UNIVERSITY OF TURKU

EFFECTS OF ARSENIC ON NESTLING DEVELOPMENT, SURVIVAL AND PHYSIOLOGY OF PASSERINES AND THE PROTECTIVE ROLE OF CALCIUM

Pablo Sánchez Virosta

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. AII OSA - TOM. 355 | BIOLOGICA - GEOGRAPHICA - GEOLOGICA | TURKU 2019

UNIVERSITY OF TURKU

EFFECTS OF ARSENIC ON NESTLING DEVELOPMENT, SURVIVAL AND PHYSIOLOGY OF PASSERINES AND THE PROTECTIVE ROLE OF CALCIUM

Pablo Sánchez Virosta

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. AII OSA - TOM. 355 | BIOLOGICA – GEOGRAPHICA – GEOLOGICA | TURKU 2019

University of Turku

Faculty of Science and Engineering
Department of Biology
Biology
Doctoral Program in Biology, Geography and Geology

Supervised by

Dr. Tapio Eeva
Department of Biology
University of Turku
Finland

Prof. Antonio J. García-Fernández
Area of Toxicology
Department of Health Sciences
University of Murcia
Spain

Reviewed by

Dr. Caroline Isaksson
Department of Biology
Lund University
Sweden

Dr. Kim Fernie
Ecotoxicology and Wildlife Health Division
Environment & Climate Change
Canada (ECCC)
Canada

Opponent

Prof. Miguel A. Mora
Department of Wildlife and Fisheries
Sciences
Texas A&M University
USA

The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

Cover Image: Silvia Espín

ISBN 978-951-29-7670-6 (PRINT)
ISBN 978-951-29-7671-3 (PDF)
ISSN 0082-6979 (Print)
ISSN 2343-3183 (Online)
Grano Oy – Turku, Finland 2019

TABLE OF CONTENTS

ABSTRACT

Arsenic (As) has been ranked first place in the Substance Priority List of hazardous substances for environmental health by the Agency for Toxic Substances and Disease Registry (ATSDR) of the USA during the last 20 years. This metalloid is of major interest because of its toxic effects on humans and animals, mainly in its inorganic form. Calcium (Ca) is an essential element for organisms and its deficiency in the diet may increase the absorption and accumulation of toxic elements, including As. In this regard, Ca administration may have a protective role against As and metal toxicity in different organisms. However, studies on this topic are rare for wild birds. The main aims of this thesis are: (i) to find out the current status and knowledge gaps of As-related research in passerine birds, (ii) to explore the effects of Ca availability on biomarkers of oxidative stress (antioxidants and oxidative damage) in passerine nestlings in relation to As and metal exposure, and (iii) to investigate if environmentally relevant As levels affect physiology, growth and survival of birds. For this purpose, I first prepared a literature review providing a broad overview of As exposure and effects in passerines, pointing out that an experimental approach is needed to explore the adverse effects of this metalloid in free-living passerines at environmental levels. To fill this gap, I performed an As manipulation experiment in great tit (*Parus major*), where nestlings inhabiting an unpolluted area were dosed with water or sodium arsenite (control, low and high As groups), whereas those living in a metal-polluted area were dosed with water (industrial control). Nestlings accumulated As in liver, bone and feathers in a dose-dependent way. Nests in the high As group produced fewer fledglings per successful nest, whereas chicks in the low As group showed slower wing growth, which could have negative post-fledging fitness effects. I found limited effects on the blood biochemical parameters. Non-significantly increased oxidative stress biomarker values in the high As group suggest that the exposure was close to the level altering reduction-oxidation balance. The effects of Ca availability on oxidative stress in great tit nestlings in relation to As/metal exposure were evaluated during a Ca supplementation experiment, where *ad libitum* Ca was provided to some nests (Ca-supplemented group) or no Ca to others (control group) in a metal-polluted and a control zone. The Ca availability had very limited effects on the antioxidant status (only on catalase activity). However, blood antioxidant levels changed over the range of metal concentrations depending on the Ca levels in plasma, suggesting that higher Ca levels stimulate antioxidants and mitigate the impacts of metals. Altogether, this thesis compiles the existing pool of knowledge regarding As exposure and effects in passerines, and identifies and fills the main gaps, elucidating the physiological and developmental effects of environmentally relevant As levels, and the role of Ca as modulator of metal/As-related oxidative stress in wild passerines.

