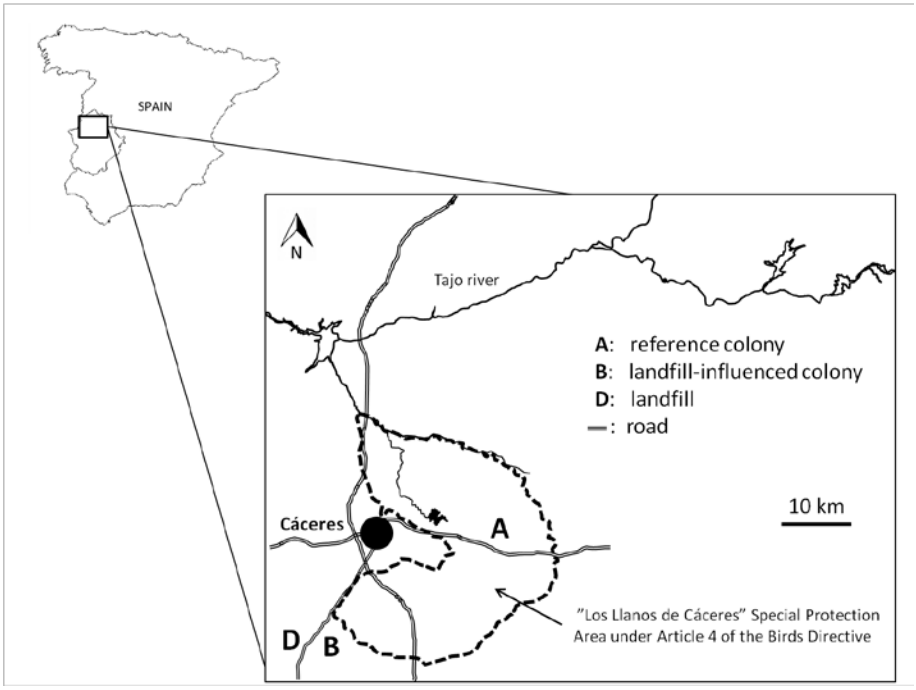



**Figure 1.** Sampling sites around the city of Cáceres (Spain).

### 2.3 Determination of metals and metalloids

Analyses were done in the Elemental and Molecular Analysis Laboratory of the Research Support Service (SAIUEX, accredited by ISO 9001:2008) from the University of Extremadura. Blood samples were thawed and shaken using an orbital shaker during 20 s for homogenization. An aliquot of 200 µl of blood was taken and then 50 µl of isopropanol and 25 µl of the internal standard solution were added. This mix was filled up to 5 ml with an aqueous solution containing $NH_4OH$ (0.7 mM), Triton X-100 (0.07 % v/v) and EDTA (0.01 mM) and finally vortexed. A Platform collision cell inductively coupled plasma mass spectrometer ICP-MS NexION 300D equipped with an S10 automatic autosampler (PerkinElmer, Inc., Shelton, CT) was used for element determination. For an optimal nebulization of the sample a Peltier-cooled (2 ºC) cyclonic chamber and a low-flow (0.25 ml/min) Meinhard concentric nebulizer was employed to generate homogeneous sample aerosols. ICP-MS is generally used for the determination of metals because it provides sufficiently low detection limits and allows the simultaneous determination of several metals. The limits of detection (DL) of the elements referred to blood were 0.21 µg/L for lead (Pb), 3.01 µg/L for mercury (Hg), and 0.5 µg/L for arsenic (As). Calibrating solutions were daily prepared from a 10 mg/L Multielement Calibration Standard 3 (PerkinElmer, Inc., Shelton, CT) and assayed as the

samples. A whole blood certified reference sample (Seronorm® Trace Elements Whole Blood) was used for elemental accuracy. Recoveries obtained were 96.04, 103.20 and 90.94 % (Pb, As and Hg respectively) and coefficients of variation for replicate samples (n=5) were below 6.5 %.

### 2.4 Blood glutathione S-transferase (GST) activity, reduced glutathione (GSH) and malondialdehyde (MDA) levels

Blood GST activity was determined using the method of Cohen *et al.,* (1964) modified by Habig *et al.,* (1974) with 1-Chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction was measured spectrophotometrically at 340 nm each 20 s during 5 min. GSH levels were measured using a fluoromethric method (Hissin and Hilf, 1976) at λex= 350 nm and λem=425 nm. Accumulation of MDA was used as an index of lipid peroxidation and evaluated spectrophotometrically at 550 nm by measuring the presence of thiobarbituric acid reactive substances (TBARS), according to the method proposed by Recknagel *et al.,* (1982) using a microplate reader.

### 2.5 Statistical analysis

Data were analyzed using statistical software Prism 5 version 5.03 for Windows (GraphPad software, Inc., CA). Results were expressed as mean ± S.E., and the level for statistical significance was defined as $P<0.05$. Since data did not show a normal distribution and the variances were not homogeneous, the statistical analyses were performed using a non-parametric Kruskal-Wallis test (Zar, 1984). Differences between colonies were determined with the Dunn´s test. Moreover, a Spearman test was used to determine correlations between metals and biomarkers.

## 3. RESULTS AND DISCUSSION

Figure 2 shows metal concentrations in blood samples of White stork nestlings, expressed in µg/l. All measured elements were detected in the blood of White stork nestlings.

Chicks from the reference colony (A) showed lower blood concentrations of the toxic elements Pb, As and Hg than those from the site located close to the landfill (B). At site B, mean blood concentrations of these three elements were respectively 4.5, 10.2 and 2.8 times higher than at the reference site A.

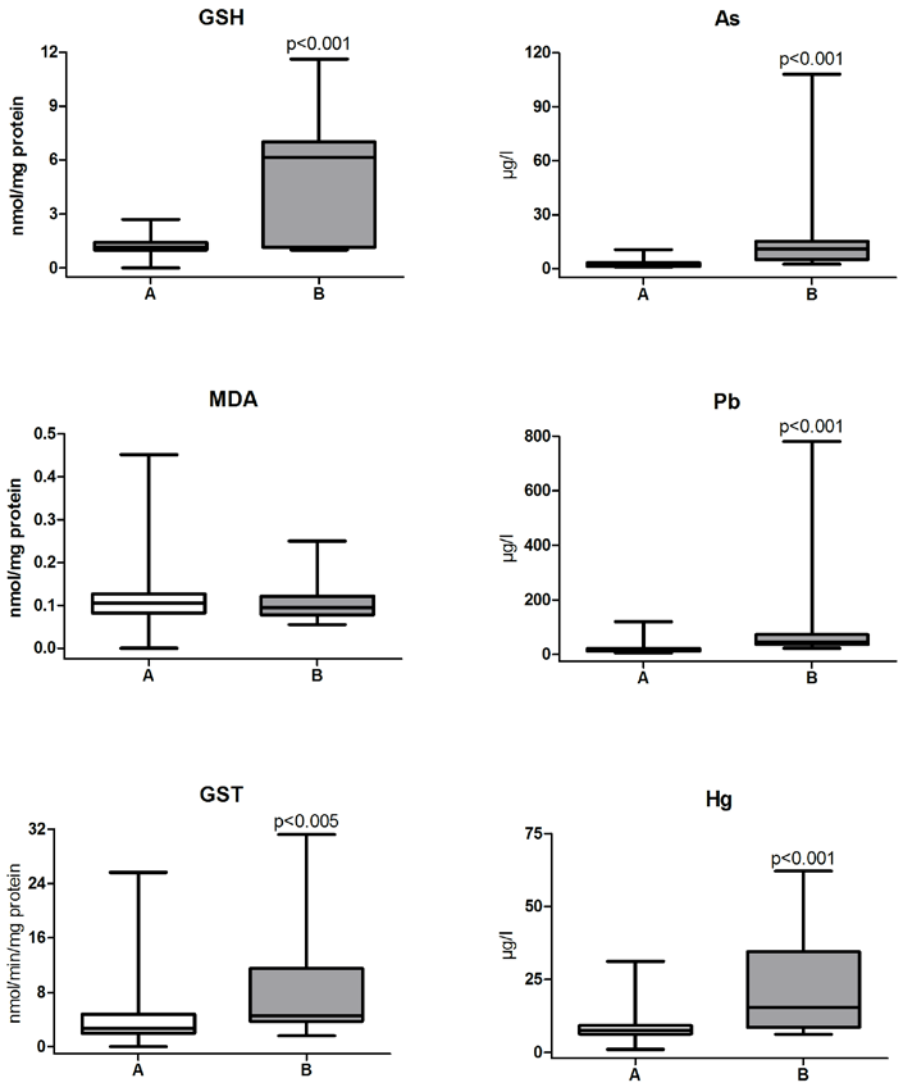



**Figure 2.** Heavy metal content and biomarker levels in blood samples of White stork chicks from colonies A and B. Box-plots illustrate the 10, 25, 50 (median), 75 and 90 % percentiles.

Mean blood Pb concentrations were 23.04 ± 4.56 µg/l in the reference site, with a maximum value of 119.50 µg/l. In contrast, mean Pb concentrations in sampling site B were of 103.30 ± 35.77 µg/l, with a maximum of 780.50 µg/l in one animal. For this toxic metal, the difference between both colonies was statistically significant ($P$<0.001).

Mercury levels were also significantly higher at sampling site B when compared to site A ($P$<0.001). Maximum values up to 62 and 31 µg/l were quantified in colonies B and

A, respectively. Mean values in colony A were of 8.39 ± 1.15 µg/l, whereas in colony B they were of 23.55 ± 4.13 µg/l.

For As again there was a significant effect of sampling site ($P<0.001$). Significant highest arsenic levels were measured in blood samples collected from colony B (maximum value of 108.10 µg/l) than in colony A (maximum of 10.60 µg/l). Mean values were situated on the range of 21.58 ± 6.68 and 2.34 ± 0.37 µg/l, respectively for colonies B and A.

Mean blood Pb concentrations in White storks from Spain were lower to those in the same species from Poland (Kamiński *et al.*, 2008, 2009b), with mean blood values ranging from 1.34 to 7.16 mg/L. Nevertheless, the concentrations of this toxic heavy metal were higher in the blood of White stork chicks from polluted and suburban environments than in those nesting in relatively unpolluted areas, also observed in the present study. Blood Pb levels in waterfowl are considered elevated at concentrations above 200 µg/L wet wt., and concentrations above 400 µg/l are indicative of poisoning (Pain, 1996). Moreover, Scheuhammer, (1987) established that values≤ 150 µg/L in blood indicate an absence of abnormal Pb exposure. The median lead level in the blood of stork chicks analyzed in the present study was 23.04 and 103.3 µg/L (respectively for colony A and B), below what is considered as harmful for a range of bird species, although there is not much data about Pb toxicity thresholds in Ciconiiformes (Franson, 1996; Meharg *et al.*, 2002). Pb concentrations obtained in this study are similar to those obtained in White stork chicks from Puebla del Rio (Sevilla, Spain), at 1 km from the area affected by the Aznalcóllar mine spill occurred in 1998 (Baos *et al.*, 2012). Pb, as well as As and Hg, exert toxic effects when they enter into biochemical reactions in which they are not normally involved. The threshold concentration at which such deleterious effects occur is usually higher for essential elements than for non-essential (Pb, Hg, As) although the "window for essentiality" for some ones is quite narrow (Kamiński *et al.*, 2008).

There are only few papers where the concentrations of Hg in blood of terrestrial birds have been studied. Some reports have documented Hg concentrations in blood of vulture and owl species (Shlosberg *et al.*, 2012; Espín *et al.*, 2014), but in all cases Hg concentrations in blood seemed to be too low to cause any adverse effect on raptors (mean values never exceed 30 µg/L). In those studies, the concentrations were markedly higher than those found in the present study, with a maximum value of 62.10 µg/L, but mean values situated in the range of 7-15 µg/L. In any case, the concentration of mercury was below 1000 µg/L, which has been reported as the threshold of high risk for birds (Evers *et al.*, 2008; Álvarez *et al.*, 2013).

At last, regarding As concentrations in blood of birds, the obtained results were similar to those quantified in blood of stork nestlings sampled during four years at the Doñana National Park area (Baos *et al.,* 2012). Shlosberg *et al.,* (2012) quantified this element in blood of juvenile and adult griffon vulture from Israel. Mean As levels were always below the limit of quantification (10 µg/L), in all cases being markedly lower than As concentrations from White stork sampled at the anthropogenically influenced area (landfill) of Caceres. For this metalloid, information regarding the threshold value in nestling blood is quite limited. Burger and Gochfeld (1997) reported an As value of 18 µg/L in young Franklin's gulls from uncontaminated areas, being considered that value as reference in some studies (Burger and Gochfeld, 1997; Benito *et al.,* 1999).

Species and its trophic situation, sex, time of exposure and biomass are the parameters with the biggest influence on trace elements accumulation in precocial and semiprecocial birds (Benito *et al.,* 1999). These authors emphasized that metals level in blood of chicks may be influenced by physiological response of species to distinct metals, and by the greater or lesser bioavailability of these metals. The reference values should be interpreted carefully, since they do not refer to the same type of species in particular environments (Kamiński *et al.,* 2008).

Regarding the antioxidant biomarkers, and as observed in figure 2, in colony B, White stork nestlings displayed significantly higher blood activities of GSH ($P<0.001$) and GST ($P<0.005$), compared to birds from the reference site. In fact, mean value of GSH in colony B was approximately four times higher than in colony A (4.69 and 1.19 nmol/min, respectively). Similarly, mean GST in colony B was twice that of colony A (8.45 and 4.51 nmol/min/mg prot, respectively).

However, there were no statistically significant differences in blood MDA levels between White stork nestlings from colonies A and B, with mean values 0.11 nmol/min/mg prot, in both cases.

Several times the alteration of biochemical parameters have been studied, as well in field studies as laboratorial ones, trying to identify the direct effect of xenobiotics in the normal organism functionality. Therefore, in the present study, the relationship between surveyed biomarker parameters and blood metal concentrations has been evaluated by means of a Spearman correlation analysis (Table 1). Some significant interactions among elements and biochemical measures were observed. To note the predominance of GSH in element–enzyme interactions is important, with Hg and As having a significant impact on the biochemical activity of this tripeptide. Simultaneously, a significant and positive interrelationship among the three considered elements was clearly observed, and also between GST and MDA.

**Table 1.** Correlation results concerning the different biomarkers and metals considered in the present study. **:*P*<0.01, ***:*P*<0.001).

| | | Hg | As | GST | GSH | MDA |
|---|---|---|---|---|---|---|
| Pb | Rho Spearman | 0.3902 | 0.6261 | 0.1774 | 0.2358 | -0.0339 |
| | *P value* | 0.0056** | 0.0000*** | 0.2225 | 0.1028 | 0.8166 |
| Hg | Rho Spearman | | 0.5773 | 0.1576 | 0.4030 | -0.0332 |
| | *P value* | | 0.0000*** | 0.2793 | 0.0041** | 0.8208 |
| As | Rho Spearman | | | 0.2765 | 0.4178 | 0.1212 |
| | *P value* | | | 0.0544 | 0.0028** | 0.4066 |
| GST | Rho Spearman | | | | -0.0603 | 0.3652 |
| | *P value* | | | | 0.6803 | 0.0098** |
| GSH | Rho Spearman | | | | | -0.0101 |
| | *P value* | | | | | 0.9451 |

One of the initial studies focused on the blood of birds, oxidative stress and inorganic elements was developed in Emperor geese (*Chen canagica*) from Alaska, where a positive correlation between plasma glutathione peroxidase activity and blood Se concentration was found (Franson *et al.,* 2002).

Similarly, Pied flycatcher nestlings (*Ficedula hypoleuca*) that grew up close to a sulfide ore smelter plant located in the northern part of Sweden had accumulated amounts of As, Cd, Hg, Pb, Fe and Zn in their liver tissue. Nestlings from this contaminated areas showed signs of oxidative stress evidenced by slightly elevated lipid peroxidation (MDA levels) and glutathione-S-transferase (GST) activities, and a positive relationship was found between GST and Pb (Berglund *et al.,* 2007). However the relevance of this study is limited since the whole study was conducted with liver samples. Moreover, there are no known threshold concentrations at which metals can affect antioxidant systems, and low metal levels may have an effect in antioxidant biomolecules (Espín *et al.,* 2014).

When considering MDA levels and ecotoxicological studies, significant interactions between toxic heavy metals (Cd) and the content of TBARS (measured as MDA) occurred in blood of White stork chicks (Kamiński *et al.,* 2007). These results indicated changes in oxidative stress intensity in chicks in response to environmental differentiation (Kamiński *et al.,* 2009b). These significant element-enzyme interactions which predominated in storks from polluted areas could be explained by the intensive and prevailing access of toxic metals in free-radical reactions scavengers, reflected by their influence upon the enzymatic activity of antioxidant enzymes and lipid peroxidation (Kamiński *et al.,* 2009a). Kurhalyuk *et al.,* (2009), reported similar results: in their study

in urban pigeons (*Columba livia*), the presence of Pb and Cd was related with an increase of oxidative damage. In fact, the increase in lipid peroxidation was about 4 times higher ($P$<0.001) in exposed areas when compared to control, being directly related to the Pb concentration. However, other researchers determined that blood oxidative damage (measured as MDA) of house sparrow was only slightly lower in urban areas when compared to relatively unpolluted areas (103.06±17.55 and 109.99±23.13 nmol/ml, respectively) (Herrera-Dueñas *et al.,* 2013).

In the present study, the GSH levels varied considerably between sampling areas. Similarly, domestic ducks (*Shaoxing duck*) exposed to Hg and Se in a field study showed increased GSH levels but no evidence of lipid peroxidation (measured as MDA) (Ji *et al.,* 2006). Mateo and Hoffman (2001) found that young mallards and Canada geese exposed to Pb-contaminated sediments showed increased lipid peroxidation and GSH levels. In the same direction, GSH levels increased significantly with increasing fecal metal concentration in Pied flycatcher nestlings (Rainio *et al.,* 2013). Conversely to these results, a high level of GSH content in White stork chicks from relatively unpolluted areas of Poland was quantified when compared to both urban and polluted ecosystems (Kamiński *et al.,* 2009a). Similarly, the total content of GSH did not differ between Pied flycatcher nestlings from polluted and reference sites (Berglund *et al.,* 2007), founding a lot of variation between individuals in total GSH level. That variation was also shown in Great tits in a comparison between urban and rural environments (Isaksson *et al.,* 2005). A time-dependent depletion in the amount of GSH was observed in Japanese quails (*Coturnix coturnix japonica*) exposed to paraquat, and suggesting that GSH could be used to detect exposure to free radical-generating agents that may cause oxidative stress (Galvani *et al.,* 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 (Espín *et al.,* 2014). In addition to tissues (e.g. liver and kidney), GSH can be measured in blood without terminal sampling of the animals, therefore being a useful tool in studies of free-living bird species (Koivula and Eeva, 2010).

GST activities in the present study were statistically increased in sampling site B, directly associated to the heavy metal content. GST catalyzes the conjugation of GSH with pollutants. Hence, the observed induction of GST activity could be indicative of a detoxification process (Jemec *et al.,* 2007), and the higher GST activity in storks from

the landfill influenced area could be associated to increased GSH concentrations due to higher GSH requirements for conjugation reactions (Espín *et al.,* 2014).

Similarly to our present results, blood GST activities in Blue tits (*Cyanistes caeruleus*) nestlings increased significantly with increasing fecal metal concentration (Rainio *et al.,* 2013). However, our results are not in accordance with those in Great tit (*Parus major*) (Isaksson *et al.,* 2009; Koivula *et al.,* 2011), where the effect of the habitat (urban *vs* rural) in the oxidative stress levels was studied, and where they neither found any difference in the GST antioxidant enzyme activity.

The fact that all those biochemical measures varied on a different way according to species could suggest that there is significant flexibility about how different species regulate their oxidative state (Rainio *et al.,* 2013). Similarly, according to other studies (Beyer *et al.,* 1988; Mateo and Hoffman 2001), species react so differently to heavy metal exposure that extrapolation of responses between species would be unwise. Even more, antioxidant defense seems to respond differently depending on pollution situation (Berglund *et al.,* 2007). According to different authors, to select one single ‘‘standard’’ biomarker for oxidative stress to be used in environmental assessment work is inadequate. This is also supported by Halliwell and Gutteridge (1999), who concluded that several different antioxidants are needed to protect against ROS and there is not a universal adequate biomarker for oxidative stress (Berglund *et al.,* 2007). However, along with the measure of metals in blood, these biochemical measures may constitute non-invasive biomarkers which represent an important criteria for long term monitoring of wildlife species.

## ACKNOWLEDGEMENTS

Irene de la Casa-Resino was supported by fellowship PRE09001 from Consejería de Empleo, Empresa e Innovación (Gobierno de Extremadura, Spain).

## REFERENCES

Álvarez, C.R., Moreno, M.J., Alonso, L.L., Gómara, B., Bernardo, F.J.G., Martín-Doimeadios, R.C.R., González, M.J. (2013). Mercury, methylmercury, and selenium in blood of bird species from Doñana National Park (Southwestern Spain) after a mining accident. Environmental Science and Pollution Research 20, 5361-5372.

Baos, R., Jovani, R., Serrano, D., Tella, J. L., Hiraldo, F. (2012). Developmental exposure to a toxic spill compromises long-term reproductive performance in a wild, long-lived bird: the White stork (*Ciconia ciconia*). Public Library of Science ONE 7, e34716.