TIIVISTELMÄ

Arseeni (As) on luokiteltu ensimmäiselle sijalle USA:n myrkyllisten aineiden ja sairauksien rekisteröintitoimiston (ATSDR) ympäristöterveydelle vaarallisten aineiden listassa viimeiset 20 vuotta. Erityisesti tämän metalloidin epäorgaaniset muodot ovat tunnettuja myrkkyvaikutuksistaan ihmisiin ja eläimiin. Kalsium (Ca) on eliöille tärkeä ravinne ja sen puute ravinnossa saattaa lisätä myrkyllisten alkuaineiden, kuten arseenin, imeytymistä ja kertymistä elimistössä. Ravinnon lisäkalsium saattaa siten suojata eliöitä arseenin ja metallien haittavaikutuksilta. Arseenin vaikutuksista luonnonvaraisiin lintuihin löytyy kuitenkin niukasti tietoa. Väitöskirjani päätavoitteet ovat: (i) selvittää mikä on varpuslintuihin liittyvän arseenitutkimuksen nykytila ja suurimmat tiedonpuutteet, (ii) tutkia kalsiumin saatavuuden vaikutusta varpuslintujen oksidatiivisen stressin biomarkkereihin (antioksidantit ja kudosvauriot) suhteessa arseeni- ja metallialtistukseen, ja (iii) selvittää miten ympäristössä esiintyvät arseenitasot vaikuttavat lintujen fysiologiaan, kasvuun ja elossasäilymiseen. Tätä varten laatimani kirjallisuuskatsaus antoi hyvän kokonaiskuvan arseenialtistuksesta ja sen vaikutuksista varpuslintuihin, sekä osoitti että linnuille haitallisten vaikutusten selvittämiseksi tarvitaan kokeellista lähestymistapaa ja sellaisilla altistustasoilla joita esiintyy ympäristössä. Tämän vuoksi tein arseenialtistuskokeen jossa saastumattoman alueen talitiaisten (*Parus major*) poikasia altistettiin vedelle tai natriumarseniitille (kontrolli, alhainen altistus, korkea altistus) ja arseenin sekä metallien saastuttaman alueen poikasia vedelle (teollisuuskontrolli). Arseenia kertyi poikasten maksaan, luihin ja sulkiin annosta vastaavassa suhteessa. Korkean altistuksen ryhmässä tuotettujen poikasten määrä oli pienempi ja alhaisen altistuksen ryhmässä poikasten siivet kasvoivat hitaammin, mikä saattaa vaikuttaa haitallisesti poikasten myöhempään selviytymiseen. Veren biokemiallisiin muuttujiin altistuksella oli vain vähän vaikutuksia. Korkea altistus nosti eimerkitsevästi oksidatiivisen stressin markkereiden arvoja, mikä viittaa siihen että altistus oli lähellä hapetus-pelkistystasapainoon vaikuttavaa tasoa. Kalsiumin saatavuuden vaikutusta oksidatiiviseen stressiin ja sen yhteyttä arseeni/metallialtistukseen testattiin metallien saastuttamalla alueella ja vertailualueella tarjoamalla jatkuvasti lisäkalsiumia osalle poikueista (kalsiumryhmä) toisten jäädessä ilman (kontrolliryhmä). Lisäkalsiumilla oli vain vähän vaikutusta veren antioksidanttitasoihin (vain katalaasientsyymin aktiivisuus muuttui). Metallialtistus kuitenkin vaikutti veren antioksidanttitasoihin ja vaikutuksen suunta riippui veriplasman kalsiumpitoisuudesta, mikä viittaa siihen että korkea kalsiumtaso stimuloi antioksidantteja ja vähentää metallien haittavaikutuksia. Kokonaisuudessaan tämä väitöskirja kokoaa yhteen varpuslintujen arseenialtistusta käsittelevän tietämyksen ja tunnistaa sekä täyttää joitakin selkeitä tiedollisia aukkoja, valottaen luonnossa esiintyvien arseenipitoisuuksien vaikutuksia luonnonvaraisten varpuslintujen fysiologiaan ja kehitykseen, sekä kalsiumin roolia metallien/arseenin aiheuttaman oksidatiivisen stressin ehkäisyssä.

ABBREVIATIONS

ALP	Alkaline phosphatase
AO	Antioxidant
As	Arsenic
ATSDR	Agency for Toxic Substances and Disease Registry
Ca	Calcium
CAT	Catalase
Cd	Cadmium
CK	Creatine kinase
Cu	Copper
FL	Number of fledglings
FS	Fledging success
GLMM	Generalized linear mixed models
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH:GSSG ratio	Ratio of reduced glutathione to oxidized glutathione
GSH	Glutathione (reduced form)
GSSG	Glutathione disulfide (oxidized form)
GST	Glutathione-S-transferase
G6PDH	Glucose-6-phosphate dehydrogenase
Hg	Mercury
HTC	Hematocrit
LMM	Linear mixed models
LOQ	Limit of quantitation
Ni	Nickel
Pb	Lead
PC	Protein carbonylation
PC1	First principal component
PCA	Principal component analysis
PTH	Parathyroid hormone
r_p	Pearson's correlation coefficient
r_s	Spearman's correlation coefficient
RBC	Red blood cells
ROS	Reactive oxygen species
Se	Selenium
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substances
tGSH	Total glutathione (both reduced and oxidized forms)
UA	Uric acid
Vit	Vitamin
8-OHdG	8-hydroxy-2'-deoxyguanosine

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and manuscripts referred to in the text by their Roman numerals:

I. Sánchez-Virosta P, Espín S, García-Fernández AJ, Eeva T. 2015. A review on exposure and effects of arsenic in passerine birds. *Science of the Total Environment* 512-513: 506-525.

II. Sánchez-Virosta P, Espín S, Ruiz S, Stauffer J, Kanerva M, García-Fernández AJ, Eeva T. 2019. Effects of calcium supplementation on oxidative status and oxidative damage in great tit nestlings inhabiting a metal-polluted area. *Environmental Research* 171: 484-492.

III. Sánchez-Virosta P, Espín S, Ruiz S, Salminen J-P, García-Fernández AJ, Eeva T. 2018. Experimental manipulation of dietary arsenic levels in great tit nestlings: accumulation pattern and effects on growth, survival and plasma biochemistry. *Environmental Pollution* 233: 764-773.

IV. Sánchez-Virosta P, Espín S, Ruiz S, Panda B, Ilmonen P, Schultz SL, Karouna-Renier N, García-Fernández AJ, Eeva T. Arsenic-related oxidative stress in experimentally dosed wild great tit nestlings. *Manuscript*.

1. INTRODUCTION

1.1. Arsenic, toxic metals and calcium metabolism

Metals are components of the earth broadly distributed in the environment, occurring naturally in minerals and ores. Mining, industrial activities and coal combustion are important anthropogenic sources of metals such as lead (Pb), mercury (Hg), cadmium (Cd), copper (Cu) and arsenic (As) into the environment (Pacyna and Pacyna, 2001). Although As is chemically classified as a metalloid, it is commonly mentioned as a metal (ATSDR, 2007). These metals produce adverse effects including immune system suppression, skeletal, reproductive and neurological disorders on humans and animals (Evans et al., 1982; Finley and Stendell, 1978; Mateo et al., 2003; Sánchez-Virosta et al., 2015; Snoeijs et al., 2004).

The Agency for Toxic Substances and Disease Registry (ATSDR) has ranked arsenic as the first compound in the Substance Priority List during the last 20 years. This list, revised on a 2-year basis, ranks substances most commonly found and which are determined to pose the most significant potential threat to human health due to their known or suspected toxicity and potential for human exposure (ATSDR, 2017). Arsenic is of special concern due to its toxic effects on both humans and animals, mainly in its inorganic form (see review article I, Sánchez-Virosta et al., 2015). However, studies on Pb, Cd and Hg have been prioritized by the scientific community, whereas field studies evaluating As-related effects on free-living birds are scarce. Biomonitoring studies evaluating environmental pollution have successfully used different bird species all over the world (Abbasi et al., 2016; Espín et al., 2016a; Furness, 1993; García-Fernández, 2014; Vizuete et al., 2018; Whitney and Cristol, 2018; Williams et al., 2018). Wild birds are frequently exposed to a combination of toxic metals and other stressors, which pose difficulties to demonstrate a causal link between levels of specific elements and their associated health effects. Besides, environmental pollution in a long-term basis may produce changes in animal and plant communities, which may end up causing secondary effects on avian species due to changes in food quality and quantity (Eeva et al., 1997, 2005a). As part of this thesis, we developed a literature review compiling data on As exposure in passerines, including information on samples used, intra/interspecific differences on As concentrations and analytical methods applied, and data on As adverse effects on laboratory animals, humans and birds (see review article I, Sánchez-Virosta et al., 2015). This review pointed out that As manipulation experiments are needed to explore the As-related effects on growth, survival and physiology in wild bird populations at levels close to those occurring in the environment.