Becker, P.H. (2003). Biomonitoring with birds. In: Markert, B.A., Breure, A.M., Zechmeister, H.G. (Eds.). Trace Metals and other Contaminants in the Environment. Bioindicators and Biomonitors Principles, Concepts and Applications 6, 677-736. Elsevier.

Benito, V., Devesa, V., Muñoz, O., Suñer, M.A., Montoro, R., Baos, R., Hiraldo, F., Ferrer, M., Fernández, M., González, M.J. (1999). Trace elements in blood collected from birds feeding in the area around Donana National Park affected by the toxic spill from the Aznalcóllar mine. Science of the Total Environment 242, 309-323.

Berglund, Å.M.M., Sturve, J., Förlin, L., Nyholm, N.E.I. (2007). Oxidative stress in pied flycatcher (*Ficedula hypoleuca*) nestlings from metal contaminated environments in northern Sweden. Environmental Research 105, 330-339.

Bertrand, S., Alonso-Alvarez, C., Devevey, G., Faivre, B., Prost, J., Sorci, G. (2006). Carotenoids modulate the trade-off between egg production and resistance to oxidative stress in zebra finches. Oecologia 147, 576-584.

Beyer, W.N., Spann, J.W., Sileo, L., Franson, J.C. (1988). Lead poisoning in six captive avian species. Archives of Environmental Contamination and Toxicology 17, 121-130.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry 72, 248-254.

Burger, J., Gochfeld, M. (1997). Age differences in metals in the blood of Herring (*Larus argentatus*) and Franklin's (*Larus pipixcan*) gulls. Archives of Environmental Contamination and Toxicology 33, 436-440.

Cohen, A.J., Smith, J.N., Turbert, H. (1964). Comparative detoxication. 10. The enzymic conjugation of chloro compounds with glutathione in locusts and other insects. Biochemical Journal 3, 457-464.

Costantini, D., Casagrande, S., Filippis, S., Brambilla, G., Fanfani, A., Tagliavini, J., Dell'Omo, G. (2006). Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). Journal of Comparative Physiology B 176, 329-337.

Domínguez, J.A., Moreno, F.A., González, F., Pita, M.A. (2011). Informe ambiental de Extremadura 2011. Consejería de Agricultura, Desarrollo Rural, Medio Ambiente y Energía de la Junta de Extremadura. http://extremambiente.gobex.es/. Accessed 3 October 2013.

Espín, S., Martínez-López, E., Jiménez, P., María-Mojica, P., García-Fernández, A.J. (2014). Effects of heavy metals on biomarkers for oxidative stress in Griffon vulture (*Gyps fulvus*). Environmental Research 129, 59-68.

Evers, D., Savoy, L., DeSorbo, C., Yates, D., Hanson, W., Taylor, K.M., Siegel, L.S., Cooley, J.H., Bank, M.S., Major, A., Munney, K., Mower, B.F., Vogel, H.S., Schoch, N., Pokras,

M., Goodale, M.W., Fair, J. (2008). Adverse effects from environmental mercury loads on breeding common loons. Ecotoxicology 17, 69-81.

Franson, J.C. (1996). Interpretation of tissue lead residues in birds other than waterfowl. In: Beyer, W.N., Heinz, G.H., Redmon-Norwood, W. (Eds.). Environmental Contaminants in Wildlife. Interpreting Tissue Concentrations. Florida: Boca Raton, 265-280.

Franson, J.C., Hoffman, D.J., Schmutz, J.A. (2002). Blood selenium concentrations and enzyme activities related to glutathione metabolism in wild emperor geese. Environmental Toxicology and Chemistry 21, 2179-2184.

Galvani, P., Cassani, A., Fumagalli, P., Santagostino, A. (2000). Effect of paraquat on glutathione activity in Japanese quail. Bulletin of Environmental Contamination and Toxicology 64, 74-80.

Geens, A., Dauwe, T., Eens, M. (2009). Does anthropogenic metal pollution affect carotenoid colouration, antioxidative capacity and physiological condition of great tits (*Parus major*)? Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 150, 155-163.

Gravato, C., Oliveira, M., Santos, M. A. (2005). Oxidative stress and genotoxic responses to resin acids in mediterranean mussels. Ecotoxicology and Environmental Safety 61, 221-229.

Habig, W.H., Pabst, M.J., Jakoby, W.B. (1974). Glutathione S-Transferases: the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249, 7130-7139.

Halliwell, B., Gutteridge, J.M.C. (1999). Free radicals in Biology and Medicine (third ed.). New York: Oxford University Press.

Hegseth, M.N., Camus, L., Helgason, L.B., Bocchetti, R., Gabrielsen, G.W., Regoli, F. (2011). Hepatic antioxidant responses related to levels of PCBs and metals in chicks of three Arctic seabird species. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 154, 28-35.

Herrera-Dueñas, A., Pineda, J., Antonio, M.T., Aguirre, J.I. (2013). Oxidative stress of House sparrow as bioindicator of urban pollution. Ecological Indicators, *In press*, doi:10.1016/j.ecolind.2013.08.014.

Hissin, P.J., Hilf, R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry 74, 214-226.

Isaksson, C., Örnborg, J., Stephensen, E., Andersson, S. (2005). Plasma Glutathione and Carotenoid Coloration as Potential Biomarkers of Environmental Stress in Great Tits. EcoHealth 2, 138-146.

Isaksson, C., Sturve, J., Almroth, B. C., Andersson, S. (2009). The impact of urban environment on oxidative damage (TBARS) and antioxidant systems in lungs and liver of great tits, *Parus major*. Environmental Research 109, 46-50.

Jemec, A., Drobne, D., Tišler, T., Trebše, P., Roš, M., Sepčić, K. (2007). The applicability of acetylcholinesterase and glutathione S-transferase in Daphnia magna toxicity test. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 144, 303-309.

Ji, X., Hu, W., Cheng, J., Yuan, T., Xu, F., Qu, L., Wang, W. (2006). Oxidative stress on domestic ducks (*Shaoxing duck*) chronically exposed in a mercury–selenium coexisting mining area in China. Ecotoxicology and Environmental Safety 64, 171-177.

Kaluga, I. (2006). Protection of the White stork *Ciconia ciconia* in the Mazovian lowland. In: Tryjanowski, P., Sparks, T.H., Jerzak, L. (Eds.). The White Stork in Poland: studies in biology, ecology and conservation. Poznań: Bogucki Wydawnictwo Naukowe, 449-458.

Kamiński, P., Kurhalyuk, N., Jerzak, L., Kasprzak, M., Tkachenko, H., Klawe, J. J., Szady-Grad, M., Koim, B., Wiśniewska, E. (2009a). Ecophysiological determinations of antioxidant enzymes and lipoperoxidation in the blood of White stork *Ciconia ciconia* from Poland. Environmental Research 109, 29-39.

Kamiński, P., Kurhalyuk, N., Kasprzak, M., Jerzak, L., Tkachenko, H., Szady-Grad, M., Klawe, J.J., Koim, B. (2009b). The Impact of element–element interactions on antioxidant enzymatic activity in the blood of White stork (*Ciconia ciconia*) chicks. Archives of Environmental Contamination and Toxicology 56, 325-337.

Kamiński, P., Kurhalyuk, N., Szady-Grad, M. (2007). Heavy metal-induced oxidative stress and changes in physiological process of free radicals in the blood of White stork (*Ciconia ciconia*) chicks in polluted areas. Polish Journal of Environmental Studies 16, 555-562.

Kamiński, P., Kurhalyuk, N., Szady-Grad, M., Tkachenko, H., Kasprzak, M., & Jerzak, L. (2008). Chemical elements in the blood of White stork *Ciconia ciconia* chicks in differentiated regions of Poland. Medical and Biological Sciences 22, 31-37.

Kekkonen, J., Hanski, I. K., Väisänen, R. A., & Brommer, J. E. (2012). Levels of heavy metals in House Sparrows (*Passer domesticus*) from urban and rural habitats of southern Finland. Ornis Fennica 89, 91-98.

Klaassen, C.D., Bracken, W.M., Dudley, R.E., Goering, P.L., Hazelton, G.A., Hjelle, J.J. (1985). Role of sulfhydryls in the hepatotoxicity of organic and metallic compounds. Fundamental and Applied Toxicology 40, 264-271.

Koivula, M.J., Eeva, T. (2010). Metal-related oxidative stress in birds. Environmental Pollution 158, 2359-2370.

Koivula, M.J., Kanerva, M., Salminen, J.P., Nikinmaa, M., Eeva, T. (2011). Metal pollution indirectly increases oxidative stress in great tit (*Parus major*) nestlings. Environmental Research 111, 362-370.

Kurhalyuk, N., Netmanski, T., Antonowicz, J., Tkachenko, H. (2009). Oxidative stress and protein oxidation affected by toxic metals in feral pigeons (*Columba livia*) from northern Poland. Baltic Coastal Zone 13, 187-197.

Leaver, M.J., George, S.G. (1998). A piscine glutathione S-transferase which efficiently conjugates the end-products of lipid peroxidation. Marine and Environmental Research 46, 71-74.

Mateo, R., Hoffman, D.J. (2001). Differences in oxidative stress between young canada geese and mallards exposed to lead-contaminated sediment. Journal of Toxicology and Environmental Health Part A 64, 531-545.

Medina, F., Avilés, J.M., Sánchez, A. (1998). Diferencias intraespecíficas en el uso de un vertedero por parte de la cigüeña blanca (*Ciconia ciconia*) en el oeste de la Península Ibérica. Butlletí del Grup Català d'Anellament 15, 9-14.

Meharg, A.A., Pain, D.J., Ellam, R. M., Baos, R., Olive, V., Joyson, A., Powell, N., Green, A.J., Hiraldo, F. (2002). Isotopic identification of the sources of lead contamination for White storks (*Ciconia ciconia*) in a marshland ecosystem (Doñana, S.W. Spain). Science of The Total Environment 300, 81-86.

Pain, D.J. (1996). Lead in waterfowl. In: Beyer, W.N., Heinz, G.H., Redmon-Norwood, A.W. (Eds.). Environmental Contaminants in Wildlife. Interpreting Tissue Concentrations. Boca Raton, Florida: CRC Press, 251-264.

Rainio, M.J., Kanerva, M., Salminen, J.P., Nikinmaa, M., Eeva, T. (2013). Oxidative status in nestlings of three small passerine species exposed to metal pollution. Science of the Total Environment 454-455, 466-473.

Recknagel, R.O., Glende, E.A., Walker, R.L., Lowery, K. (1982). Lipid peroxidation biochemistry measurement and significance in liver cell injury. In: Plaa, G., Hewitt, W. (Eds.). Toxicology of the liver. New York: Raven Press, 213-241.

Scheuhammer, A.M. (1987). The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: a review. Environmental Pollution 46, 263-295.

Shlosberg, A., Wu, Q., Rumbeiha, W., Lehner, A., Cuneah, O., King, R., Hatzofe, O., Kannan, K., Johnson, M. (2012). Examination of Eurasian Griffon Vultures (*Gyps fulvus fulvus*) in Israel for Exposure to Environmental Toxicants Using Dried Blood Spots. Archives of Environmental Contamination and Toxicology 62, 502-511.

Swaileh, K.M., Sansur, R. (2006). Monitoring urban heavy metal pollution using the house sparrow (*Passer domesticus*). Journal of Environmental Monitoring 8, 209-213.

Tkachenko, H., Kurhaluk, N. (2012). Pollution-induced oxidative stress and biochemical parameter alterations in the blood of White stork nestlings *Ciconia ciconia* from regions with different degrees of contamination in Poland. Journal of Environmental Monitoring 14, 3182-3191.

Zar, J.H. (1984). Biostatistical analysis 2nd edition. New Jersey: Prentice Hall.

# CAPÍTULO VI

---

**Contaminantes clorados en sangre de pollos de cigüeña blanca (*Ciconia ciconia*) procedentes de diferentes colonias en España.**

I

*de la Casa Resino, I., Hernández-Moreno, D., Castellano, A., Pérez-López, M., Soler, F. Chlorinated pollutants in blood of White stork nestling (Ciconia ciconia) in different colonies in Spain. In preparation.*

**RESUMEN:** El objetivo de este estudio fue investigar los niveles de contaminantes orgánicos persistentes (COPs) en aves salvajes. Se han determinado las concentraciones de bifenilos policrorados (PCBs) y los pesticidas organoclorados (OCPs) en muestras de plasma de pollos cigüeña blanca. Las muestras fueron recogidas en tres colonias localizadas en tres ambientes distintos (dehesa, planta de tratamiento de residuos sólidos urbanos (PTRSU) y area de agricultura intensiva) en el oeste de España. No se detectaron niveles de PCBs en ninguna de las muestras estudiadas. En relación a los OCPs, el heptacloro, el *4,4'*-DDE, el endosulfán y el aldrín fueron los pesticidas detectados en mayor frecuencia, mientras que el *4,4'*-DDE y el heptacloro fueron los detectados en mayores concentraciones. La cría en una área de agricultura intensiva no tuvo niguna influencia en los niveles de OCPs, indicando que estos compuestos, ya prohibidos, no están en uso en este área. La media de los valores de OCPs encontrados en plasma de la segunda colonia (PTRSU) fue más alta que la encontrada en las otras dos colonias. Estos niveles tan altos podrían estar relacionados con un posible contacto de las madres con estos pesticidas durante su migración africana y un depósito posterior en los lípidos del huevo. Sin embargo, son necesarios más estudios para localizar el origen de estos contaminantes en relación al alimento, el suelo, el uso de estos pesticidas y la migración.

**Palabras clave:** sangre, cigüeña blanca, PCBs, pesticidas organoclorados.

**ABSTRACT:** This study aimed to investigate the levels of persistent organic pollutants (POPs) in wild birds. The concentrations of certain POPs, including polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), in plasma of White stork (*Ciconia ciconia*) nestling were measured. The blood samples were collected from three breeding colonies located in three different environments in the West of Spain (grassland, landfill and agricultural area). No detected concentrations of PCBs were found in any sample from any colony. Regarding OCPs, heptaclor, *4,4'*-DDE, endosulfan and aldrin were the most frequently detected, being *4,4'*-DDE and heptachlor the OCPs detected at the highest concentrations. Breading close to an intensive agricultural area has no influence in OCPs levels, indicating that these banned compounds are not currently used in this area. The average values of OCPs found in plasma from the second colony (next to the landfill) were higher than those from the other colonies. These high levels might be related to a potentially impregnation of the mother during its migration to Africa and subsequent convey to the egg via lipid deposit. However, more knowledge is needed regarding localized exposure in relation to food, soil, pesticide use and migration.

**Keywords:** blood, White stork, PCBs, organochlorine pesticides.

## 1. INTRODUCTION

Persistent organic pollutants (POPs) are substances that are resistant in the environment, bioaccumulate in the food web, and pose a risk of causing adverse effects to human health and the environment (Safe, 1994; Gioia *et al.*, 2013). POPs include chemicals such as dioxins/furans, polychlorinated biphenyls (PCBs), brominated flame retardants, perfluorinated compounds, and organochlorine pesticides (OCPs) such as *4,4'*-dichlorodiphenyltrichloroethane (*4,4'*-DDT) and its metabolites, among others (Dias *et al.*, 2013). Due to the accumulation of this kind of substances in tissues and fluids of birds, these animals can be useful tools for biomonitoring programs (Cunha *et al.*, 2012). In fact, terrestrial birds are well known as appropriate organisms for biomonitoring purposes because of their high trophic position in the food chain, which leads to the accumulation of POPs (van Wyk *et al.*, 2001; Gómez-Ramírez *et al.*, 2012, 2014; Luzardo *et al.*, 2014). In this sense, raptors have been widely used in biomonitoring schemes (van Wyk *et al.*, 2001; Martinez-Lopez *et al.*, 2007, 2009; Gómez-Ramírez *et al.*, 2012, 2014) however, other species less used for this purpose, like Ciconiiformes, have good qualities as bioindicators (Jimenez *et al.*, 2000; Sáez *et al.*, 2008, 2009).

White stork (*Ciconia ciconia*) is a large (2.2–4.4 kg), long-lived wading bird that breeds from North Africa to Northern Europe. At Iberian latitudes, it has a prolonged breeding season (from February to July), with chicks hatching after 33–34 days of incubation and depending on adults before fledging (Cramp, 1980). White stork is a colonial species that feeds mainly on wildlife preys, but rubbish dumps have become in the later years an important source of nutrients (Jimenez *et al.*, 2000). Contaminant levels in White stork nestlings could indicate the presence of contaminants within a colony's local environment (Blázquez, 2006). Simultaneously, as a migratory species, they can be exposed to pollutants, like chlorinated pollutants, which can be transferred from the birds to their eggs and offsprings which can provide the means for observing the effects of these pollutants on the reproductive processes of bird colonies (Burger and Gochfeld, 2004). In this sense, the effect of these substances in the reproductive success of bird species, like eggshell thinning, altered breading behavior and teratological malformations have been well documented (Cooke, 1973; McArthur *et al.*, 1983; Fry, 1995; Peakall and Lincer, 1996; Cunha *et al.*, 2012).

Choice of matrix in which the pesticides will be measured is an important point in a biomonitoring program. Non destructive samples (feathers, blood, eggs), are the most frequently collected samples, thus reflecting the importance of practical, ethical and conservational issues when sampling wild birds. However, collection of whole blood,

plasma or serum is less common than collecting feathers or eggs (Gómez-Ramírez *et al.*, 2014).

The aim of this study was to investigate the chlorinated pollutants content in White stork from Cáceres province, in order to obtain baseline data. Therefore, we determined the concentrations of selected chlorinated pollutants, including PCBs and OCPs, in blood of White stork chicks from three different environmental influences (grassland, urban landfill and agricultural area).

## 2. MATERIAL AND METHODS

### 2.1 Reagents

Isooctane (Panreac, Spain), n-hexane, sulfuric acid (Scharlau, Spain) and acetone (VWR, Germany) were residue analysis. Florisil, individual OCPs standard (chlordane and hexachlorobencene) and mixture of pesticides (EPA625/CLP Pesticides Mix) were purchased from Supelco (USA). PCBs (PCB Mix 3, PCB 169 and PCB 143) were obtained from Dr. Ehrenstorfer (Germany). EPA 625/CLP Pesticides Mix included aldrin, α-HCH, β-HCH, Δ-HCH, γ-HCH, dieldrin, α-endosulfan, β-endosulfan, endosulfan sulfate, endrin, endrin aldehyde, heptachlor, heptachlor epoxide *4,4'*-DDD, *4,4'*-DDE, *4,4'* DDT. PCBs Mix 3 contained PCBs 28, 52, 101, 118, 138, 153, 180.

### 2.2 Study area

Fieldwork was conducted in three colonies located in the province of Cáceres (Extremadura region, West of Spain) during the spring of 2011, and covering a gradient of potential contamination (Figure 1). The reference colony (A) was located in "Los Llanos de Cáceres" (39º 28' 17.53'' N, 6º 10' 35.00'' W), a natural area far from apparent sources of pollution, considered a Special Protection Area for Birds under the Directive 2009/147/EC and forming an integral part of the NATURA 2000 ecological network. It is a pseudo steppe zone crossed by 4 rivers (Tajo, Almonte, Tamuja and Salor rivers) which contains an important amount of protected steppe birds like Bustard (*Otis tarda*), Little bustard (*Tetrax tetrax*) and Lesser kestrel (*Falco naumanni*). It is characterized by rainfed crops, mainly cereal but some legumes also, that are used for feeding livestock, sheep and cattle, in an extensive farming. The second colony (B) was located in a grassland area similar to previous one but with more trees (*Quercus ilex*) (39º 18' 37.13'' N, 6º 29' 47.53'' W), at a distance of 13.4 km far from Cáceres town (95668 inhabitants, 2012) and close to (4.9 km) a solid waste rubbish dump that handled

61826 tons of solid waste in 2011 (Domínguez *et al.*, 2011) where the White storks go very frequently to feed during breeding period. The third colony (C) was located between the towns of Navalmoral de la Mata (17401 inhabitants, 2012) and Talayuela (9269 inhabitants, 2012) (39º 18' 37.13''N, 6º 29' 47.53'' W). It is situated in a grassland area with *Quercus ilex* trees used for livestock and close to an important intensive agricultural area crossed by the Tietar river which is characterized for large tobacco, pepper, tomato, asparagus and corn crops. Close to colony C (1.5 km) there is a solid waste rubbish dump that handled 39446 tons of solid waste in 2011 (Domínguez et al., 2011). The distance between colonies A and B is 26.2 km, whereas between colonies A and C it is 71.7 km. Nests in the 3 colonies were placed on trees.