Calcium (Ca) is an essential element for organisms, providing structural strength and support and playing basic roles in biochemical reactions. Its metabolism has some unique characteristics in birds mainly because they lay eggs with calcified eggshells (de Matos, 2008). Therefore, birds need Ca for successful breeding during egg laying, considering that up to 98% of the dry mass of the eggshell is calcium carbonate (Reynolds et al., 2004), and

during skeletal growth of nestlings (Starck, 1998). Ca metabolism in birds is efficiently regulated mainly by parathyroid hormone (PTH) and vitamin D3 (cholecalciferol) (Figure 1). PTH secretion is stimulated in response to hypocalcemia or a fall in plasma Ca levels, while hypercalcemia or a rise in Ca levels suppresses it (de Matos, 2008). PTH increases plasma Ca by decreasing Ca loss in the urine, increasing bone resorption and promoting the renal formation of 1,25-$(OH)_2$-D_3 (calcitriol or 1,25-dihydroxyvitamin D3). Calcitriol is the most metabolically active form of vitamin D3, playing an important role in Ca homeostasis since it induces the absorption of dietary Ca by increasing the synthesis of Ca-binding proteins in the intestine (de Matos, 2008).

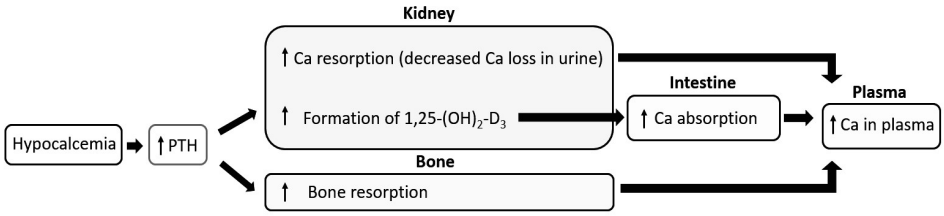

Figure 1. Restoring plasma Ca by parathyroid hormone (PTH) and 1,25-$(OH)_2$-D_3 (calcitriol). Based on de Matos (2008).

Since small passerines are unable to store enough Ca in their body for successful reproduction, they have to consume Ca-rich material to complement their normal diet, since the latter do not contain sufficient Ca (Graveland and van Gijzen, 1994). One of the main sources of dietary Ca for many passerine bird species are snail shells (Graveland, 1996; Mänd et al., 2000; Reynolds and Perrins, 2010; Tilgar et al., 1999). Environmental changes reducing the availability of this Ca-rich material may pose problems for breeding and nestling development. Evidence of Ca-limited reproduction in birds include thin-shelled eggs, decreased clutch and egg size, and diminished growth rate or number of fledglings (reviewed in Reynolds et al., 2004). Additional problems may arise when populations inhabit acidified or polluted areas. The degree of acidification of soils may affect the amount of exchangeable Ca, and consequently the abundance of snails (Graveland and van der Wal, 1996). In addition, metal pollution may affect abundance, diversity and quality of land snails, decreasing the stock of Ca-rich food items (Eeva et al., 2010). Furthermore, inadequate Ca intake may increase the absorption and accumulation of metals like Pb and Cd in birds (Dauwe et al., 2006; Scheuhammer, 1996). The higher Pb absorption in low-dietary Ca conditions is related to the synthesis of Ca-binding proteins in the intestine, when Ca is replaced by Pb (Fullmer et al., 1985). On the other hand, it is well known that Pb and Cd may modify the regulation and function of Ca (e.g. these metals may interfere with Ca transport or storage, such as Ca^{2+} transport proteins; or Ca^{2+} may be substituted with Pb^{2+}/Cd^{2+} at functionally Ca binding sites, such as calmodulin; Pounds, 1984; Suzuki et al., 2004, 1985), but also As may alter Ca metabolism (Florea and Büsselberg, 2009; Florea et al., 2005). In this regard, different publications have reported

that the administration of Ca may have a defensive or protective role against As and metal (mainly Pb and Cd) toxicity (Abdel-Hameid, 2009; Prasanthi et al., 2010; Snoeijs et al., 2005; Srivastava et al., 2010). Some studies suggest that Ca administration may decrease gastrointestinal absorption of metals and modulate metal/As-induced oxidative stress (Abdel-Hameid, 2009; Diamond et al., 1997; Jamakala and Rani, 2012; Prasanthi et al., 2010; Rai et al., 2012; Srivastava et al., 2010). However, similar studies are scarce for wild birds (Espín et al., 2017a).

1.2. Antioxidant defense and oxidative stress

Oxygen (O_2) is needed in aerobic organisms for energy production, and around 80% of that energy is produced in the mitochondria, where O_2 is reduced to H_2O in the electron transport chain (Halliwell and Gutteridge, 2007). During these oxidative reactions and other endogenous processes (e.g. oxidation of fatty acids, activity of phagocytic cells or cytochrome P450 reactions), free radicals are naturally produced (Finkel and Holbrook, 2000). The reactive oxygen species (ROS) are greatly reactive and unstable molecules produced in redox reactions, for example in fast growing nestlings due to the increased metabolic activity (Alonso-Álvarez et al., 2007; Dowling and Simmons, 2009). The term ROS involves different oxygen radicals and non-radical derivatives of O_2 (e.g. hydrogen peroxide, H_2O_2). Aerobic living beings are equipped with an antioxidant system (Figure 2) able to inhibit ROS generation and protect from oxidative damage (Koivula and Eeva, 2010). The imbalance between ROS and antioxidants, caused by excessive ROS generation, antioxidant depletion or both, is called oxidative stress (Halliwell and Gutteridge, 2007), and it may lead to oxidative damage to biomolecules (i.e. lipids, proteins and DNA). This oxidative damage may end up affecting membrane structure and properties, disrupting receptors, enzymes and proteins, affecting telomeres length or causing mutations to DNA (Halliwell and Gutteridge, 2007); which may cause cellular dysfunction and tissue injury (Rotilio et al., 1995).

The antioxidant defense consists of both endogenous and dietary antioxidants such as glutathione (GSH), antioxidant enzymes, metal-binding proteins and some vitamins (Figure 2) (Halliwell and Gutteridge, 2007). GSH is an endogenously produced tripeptide with an essential role in the cell protection since it participates in ROS and metals binding through the sulfhydryl (SH) group. Moreover, it is involved in enzymatic detoxification reactions for ROS as a cofactor for two main enzymes: glutathione S-transferase (GST) and glutathione peroxidase (GPx) (Figure 3) (Gurer and Ercal, 2000). The regulation of oxidative status by GSH involves its cycling between reduced (GSH) and oxidized (GSSG) forms, and the ratio between both forms (GSH:GSSG ratio) is considered an important indicator of the redox status (Halliwell and Gutteridge, 2007). There are other important endogenous components of the antioxidant defense, the enzymes superoxide dismutase (SOD), catalyzing the dismutation of superoxide $O_2^{\cdot -}$ to H_2O_2, and catalase (CAT) and GPx, both detoxifying H_2O_2, the former directly and the latter by oxidizing GSH (Figure 3) (Gurer and Ercal, 2000).

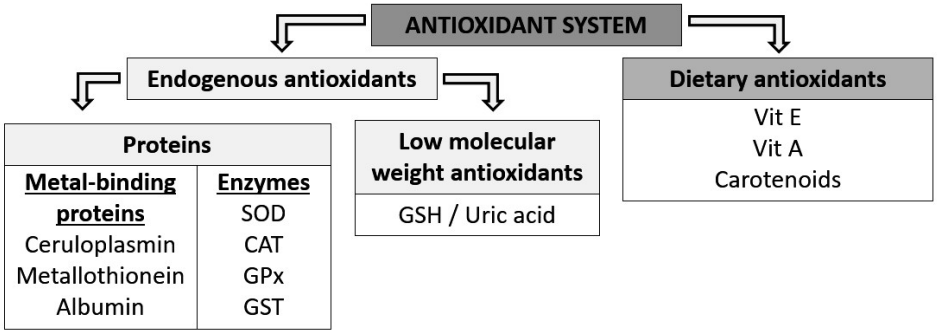

Figure 2. Endogenous and dietary components of the antioxidant system. *GSH: glutathione, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, GST: glutathione-S-transferase, Vit: vitamins.*