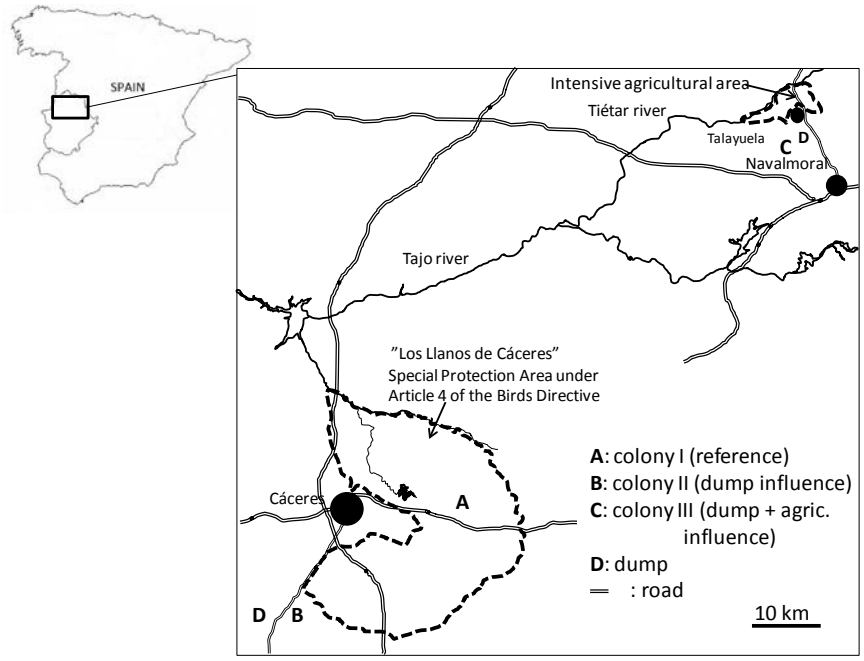



**Figure 3.** Sampling sites around the city of Cáceres (Spain).

## 2.3 Field procedure and blood sampling

During the spring of 2011, with the regional government permission (Gobierno de Extremadura CN10/0306), White stork nestlings were sampled in each colony depending on the accessibility to the nest. A total of 27, 22 and 10 animals were sampled in colonies A, B and C respectively. The number of sampled animals was as big as possible according to the accessibility to the nest, the number of nests in the colony, the weight (2.3-3 kg) and the developmental state of nestlings (3-4 weeks) and also the climate on

sampling days (avoiding the warmest moment of the day). Both standardized capture and handling method were performed in order to produce the minimal stress to the animals. All nestlings from each colony were taken down from their nests, gently restrained by hand during blood collection, physical examination, weighing and measuring (to establish the age). Blood samples were taken from the tarsal vein with heparinized 0.8x25 mm needle and 5 ml syringe. The blood samples were refrigerated till arrival to the laboratory, where the plasma was obtained after centrifugation (3000 rpm for 10 min) and frozen at -80 ºC until use.

### 2.4 Sample preparation

The PCBs and OCPs extraction procedure for plasma was adapted from the protocol used by Mateo et al. (2012). This method was based on the sulfuric acid clean-up of an *n*-hexane extraction procedure. Briefly, 3 ml of n-hexane were added to 400 µl of plasma. Then, the tubes were shaken in an orbital shaker for 10 min, sonicated during 5 min and centrifuged at 1000 g for 5 min. The procedure was repeated again and a final volume of 6 ml was obtained. This extract was divided in two aliquots of 3 ml. To the first one, 0.4 ml of sulfuric acid were added, and then horizontally shaken for 10 min and centrifuged at 1000 g for 5 min. The obtained extract was evaporated, rediluted in 200 µl of isooctane and used for PCBs determination. The second aliquot was clean up with florisil cartridge, following the protocol of Young (2009) for pesticides. The obtained extract was evaporated, resuspended in 200 µl of isooctane and used for OCPs determination. An internal standard (10 µl of PCB143 at working solution of 1 ng/µl) was added at the beginning of the extraction process. Blanks were processed among samples to assure quality of analyses.

### 2.5 GC/MS instrumentation and conditions

All samples were analyzed using a Shimadzu QP 2010 Plus GC/MS operating in negative chemical ionization (NCI) mode which was coupled to an AOC-20i Autosampler (Shimadzu, Japan). Analyte separation was achieved on a SLB 5MS column (30 m–0.25 mm, i.d. x 0.25 film thickness) (Supelco, USA). The results were analyzed using Shimadzu GCMS Solution version 2.51 software. The GC oven temperature ramp was: 100 ºC, hold 1 min, then temperature increased at a rate of 60 ºC/min to 200 ºC, to 250 ºC at a rate of 3 ºC/min, and to 300 ºC at a rate of 30 ºC/min. All injections were in splitless mode with a column flow rate of 0.89 ml/min. The injection temperature was 275 ºC. The detector and interface temperatures were 200 ºC

and 300 ºC, respectively. Quantitative evaluation of the PCBs and OCPs residues was performed by the internal standard method (PCB 143) and the calibration curves were obtained by comparing the values of the areas of the specific peaks with their concentrations. The procedures were checked for recovery. Percent recoveries of PCBs spiked samples were between 88-126 % (CV <20%, n=3). Recoveries for OCPs were between 72-148 % (CV <21%, n=3). The limit of detection (LOD) was calculated as the concentration at which the signal-to-noise ratio exceeded 3. LOD was between 0.3-0.7 µg/l for PCBs and 0.05-2.59 µg/l for OCPs and it was calculated using Shimadzu GC/MS Solution version 2.50 software; the calculation of signal-to-noise function was used. OCPs were grouped according to their structure in HCHs, and DDTs. HCHs includes α-, β-, and γ-isomers. DDTs represented the sum of *4,4'*-DDD, *4,4'*-DDE and *4,4'* DDT.

### 2.6 Statistical analysis

Data representing the concentration of contaminants are presented as mean values accompanied with standard deviation, median, minimum and maximum. A value half the LOD was assigned to samples with undetectable contaminant concentration. All statistical assessment was limited to the chemicals that were detected in more than 50 % of all samples. Due to the non normal distribution of the data (Kolmogorov–Smirnov and Shapiro–Wilk test) the statistical analyses were performed using a non-parametric Kruskal-Wallis test. Differences among colonies were determined with the Dunn´s test ($P<0.05$).

## 3. RESULTS AND DISCUSSION

Residues of PCBs and OCPs were quantified in 59 White stork chicks from three different locations in Extremadura (W of Spain).

No detected concentrations of PCBs were found in any sample. Plasma PCBs were found in an amount of 1.65 ng/g (Sáez *et al.*, 2009) and also in a a range of 2.91-3.51 ng/g (Sáez *et al.*, 2008) in two previous studies in White stork nestlings from Spain. Moreover, concentrations of PCBs in serum of Egyptian vulture (*Neophron percnopterus*) from Spain appeared between 3.2-97 ng/ml (Gómara *et al.*, 2004). Kannan et al. (2002) found serum PCBs levels ranging between 46 and 67 ng/g in Bald eagle (*Haliaeetus leucocephalus*) nestlings from Michigan (USA). In other study, an increase in the plasma ∑PCBs was found during the years 2008, 2009 and 2010 in Golden eagles, Northern goshawks and White-tailed eagles from northern Norway (Sonne *et al.*, 2012).

Kannan et al. (2002) reported similar PCBs concentrations in eggs and blood samples. During the 1980s and 1990s a decrease in the levels of PCBs have been observed in eggs of White stork and other birds species in Spain (Hernandez *et al.*, 1986, 1988, 1989; Jimenez *et al.*, 2000). Furthermore, and similarly to that observed with plasma samples, the results found in eggs of these species in Spain were much lower than the ones obtained in other countries during the same period (Ramesh *et al.*, 1992; Tanabe *et al.*, 1998). The results of the present study reflect the decrease of PCBs levels in the environment after their restriction of use in Spain (Real Decreto 1378/1999). Likewise, these results indicate that the presence of landfills or intensive agricultural areas have no influence in the levels of these contaminants, due to their absence in the studied samples.

Concentrations of OCPs (µg/l) found in plasma of White stork are reported in Table 1 and 2. Residue pattern of OCPs in all the studied chicks presented the following order: heptachlor > endosulfan > ∑DDTs > endosulfan sulfate > ∑HCHs > endrin > aldrin > dieldrin. Heptaclor, *4,4'*-DDE, endosulfan and aldrin were the most frequently detected, being *4,4'*-DDE and heptachlor detected at the highest concentrations. A high dispersion in the levels of residues among colonies was observed (Table 1). Animals from colony B (close to a landfill) showed the highest mean values for all the studied OCPs. Moreover, in chicks from colony C (close to a landfill and intensive agricultural area) the lowest mean values of those OCPs were quantified (all of them were below LOD).

Total ∑DDTs was 2.96 ± 15.43 µg/l. The analysis did not reveal the presence of quantifiable levels of *4,4'*-DDT, being influenced this∑DDTs mainly by *4,4'*-DDE concentrations. The highest concentration was found for *4,4'*-DDE, with an average of 5.59 ± 21.56 µg/l. When *4,4'*-DDT is released into the environment, it degrades into *4,4'*-DDE and *4,4'*-DDD. Traditionally, the ratio of *4,4'*-DDE/DDTs has been used to discriminate between recent and past usages of DDT, where low ratios indicate the recent use of *4,4'*-DDT (Cunha, 2012). *4,4'*-DDE levels detected in the present study were much higher than those obtained by other authors in blood of White stork nestlings. For example, levels ranging 0.04-0.23 ng/g (Sáez *et al.*, 2008, 2009) have been found in White stork nestlings from Madrid (central Spain). However, in other bird species (i.e. vultures) a maximum value of *4,4'*-DDE of 62.86 µg/l in blood has been reported, being mean values notably higher in captivity birds (11.33 µg/l) than in birds from natural populations (5.7 µg/l) (van Wyk *et al.*, 2001). The data obtained for *4,4'*-DDE in natural populations of vultures (van Wyk *et al.*, 2001) are similar to those found in the present study for the same compound. Concerning *4,4'*-DDD, mean levels reported in blood samples of Little stints (*Calidris minuta*, 6.54 µg/l) and Threebanded plovers (*Charadrius tricollaris*, 2.41 µg/l) from the North-West Province of South Africa (Lötter and Bouwman, 1997) are markedly higher than results found in the present study. Some

studies in eggs of raptors (Bustnes *et al.*, 2007; García-Fernández *et al.*, 2008; Gómez-Ramírez *et al.*, 2012) and blood of White stork (Sáez *et al.*, 2009) have found a stabilizing or even increasing concentration of *4,4'*-DDE during the last years. This fact could be associated to local current environmental inputs of diphenylaliphatics such as dicofol (Martínez-Lopez *et al.*, 2007; García-Fernández *et al.*, 2008; Gómez-Ramírez *et al.*, 2012). Besides the high concentrations found, it is also important to note that *4,4'*-DDE levels were highly variable among samples (standard deviations were higher than average values; see Table 1). Since we knew the exact location where each sample was collected, we tried to identify common patterns of variation among sampling locations. The results showed that *4,4'*-DDE and *4,4'*-DDD concentrations were much higher in blood from the colony B compared with samples from colonies A and C. However, no statistical analysis between sampling location was done because in colonies A and C more than 50 % of the results were below the detection limit.

The high concentrations of heptachlor and endosulfan (Table 1 and 2) compared with the other pesticides, make cyclodienes the second main group of our study. Heptaclor, with a total mean of 21.31 ± 61.43 µg/l was the only OCPs detected in colonies A and B in more than 70% of the samples, appearing a statistically significant difference between both colonies ($P<0.001$). In vertebrates, heptachlor is readily metabolized to heptachlor epoxide (Elliott and Bishop, 2011). The absence of this metabolite could indicate a recent exposure to the pesticide heptachlor. The values obtained in the present study are similar to those obtained by van Wyk et al. (2001) for heptachlor epoxide in blood of vultures (1.03-39.49 µg/l). These authors also found the highest values in wild vultures when compared with those from a natural reserve.

With a total mean of 3.56 ± 14.43 µg/l, endosulfan was the third highest-leveled OCP detected. The highest levels of endosulfan were found in colony B, being detected in less than 50 % of the samples from the two remaining colonies. Although certain studies have considered endosulfan sulfate the main metabolite of endosulfan (Antonious and Byers, 1997) in the present study endosulfan sulfate was only detected in colony B (14.29 %).

Endosulfan results are similar to those obtained by different authors in whole blood (1.28-13.29 µg/l) of wild African Whitebacked vultures (van Wyk *et al.*, 2001). Moreover, the study developed by Martínez-López (2009) during the years 1999-2003 in whole blood of Booted eagle (*Hieraaetus pennatus*) showed higher mean values (43.06 ± 55.07 µg/l) than those obtained in the present study, observing an increasing trend along the considered period. Endosulfan is not persistent in warm-blooded organisms, being partly converted by hydrolysis to dialcohol and partly oxidized to endosulfan sulfate (Martínez-López *et al.*, 2009). In the present study, endosulfan sulfate levels found in

**Table 1.** Concentrations of OCPs in plasma of White stork in the three colonies. Data are expressed in µg/l. ND: not detected. SD: standard deviation. %: percentage of the samples where the pesticide has been detected.

| | A (n=27) | | | | B (n = 22) | | | | C (n = 10) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *mean ± SD* | Range | median | % | *mean ± SD* | Range | median | % | *mean ± SD* | Range | median | % |
| **Heptachlor** | *0.93 ± 1.56* | ND-7.71 | 0.63 | 74 | *55.98 ± 91.72* | 0.09-300 | 9.42 | 100 | *ND* | - | ND | 0 |
| **Aldrin** | *0.05 ± 0.08* | ND-0.41 | ND | 7 | *0.40 ± 0.59* | ND-2.14 | 0.09 | 59 | *ND* | - | ND | 0 |
| **Endosulfan** | *0.06 ± 0.06* | ND-0.38 | ND | 4 | *9.45 ± 22.74* | ND-93.6 | 0.05 | 50 | *ND* | - | ND | 0 |
| **Dieldrin** | *ND* | - | ND | 0 | *0.11 ±0.37* | ND-1.76 | 0.03 | 9 | *ND* | - | ND | 0 |
| **Endrin** | *0.24 ± 0.78* | ND-1.29 | ND | 4 | *0.50 ± 1.23* | ND-5.36 | 0.09 | 13 | *ND* | - | ND | 0 |
| **Endosul sulf** | *ND* | - | ND | 0 | 1.45 ± 0.52 | ND-3.26 | ND | 1 | *ND* | - | ND | 0 |
| ***4.4'*-DDE** | *0.38 ± 1.17* | ND- 4.69 | 0.052 | 7 | *14.50 ± 33.90* | ND-121 | 0.66 | 68 | *ND* | - | ND | 0 |
| ***4.4'*-DDD** | *ND* | - | ND | 0 | *0.72 ± 1.95* | ND-8.9 | 0.11 | 13 | *ND* | - | ND | 0 |
| **∑DDTs** | *0.24 ± 0.83* | ND-4.69 | 0.1065 | 6 | *7.61 ± 24.73* | ND-121 | 0.11 | 41 | *ND* | - | ND | 0 |
| **β-HCH** | *0.17 ± 0.03* | ND-0.34 | ND | 4 | *0.84 ± 1.86* | ND-8.55 | 0.17 | 36 | *0.24 ± 0.23* | ND-0.88 | ND | 10 |
| **γ-HCH** | *0.08 ± 0.01* | ND-0.12 | ND | 4 | *1.48 ± 2.57* | ND-9.14 | 0.08 | 41 | *ND* | - | ND | 0 |
| **∑HCHs** | *0.13 ± 0.05* | ND-0.34 | ND | 6 | *1.16 ± 2.25* | ND-9.14 | 0.17 | 36 | *ND* | - | ND | 5 |

colony B (although much lower than endosulfan) could be related with the high levels of endosulfan quantified in this colony, indicating maybe a recent exposure to this pesticide (Martínez-López *et al.*, 2009).

Dieldrin has been labelled as a commonly detected environmental contaminant due to the stability and relative ease of measurement (Santerre *et al.*, 1997). However, in the present study, dieldrin occurred in the lowest concentration (0.06 ± 0.22 µg/l). In fact, van Wyk *et al.* (2001) reported higher blood levels (26.95 µg/l) of dieldrin in vultures from South Africa. Other studies in wading birds (Little stint and Kittlitz's plover) also exceed the results of the present study, founding mean levels of 80.76 µg/l in blood samples (Lötter and Bouwman, 1997). The latter values are, however, lower than those reported in Cape griffon vulture eggs analysed between 1973 and 1980 in Southern Africa (50-3800 µg/kg) (Mundy *et al.*, 1982).

**Table 2.** Total mean concentration of OCPs in plasma of White stork. Data expressed in µg/l. ND: not detetcted. SD: Standard deviation. %: percentage of the samples where the pesticide has been detected.

| | *n* = 59 | | | |
|---|---|---|---|---|
| | *mean* ± *SD* | Range | median | % |
| **Heptachlor** | *21.31* ± *61.4* | ND-300 | 0.74 | 71 |
| **Aldrin** | *0.18* ± *0.40* | ND-2.14 | 0.03 | 25 |
| **Endosulfan** | *3.56* ± *14.4* | ND-93.56 | 0.05 | 20 |
| **Dieldrin** | *0.06* ± *0.22* | ND-1.76 | 0.03 | 3 |
| **Endrin** | *0.31* ± *0.92* | ND-5.36 | 0.09 | 7 |
| **Endosul sulf** | *1.35* ± *0.32* | ND-3.25 | 1.30 | 3 |
| ***4,4'*-DDE** | *5.59* ± *21.6* | ND-121 | 0.05 | 29 |
| ***4,4'*-DDD** | *0.33* ± *1.21* | ND-8.90 | 0.11 | 5 |
| **∑DDTs** | *2.96* ± *15.4* | ND-121 | 0.11 | 17 |
| **β-HCH** | *0.43* ± *1.17* | ND-8.54 | 0.17 | 16 |
| **γ-HCH** | *0.60* ± *1.69* | ND-9.13 | 0.08 | 17 |
| **∑HCHs** | *0.52* ± *1.45* | ND-9.13 | 0.17 | 17 |

Regarding HCHs, the average value obtained was 0.52 ± 1.45 µg/l ranging from not detected (ND) to 9.13 µg/l, and being *β*-HCH and *γ*-HCH (lindane) the only detected. Normally, the exposure to lindane is accompanied by the exposure to other isomers, being *β*-HCH the most persistent (Li, 1998). When comparing among colonies, more than 90 % of the results in A and C were below the detection limit. In colony B, detectable levels of *β*-HCH and lindane (36 % and 41 %, respectively) were found. Levels of lindane were higher than *β*-HCH in this colony (B) (1.48 ± 2.57 and 0.84 ±

1.86 µg/l, respectively). These results are different to those obtained by van Wyk *et al.* (2001), who found higher levels of *β*-HCH than of lindane in whole blood samples from African Whitebacked vultures. *β*-HCH and lindane concentrations found in the present study are lower than those described by Martínez-López *et al.* (2009) (6.19 ± 13.59 µg/l and 10.10 ± 20.16 µg/l, respectively) in Booted eagle nestling blood over five breading seasons (1999-2003) in the NW of Murcia (Spain). Also van Wyk *et al.* (2001) found levels of these pesticides in blood of wild African Whitebacked vulture of 4.32-55.94 µg/l for *β*-HCH and 1.20-29.45 µg/l for lindane. The accumulated *β*-isomer in fatty tissue is 10-30 times higher than lindane, and its metabolization is slower (Martínez-López *et al.*, 2009). This bigger quantity in fatty tissue, together with the restriction imposed on the use of pesticides, such as lindane (Decision 2000/801/EC), is likely the reason explaining the greater levels of *β*-HCH comparing with lindane in our samples.

The effect of the environment where birds live (landfill or intensive agricultural area + landfill) in the levels of OCPs was also evaluated. According to the obtained results in colony C, where no residues of any pesticide were detected, it seems that landfills and agricultural areas might no influence the levels of these pesticides. In contrast, previous studies in blood of Booted eagle nestlings from agricultural areas (associated with farming areas with grains, vineyards, almonds and olives) have reported an increase in the endosulfan levels along the years (1999-2003) and a decrease in the levels of lindane after its ban by the European Union (Martínez-López *et al.*, 2009). Furthermore, the concentrations of pesticides, like DDT, reported in soils from agricultural areas (with strawberry, citrus, rice, cotton, vineyard and olive grove crops) in the SW of Spain are relatively low (0.08-11.1 ng/g d.w.) when compared with those found in Mexico, Nigeria and China (70.5, 70.3 and 186 ng/g d.w., respectively, Muñoz-Arnanz and Jiménez, 2011). This pesticide is a ubiquitous contaminant whose environmental concentrations are reported not to decline in some areas (Muñoz-Arnanz and Jiménez, 2011). The current level of contamination by∑DDTs in colony B is surprisingly high when compared with the other two colonies, because in Spain, as well as in many other developed countries, the usage of OCPs ceased in the late 1970s or the beginning of the 1980s (Luzardo *et al.*, 2014). These results could be explained, at least in part, by two facts: the proximity to the African continent and the extreme environmental persistence of these compounds. The first factor is most likely to be the major cause because of the migration of these birds to the African continent. Several banned OCPs remain under use in developing countries, therefore, high levels of contamination are found in these countries, and also due to the existence of considerable stocks of uncontrolled obsolete pesticides, which makes them available for people and wildlife (Nweke and Sanders, 2009; Nájera *et al.*, 2011; Gioia *et al.*, 2013). It has been reported that migratory birds

acquire most POPs, particularly *4,4'*-DDE, on their wintering grounds (Mora, 1997). Many studies made in North America with birds migrating to Latin America, have found a higher accumulation of POPs while on their wintering grounds in Latin America than on their breeding grounds in North America (Henny *et al.*, 1982; Fyfe *et al.*, 1990; Banasch *et al.*, 1992; Elliott and Shutt, 1993; Elliott and Martin, 1994). However, a study made in birds of prey in Spain did not found any difference in the levels of these pollutants between the species that migrate to South or North of the Sahara (or do not migrate) (van Drooge *et al.*, 2008). The *4,4'*-DDT is metabolized in the liver, mainly to *4,4'*-DDE and *4,4'*-DDD. Therefore, higher hepatic *4,4'*-DDE concentrations could indicate the ability to convert *4,4'*-DDT into *4,4'*-DDE (Tanabe *et al.*, 1998). The high levels of *4,4'*-DDE found in the present study in colony B, and the absence of *4,4'*-DDT residues could mean an exposure to the second one during the migration and its metabolism to *4,4'*-DDE. Alternatively, the widely use of pesticides in the past and their extreme persistence could be another cause for the presence of these pollutants in the stork chicks. OCPs are conveyed from the mother to the eggs via lipids in varying quantities, depending on the species (Aurigi *et al.*, 2000). It has been demonstrated in the field by Bogan and Newton (1977) that 52 % of the body burden of DDE was eliminated in a clutch of six eggs laid by a European sparrowhawk. The exposure of the mother to these pesticides (in wintering or breeding grounds) could produce an increase in the levels of these pesticides in the eggs and in the future chicks.

## 4. CONCLUSIONS

Our data deserve particular attention not only because of their significance but especially because they were recorded in the West of Spain, a region with very low risk of OCP pollution due to the shortage of industries. However, the presence of OCP concentrations found in White stork nestlings indicates the persistence of these compounds against degradation and elimination from the body of these birds. The agricultural activity and landfills might have no influence in the blood levels of OCPs in White storks chicks according to the results obtained in colony C. However, more studies are necessary to evaluate the OCPs content in the White stork food and soils. Therefore, special attention should be paid to the sources and fate of these chemicals in colony B considering also transboundary pollution in adults. In this regard, further investigations should be undertaken to find out whether OCPs contamination exists in other species in the area or not, as well as the source and their potential health effects on individuals and/or populations.

**ACKNOWLEDGMENTS**

Irene de la Casa-Resino was supported by fellowship PRE09001 from Consejería de Empleo, Empresa e Innovación (Gobierno de Extremadura, Spain).

## REFERENCES

Antonious, G.F., Byers, M.E. (1997). Fate and movement of endosulfan under field conditions. Environmental Toxicology and Chemistry 16, 644-649.

Aurigi, S., Focardi, S., Hulea, D., Renzoni, A. (2000). Organochlorine contamination in bird's eggs from the Danube Delta. Environmental Pollution 109, 61-67.

Banasch, U., Goossen, J.P., Einstein Riez, A., Casler, C., Dominguez Barradas, R. (1992). Organochlorine contaminants in migrant and resident prey of Peregrine Falcons, *Falco peregrinus*, in Panama, Venezuela, and Mexico. Canadian Field-Naturalist 106, 493-498.

Blázquez, E., Aguirre, J.I., Martínez-Haro, M., Mateo, R., Jiménez, B. (2006). The use of White stork (*Ciconia ciconia*) nestling in a biomonitoring programme for organochlorines through the region of Madrid (Spain). Organohalogen Compounds 68, 2081-2084.

Bogan, J., Newton, I. (1977). Redistribution of DDE in sparrowhawks during starvation. Bulletin of Environmental Contamination and Toxicology 18, 317-321.

Burger, J., Gochfeld, M. (2004). Marine birds as sentinels of environmental pollution. EcoHealth 1, 263-274.

Bustnes, J.O., Yoccoz, N.G., Bangjord, G., Polder, A., Skaare, J.U. (2007). Temporal trends (1986–2004) of organochlorines and brominated flame retardants in Tawny owl Eggs from Northern Europe. Environmental Science and Technology 41, 8491-8497.

Cooke, A. (1973). Shell thinning in avian eggs by environmental pollutants. Environmental Pollution 4, 85-152.

Cramp, S., Simmons, K.E.L. (1980). The Birds of the Western Palearctic., Oxford, UK, Oxford University Press.

Cunha, L., Torres, J., Muñoz-Arnanz, J., Jimenez, B. (2012). Evaluation of the possible adverse effects of legacy persistent organic pollutants (POPs) on the brown booby (*Sula leucogaster*) along the Brazilian coast. Chemosphere 87, 1039-1044.

Decision 2000/801/EC concerning the non-inclusion of lindane in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant-rotection products containing this active substance. Official Journal of the European Communities 324, 42-43.

Dias, P.S., Cipro, C.V., Taniguchi, S., Montone, R.C. (2013). Persistent organic pollutants in marine biota of São Pedro and São Paulo Archipelago, Brazil. Marine Pollution Bulletin 74, 435-440.

Directive 2009/147/EC of the European Parliament and of the Council of 30 November 2009 on the conservation of wild birds. Official Journal of the European Union 20, 7-25.

Domínguez, J., Moreno, F., Gonzalez, F., Pita, M. (2011). Informe ambiental de Extremadura. Consejería de Agricultura, Desarrollo Rural, Medio Ambiente y Energía de la Junta de Extremadura. http://extremambiente.gobex.es/. Accessed 3 October 2013.

Elliott, J.E., Bishop, C.A. (2011). Cyclodiene and other organochlorine pesticides in birds. Environmental Contaminants in Biota. CRC Press.

Elliott, J.E., Martin, P.A. (1994). Chlorinated hydrocarbons and shell thinning in eggs of (*Accipiter*) hawks in Ontario, 1986–1989. Environmental Pollution 86, 189-200.

Elliott, J.E., Shutt, L. (1993). Monitoring organochlorines in blood of sharp-shinned hawks (*Accipiter striatus*) migrating through the great lakes. Environmental Toxicology and Chemistry 12, 241-250.

Fry, D.M. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. Environmental Health Perspectives 103, 165.

Fyfe, R., Banasch, U., Benavides, V., Hilgert de Benavides, N., Luscombe, A., Sánchez-Pérez, J.E. (1990). Organochlorine residues in potential prey of peregrine falcons, *Falco peregrinus*, in Latin America. Residuos de organoclorados en presas potenciales de halcones peregrinos, *Falco peregrinus*, en Latinoamérica. Canadian Field Naturalist. 104, 285-292.

García-Fernández, A.J., Calvo, J.F., Martínez-López, E., María-Mojica, P., Martínez, J.E. (2008). Raptor Ecotoxicology in Spain: A Review on Persistent Environmental Contaminants. AMBIO: A Journal of the Human Environment 37, 432-439.

Gioia, R., Akindele, A., Adebusoye, S., Asante, K., Tanabe, S., Buekens, A., Sasco, A. (2013). Polychlorinated biphenyls (PCBs) in Africa: a review of environmental levels. Environmental Science and Pollution Research, DOI 10.1007/s11356-013-1739-1.

Gómara, B., Ramos, L., Gangoso, L., Donázar, J., González, M. (2004). Levels of polychlorinated biphenyls and organochlorine pesticides in serum samples of Egyptian Vulture (*Neophron percnopterus*) from Spain. Chemosphere 55, 577-583.

Gómez-Ramírez, P., Martínez-López, E., García-Fernández, A., Zweers, A., van den Brink, N. (2012). Organohalogen exposure in a Eurasian Eagle owl (*Bubo bubo*) population from Southeastern Spain: Temporal–spatial trends and risk assessment. Chemosphere 88, 903-911.

Gómez-Ramírez, P., Shore, R.F., van den Brink, N.W., van Hattum, B., Bustnes, J.O., Duke, G., Fritsch, C., García-Fernández, A.J., Helander, B.O., Jaspers, V., Krone, O., Martínez-López, E., Mateo, R., Movalli, P., Sonne, C. (2014). An overview of existing raptor contaminant monitoring activities in Europe. Environment International 67, 12-21.

Henny, C.J., Ward, F.P., Riddle, K.E., Prouty, R.M. (1982). Migratory Peregrine falcons, *Falco peregrinus*, accumulate pesticides in Latin America during winter 96, 333-338.

Hernández, L., Fernández, M., González, M.J. (1989). Total PCBs and PCB congeners in spanish Imperial eagle eggs. Bulletin of Environmental Contamination and Toxicology 43, 725-732.

Hernández, L., González, M.J., Rico, M.C., Fernández, M., Aranda, A. (1988). Organochlorine and heavy metal residues in Falconiforme and Ciconiforme eggs (Spain). Bulletin of Environmental Contamination and Toxicology 40, 86-93.

Hernandez, L.M., Rico, M.C., Gonzalez, M.J., Hernan, M.A., Fernandez, M.A. (1986). Presence and time trends of organochlorine pollutants and heavy metals in eggs of predatory birds of Spain. Journal of Field Ornithology 57, 270-282.

Jimenez, B., Gomara, B., Baos, R., Hiraldo, F., Eljarrat, E., Rivera, J., Gonzalez, M.J. (2000). An Study of the Toxic Equivalents Derived from PCDDs, PCDFs and Dioxin-Like PCBs in Two Bird Species (*Ciconia Ciconia* and *Milvus Migrans*) Nesting in a Protected Area (Donana National Park, Spain). Organohalogen Compounds 46, 542-545.

Kannan, K., Kumar, K.S., Takasuga, T., Giesy, J.P., Masunaga, S. (2002). Accumulation and elimination of PCDD/DFS, dioxin-like PCBs and organochlorine pesticides in bald eagles from Michigan, USA. Organohalogen Compounds 57, 431-434.

Li, Y., Cai, D., Singh, A. (1998). Technical hexachlorocyclohexane use trends in China and their impact on the environment. Archives of Environmental Contamination and Toxicology 35, 688-697.

Lötter, L., Bouwman, H. (1997). DDT levels in the blood of waders (Aves, Charadriidae) from the North-West Province (South Africa). Journal of African Zoology 111, 85-93.

Luzardo, O.P., Ruiz-Suárez, N., Henríquez-Hernández, L.A., Valerón, P.F., Camacho, M., Zumbado, M., Boada, L.D. (2014). Assessment of the exposure to organochlorine pesticides, PCBs and PAHs in six species of predatory birds of the Canary Islands, Spain. Science of The Total Environment 472, 146-153.

Martinez-Lopez, E., Maria-Mojica, P., Martinez, J.E., Calvo, J.F., Wright, J., Shore, R.F., Romero, D., Garcia-Fernandez, A.J. (2007). Organochlorine residues in booted eagle (*Hieraaetus pennatus*) and goshawk (*Accipiter gentilis*) eggs from southeastern Spain. Environmental Toxicology and Chemistry 26, 2373-2378.

Martínez-López, E., Romero, D., María-Mojica, P., Martínez, J.E., Calvo, J.F., García-Fernández, A.J. (2009). Changes in blood pesticide levels in booted eagle (*Hieraaetus pennatus*) associated with agricultural land practices. Ecotoxicology and Environmental Safety 72, 45-50.

Mateo, R., Millán, J., Rodríguez-Estival, J., Camarero, P.R., Palomares, F., Ortiz-Santaliestra, M.E. (2012). Levels of organochlorine pesticides and polychlorinated biphenyls in the

critically endangered Iberian lynx and other sympatric carnivores in Spain. Chemosphere 86, 691-700.

McArthur, M., Fox, G., Peakall, D., Philogene, B. (1983). Ecological significance of behavioral and hormonal abnormalities in breeding ring doves fed an organochlorine chemical mixture. Archives of Environmental Contamination and Toxicology 12, 343-353.

Mora, M.A. (1997). Transboundary pollution: persistent organochlorine pesticides in migrant birds of the southwestern United States and Mexico. Environmental Toxicology and Chemistry 16, 3-11.

Mundy, P.J., Grant, K.I., Tannock, J., Wessels, C.L. (1982). Pesticide residues and eggshell thickness of griffon vulture eggs in southern Africa. The Journal of Wildlife Management 46, 769-773.

Muñoz-Arnanz, J., Jiménez, B. (2011). New DDT inputs after 30 years of prohibition in Spain. A case study in agricultural soils from south-western Spain. Environmental Pollution 159, 3640-3646.

Nájera, J.A., González-Silva, M., Alonso, P.L. (2011). Some lessons for the future from the Global Malaria Eradication Programme (1955–1969). PLoS Medicine 8, e1000412.

Nweke, O.C., Sanders, W.H. (2009). Modern environmental health hazards: a public health issue of increasing significance in Africa. Environmental Health Perspectives 117, 863.

Peakall, D.B., Lincer, J.L. (1996). Do PCBs cause eggshell thinning? Environmental Pollution 91, 127-129.

Ramesh, A., Tanabe, S., Kannan, K., Subramanian, A., Kumaran, P., Tatsukawa, R. (1992). Characteristic trend of persistent organochlorine contamination in wildlife from a tropical agricultural watershed, South India. Archives of Environmental Contamination and Toxicology 23, 26-36.

Real Decreto 1378/1999 de 27 de agosto, por el que se establecen medidas para la eliminación y gestión de los policlorobifenilos, policloroterfenilos y aparatos que los contengan. Boletin Oficial del Estado 206, 31911-31914.

Sáez, M., Aguirre, J.I., Blázquez, E., Jiménez, B. (2008). Organochlorines in White stork (*Ciconia ciconia*): a comparison of levels in eggs and nestlings. Organohalogen Compounds 70, 1133-1136.

Sáez, M., Aguirre, J.I., Jiménez, B. (2009). Organochlorines in plasma of nestlings and adults White stork (*Ciconia ciconia*) from Madrid, Spain. Organohalogen Compounds 71, 2915-2918.

Safe, S.H. (1994). Polychlorinated Biphenyls (PCBs): Environmental impact, biochemical and toxic responses, and implications for risk assessment. Critical Reviews in Toxicology 24, 87-149.

Santerre, C., Blazer, V., Khanna, N., Reinert, R., Barrows, F. (1997). Absorption of dietary dieldrin by striped bass. Bulletin of Environmental Contamination and Toxicology 58, 334-340.

Sonne, C., Bustnes, J.O., Herzke, D., Jaspers, V.L., Covaci, A., Eulaers, I., Halley, D.J., Moum, T., Ballesteros, M., Eens, M. (2012). Blood plasma clinical–chemical parameters as biomarker endpoints for organohalogen contaminant exposure in Norwegian raptor nestlings. Ecotoxicology and Environmental Safety 80, 76-83.

Tanabe, S., Senthilkumar, K., Kannan, K., Subramanian, A. (1998). Accumulation features of polychlorinated biphenyls and organochlorine pesticides in resident and migratory birds from South India. Archives of Environmental Contamination and Toxicology 34, 387-397.

van Drooge, B., Mateo, R., Vives, Í., Cardiel, I., Guitart, R. (2008). Organochlorine residue levels in livers of birds of prey from Spain: Inter-species comparison in relation with diet and migratory patterns. Environmental Pollution 153, 84-91.

van Wyk, E., Bouwman, H., van der Bank, H., Verdoorn, G.H., Hofmann, D. (2001). Persistent organochlorine pesticides detected in blood and tissue samples of vultures from different localities in South Africa. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 129, 243-264.

Young, M.S. 2009. Certified SEP-PAK florisil for sample cleanup organochlorine pesticides and PCBs, Milford, U.S.A., Waters Corporation.

# DISCUSIÓN GENERAL

El incremento de la población humana, la demanda de recursos y el avance de las tecnologías y el desarrollo industrial, entre otros muchos factores, tienen como consecuencia la liberación de sustancias al medio ambiente que, intencionadamente o no, generan un serio impacto en el ecosistema y en las especies que en él habitan. Como consecuencia de esta problemática, la necesidad de monitorizar el medio ambiente se ha incrementado en los últimos años.

Extremadura, por sus circunstancias ambientales y su extraordinaria riqueza en aves de alta importancia ecológica, constituye uno de los destinos ornitológicos más importantes de Europa. Más de un millón de hectáreas en la comunidad han merecido la declaración de Zona de Especial Protección para Aves (ZEPA), superando el 25 % de la superficie regional (Palacios *et al*., 2008). Dada su riqueza en fauna aviar, entre ellas diversas parejas de águila imperial y cigüeña negra, se hace necesaria su protección frente a posibles contaminantes ambientales que disminuyan o pongan en peligro estas poblaciones, y es aquí, a su vez, donde las aves juegan un papel muy importante como bioindicadores.

Las aves son uno de los grupos de seres vivos más ampliamente estudiados, y cualquier cambio es su biología normal se detecta rápidamente. Además aquellas especies que se encuentran en lo alto de la cadena trófica tienden a acumular contaminantes químicos a altas concentraciones, los cuales pueden causar alteraciones fisiológicas, reproductivas o incluso la muerte y el declive de la población, provocando que muchas de estas especies se hayan visto en serio peligro de extinción. Los factores que más han contribuido a la reducción de las poblaciones de aves son variados, englobando por ejemplo a la agricultura y la pesca, la caza y persecución directa, el cambio climático y el caso que en esta Tesis Doctoral nos ocupa, los contaminantes ambientales (Becker, 2003).

Dos de los grupos de contaminantes más preocupantes ambientalmente lo constituyen los metales pesados y los compuestos orgánicos persistentes (COPs), como los PCBs y los plaguicidas clorados, debido, fundamentalmente a su alta persistencia en el medio ambiente lo que hace que tengan una alta capacidad de bioacumulación. No obstante, ¿cómo influyen estos contaminantes químicos sobre las aves? Numerosas referencias bibliográficas han estudiado los niveles de estos compuestos químicos en la aves (Bortolotti *et al.*, 2003; Baos *et al.*, 2006a,b, 2012; Sáez *et al.*, 2008, 2009), sin embargo, son más escasos los estudios que evalúan el efecto de los mismos en estos animales (Elliott *et al.*, 2001; Kamiński *et al.*, 2009a,b; Koivula and Eeva, 2010). Dichos efectos pueden ser letales o subletales, y su estudio se puede abordar en función de las consecuencias sobre la fisiología del individuo, sobre la reproducción o en función de las repercusiones sobre las poblaciones (Becker, 2003). Tradicionalmente se han utilizado las alteraciones bioquímicas en los distintos tejidos, como los cambios en actividades enzimáticas o los niveles de radicales libres y estrés oxidativo como biomarcadores para detectar la exposición o el efecto de estos compuestos en las aves (Kamiński *et al.*, 2007; Koivula and Eeva, 2010). No obstante, en los últimos años han cobrado importancia los biomarcadores que nos indican el efecto que algunos contaminantes tienen sobre el sistema endocrino de las aves. Y es que existen multitud de compuestos químicos que ejercen su efecto provocando alteraciones en la función endocrina. Estas sustancias se han dado en llamar alteradores o perturbadores endocrinos (AE[1], en inglés “endocrine disrupters”). Entre estos

[1] La traducción literal de la denominación inglesa, *Endocrine Disruptor* (ED), al español no está oficialmente aceptada, por lo cual los autores han optado por la forma más correcta *Alterador Endocrino* (AE).

alteradores endocrinos se encuentran los PCBs, los plaguicidas clorados como el DDT, o los herbicidas triazínicos, como la ATZ.

Los trabajos que componen la presente Tesis Doctoral se desarrollaron en este contexto, y constituyen una aproximación para determinar la validez de distintos biomarcadores así como los niveles y efectos de los contaminantes persistentes (orgánicos e inorgánicos) en aves en la provincia de Cáceres (Extremadura). Se han utilizado como bioindicadoras dos de las especies más representativas del ecosistema extremeño, la codorniz (*Coturnix coturnix*) y la cigüeña blanca (*Ciconia ciconia*). Se han determinado los efectos de la ATZ a nivel de distintos receptores celulares y en tejidos biológicos tan importantes como el hígado, la sangre o las heces (estos últimos de vital importancia al no ser muestras invasivas). Además, se ha evaluado el efecto de este herbicida como AE en aves, hecho no totalmente elucidado en estudios anteriores centrados en esta especie (Wilhelms et al., 2005, 2006a,b) (**Capítulos 1-3**). Por otra parte, también se ha llevado a cabo un estudio de biomonitorización de metales pesados y COPs en cigüeña blanca (**Capítulos 4 y 6**). Además se ha podido observar que los niveles de ciertos metales tóxicos pueden estar íntimamente relacionados con algunos biomarcadores de estrés oxidativo (**Capítulo 5**). Con todo ello se pretende hacer ver la necesidad de establecer una serie de biomarcadores útiles para la monitorización del medio ambiente mediante el empleo de muestras no invasivas, así como evaluar el estado actual del medio ambiente extremeño.

## Parte 1. Efecto de los herbicidas triazínicos en ensayos *in vitro* en cultivos celulares e *in vivo* en codorniz (*Coturnix coturnix*)

Dentro del grupo de los herbicidas triazínicos la ATZ, la SIM, la PRZ y la PRT son o han sido las más ampliamente comercializadas. Aunque actualmente se encuentra prohibido su uso en la Unión Europea (Directiva 91/414/CEE), son todavía ampliamente utilizadas en EE.UU., con una tasa de producción de ATZ de 33-36 millones de kg/año (Kiely *et al*., 2004).