Metal-binding proteins (e.g. metallothionein, ceruloplasmin, albumin) are considered part of the antioxidant system since, by chelating metals, ROS generation is decreased (Koivula and Eeva, 2010). In addition, vitamins (E and A) and carotenoids are also considered antioxidants, although they are dietary antioxidants since animals are unable to synthesize them *de novo* (Halliwell and Gutteridge, 2007). Carotenoids have different functions: they are precursors of vitamin A, they are involved in cell proliferation and differentiation, act as pigments in feathers, they play various roles in the immune and endocrine systems, and have been considered as part of the antioxidant system (Eeva et al., 2008; Møller et al., 2000; Surai et al., 2001); however, their role as antioxidants in birds is not clear (e.g. Isaksson and Andersson, 2008). Vitamin E or α-tocopherol is a fat-soluble vitamin considered the main membrane-bound antioxidant in the cell, protecting against lipid peroxidation (Valko et al., 2006).

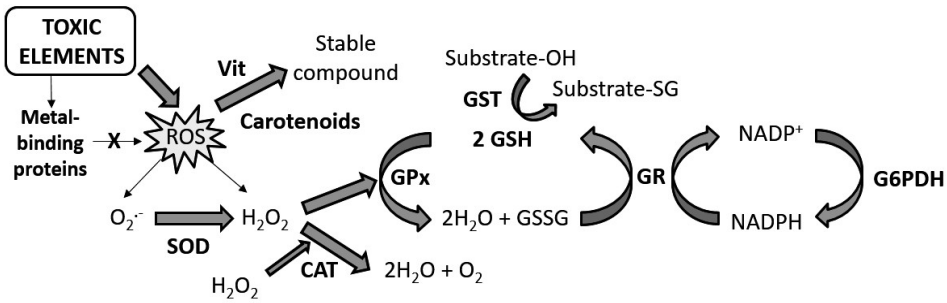

Figure 3. The antioxidant defense system. ROS: reactive oxygen species, *SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, GST: glutathione-S-transferase, GSH: glutathione (reduced form), GSSG: glutathione (oxidized form), GR: glutathione reductase, G6PDH: glucose-6-phosphate dehydrogenase, Vit: vitamins.*

Although some metals (e.g. Cu or zinc, Zn) are essential for various biological processes of cells (Flora et al., 2008), it is known that elements such as As, Pb, Cd and Hg can alter biochemical processes, reproductive performance, behavior, development, and immune response (Burger and Gochfeld, 2000; García-Fernández, 2014; Koivula and Eeva, 2010; Marettová et al., 2015; Sánchez-Virosta et al., 2015; Wolfe et al., 1998). Oxidative stress is precisely one of the mechanisms responsible of As and metal toxicity (Figure 4). This metal-related oxidative stress is induced by: (i) the depletion of main antioxidants of cells, such as glutathione (GSH) or vitamin E; (ii) the alteration of enzymes involved in the antioxidant system such as SOD, CAT, GPx, glutathione reductase (GR), and GST; and (iii) the production of ROS; which may lead to damage in key components of the cells, including lipids, proteins and DNA (Ercal et al., 2001; Flora et al., 2008; Koivula and Eeva, 2010). This process may culminate in damage to liver (hepatotoxicity), kidney (nephrotoxicity), central nervous system (neurotoxicity) and DNA (genotoxicity) (Sharma et al., 2014; Stohs and Bagchi, 1995).

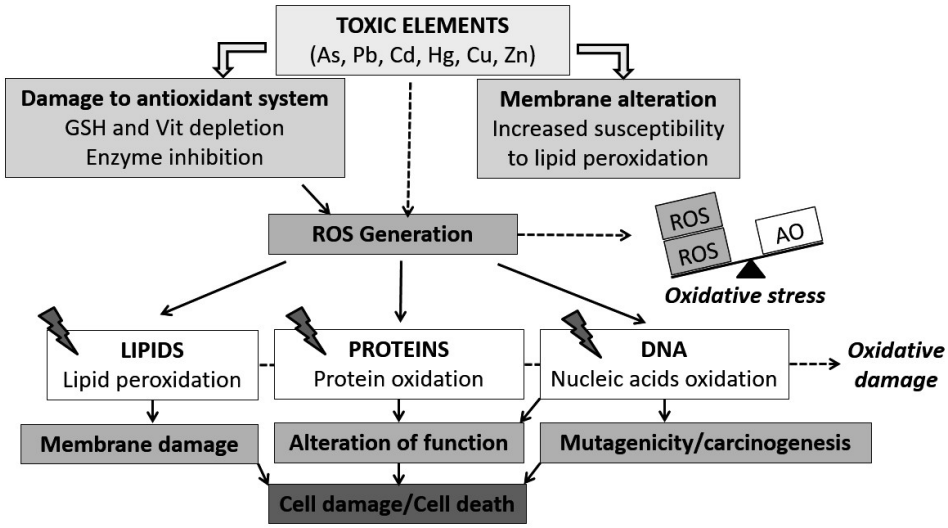

Figure 4. Mechanisms of As/metal-induced oxidative stress and oxidative damage. *GSH: glutathione, Vit: vitamins, ROS: reactive oxygen species, AO: antioxidants.*