Las triazinas se han considerado tradicionalmente como AE debido a los efectos producidos sobre distintos organismos. Aunque estos herbicidas no tienen una estructura química similar a las hormonas esteroideas algunos estudios sugieren que producen la alteración de la expresión del ER actuando como agonistas o antagonistas del mismo (Eldridge *et al.*, 1994; Tennant *et al.*, 1994; Connor *et al.*, 1996; Tran *et al.*, 1996; Graumann *et al.*, 1999). Así mismo, dichas alteraciones estrogénicas o antiestrogénicas también pueden estar mediadas por el AhR, ya que la ATZ es metabolizada a través del CYP1A, cuya expresión depende directamente de la activación del AhR. Por otro lado, puesto que las hormonas tiroideas poseen un papel fundamental en la regulación y mantenimiento de la homeostasis de numerosas funciones biológicas en los organismos, particularmente de los procesos de desarrollo y metamorfosis, también se ha sugerido que la ATZ podría causar daños generales al interferir con la función de estas hormonas. Sin embargo, diversos estudios llevados a cabo en ratas expuestas a ATZ no han mostrado alteraciones en los niveles plasmáticos de hormonas tiroideas, triyodotironina (T3), tiroxina (T4) y hormona estimulante del tiroides (TSH) (Laws *et al*., 2000; Stoker *et al.*, 2002). No obstante, no existen estudios de referencia sobre el efecto de los restantes herbicidas triazínicos en las hormonas tiroideas o el TR.

Los resultados de la presente tesis mostraron que las triazinas no ejercen su acción *in vitro* a través de los ER, AhR y TR (**Capítulo 1**). No obstante, los estudios realizados posteriormente *in vivo* exponiendo a codornices a dosis de ATZ de 25 y 100 mg/kg mostraron resultados ligeramente diferentes (**Capítulo 2**). En relación a los biomarcadores de alteración endocrina, la exposición a ATZ a las dosis indicadas supuso un incremento en la expresión de ER hepático y de los niveles de E2 plasmáticos en el día 30 de experimento. Además, en el mismo día se observó un incremento de los niveles plasmáticos de VTG (**Capítulo 2**). Dicho incremento de los niveles de E2 puede estar relacionado con la estimulación de la actividad aromatasa que transforma el andrógeno T a E2. Este mecanismo parece ser la explicación de los procesos de feminización observados en peces y anfibios (Sanderson *et al.*, 2000; Hayes *et al.*, 2002, 2003). La expresión de la enzima aromatasa está controlada por la inducción del ER (Sanderson *et al.*, 2000; Hayes *et al.*, 2002), y aunque la ATZ parece no ser un agonista directo del mismo (**Capítulo 1**), otros mecanismos pueden explicar la inducción de dicha enzima. Así, en los estudios realizados en la línea celular de carcinoma adrenocortical humano H295R, la exposición a ATZ produjo un incremento de los niveles de aromatasa paralelos a un incremento en la concentración de adenosín monofosfato cíclico (AMPc) (Sanderson *et al.*, 2000, 2001). Roberge *et al*. (2004) evidenciaron que la ATZ era capaz de interaccionar con la familia de enzimas fosfodiesterasas (PDEs) que hidrolizan el AMPc a 5-AMP. Dicha interacción inhibiría a las enzimas PDEs produciéndose un incremento en los niveles de AMPc, que puede resultar en un incremento de los niveles de expresión de la enzima aromatasa (Sanderson *et al*., 2000; Mehats *et al*., 2002). Serían necesarios más estudios para evaluar si esta cascada de mecanismos tiene lugar en las aves. No obstante, en la presente Tesis Doctoral (**Capítulo 2**) se ha observado un incremento de los niveles de E2 en el día 30 de experimento (30 días después del inicio de la administración de ATZ y 20 días después de la última administración). Esto parecería indicar que es necesaria una cascada de reacciones que llevarían algún tiempo hasta hacerse efectivas, lo que podría estar relacionado con reacciones metabólicas que implicaran inhibición de las PDEs y el aumento de la actividad enzimática aromatasa en vez de una acción directa sobre el ER.

Con el objetivo de evaluar el efecto de la ATZ sobre el CYP450, se midió la posible inducción del CYP1A4 y su actividad enzimática asociada EROD en las muestras de hígado de codorniz, así como también los niveles de porfirinas fecales (**Capítulos 2 y 3**). Las porfirinas conforman un grupo de derivados tetrapirrólicos cíclicos necesarios para la síntesis del grupo hemo, que a su vez conforma la estructura central del CYP450. Cualquier alteración producida por la ATZ en la síntesis de porfirinas podría tener consecuencias en la síntesis del grupo hemo, y en última instancia en la producción del CYP450. No se observaron cambios en la expresión del AhR, ni en la actividad EROD en ninguno de los días de ensayo (15, 30 y 45) a ninguna de las dosis estudiadas (25 y 100 mg/kg). Estos resultados estarían relacionados con la falta de interacción de las triazinas con el AhR observada en el **Capítulo 1** de la presente Tesis Doctoral. Sin embargo, la exposición a ATZ produjo alteraciones en los niveles de porfirinas fecales en los días 5 y 10 de experimento a las dosis más bajas, intuyéndose por tanto una posible efecto de la ATZ sobre estas moléculas. Más estudios son necesarios para establecer las causas de estos resultados y la relación entre la ATR y las alteraciones en los niveles de porfirinas.

Por otra parte, los resultados de la presente Tesis Doctoral ponen de manifiesto que el estrés oxidativo no es uno de los mecanismos por los que la ATZ ejerce su toxicidad en aves. La exposición a ATZ no produjo alteraciones en los niveles de GSH y GR a ninguna de las dosis estudiadas en ninguno de los días de ensayo. Sin embargo, se observó una inhibición significativa de los niveles de MDA 30 días después del inicio del experimento a la dosis más alta, hecho que podría correlacionarse con un incremento de los niveles de GSH durante ese mismo día de ensayo (aunque en este último caso la diferencia no fue significativa). No ha sido posible localizar estudios en la bibliografía que evalúen el impacto de la ATZ en los niveles de estrés oxidativo en aves. Sin embargo, en peces se ha observado una disminución en los niveles de GSH y un incremento de los niveles de peroxidación lipídica en áreas contaminadas por agroquímicos como la SIM, la ATZ y su metabolito desetil-atrazina (Dorval *et al*., 2005). Los estudios realizados en mamíferos han mostrado también la capacidad de estos herbicidas para producir estrés oxidativo. En ratas, se ha observado un incremento de los niveles de GSH y GST tras la exposición a ATZ a dosis de 200 mg/kg durante 7 y 16 días. A su vez, también se observó un incremento de los niveles de MDA tras 16 días de exposición a la dosis antes indicada (Abarikwu *et al*., 2010). Los resultados obtenidos en el **Capítulo 3** del presente trabajo nos permiten concluir que estas moléculas no son biomarcadores eficaces de la presencia de ATZ a las dosis y tiempos ensayados.

**Parte 2. Estudio de biomarcadores de estrés oxidativo, metales y contaminantes orgánicos persistentes en sangre de pollos de cigüeña blanca (*Ciconia ciconia*)**

En esta segunda parte de la Tesis Doctoral, se ha llevado a cabo un estudio de biomonitorización en pollos de cigüeña blanca. Se han evaluado los niveles de metales pesados y contaminantes orgánicos persistentes en 3 colonias distintas, con distinta influencia ambiental (dehesa, planta de tratamiento de residuos sólidos urbanos (PTRSU) y zona de agricultura intensiva con una PTRSU) distribuidas en el ecosistema extremeño (**Capítulos 4, 5 y 6**).

Al trabajar con animales salvajes una de las mayores dificultades es encontrar una población en una zona control no contaminada, y por ello seguramente la mejor opción es establecer una población de referencia para comparar, ya que la amplia distribución de contaminantes en el medio ambiente hace muy difícil, si no imposible, encontrar una población control (Norris, 2000). En la presente Tesis Doctoral, se ha establecido como población de referencia una colonia (A) situada en la ZEPA de Llanos de Cáceres y Sierra de Fuentes, lejos de aparentes fuentes de contaminación.

En el caso de los metales, numerosos estudios han determinado la validez de los pollos de cigüeña como bioindicadores (Baos *et al.*, 2006a,b, 2012; Goutner *et al.*, 2011; Tkachenko and Kurhaluk, 2012). Los niveles de metales en los tejidos de estos animales pueden provenir del medio ambiente que les rodea o del huevo, aunque en el caso de los metales, a diferencia de lo que ocurre con los contaminantes orgánicos como los PCBs y los OCPs, la cantidad que proviene de la madre a través del huevo es mínima. Además, numerosos estudios han permitido llegar a la conclusión de que los niveles de metales en la sangre de los pollos reflejan una exposición reciente que proviene del medio ambiente local (García-Fernández *et al.*, 1996; Burger and Gochfeld, 1997; Blanco *et al.*, 2003; Blázquez *et al.*, 2006).

De todos los elementos metálicos analizados, los niveles más altos se cuantificaron para el Zn y el Fe (dato esperado, dado que ambos poseen una gran relevancia fisiológica como elementos esenciales), seguidos por Pb>Se>Hg>As>Cd. Los niveles de metales detectados en sangre en las tres zonas siempre estuvieron dentro del rango de concentraciones establecido como basal en aves, a excepción de 3 animales de la colonia B (cercana a la PTRSU) y 2 animales de la colonia C (situada en una zona de agricultura intensiva y a una PTRSU) en el caso de Pb. Estos animales presentaron valores superiores a 200 µg/l, nivel a partir del cual se considera que puede haber una exposición a Pb que potencialmente pueda causar efectos tóxicos evidentes.

En todos los casos los niveles más bajos se encontraron en la colonia A (de referencia), seguidos por la colonia B y la C (a excepción del Fe donde la colonia C presentó niveles más altos que la B). Estas diferencias fueron estadísticamente significativas con respecto a la colonia A en el caso de los elementos inorgánicos tóxicos (Pb, Hg y As) y el Se (**Capítulo 4**).

Está comprobado científicamente que las poblaciones de cigüeña blanca se han incrementado en los últimos años, y este hecho se ha atribuido al crecimiento del aporte de alimentos que ofrecen los basureros (Peris, 2003; Blázquez *et al.*, 2006). Sin embargo, el origen de los metales en el medio ambiente puede ser muy variado y deben considerarse otras posibles fuentes. Dado que las zonas donde se situaban las colonias no eran industriales, esta posible fuente de contaminación por metales quedó descartada. Sin embargo, es necesario evaluar los niveles de metales que pueden tener su origen en el suelo cercano a las colonias. Desde el suelo, los metales se incorporan a la cadena alimentaria a través de plantas o pequeñas presas y finalmente llegan a las aves. La agricultura también puede ser una posible fuente de contaminación por metales, que podrían provenir del uso como enmienda agrícola de residuos sólidos urbanos, así como también de la aplicación de abonos, fertilizantes y pesticidas (Hargreaves *et al.*, 2008; Khan *et al.*, 2008, 2013). Con el objetivo de comparar los resultados obtenidos en la sangre de las cigüeñas (**Capítulo 4**) con los niveles obtenidos en suelos cercanos a las colonias, se utilizaron los valores de referencia en el suelo publicados por López-Arias y Grau-Corbí (2005). Las concentración media de Cd (<LD), Pb (6 mg/kg) y Zn (18 mg/kg) en el suelo cercano (1,2 km) a la colonia C fueron inferiores que las obtenidas en el suelo cercano (7,5 km) a la colonia A (0,10, 20 y 91 mg/kg para el Cd, Pb y Zn respectivamente) y en el suelo cercano (3,5 km) a la colonia B (<LOD, 19 y 98 mg/kg para el Cd, Pb y Zn respectivamente). El Cd fue detectado sólo en el suelo de la colonia A, pero no en la sangre de sus pollos. En el caso del Pb, los niveles más altos en el suelo se encontraron en el de la colonia A, mientras que los animales de dicha colonia presentaron los niveles más bajos. Lo mismo ocurre en el caso del Zn. Teniendo en cuenta esta composición metálica del suelo, la actividad agrícola no parece tener influencia en los niveles de estos metales (Cd, Pb y Zn), y la presencia de basureros cercanos a las colonias B y C parecen ser las principales fuentes de contaminación por estos metales en los pollos. En cambio, en el caso del Hg los niveles detectados en el suelo de las colonias B (0,02 mg/kg) y C (0,02 mg/kg) fueron superiores a los detectados en la colonia A (<LD), lo que parece indicar que para este metal el suelo debe tenerse en cuenta como posible fuente de contaminación. Sin embargo, son necesarios más estudios para conocer los niveles en el suelo de los restantes elementos estudiados en la presente tesis (Fe, As y Se), y evaluar su impacto en los niveles sanguíneos de estas aves.

Algunos estudios han puesto de manifiesto el efecto de los metales sobre el sistema antioxidante de las aves (Kamiński *et al.*, 2007; 2009a,b). Con el objetivo de evaluar el impacto de los mismos sobre el sistema antioxidante de las cigüeñas, se analizaron en sangre los niveles de GSH, GST y MDA (**Capítulo 5**). En la colonia B se cuantificaron los valores más altos de GSH y GST ($P$<0,001 y $P$<0,005, respectivamente) en comparación con los detectados en la colonia de referencia (A). Sin embargo, en relación al MDA no se encontraron diferencias entre ambas colonias. Además, se encontró una correlación positiva y altamente significativa entre los niveles de GSH-Hg y GSH-As. En este sentido, la bibliografía consultada permitió confirmar que las aves pueden experimentar estrés oxidativo inducido por metales pesados, que pueden dañar distintos sistemas orgánicos y causar alteraciones orgánicas (Kamiński *et al.*, 2009a,b; Tkachenko and Kurhaluk, 2012). El GSH es uno de los mayores antioxidantes en los organismos aeróbicos, con un papel muy importante en la protección celular, ya que se une a los radicales libres y muchos metales facilitando su eliminación (Klaassen *et al*., 1985). Un incremento en los niveles de GSH puede ser interpretado como una respuesta protectora contra la presencia de EROs o metales (Espín *et al*., 2014). Por otra parte, como se ha indicado anteriormente las enzimas GST catalizan la conjugación del GSH con los contaminantes. Por lo tanto, el incremento observado en la colonia B tanto para el GSH como para las enzimas GST podría ser indicativo de la existencia de un proceso de detoxificación (Jemec *et al*., 2007; Espín *et al*., 2014). Otros estudios realizados en aves mostraron que estos animales reaccionan al estrés oxidativo de manera diferente en función de la especie y del grado de contaminación (Beyer *et al*., 1988; Mateo and Hoffman, 2001; Berglund *et al*., 2007; Rainio *et al*., 2013). Teniendo esto en cuenta, parece inadecuado elegir un único biomarcador "estándar" de estrés oxidativo en aves. Debido a que son necesarios numerosos mecanismos de defensa antioxidante para proteger correctamente un organismo vivo frente al efecto de las EROs y tal como han concluido otros autores, no existe seguramente un único biomarcador para evaluar el estrés oxidativo (Halliwell and Gutteridge, 1999; Berglund *et al.*, 2007) y probablemente lo más acertado sea elegir una batería de ellos.

En el caso de los compuestos clorados son más escasos los estudios realizados en cigüeña blanca (Sáez *et al*., 2008, 2009). En el **Capítulo 6** del presente trabajo se estudiaron los niveles de PCBs y OCPs en el plasma disponible de los pollos de cigüeña blanca.

No se detectaron residuos de PCBs en ninguna de las muestras estudiadas. Los resultados del presente trabajo parecen indicar que ni la presencia de la PTRSU ni la actividad agrícola poseen ninguna influencia en los niveles de este grupo de contaminantes. Otros estudios, también han observado una reducción en los niveles de estos pesticidas en aves a lo largo del siglo XX (Hernandez *et al.*, 1986, 1988, 1989; Jimenez *et al.*, 2000) lo cual está probablemente relacionado con la reducción efectiva de su utilización a partir de la prohibición de su uso.

En relación a la presencia de OCPs, los niveles de residuos detectados en el plasma de estos animales siguió el siguiente orden: DDTs > dieldrín > endosulfan > endrín > heptaclor > endosulfan sulfato > HCHs > aldrín. El heptacloro, el *4,4'*-DDE, el endosulfan y el aldrín fueron los pesticidas más frecuentemente detectados, mientras que el *4,4'*-DDE y el dieldrín fueron los detectados en mayor concentración, al igual que ocurre en otros estudios realizados en aves (Van Drooge *et al.*, 2008; Gómez-Ramírez *et al.*, 2012).

Los niveles detectados de DDTs y dieldrín fueron sorprendentemente altos, más si cabe porque en España, al igual que en otros países, su utilización se encuentra prohibida desde hace ya varios años (Luzardo *et al.*, 2014). Estos resultados pueden ser explicados en parte por dos hechos: la proximidad al continente africano y la elevada persistencia ambiental de estos compuestos. El primero de ellos parece ser el más acertado, ya que estos animales realizan su migración invernal a este continente. Muchos de estos pesticidas se mantienen aún en uso en estos países, detectándose altos niveles de contaminación por estos componentes (Nweke and Sanders, 2009; Nájera *et al.*, 2011; Gioia *et al.*, 2013). Algunos estudios han demostrado que las aves migrantes adquieren los mayores niveles de COPs durante sus migraciones invernales (Mora, 1997). Los estudios realizados en aves de Norteamérica que migran durante el invierno hacia Latinoamérica han encontrado altos niveles de COPs después de dicha migración (Henny *et al.*, 1982; Fyfe *et al.*, 1990; Banasch *et al.*, 1992; Elliott and Shutt, 1993, Elliott and Martin, 1994). Sin embargo, estudios más recientes en aves en España no han mostrado ninguna diferencia en los niveles de estos contaminantes entre las especies que migran al sur del Sahara y las que migran al norte del Sahara (o no migran) (Van Drooge *et al*., 2008). Por otra parte, la extrema persistencia de estos pesticidas y su amplio uso en el pasado pueden ser otra causa de su presencia en la sangre de los pollos de cigüeña blanca. Los COPs se transportan desde la madre hacia los lípidos del huevo, en distintas cantidades dependiendo de la especie (Aurigi *et al*., 2000). La exposición de la madre a estos pesticidas (en sus migraciones o en sus zonas de cría) puede producir un incremento de los mismos en los huevos y en el futuro pollo.

**Una mirada hacia el futuro**

Esta Tesis constituye una aproximación a los efectos que los herbicidas triazínicos pueden tener en las aves (especialmente la ATZ). Sin embargo, tan sólo es una pequeña aproximación. Se deberían estudiar con más profundidad los efectos tanto celulares, como *in vivo*, que tienen estos herbicidas en las aves, así como, los mecanismos moleculares subyacentes a dichos efectos.

Por otra parte, se ha intentado dar un punto de vista general sobre los efectos que las PTRSU y las zonas agrícolas pueden tener en los niveles de metales pesados y pesticidas en las aves que desarrollan su actividad y se alimentan cerca de estas zonas. Los análisis realizados muestran una clara influencia de las PTRSU sobre los niveles de metales, pero son necesarios más estudios sobre las otras posibles fuentes de procedencia de estos metales (suelo), así como un examen de la frecuencia con la que visitan estos animales dichas plantas de tratamiento. Con todos estos datos podremos llegar a un conocimiento más real sobre las fuentes de estos metales y establecer soluciones que eviten daños a largo plazo en estas aves.

Además, no se ha podido establecer exactamente la procedencia de los niveles tan altos de algunos pesticidas (como el DDE) en la colonia B (cercana a la PTRSU). Es muy posible que dichos niveles altos en los pollos procedan de la migración de los adultos al continente africano (y posterior transferencia de los pesticidas a los pollos a través de los lípidos del huevo). No obstante, serían necesarios más estudios en la zona (evaluando los niveles de estos pesticidas en el alimento de estos animales, suelo…) para clarificar estos resultados.

Para terminar, y teniendo en cuenta el efecto como AE de muchos de los pesticidas aquí estudiados, está pendiente la publicación de un estudio sobre los niveles de hormonas

esteroideas y tiroideas en el plasma de cigüeña blanca, así como un estudio y puesta a punto de la técnica de detección de VTG en estas aves (hasta ahora sólo realizada en codorniz). Con esta última parte se pretenden detectar alteraciones endocrinas que puedan ser asociadas a la presencia de estos contaminantes y que puedan llegar a comprometer a largo plazo la viabilidad de la población.

## REFERENCIAS

Abarikwu, S., Adesiyan, A., Oyeloja, T., Oyeyemi, M., Farombi, E. (2010). Changes in sperm characteristics and induction of oxidative stress in the testis and epididymis of experimental rats by a herbicide, atrazine. Archives of Environmental Contamination and Toxicology 58, 874-882.

Aurigi, S., Focardi, S., Hulea, D., Renzoni, A. (2000). Organochlorine contamination in bird's eggs from the Danube Delta. Environmental Pollution 109, 61-67.

Banasch, U., Goossen, J.P., Einstein Riez, A., Casler, C., Dominguez Barradas, R. (1992). Organochlorine contaminants in migrant and resident prey of Peregrine Falcons, *Falco peregrinus*, in Panama, Venezuela, and Mexico. Canadian field-naturalist 106, 493-498.

Baos, R., Blas, J., Bortolotti, G.R., Marchant, T.A., Hiraldo, F. (2006a). Adrenocortical response to stress and thyroid hormone status in free-living nestling White storks (*Ciconia ciconia*) exposed to heavy metal and arsenic contamination. Environmental Health Perspectives 114, 1497-1501.

Baos, R., Forero, M.G., Gómez, G., Gónzalez, M.J., Hiraldo, F., Jiménez, B., Jovani, R., Tella, J.L. (2006b). Relationships between T-cell mediated immnune response and Pb, Zn. Cu, Cd, and as concetration in blood of nestling White storks (*Ciconia ciconia*) and Black kites (*Milvus migrans*) from Doñana (Southwestern Spain) after the Aznalcollar toxic spill. Environmental Toxicology and Chemistry 25, 1153-1159.

Baos, R., Jovani, R., Serrano, D., Tella, J.L., Hiraldo, F. (2012). Developmental exposure to a toxic spill compromises long-term reproductive performance in a wild, long-lived bird: the White stork (*Ciconia ciconia*). PloS One 7, e34716.

Becker, P.H. (2003). Biomonitoring with birds. Trace Metals and other Contaminants in the Environment 6, 677-736.

Berglund, Å.M.M., Sturve, J., Förlin, L., Nyholm, N.E.I. (2007). Oxidative stress in Pied flycatcher (*Ficedula hypoleuca*) nestlings from metal contaminated environments in northern Sweden. Environmental Research 105, 330-339.

Beyer, W.N., Spann, J.W., Sileo, L., Franson, J.C. (1988). Lead poisoning in six captive avian species. Archives of Environmental Contamination and Toxicology 17, 121-130.

Blanco, G., Frías, O., Jiménez, B., Gómez, G. (2003). Factors influencing variability and potential uptake routes of heavy metals in Black kites exposed to emissions from a solid-waste incinerator. Environmental Toxicology and Chemistry 22, 2711-2718.

Blázquez, E., Aguirre, J., Martínez-Haro, M., Mateo, R., Jiménez, B. (2006). The use of White stork (*Ciconia ciconia*) nestlings in a biomonitoring programme for organochlorines through the region of Madrid (Spain). Organohalogen Compounds 68, 2081-2085.

Bortolotti, G.R., Fernie, K.J., Smits, J.E. (2003). Carotenoid concentration and coloration of American Kestrels (*Falco sparverius*) disrupted by experimental exposure to PCBs. Functional Ecology 17, 651-657.

Burger, J., Gochfeld, M. (1997). Age differences in metals in the blood of herring (*Larus argentatus*) and Franklin's (*Larus pipixcan*) gulls. Archives of Environmental Contamination and Toxicology 33, 436-440.

Connor, K., Howell, J., Chen, I., Liu, H., Berhane, K., Sciarretta, C., Safe, S., Zacharewski, T. (1996). Failure of chloro-s-triazine-derived compounds to induce estrogen receptor-mediated responses *in vivo* and *in vitro*. Toxicological Sciences 30, 93-101.

Directiva 91/414/CEE. Decisión de la comisión de 10 de marzo de 2004 relativa a la no inclusión de la atrazina en el anexo I de la Directiva 91/414/CEE del Consejo y a la retirada de las autorizaciones de los productos fitosanitarios que contengan esta sustancia activa. Diario Oficial de la Unión Europea 78, 53-55.

Dorval, J., Leblond, V., Deblois, C., Hontela, A. (2005). Oxidative stress and endocrine endpoints in White sucker (*Catostomus commersoni*) from a river impacted by agricultural chemicals. Environmental Toxicology and Chemistry 24, 1273-1280.

Eldridge, J.C., Fleenor-Heyser, D.G., Extrom, P.C., Wetzel, L.T., Breckenridge, C.B., Gillis, J.H., Luempert III, L.G., Stevens, J.T. (1994). Short-term effects of chlorotriazines on estrus in female Sprague-Dawley and Fischer 344 rats. Journal of Toxicology and Environmental Health, Part A 43, 155-167.

Elliott, J.E., Martin, P.A. (1994). Chlorinated hydrocarbons and shell thinning in eggs of (*Accipiter*) hawks in Ontario, 1986–1989. Environmental Pollution 86, 189-200.

Elliott, J.E., Shutt, L. (1993). Monitoring organochlorines in blood of sharp-shinned hawks (*Accipiter striatus*) migrating through the great lakes. Environmental Toxicology and Chemistry 12, 241-250.

Elliott, J.E., Wilson, L.K., Henny, C.J., Trudeau, S.F., Leighton, F.A., Kennedy, S.W., Cheng, K.M. (2001). Assessment of biological effects of chlorinated hydrocarbons in osprey chicks. Environmental Toxicology and Chemistry 20, 866-879.

Espín, S., Martínez-López, E., Jiménez, P., María-Mojica, P., García-Fernández, A.J. (2014). Effects of heavy metals on biomarkers for oxidative stress in Griffon vulture (*Gyps fulvus*). Environmental Research 129, 59-68.

Fyfe, R., Banasch, U., Benavides, V., Hilgert de Benavides, N., Luscombe, A., Sánchez-Pérez, J.E. (1990). Organochlorine residues in potential prey of peregrine falcons, *Falco peregrinu*s, in Latin America. Residuos de organoclorados en presas potenciales de halcones peregrinos, *Falco peregrinus*, en Latinoamérica. Canadian Field Naturalist 104, 285-292.

García-Fernández, A.J., Sanchez-Garcia, J.A., Gomez-Zapata, M., Luna, A. (1996). Distribution of cadmium in blood and tissues of wild birds. Archives of Environmental Contamination and Toxicology 30, 252-258.

Gioia, R., Akindele, A., Adebusoye, S., Asante, K., Tanabe, S., Buekens, A., Sasco, A. (2013). Polychlorinated biphenyls (PCBs) in Africa: a review of environmental levels. Environmental Science and Pollution Research, DOI 10.1007/s11356-013-1739-1.

Gómez-Ramírez, P., Martínez-López, E., García-Fernández, A., Zweers, A., Van Den Brink, N. (2012). Organohalogen exposure in a Eurasian Eagle owl (*Bubo bubo*) population from Southeastern Spain: Temporal-spatial trends and risk assessment. Chemosphere 88, 903-911.

Goutner, V., Becker, P., Liordos, V., Tsachalidis, E. (2011). Mercury in White Stork (*Ciconia ciconia*) chick feathers from Northeastern mediterranean areas in relation to age, brood size, and hatching order. Archives of Environmental Contamination and Toxicology 61, 327-336.

Graumann, K., Breithofer, A., Jungbauer, A. (1999). Monitoring of estrogen mimics by a recombinant yeast assay: synergy between natural and synthetic compounds? Science of the Total Environment 225, 69-79.

Halliwell, B., Gutteridge, J.M.C. (1999). Free radicals in Biology and Medicine, New York, Oxford University Press.

Hargreaves, J., Adl, M., Warman, P. (2008). A review of the use of composted municipal solid waste in agriculture. Agriculture, Ecosystems and Environment 123, 1-14.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A. (2002). Herbicides: feminization of male frogs in the wild. Nature 419, 895-896.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A. (2003). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environmental Health Perspectives 111, 568-575.

Henny, C.J., Ward, F.P., Riddle, K.E., Prouty, R.M. (1982). Migratory Peregrine falcons, *Falco peregrinus*, accumulate pesticides in Latin America during winter. Canadian Field-Naturalist 96, 333-338.

Hernández, L., Fernández, M., González, M.J. (1989). Total PCBs and PCB congeners in Spanish Imperial eagle eggs. Bulletin of Environmental Contamination and Toxicology 43, 725-732.

Hernández, L., González, M.J., Rico, M.C., Fernández, M., Aranda, A. (1988). Organochlorine and heavy metal residues in Falconiforme and Ciconiforme eggs (Spain). Bulletin of Environmental Contamination and Toxicology 40, 86-93.

Hernández, L.M., Rico, M.C., González, M.J., Hernán, M.A., Fernández, M.A. (1986). Presence and time trends of organochlorine pollutants and heavy metals in eggs of predatory birds of Spain. Journal of Field Ornithology, 270-282.

Jemec, A., Drobne, D., Tišler, T., Trebše, P., Roš, M., Sepčić, K. (2007). The applicability of acetylcholinesterase and glutathione S-transferase in Daphnia magna toxicity test. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 144, 303-309.

Jiménez, B., Gómara, B., Baos, R., Hiraldo, F., Eljarrat, E., Rivera, J., Gonzalez, M.J. (2000). A study of the toxic equivalents derived from PCDDs, PCDFs and dioxin-like PCBs in two bird species (*Ciconia Ciconia* and *Milvus Migrans*) nesting in a protected area (Donana National Park, Spain). Organohalogen Compounds 46, 542-545.

Kamiński, P., Kurhalyuk, N., Jerzak, L., Kasprzak, M., Tkachenko, H., Klawe, J.J., Szady-Grad, M., Koim, B., Wiśniewska, E. (2009a). Ecophysiological determinations of antioxidant enzymes and lipoperoxidation in the blood of White Stork *Ciconia ciconia* from Poland. Environmental Research 109, 29-39.

Kamiński, P., Kurhalyuk, N., Kasprzak, M., Jerzak, L., Tkachenko, H., Szady-Grad, M., Klawe, J.J., Koim, B. (2009b). The impact of element–element interactions on antioxidant enzymatic activity in the blood of White stork (*Ciconia ciconia*) chicks. Archives of Environmental Contamination and Toxicology 56, 325-337.

Kamiński, P., Kurhalyuk, N., Szady-Grad, M. (2007). Heavy Metal-Induced Oxidative Stress and Changes in physiological process of free radicals in the blood of White stork (*Ciconia ciconia*) chicks in polluted areas. Polish Journal of Environmental Studies 16, 555-562.

Khan, K., Lu, Y., Khan, H., Ishtiaq, M., Khan, S., Waqas, M., Wei, L., Wang, T. (2013). Heavy metals in agricultural soils and crops and their health risks in Swat District, Northern Pakistan. Food and Chemical Toxicology 58, 449-458.

Khan, S., Cao, Q., Zheng, Y., Huang, Y., Zhu, Y. (2008). Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. Environmental Pollution 152, 686-692.

Kiely, T., Donaldson, D., Grube, A., (2004). Pesticide Industry Sales And Usage -2000 and 2001 Market Estimates. U.S. Environmental Protection Agency, Office EPA-733-R-04-001, p. 33.

Klaassen, C.D., Bracken, W.M., Dudley, R.E., Goering, P.L., Hazelton, G.A., Hjelle, J.J. (1985). Role of sulfhydryls in the hepatotoxicity of organic and metallic compounds. Toxicological Sciences 5, 806-815.

Koivula, M.J., Eeva, T. (2010). Metal-related oxidative stress in birds. Environmental Pollution 158, 2359-2370.

Laws, S.C., Ferrell, J.M., Stoker, T.E., Schmid, J., Cooper, R.L. (2000). The effects of atrazine on female Wistar rats: an evaluation of the protocol for assessing pubertal development and thyroid function. Toxicological Sciences 58, 366-376.

López-Arias, M., Grau-Corbí, J.M. (2005). Metales pesados, materia orgánica y otros parámetros de la capa superficial de los suelos agrícolas y de pastos de la España Peninsular. II: Resultados provinciales y puntuales. Madrid, INIA.

Luzardo, O.P., Ruiz-Suárez, N., Henríquez-Hernández, L.A., Valerón, P.F., Camacho, M., Zumbado, M., Boada, L.D. (2014). Assessment of the exposure to organochlorine pesticides, PCBs and PAHs in six species of predatory birds of the Canary Islands, Spain. Science of The Total Environment 472, 146-153.

Mateo, R., Hoffman, D.J. (2001). Differences in oxidative stress between young Canada geese and mallards exposed to lead-contaminated sediment. Journal of Toxicology and Environmental Health, Part A 64, 531-545.

Mehats, C., Andersen, C.B., Filopanti, M., Jin, S., Conti, M. (2002). Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. Trends in Endocrinology and Metabolism 13, 29-35.

Mora, M.A. (1997). Transboundary pollution: persistent organochlorine pesticides in migrant birds of the southwestern United States and Mexico. Environmental Toxicology and Chemistry 16, 3-11.

Nájera, J.A., González-Silva, M., Alonso, P.L. (2011). Some lessons for the future from the Global Malaria Eradication Programme (1955–1969). PLoS Medicine 8, e1000412.

Norris, D.O. (2000). Endocrine disruptors of the stress axis in natural populations: How can we tell? American Zoologist 40, 393-401.

Nweke, O.C., Sanders, W.H. (2009). Modern environmental health hazards: a public health issue of increasing significance in Africa. Environmental Health Perspectives 117, 863.

Palacios, M.J., Prieto, J.P., Muñoz, P., Sánchez, A., Miranzo, J.C., Andújar, J.P., Machón, D., Dávalos, J. (2008). Catalogo regional de especies amenazadas de Extremadura, Fauna II, Clase Aves. Consejería de Industria, Energía y Medio Ambiente. Junta de Extremadura.

Peris, S.J. (2003). Feeding in urban refuse dumps: ingestion of plastic objects by the White Stork (*Ciconia ciconia*). Ardeola 50, 81-84.

Rainio, M.J., Kanerva, M., Salminen, J.-P., Nikinmaa, M., Eeva, T. (2013). Oxidative status in nestlings of three small passerine species exposed to metal pollution. Science of the Total Environment 454–455, 466-473.

Roberge, M., Hakk, H., Larsen, G. (2004). Atrazine is a competitive inhibitor of phosphodiesterase but does not affect the estrogen receptor. Toxicology Letters 154, 61-68.

Sáez, M., Aguirre, J.I., Blázquez, E., Jiménez, B. (2008). Organochlorines in White stork (*Ciconia ciconia*): a comparison of levels in eggs and nestlings. Organohalogen Compounds 70, 1133-1136.

Sáez, M., Aguirre, J.I., Jiménez, B. (2009). Organochlorines in plasma of nestlings and adults White stork (*Ciconia ciconia*) from Madrid, Spain. Organohalogen Compounds 71, 2915-2918.

Sanderson, J.T., Letcher, R.J., Heneweer, M., Giesy, J., Van Den Berg, M. (2001). Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environmental Health Perspectives 109, 1027-1031.

Sanderson, J.T., Seinen, W., Giesy, J.P., Van Den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicological Sciences 54, 121-127.

Stoker, T., Guidici, D., Laws, S., Cooper, R. (2002). The effects of atrazine metabolites on puberty and thyroid function in the male Wistar rat. Toxicological Sciences 67, 198-206.

Tennant, M.K., Hill, D.S., Eldridge, J.C., Wetzel, L.T., Breckenridge, C.B., Stevens, J.T. (1994). Chloro-s-triazine antagonism of estrogen action: limited interaction with estrogen receptor binding. Journal of Toxicology and Environmental Health, Part A 43, 197-211.

Tkachenko, H., Kurhaluk, N. (2012). Pollution-induced oxidative stress and biochemical parameter alterations in the blood of White stork nestlings *Ciconia ciconia* from regions with different degrees of contamination in Poland. Journal of Environmental Monitoring 14, 3182-3191.

Tran, D.Q., Kow, K.Y., McLachlan, J.A., Arnold, S.F. (1996). The inhibition of estrogen receptor-mediated responses by Chloro-s-triazine-Derived compounds is dependent on estradiol concentration in yeast. Biochemical and Biophysical Research Communications 227, 140-146.

Van Drooge, B., Mateo, R., Vives, Í., Cardiel, I., Guitart, R. (2008). Organochlorine residue levels in livers of birds of prey from Spain: Inter-species comparison in relation with diet and migratory patterns. Environmental Pollution 153, 84-91.

Wilhelms, K.W., Cutler, S.A., Proudman, J.A. Carsia, R.V. Anderson, L.L., Scanes, C.G., (2006a). Lack of effects of atrazine on estrogen-responsive organs and circulating hormone concentrations in sexually immature female Japanese quail (*Coturnix coturnix japonica*). Chemosphere 65, 674-681.

Wilhelms, K.W., Cutler, S.A., Proudman, J.A., Anderson, L.L., Scanes, C.G. (2005). Atrazine and the hypothalamo-pituitary-gonadal axis in sexually maturing precocial birds: studies in male Japanese quail. Toxicological Sciences 86, 152-160.

Wilhelms, K.W., Fitzpatrick, K.F., Scanes, C.G., Anderson, L.L., (2006b). *In ovo* exposure to a triazine herbicide: effects of atrazine on circulating reproductive hormones and gonadal histology in young Japanese quail. Archives of Environmental Contamination and Toxicology 51, 117-122.

# CONCLUSIONES

1. Las clorotriazinas y sus principales metabolitos no ejercen su efecto a través del receptor de hidrocarburos aromáticos (AhR), del receptor de estrógenos (ER) y del receptor de hormonas tiroiedas (TR) en ensayos *in vitro* sobre cultivos de diversas líneas celulares.

2. En ensayos *in vivo*, la administración oral de atrazina a dosis bajas produce una alteración del sistema endocrino en codorniz, caracterizada por un incremento de los niveles hepáticos del ER y de los niveles plasmáticos de estradiol y vitelogenina.

3. La administración oral de atrazina a dosis bajas no causa ningún efecto en la expresión hepática del CYP1A o en su actividad enzimática asociada, EROD, en codorniz. No obstante, parece tener un efecto inductor sobre los niveles de porfirinas fecales a las dosis más bajas en exposiciones recientes.

4. Los niveles de distintos antioxidantes endógenos (glutatión reducido, glutatión reductasa y glutatión S-transferasa) no se ven afectados por la administración oral de dosis bajas de atrazina a lo largo del tiempo en codorniz.

5. Existe una clara influencia del ambiente en los niveles sanguíneos de metales y metaloides en los pollos de cigüeña blanca, siendo el anidamiento en las proximidades a las plantas de tratamiento de residuos sólidos urbanos (PTRSU) una de las posibles fuentes principales de contaminación.

6. La exposición a los metales estudiados (Pb, Hg y As) parece producir una estimulación en el mecanismo antioxidante de los pollos (marcado por un incremento de los niveles de GSH y GST). No obstante, es aconsejable el uso de una batería de

biomarcadores para esclarecer el efecto que estos contaminantes tienen sobre la fisiología de estas aves.

7. No se han detectado niveles de policlorobifenilos (PCBs) en la sangre de ninguno de los pollos de cigüeña blanca muestreados. Las PTRSU o la agricultura intensiva no suponen, por lo tanto, una fuente de contaminación por estos compuestos químicos clorados ya prohibidos.

8. La contaminación por pesticidas organoclorados (OCPs), y en concreto por *4,4'*-DDE, es mucho más alta en la zona de anidamiento cercana a la PTRSU que en las zonas de dehesa y agricultura intensiva + PTRSU. Las PTRSU o la agricultura intensiva no suponen, una fuente de contaminación por este pesticida. Es necesario, por lo tanto, estudiar en profundidad la procedencia de dichos niveles elevados de *4,4'*-DDE, y en particular la posible influencia de la migración al continente africano de los progenitores, ya que el DDT aún se puede seguir usando en algunos de estos países.

# EXTENDED ABSTRACT

**English title:** New horizonts in Ecotoxicology: destructive and non destructive biomarkers in European quail (*Coturnix coturnix*) and White stork (*Ciconia ciconia*).

**Author:** Irene de la Casa Resino

**Directors:** Marcos Pérez López, Francisco Soler Rodríguez and José María Navas Antón.

## INTRODUCTION

Increasing human population, greater demands on resources, technological and industrial developments and the input of various foreign substances into the environment both intentionally and unintentionally, result in serious threats to the natural environment. These impacts can trigger important negative consequences on the ecosystems and species. As a consequence, the need for environmental monitoring studies has never been higher (Becker, 2003).

Birds have often played an important role as indicators of environmental problems. They are relatively easy to observe and object of considerable public attention, which makes that discernible changes in their biology seldom go unremarked. Birds are one of the best-studied and best-known groups of organisms. Furthermore, they are threatened by toxic chemicals, i.e. persistent compounds, that accumulate to such high levels that physiology and reproduction can be affected, even, causing death, and leading to population declines (Becker, 2003; Gómez-Ramírez *et al.*, 2014). Terrestrial birds are well known as appropriate organisms for biomonitoring purposes because of their high trophic position in the food chain, which leads to the accumulation of pollutants (Van Wyk *et al.*, 2001; Baos *et al.*, 2006b, 2012; Gómez-Ramírez *et al.*, 2012, 2014; Luzardo *et al.*, 2014). The most striking examples of the value of birds as biomonitors come from their use as qualitative and quantitative accumulative indicators of pesticides and heavy metals. Studies are based on logistically convenient and non-destructive avian matrices such as eggs, feathers or blood, and on high biomagnification rates in dose-dependent responses (Becker, 2003).

During the last decades, increasing attention has been paid to the to the evaluation on the adverse effects of endocrine disrupting chemicals (EDCs) evaluation. These are defined as “exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, its progeny, or (sub) populations” (WHO/IPCS, 2002). These chemicals can mimic the action of sex steroid hormones, estrogens and androgens, by binding to hormone receptors or influencing cell signaling pathways. In addition, they may alter production and breakdown of natural hormones or modify hormone receptors expression levels and function (Soto *et al.*, 1995). One important group of chemicals that can provoke alterations on the endocrine function of organisms, therefore being considered EDs are chlorotriazines. This group of chemicals includes a number of widely used herbicides such as atrazine (ATZ), prometryn (PRT), propazine (PRZ) and simazine (SIM). Considering their potentially deleterious effects they have been excluded from the EU registration process for use on crops. However,

this is one of the most widely used agricultural groups of pesticides in the U.S.A. that may be applied before and after planting for broadleaf and grassy weeds control.

It is also important to note that many persistent organic pollutants (POPs) have shown endocrine disrupting effects in *in vitro* and/or *in vivo* assays (Geyer *et al.*, 2000). The main characteristics of these pesticides are that they are persistent in the environment, bioaccumulate in the food web, and pose a risk of causing adverse effects to human health and the environment (Safe, 1994; Gioia *et al.*, 2013). POPs include chemicals such as dioxins/furans, polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) such as *4,4'*-dichlorodiphenyltrichloroethane (*4,4'*-DDT) and its metabolites, brominated flame retardants, and perfluorinated compounds, among others (Dias *et al.*, 2013). The tendency of these pesticides to persist in the environment is a serious problem, due to the increasing exposure to wildlife and humans through direct contact or to secondary exposure after contaminated food items ingestion, suffering also bioaccumulation processes (Oxynos *et al.*, 1993; Van Den Berg *et al.*, 1994).

Metals and metalloids are natural components in the environment and many of them are essential micronutrients for organisms. In general, they could be concentrated through food webs, and thus the species situated on the top accumulate high levels of metals (Hernández *et al.*, 1999; Koivula and Eeva, 2010). Inputs of metals to the environment as a result of anthropogenic activities are difficult to measure due to the wide natural inputs from the erosion of rocks, wind-blowing dusts, volcanic activity and forest fires. Moreover, many metals are essential to live organisms (i.e. Fe, Se and Cu), but they can become toxic at high concentrations. However, some metals and metalloids such as Pb, Hg, Ni or As are generally not required for metabolic activity and are toxic to living organisms at low concentrations (Merian, 1991).

With the previous considerations, the main goal of this research was to study the usefulness of different biomarkers and to determine de levels and effects of pollution in birds from Extremadura (West of Spain). The European quail (*Coturnix coturnix*) and the White stork (*Ciconia ciconia*) have been used as bioindicator species, due to their importance in the environment of Extremadura. The effect of the herbicide ATZ on different cellular receptors, as well as on different tissues (liver, blood and feces), has been evaluated. Moreover, the effect of this herbicide as endocrine disruptor has been studied (**Chapters 1-3**). On the other hand, a biomonitoring study with metals and POPs in White stork has been developed (**Chapters 4 and 6**). Furthermore, the relationship between metals and blood oxidative stress biomarkers has been evaluated (**Chapter 5**). To sum up, the present study wants to highlight the need to set up some useful

biomarkers for environmental monitoring by using noninvasive samples, as well as evaluate the pollution status in the natural environment of Extremadura.

## OBJECTIVES

This thesis has been divided into two different parts, including three chapters each one. Three of the chapters are already published papers (de la Casa-Resino *et al*. 2012, 2013, 2014), whereas chapters 4 and 5 have already been sent to the editor-in-chief of different Journals, in order to evaluate if they are suitable for publication (chapter 4 has just been accepted).

### Part 1. Effects of triazine herbicides in *in vitro* assays and in the European quail (*Coturnix coturnix*).

*Chapter I. Chlorotriazines do not activate the aryl hydrocarbon receptor, the oestrogen receptor or the thyroid receptor in in vitro assays (de la Casa-Resino et al., 2014).*

Given the lack of information on any chlorotriazines other than ATZ, the aim of the this study was to employ an array of *in vitro* bioassays to assess whether ATZ, SIM, PRZ, PRT and the ATZ metabolites, desethyl-*s*-chloro- triazine (DEA) and desisopropyl-s-chlorotriazine (DIA), might cause the transactivation of the aryl hydrocarbon receptor (Ahr), the oestrogen receptor (Er) or the thyroid receptor (Tr).

*Chapter II. Endocrine disruption caused by oral administration of atrazine in European quail (Coturnix coturnix coturnix) (de la Casa-Resino et al., 2012).*

The main goal of this study was to determine the possible effects of an intermittent administration of single doses of ATZ under realistic conditions on the hormonal control of reproduction of European quail. Since the activation of detoxification pathways caused by ATZ can also influence possible hormonal alterations, the induction of CYP1A4 was also estimated at the transcriptional and enzymatic levels.

*Chapter III. Non-destructive multibiomarker approach in European quail (Coturnix coturnix coturnix) exposed to the herbicide atrazine (de la Casa-Resino et al., 2013).*

The objective of this study was to investigate if ATZ produced alteration in profiles of different biomarkers. Levels of fecal porphyrins were quantified to evaluate a possible affectation of the heme group by ATZ related to phase I metabolism. In addition, blood

glutathione S-transferase (GST) and GR activities, as well as reduced glutathione (GSH) levels, were studied to determine a possible effect of ATZ in the oxidative stress induction. Moreover, the effect of the herbicide on membranes was examined based on the measurement of lipid peroxidation in terms of malondialdehyde (MDA).

**Part 2. Study of oxidative stress biomarkers, metals and chlorinated pollutants in blood of White stork (*Ciconia ciconia*) nestlings.**

*Chapter IV. Breeding near a landfill may influence blood metals (Cd, Pb, Hg, Fe, Zn) and metalloids (Se, As) in White stork (Ciconia ciconia) nestlings.*

This study aimed to provide data on the incidence of persistent toxic substances in White stork nestlings from three different colonies in Extremadura (West of Spain), representative of three different environmental influences (grassland, urban landfill and intensive agricultural area + urban landfill). Specifically, the presence of selected metals (Cd, Pb, Hg, Fe, and Zn) and metalloids (Se and As) was assessed in blood of 59 chicks to study how the local environment where their parents were feeding could influence their exposure to these compounds.

*Chapter V. Biomarkers of oxidative stress associated to metal pollution in blood of White stork (Ciconia ciconia) from Spain.*

The aim of this study was to characterize antioxidant defenses (GSH, GST) and MDA in the blood of White stork chicks breeding in two different types of environments (grassland and urban landfill). Simultaneously, the levels of three toxic elements (Pb, As and Hg) were quantified in blood samples. Finally, we evaluate the impact of the considered environmental contaminants in the activity and levels of the antioxidant defense of free-living White storks.

*Chapter VI. Chlorinated pollutants in blood of White stork nestlings (Ciconia ciconia) in different colonies in Spain.*

The goal of this study was to investigate the levels of pollution by chlorinated compounds in Cáceres province to improve current knowledge and obtain baseline information. Therefore, we determined the concentrations of selected chlorinated pollutants, including PCBs and OCPs, in the blood of White stork chicks from three different environmental influences (grassland, urban landfill and intensive agricultural area + urban landfill).

## MATERIALS AND METHODS

### Part 1. Effects of triazine herbicides in *in vitro* assays and in the European quail (*Coturnix coturnix*).

*Cell culture and exposure to test chemicals*

Three cell lines were used for the exposure assays. The fibroblast-like RTG-2, HERLUC and PC-DR-LUC cell lines were used to evaluate the activation of the AhR, ER and TR respectively. In all cases, the cells were exposed for 24 hours to serially doubling dilutions of the six compounds under investigation (ATZ, SIM, PRZ, PRT and the ATZ metabolites, DEA and DIA). The exposure concentrations ranged from 0.78 to 100 μM. Control cells received the maximal vehicle (DMSO) concentration that was used with the exposed cells (0.3% v/v). β-Naphthoflavone (0.25 μM), E2 (1 μM) and T3 (1 nM) were included in each assay as positive controls, since these compounds are potent inducers of the AhR, ER and TR, respectively. Three independent experiments were performed for each compound in the different cell lines. For each experiment, each concentration of the test or control chemicals was applied in triplicate.

*Animals, treatment and assays*

A total of 45 adult female European quails (*C. coturnix*), free from apparent clinical ailment, were obtained from a local quail farm. The birds were placed in cages under constant temperature, humidity and lighting conditions (12 h light/dark cycle). Feed and water were offered *ad libitum* for the duration of the experiment. After two weeks of acclimatization, birds were divided into three groups of 15 animals (C, D1 and D2). Birds of group C received only the vehicle solvent (corn oil) and served as control. ATZ, dissolved in corn oil, was orally administered (through crop tubing) at days 0, 5 and 10 of the experiment at a dose of 25 mg/kg body mass (group D1), or 100 mg/kg body mass (group D2). The birds were monitored daily for clinical signs of discomfort.

At days 15, 30 and 45 of the experiment, 5 animals of each group were sacrificed in a $CO_2$ chamber. The blood was collected from the heart of each bird and centrifuged at 3000 rpm for 10 min to obtain the plasma and erythrocyte pellet. The plasma was used to determine vitellogenin (VTG) and 17ß-estradiol (E2) levels. The pellet was homogenized in 0.1 M of phosphate buffer ($KH_2PO_4/K_2HPO_4$ [pH 7.4]) and used to determine GSH (Hissin and Hilf, 1976) and MDA (Recknagel *et al.*, 1982) levels. 2 mL of the pellet homogenate were centrifuged at 13200 x g for 20 min to obtain the postmitochondrial

fraction. The pellet was discarded, and the supernatant was used to determine protein content (Bradford, 1976) and GST activity (Habig *et al.*, 1974).

Livers were removed immediately and stored at −80 °C, and they were used to determine ethoxyresorufin-*O*-deethylase (EROD, an enzyme activity dependent on CYP1A4) induction and mRNA levels corresponding to vitellogenin, ER and CYP1A4 (de la Casa-Resino *et al.*, 2012).

At days 0, 5, 10, 15, 30 and 45 of the experiment, feces were collected from the cages of each working group and homogenized before analysis. They were used for porphyrin analyses according to the method of Mateo *et al*. (2004).

*Statistical analysis*

Data were analyzed using statistical software Prism 5 version 5.03 for Windows (GraphPad, CA). Data were tested for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and homoscedasticity (Levene's test). Because data did not show a normal distribution, and the variances were not homogeneous, statistical analyses were performed using nonparametric Kruskal–Wallis test. To compare each treatment (dose) with its own control, Dunn's test was applied ($P<0.05$). Moreover, Spearman test was used to determine correlations between GST, GSH, and MDA levels on the three sampling days (15, 30, and 45).

**Part 2. Study of oxidative stress biomarkers, metals and chlorinated pollutants in blood of White stork (*Ciconia ciconia*) nestlings.**

*Animals, study area and sampling*

Fieldwork was conducted in three colonies located in Cáceres province (Extremadura region, Spain) during spring 2011 covering a gradient of potential contamination. The reference colony (colony A) was located in "Los Llanos de Cáceres y Sierra de Fuentes" (39º 28' 17.53'' N, 6º 10' 35.00'' W), a natural area far from apparent sources of pollution. This area is considered a Special Protection Area for Birds under the Directive 2009/147/EC and forms an integral part of the NATURA 2000 ecological network. It is a pseudo steppe zone crossed by 4 rivers (Tajo, Almonte, Tamuja and Salor) that contains an important number of protected steppe birds like Bustard (*Otis tarda*), Little bustard (*Tetrax tetrax*) and Lesser kestrel (*Falco naumanni*). It is characterized by rain-fed crops, mainly cereals but some legumes too, that are used for feeding livestock, sheep and cattle, in an extensive farming.

The second colony (colony B) was located in a grassland area similar to the colony A but with more trees (Holm oak, *Quercus ilex*) (39º 18' 37.13'' N, 6º 29' 47.53'' W), at a distance of 13.4 km far from Cáceres town (95668 inhabitants, 2012) and close to (4.9 km) a landfill that handled 61826 tons of solid waste in 2011 (Domínguez *et al.*, 2011) where the White storks frequently go to feed during their breeding period (Medina *et al.* 1998). An important road ("national" road) was 15 m far from the nearest nest in this colony. The third colony (colony C) was located between the towns of Navalmoral de la Mata (17401 inhabitants, 2012) and Talayuela (9269 inhabitants, 2012) (39º 18' 37.13''N, 6º 29' 47.53'' W). It was situated in a grassland area with *Quercus ilex* trees used for livestock and next to an important intensive agricultural area crossed by the Tietar river which is characterized for large tobacco, pepper, tomato, asparagus and corn crops. Close to colony C (1.5 km) there was a landfill that handled 39446 tons of solid waste in 2011 (Domínguez *et al.*, 2011). The distance between colonies A and B was 26.2 km and between colonies A and C was 71.7 km. The distance between colonies B and C was 97 km. All nests were placed on trees.

*Field procedure and blood sampling*

During the spring of 2011, with the regional government permission (Gobierno de Extremadura CN10/0306), White stork nests were selected and sampled in each colony depending on its accessibility (A, $n = 11$; B, $n = 8$; C, $n = 6$). The sampled nests were dispersed into the colony. Both standardized capture and handling method were performed in order to produce the minimal stress to the animals. All nestlings from each colony were taken down from their nests, gently restrained by hand during blood collection, physical examination, weighing and measuring.

Blood samples were taken from the tarsal vein with heparinized 0.8x25 mm needle and 5 mL syringe. The whole blood samples were refrigerated until arriving to the laboratory, where they were stored at - 80ºC in 1.5 mL plastic tubes previously washed with $HNO_3$ 2%.

*Determination of metals and metalloids*

Analyses were done in the Elemental and Molecular Analysis Laboratory of the Research Support Service (SAIUEX, accredited by ISO 9001:2008) from the University of Extremadura. A Platform collision cell inductively coupled plasma mass spectrometer ICP-MS NexION 300D equipped with an S10 automatic autosampler (PerkinElmer, Inc., Shelton, CT) was used for element determination.

*Determination of OCPs and PCBs*

The extraction procedure was based on the protocol used by Mateo *et al*. (2012) in plasma samples. All samples were analyzed using a Shimadzu QP 2010 Plus GC/MS operating in negative chemical ionization (NCI) mode which was coupled to an AOC-20i Autosampler (Shimadzu, Japan). Analyte separation was achieved on a SLB 5MS column (30 m–0.25 mm, i.d. x 0.25 film thickness) (Supelco, USA).

*Statistical analysis*

Data were grouped by colony in two ways. Firstly, the nestling was used as the sampling unit. Secondly, the nest was used as the sampling unit since in several nests more than one nestling was present. Data were statistically treated as previously described. Differences among colonies were determined with the Dunn´s test ($P<0.05$). Moreover, a Spearman test was used to determine correlations between metals and oxidative stress parameters.

## RESULTS AND DISCUSION

### Part 1. Effects of triazine herbicides in *in vitro* assays and in the European quail (*Coturnix coturnix*).

Triazines have been considered as endocrine disruptors (ED) due to alterations observed in endocrine activities from different organisms exposed to these substances. However, the mechanisms of action that explain these disruptive actions have not been fully elucidated. Although these herbicides have no similar structure to steroid hormones some studies suggest that they alter the expression of the oestrogen receptor (ER) by acting as agonists or antagonists (Eldridge *et al.*, 1994; Tennant *et al.*, 1994; Connor *et al.*, 1996; Tran *et al.*, 1996; Graumann *et al.*, 1999). Moreover, these estrogenic or antiestrogenic alterations may be mediated by aryl hydrocarbon receptor (AhR), since ATZ is metabolized by cytochrome P450 (CYP1A) whose expression is directly dependent on the activation of the AhR. In addition, thyroid hormones play an important role in regulating and maintaining homeostasis of numerous biological functions in organisms. Changes in plasma levels of thyroid hormones (triiodothyronine, T3; thyroxine, T4; and thyroid stimulating hormone, TSH) were not observed in some studies conducted in rats exposed to ATZ (Laws *et al.*, 2000; Stoker *et al.*, 2002). However, there are no previous studies of the effect of triazine herbicides in the thyroid hormones in birds.

The results of the present thesis showed that triazines do not interact *in vitro* with the ER, the AhR and the TR (**Chapter 1**). However, the studies performed *in vivo* in European quail exposed to 25 and 100 mg/kg of ATZ showed slightly different results. **Chapter 2** clearly shows that ATZ provokes an increase of 17$\beta$-estradiol (E2) plasma levels that is associated with typical estrogenic effects as the increase in ER mRNA levels in liver or the increase of VTG plasma concentrations. The increase in E2 plasma levels observed in this and in other works (Sanderson *et al.*, 2000; Hayes *et al.*, 2002, 2003) could be related to the stimulation of the aromatase activity, which transforms the androgen testosterone into E2. Aromatase expression is controlled by the induction of the ER (Sanderson *et al.*, 2000; Hayes *et al.*, 2002), and although ATZ appears not to be a direct ER agonist (**Chapter 1)** (Connor *et al.*, 1996; Roberge *et al.*, 2004) other mechanisms different than ER activation by ATZ could explain the induction of aromatase activity. For instance, in the H295R human adrenocortical carcinoma cell line, exposure to ATZ led to an elevation of aromatase activity in parallel to an increase of the concentration of cyclic adenosine monophosphate (cAMP) (Sanderson *et al.*, 2000, 2001). The phosphodiesterase (PDE) family of enzymes hydrolyzes cAMP to 5-AMP. By means of fluorescence polarization, Roberge *et al.* (2004) evidenced that ATZ was able to interact with PDE. This interaction would inhibit PDE activity resulting in elevated levels of cAMP, which may result in elevated aromatase mRNA (Sanderson *et al.*, 2000; Mehats *et al.*, 2002). Suzawa and Ingraham (2008) proposed a complex mechanism in zebrafish involving NR5A receptor activation, as well as receptor phosphorylation, amplification of cAMP, and PI3K signaling. Whether these mechanisms are acting also in the case of quail should be determined in future studies. The induction of E2 production observed in female European quails after administration of ATZ was not immediate and could only be observed after 30 days of the start of exposure and 20 days after the third and last administration of ATZ. All this suggests that ATZ could act through a cascade of mechanisms that need some time to be effective.

In order to determine a possible modulatory effect of catabolic activities on ATZ action, the induction of CYP1A4 by ATZ was also studied. No significant changes in the CYP1A4 mRNA expression or in the CYP1A4-dependent EROD activity were observed at any dose or day of experiment compared to control (**Chapter 2**). Moreover, in order to evaluate a possible alteration of the heme group related with CYP metabolism, levels of fecal porphyrins were evaluated (**Chapter 3**). Increased concentrations of uroporphyrin I (UPI) and coproporphyrin III (CPIII) were observed 5 days after the first administration of ATZ (at the beginning of the experiment) at the low dose used (25 mg/kg bw). An increase of coproporphyrin I (CPI) also appeared with the same dose 20 days after the last administration of ATZ. The presence of ATZ in the avian organism could trigger an

increase in the heme synthesis necessary for the CYP production. CYP activity was assayed at day 15 of the experiment after the last ATZ administration showing no differences between control and dose groups, whereas the most significant porphyrin differences were found previously, at days 5 and 10. The results obtained in the present study seem to indicate a clear porphyrinogenic action of ATZ at low doses. However, further studies are necessary to clarify these points and to establish the causality of these findings.

In **Chapter 3,** blood GST and GR activities, as well as GSH levels, were studied to determine a possible effect of ATZ on the induction of oxidative stress. The effect of the herbicide on membranes based on the measurement of lipid peroxidation in terms of MDA was also examined. In blood, normal erythrocyte function depends on the intactness of cell membrane, which is the target for many toxic factors, including pesticides (Banerjee *et al.*, 1999). MDA and GSH levels were slightly modified in blood 20 days after the last administration (day 30 of the experiment). Moreover, the dose-dependent increase of GST activity at day 30 might be partly related to the increased availability of the substrate, GSH, although it was not statistically significant in any case.

**Part 2. Study of oxidative stress biomarkers, metals and chlorinated pollutants in blood of White stork (*Ciconia ciconia*) nestlings.**

The second part of the present study was focused in a biomonitoring study in wild White stork nestling. The levels of metals and POPs in 3 different colonies with different environmental influences (grassland, landfill and intensive agricultural area + landfill) were evaluated (**Chapters 4** and **6**). Moreover, the relationship between metals and oxidative stress biomarkers was also assessed (**Chapter 5**).

One of the major difficulties when working with wild animals is to find a population under controlled conditions. Therefore, the best option is probably to establish a reference population for comparison, since the wide distribution of contaminants in the environment makes difficult, if not impossible, to find a control population (Norris, 2000). The stork colony considered as reference was in a natural area far from apparent sources of contamination. Since breeding storks generally forage near their nesting sites, animals from colony A were very unlikely to have foraged in potentially contaminated areas, which supports their validity as a reference colony.

Numerous studies have established the validity of the stork chicks as bioindicators of metal pollution (Baos *et al.*, 2006a,b, 2012; Goutner *et al.*, 2011; Tkachenko and Kurhaluk, 2012). Metal concentrations in blood of nestlings can be derived from local

exposure to food and water as well as from the egg content. However, the metal content derived from eggs (which comes from the mother) has been reported to be minimal compared with that from the nestling exposure by the food since hatching to fledging. Therefore, it is considered that metal levels in chick blood reflect concentrations of metals recently absorbed from local exposure and they are very useful tools in biomonitoring studies (García-Fernández *et al.*, 1996; Burger and Gochfeld, 1997; Blanco *et al.*, 2003; Blázquez *et al.*, 2006).

When considering all the metallic elements analyzed, Zn and Fe showed the highest concentrations, probably due to their relevance as essential elements with a wide range of physiological functions, followed by Pb>Se>Hg>As>Cd. The metal levels in the three colonies were generally below those considered to cause toxicological effects in birds according to the literature. The lowest levels were found in colony A (reference colony), followed by colony B (landfill) and colony C (agricultural area + landfill). These differences were statistically significant with respect to the colony A in the case of toxic metals (Pb, Hg and As) and Se (**Chapter 4**).

It is scientifically proven that White stork populations have increased in recent years, and this growth has been attributed to the contribution of food offered by landfills (Peris, 2003; Blázquez *et al.*, 2006). Since metals are ubiquitous, special care should be taken to identify the source, therefore the presence of landfill or agricultural activity was evaluated in our study. However, the origin of toxic metals is diverse and other potential sources need to be considered. There is no industrial activity around the colonies considered, but the metal content in soil of the studied areas must be taken into account. From soils, metals may be transferred into the food chain through plants or small prey and finally reach birds. Agriculture is a source of contamination for soil and crops, providing different metals and metalloids coming from the application of municipal solid waste compost, sewage irrigation, addition of manures, fertilizer and pesticide applications, etc. (Hargreaves *et al.*, 2008; Khan *et al.*, 2008, 2013). Since metal concentrations in ingested soil may be higher than in prey items, soil can be an important pathway of exposure to birds. In order to compare the metal concentrations in blood with those in soils, data of Cd, Hg, Pb and Zn in soils of Spain reported by López-Arias and Grau-Corbí, (2005), including the areas of the present study, were used as reference (**Chapter 4**).

Some studies have shown the effect of metals on the antioxidant system of birds (Kamiński *et al.*, 2007, 2009a,b; Tkachenko and Kurhaluk, 2012). In order to determine how metal levels affect blood oxidative stress, GSH, GST and MDA levels were evaluated. White stork nestlings displayed significantly higher blood GSH levels

($P$<0.001) and GST activity ($P$<0.005) in colony B, compared to birds from the reference site. In fact, the mean value of GSH in colony B was approximately four times higher than in colony A (4.69 and 1.19 nmol/min, respectively). Similarly, mean GST in colony B was twice that of colony A (8.45 and 4.51 nmol/min/mg prot, respectively). However, there were no statistically significant differences in blood MDA levels between White stork nestlings from colonies A and B, with mean values of 0.11 nmol/mg prot in both cases. Some significant interactions among elements and biochemical measures were observed. A significant and positive interrelationship among the three considered elements was clearly observed, and also between GST and MDA. GSH is a major antioxidant in aerobic organisms with an important role in cells protection, 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 reactive oxygen species (ROS, Espín *et al.*, 2014). In an attempt to avoid the use of tissues (e.g. liver and kidney), GSH can be measured in blood without terminal sampling of the animals, therefore being a useful tool in studies of free-living bird species (Koivula and Eeva, 2010). GST catalyzes the conjugation of GSH with pollutants. Hence, the observed induction of GST activity could be indicative of a detoxification process (Jemec *et al.*, 2007), and the high GST activity in storks from the landfill-influenced area could be associated to increased GSH concentrations due to greater GSH requirements for conjugation reactions (Espín *et al.*, 2014) (**Chapter 5**).

Furthermore, in **Chapter 6** the residues of PCBs and OCPs in blood of White stork were studied. No detected concentrations of PCBs were found in any sample from any colony, probably due to the reduction in their use during the twentieth century (Hernandez *et al.*, 1986, 1988, 1989; Jimenez *et al.*, 2000). These results indicate that the presence of landfills or intensive agricultural areas has no influence in the levels of these contaminants.

OCPs concentrations in all the chicks studied in the **Chapter 6** followed the order heptachlor> endosulfan>∑DDTs> e ndosulfan sulfate> ∑HCHs > endrin> aldrin> dieldrin. Heptachlor, *4,4'*-DDE, endosulfan and aldrin were the most frequently detected substances, being *4,4'*-DDE and heptachlor the chemicals detected at the highest concentrations. A high dispersion in the levels of residues among colonies was observed. Animals from colony B presented the highest mean values of all the OCPs studied. The effect of the environment where the birds live (landfill and intensive agricultural area + landfill) in the levels of OCPs was also evaluated. Since no residues of any pesticide were detected in colony C, it seems that landfills and agricultural areas might have no influence in the levels of these pesticides. The level of contamination by ∑DDTs in colony B is surprisingly high in comparison with the other colonies, mainly because in

Spain the usage of OCPs ceased in the late 1970s or the beginning of the 1980s (Luzardo *et al*., 2014). These results could be explained by two facts: the proximity to the African continent and the extreme environmental persistence of these compounds. The first factor is most likely to be the major cause because the migration of this birds to the African continent during the winter season. Several prohibited OCPs are still in use in developing countries, thus high levels of contamination are found in those countries. Another source can be the existence of considerable stocks of uncontrolled obsolete pesticides, which makes possible the direct contact with these chemical for people and wildlife living near the place where they are used (Nweke and Sanders, 2009; Nájera *et al.*, 2011; Gioia *et al.*, 2013). Alternatively, the widely use of pesticides in the past and their extreme persistence could be another cause of the presence of these pollutants in the stork chicks. OCPs are conveyed from the mother to the eggs via lipids in varying quantities, depending on the species (Aurigi *et al.*, 2000). It has been demonstrated in field studies by Bogan and Newton (1977) that 52 % of the body burden of DDE is eliminated in a clutch of six eggs laid by an European sparrowhawk. The exposure of the mother to these pesticides (in wintering grounds or breeding grounds) could produce an increase in the levels of these pesticides in the eggs and in the future chicks.

## REFERENCES

Aurigi, S., Focardi, S., Hulea, D., Renzoni, A. (2000). Organochlorine contamination in bird's eggs from the Danube Delta. Environmental Pollution 109, 61-67.

Banerjee, B., Seth, V., Bhattacharya, A., Pasha, S., Chakraborty, A. (1999). Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. Toxicology Letters 107, 33-47.

Baos, R., Blas, J., Bortolotti, G. R., Marchant, T. A., Hiraldo, F. (2006a). Adrenocortical response to stress and thyroid hormone status in free-living nestling White storks (*Ciconia ciconia*) exposed to heavy metal and arsenic contamination. Environmental Health Perspectives 114, 1497-1501.

Baos, R., Forero, M. G., Gómez, G., Gónzalez, M. J., Hiraldo, F., Jiménez, B., Jovani, R., Tella, J. L. (2006b). Relationships between T-cell mediated immnune response and Pb, Zn. Cu, Cd, and as concentration in blood of nestling White storks (*Ciconia ciconia*) and Black kites (*Milvus migrans*) from Doñana (Southwestern Spain) after the Aznalcóllar toxic spill. Environmental Toxicology and Chemistry 25, 1153-1159.

Baos, R., Jovani, R., Serrano, D., Tella, J. L., Hiraldo, F. (2012). Developmental exposure to a toxic spill compromises long-term reproductive performance in a wild, long-lived bird: The white stork (*Ciconia ciconia*). PloS One 7, e34716.

Becker, P.H. (2003). Biomonitoring with birds. In: Markert, B.A., Breure, A.M., Zechmeister, H.G. (Eds.). Trace Metals and other Contaminants in the Environment. Bioindicators and Biomonitors Principles, Concepts and Applications 6, 677-736. Elsevier.

Blanco, G., Frías, O., Jiménez, B., Gómez, G. (2003). Factors influencing variability and potential uptake routes of heavy metals in Black kites exposed to emissions from a solid waste incinerator. Environmental Toxicology and Chemistry 22, 2711-2718.

Blázquez, E., Aguirre, J., Martínez-Haro, M., Mateo, R., Jiménez, B. (2006). The use of White stork (*Ciconia ciconia*) nestlings in a biomonitoring programme for organochlorines through the region of Madrid (Spain). Organohalogen Compounds 68, 2081-2085.

Bogan, J., Newton, I. (1977). Redistribution of DDE in sparrowhawks during starvation. Bulletin of Environmental Contamination and Toxicology 18, 317-321.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Burger, J., Gochfeld, M. (1997). Age differences in metals in the blood of Herring (*Larus argentatus*) and Franklin's (*Larus pipixcan*) gulls. Archives of Environmental Contamination and Toxicology 33, 436-440.

Connor, K., Howell, J., Chen, I., Liu, H., Berhane, K., Sciarretta, C., Safe, S., Zacharewski, T. (1996). Failure of chloro-s-triazine-derived compounds to induce estrogen receptor-mediated responses *in vivo* and *in vitro*. Toxicological Sciences 30, 93-101.

de la Casa-Resino, I., Valdehita, A., Soler, F., Navas, J.M., Pérez-López, M. (2012). Endocrine disruption caused by oral administration of atrazine in European quail (*Coturnix coturnix*). Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 156, 159-165.

Dias, P.S., Cipro, C.V., Taniguchi, S., Montone, R.C. (2013). Persistent organic pollutants in marine biota of São Pedro and São Paulo Archipelago, Brazil. Marine Pollution Bulletin 74, 435-440.

Directive 2009/147/EC of the European Parliament and of the Council of 30 November 2009 on the conservation of wild birds. Official Journal of the European Union 20, 7-25

Domínguez, J., Moreno, F., Gonzalez, F., Pita, M. (2011). Informe ambiental de Extremadura. Consejería de Agricultura, Desarrollo Rural, Medio Ambiente y Energía de la Junta de Extremadura. http://extremambiente.gobex.es/. Accessed October 3, 2013.

Eldridge, J.C., Fleenor-Heyser, D.G., Extrom, P.C., Wetzel, L.T., Breckenridge, C.B., Gillis, J. H., Luempert III, L. G., Stevens, J. T. (1994). Short-term effects of chlorotriazines on estrus in female Sprague-Dawley and Fischer 344 rats. Journal of Toxicology and Environmental Health 43, 155-167.

Espín, S., Martínez-López, E., Jiménez, P., María-Mojica, P., García-Fernández, A.J. (2014). Effects of heavy metals on biomarkers for oxidative stress in Griffon vulture (*Gyps fulvus*). Environmental Research 129, 59-68.

García-Fernández, A.J., Sánchez-García, J.A., Gómez-Zapata, M., Luna, A. (1996). Distribution of cadmium in blood and tissues of wild birds. Archives of Environmental Contamination and Toxicology 30, 252-258.

Geyer, H.J., Rimkus, G.G., Scheunert, I., Kaune, A., Schramm, K.-W., Kettrup, A., Zeeman, M., Muir, D.C., Hansen, L.G., Mackay, D. (2000). Bioaccumulation and occurrence of endocrine-disrupting chemicals (EDCs), persistent organic pollutants (POPs), and other organic compounds in fish and other organisms including humans. Bioaccumulation-New Aspects and Developments. Springer.

Gioia, R., Akindele, A., Adebusoye, S., Asante, K., Tanabe, S., Buekens, A., Sasco, A. (2013). Polychlorinated biphenyls (PCBs) in Africa: a review of environmental levels. Environmental Science and Pollution Research, DOI 10.1007/s11356-013-1739-1.

Gómez-Ramírez, P., Martínez-López, E., García-Fernández, A., Zweers, A., Van Den Brink, N. (2012). Organohalogen exposure in a Eurasian Eagle owl (*Bubo bubo*) population from Southeastern Spain: Temporal-spatial trends and risk assessment. Chemosphere 88, 903-911.

Gómez-Ramírez, P., Shore, R.F., Van Den Brink, N.W., Van Hattum, B., Bustnes, J.O., Duke, G., Fritsch, C., García-Fernández, A.J., Helander, B.O., Jaspers, V., Krone, O., Martínez-López, E., Mateo, R., Movalli, P., Sonne, C. (2014). An overview of existing raptor contaminant monitoring activities in Europe. Environment International 67, 12-21.

Goutner, V., Becker, P., Liordos, V., Tsachalidis, E. (2011). Mercury in White Stork (*Ciconia ciconia*) chick feathers from Northeastern Mediterranean areas in relation to age, brood size, and hatching order. Archives of Environmental Contamination and Toxicology 61, 327-336.

Graumann, K., Breithofer, A., Jungbauer, A. (1999). Monitoring of estrogen mimics by a recombinant yeast assay: synergy between natural and synthetic compounds? Science of the Total Environment 225, 69-79.

Habig, W.H., Pabst, M.J., Jakoby, W.B. (1974). Glutathione S-transferases the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249, 7130-7139.

Hargreaves, J., Adl, M., Warman, P. (2008). A review of the use of composted municipal solid waste in agriculture. Agriculture, Ecosystems and Environment 123, 1-14.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A. (2002). Herbicides: feminization of male frogs in the wild. Nature 419, 895-896.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A. (2003). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environmental Health Perspectives 111, 568-575.

Hernández, L., Fernández, M., González, M.J. (1989). Total PCBs and PCB congeners in Spanish Imperial eagle eggs. Bulletin of Environmental Contamination and Toxicology 43, 725-732.

Hernández, L., González, M.J., Rico, M.C., Fernández, M., Aranda, A. (1988). Organochlorine and heavy metal residues in Falconiforme and Ciconiforme eggs (Spain). Bulletin of Environmental Contamination and Toxicology 40, 86-93.

Hernández, L.M., Gómara, B., Fernández, M., Jiménez, B., González, M.J., Baos, R., Hiraldo, F., Ferrer, M., Benito, V., Suñer, M.A., Devesa, V., Muñoz, O., Montoro, R. (1999). Accumulation of heavy metals and As in wetland birds in the area around Doñana National Park affected by the Aznalcollar toxic spill. Science of the Total Environment 242, 293-308.

Hernández, L.M., Rico, M.C., González, M.J., Hernán, M.A., Fernández, M.A. (1986). Presence and time trends of organochlorine pollutants and heavy metals in eggs of predatory birds of Spain. Journal of Field Ornithology 57, 270-282.

Hissin, P. J., Hilf, R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry 74, 214-226.

Jemec, A., Drobne, D., Tišler, T., Trebše, P., Roš, M., Sepčić, K. (2007). The applicability of acetylcholinesterase and glutathione S-transferase in Daphnia magna toxicity test. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 144, 303-309.

Jiménez, B., Gómara, B., Baos, R., Hiraldo, F., Eljarrat, E., Rivera, J., González, M.J. (2000). A study of the toxic equivalents derived from PCDDs, PCDFs and dioxin-like PCBs in two bird species (*Ciconia Ciconia* and *Milvus Migrans*) nesting in a protected area (Doñana National Park, Spain). Organohalogen Compounds 46, 542-545.

Kamiński, P., Kurhalyuk, N., Jerzak, L., Kasprzak, M., Tkachenko, H., Klawe, J.J., Szady-Grad, M., Koim, B., Wiśniewska, E. (2009a). Ecophysiological determinations of antioxidant enzymes and lipoperoxidation in the blood of White Stork *Ciconia ciconia* from Poland. Environmental Research 109, 29-39.

Kamiński, P., Kurhalyuk, N., Kasprzak, M., Jerzak, L., Tkachenko, H., Szady-Grad, M., Klawe, J. J., Koim, B. (2009b). The impact of element-element interactions on antioxidant enzymatic activity in the blood of White stork (*Ciconia ciconia*) chicks. Archives of Environmental Contamination and Toxicology 56, 325-337.

Kamiński, P., Kurhalyuk, N., Szady-Grad, M. (2007). Heavy metal-induced oxidative stress and changes in physiological process of free radicals in the blood of White Stork (*Ciconia ciconia*) chicks in polluted areas. Polish Journal of Environmental Studies 16, 555-562.

Khan, K., Lu, Y., Khan, H., Ishtiaq, M., Khan, S., Waqas, M., Wei, L., Wang, T. (2013). Heavy metals in agricultural soils and crops and their health risks in Swat District, Northern Pakistan. Food and Chemical Toxicology 58, 449-458.

Khan, S., Cao, Q., Zheng, Y., Huang, Y., Zhu, Y. (2008). Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. Environmental Pollution 152, 686-692.

Klaassen, C.D., Bracken, W.M., Dudley, R.E., Goering, P.L., Hazelton, G.A., Hjelle, J.J. (1985). Role of sulfhydryls in the hepatotoxicity of organic and metallic compounds. Toxicological Sciences 5, 806-815.

Koivula, M.J., Eeva, T. (2010). Metal-related oxidative stress in birds. Environmental Pollution 158, 2359-2370.

Laws, S.C., Ferrell, J.M., Stoker, T.E., Schmid, J., Cooper, R.L. (2000). The effects of atrazine on female Wistar rats: an evaluation of the protocol for assessing pubertal development and thyroid function. Toxicological Sciences 58, 366-376.

López-Arias, M., Grau-Corbí, J. M. 2005. Metales pesados, materia orgánica y otros parámetros de la capa superficial de los suelos agrícolas y de pastos de la España Peninsular. II: Resultados provinciales y puntuales. INIA, Madrid.

Luzardo, O.P., Ruiz-Suárez, N., Henríquez-Hernández, L.A., Valerón, P.F., Camacho, M., Zumbado, M., Boada, L.D. (2014). Assessment of the exposure to organochlorine pesticides, PCBs and PAHs in six species of predatory birds of the Canary Islands, Spain. Science of the Total Environment 472, 146-153.

Mateo, R., Castells, G., Green, A.J., Godoy, C., Cristòfol, C. (2004). Determination of porphyrins and biliverdin in bile and excreta of birds by a single liquid chromatography-ultraviolet detection analysis. Journal of Chromatography B 810, 305-311.

Mateo, R., Millán, J., Rodríguez-Estival, J., Camarero, P.R., Palomares, F., Ortiz-Santaliestra, M. E. (2012). Levels of organochlorine pesticides and polychlorinated biphenyls in the critically endangered Iberian lynx and other sympatric carnivores in Spain. Chemosphere 86, 691-700.

Mehats, C., Andersen, C.B., Filopanti, M., Jin, S., Conti, M. (2002). Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. Trends in Endocrinology and Metabolism 13, 29-35.

Merian, E. 1991. Metals and their compounds in the environment: occurrence, analysis and biological relevance, VCH, Weinheim.

Nájera, J.A., González-Silva, M., Alonso, P.L. (2011). Some lessons for the future from the Global Malaria Eradication Programme (1955–1969). PLoS Medicine 8, e1000412.

Norris, D.O. (2000). Endocrine disruptors of the stress axis in natural populations: How can we tell? American Zoologist 40, 393-401.

Nweke, O.C., Sanders, W.H. (2009). Modern environmental health hazards: a public health issue of increasing significance in Africa. Environmental Health Perspectives 117, 863-870.

Oxynos, K., Schmitzer, J., Kettrup, A. (1993). Herring gull eggs as bioindicators for chlorinated hydrocarbons (contribution to the German Federal Environmental Specimen Bank). Science of the Total Environment 139, 387-398.

Peris, S.J. (2003). Feeding in urban refuse dumps: ingestion of plastic objects by the White Stork (*Ciconia ciconia*). Ardeola 50, 81-84.

Recknagel, R.O., Glende, E.A., Jr., Waller, R.L., Lowrey, K. (1982). Lipid peroxidation: biochemistry, measurement and significance in liver cell injury. In: Plaa, G., Hewitt, H.R. (eds.). Toxicology of the Liver. New York: Raven Press.

Roberge, M., Hakk, H., Larsen, G. (2004). Atrazine is a competitive inhibitor of phosphodiesterase but does not affect the estrogen receptor. Toxicology Letters 154, 61-68.

Safe, S.H. (1994). Polychlorinated Biphenyls (PCBs): Environmental Impact, Biochemical and Toxic Responses, and Implications for Risk Assessment. Critical Reviews in Toxicology 24, 87-149.

Sanderson, J.T., Letcher, R.J., Heneweer, M., Giesy, J., Van Den Berg, M. (2001). Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environmental Health Perspectives 109, 1027-1031.

Sanderson, J.T., Seinen, W., Giesy, J.P., Van Den Berg, M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicological Sciences 54, 121-127.

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Serrano, F.O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental Health Perspectives 103, 113-122.

Stoker, T., Guidici, D., Laws, S., Cooper, R. (2002). The effects of atrazine metabolites on puberty and thyroid function in the male Wistar rat. Toxicological Sciences 67, 198-206.

Suzawa, M., Ingraham, H.A. (2008). The herbicide atrazine activates endocrine gene networks via non-steroidal NR5A nuclear receptors in fish and mammalian cells. PloS One 3, e2117.

Tennant, M.K., Hill, D.S., Eldridge, J.C., Wetzel, L.T., Breckenridge, C.B., Stevens, J.T. (1994). Chloro-s-triazine antagonism of estrogen action: Limited interaction with estrogen receptor binding. Journal of Toxicology and Environmental Health 43, 197-211.

Tkachenko, H., Kurhaluk, N. (2012). Pollution-induced oxidative stress and biochemical parameter alterations in the blood of White stork nestlings *Ciconia ciconia* from regions with different degrees of contamination in Poland. Journal of Environmental Monitoring 14, 3182-3191.

Tran, D.Q., Kow, K.Y., McLachlan, J.A., Arnold, S.F. (1996). The Inhibition of estrogen receptor-mediated responses by chloro-s-triazine-derived compounds is dependent on estradiol concentration in yeast. Biochemical and Biophysical Research Communications 227, 140-146.

Van Den Berg, M., Craane, B. L., Sinnige, T., VanMourik, S., Van Der Gaag, M., Lutke-Schipholt, I.J., Spenkelink, B., Brouwer, A., Dirksen, S., Boudewun, T. (1994). Biochemical and toxic effects of polychlorinated biphenyls (PCBs), dibenzo-P-dioxins (PCDDs) and dibenzofurans (PCDFs) in the cormorant (*Phalacrocorax carbo*) after *in ovo* exposure. Environmental Toxicology and Chemistry 13, 803-816.

Van Wyk, E., Bouwman, H., Van Der Bank, H., Verdoorn, G.H., Hofmann, D. (2001). Persistent organochlorine pesticides detected in blood and tissue samples of vultures from different localities in South Africa. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 129, 243-264.

WHO/IPCS (World Health Organization/International Petroleum Chemical Society), 2002. In: Damstra, T., Barlow, S., Bergman, A., Kavlock, R., Van Der Kraak, G. (Eds.), Global assessment of the state-of-the-science of endocrine disruptors. WHO/IPCS/EDC/02.2. World Healh Organization, Geneva, Switzerland.

***Fotos: Francisco E. Martín Cano***