1.3. Aims of the thesis

Taking into account the background information available regarding As exposure and effects in passerine birds, the potential of Ca to modulate metal (As)-induced oxidative stress, and the lack of data regarding the effects of As on physiology, growth and survival in free-living passerines, the following three aims were set for this thesis:

(i) The first aim of the thesis was to collect and discuss available information on As-related research in passerine birds. For this purpose, we prepared a

database containing As concentrations in different matrix types of passerines, such as feces, eggs, feathers, blood and internal tissues, and the As-related effects in humans, laboratory animal, and passerines and other bird species. All this data is compiled and discussed in a literature review (see review article I, Sánchez-Virosta et al., 2015).

(ii) The second aim was to explore the effects of Ca availability on oxidative stress biomarkers in great tit (*Parus major*) nestlings in relation to As and metal exposure. To do so, we designed a Ca-manipulation experiment (breeding season of 2014) in a metal/As-polluted and a control zone in Finland, evaluating metal and As exposure, Ca levels in the organism and a set of blood antioxidant molecules and oxidative damage biomarkers. We studied the effect of Ca consumption on the antioxidant status and its potential protection against metal toxicity. Additionally, we assessed how the nestling growth and some breeding parameters were affected by the antioxidant status. Our main hypothesis was that nestlings inhabiting the metal-polluted environment would show higher antioxidant and oxidative damage levels due to higher oxidative challenge compared to the unpolluted/control zone, and the oxidative effect would be reduced in the Ca-supplemented nestlings (see article II, Sánchez-Virosta et al., 2019).

(iii) The third aim of this thesis was to investigate if environmentally relevant As levels affect physiology, growth and survival of great tit nestlings. For this purpose, we performed a dietary As manipulation experiment (breeding season of 2015) in wild great tit nestlings inhabiting a metal/As-polluted and control zone in Finland, and they were measured in terms of fecal As concentrations, breeding and growth parameters, and blood physiological biomarkers (vitamins, carotenoids, oxidative stress, and other biochemical parameters). In order to study As accumulation, dead nestlings were necropsied and As concentrations were measured in liver, bone and feathers. Based on the physiological, developmental and reproductive effects reported in As-manipulation experiments in other organisms, we hypothesized that As would alter antioxidant levels, produce oxidative damage to the main biomolecules, and would decrease nestling growth and survival (see article III, Sánchez-Virosta et al., 2018, and manuscript IV).

2. MATERIAL AND METHODS

2.1. Literature review (article I)

A literature review was prepared to compile, provide and discuss available data on As-related research in passerine birds. An extensive search of the literature available was done using databases (PubMed, Springer, Science Direct and Web of Science). In addition, the bibliography list of each article was scanned to identify documents that had been missed during the search in databases. The following keywords and combinations of terms were used: `arsenic´, `trace element´, `metalloid´, `heavy metals´, `pollution´, `songbird´, `passerine´, `bird´, `great tit´, `parus major´, `pied flycatcher´, `effects´, `oxidative stress´, `status´, `health´, `reproductive´, `breeding´, `condition´, `performance´, `success´, `survival´, `egg´, `feathers´, `excrement´, `feces´ and `tissues´. In order to look for project reports and documents that are not found in the main databases, google searches were also done.

2.2. Study area and species (articles II and III, and manuscript IV)

This thesis is focused on the copper-nickel (Cu-Ni) smelter of Harjavalta in the Satakunta region, Western Finland (Figure 5). The smelter (61°20'N, 22°10'E) was built in 1944. All the toxic metals and sulphuric oxides were emitted into the surroundings without filtering. Later, most of the sulphuric oxides were filtered and dust emissions decreased (Eeva et al., 2006). However, elevated metal (mainly Cu, Ni, Pb, As, Zn) concentrations still occur in the area because of current and long-term deposition (Rainio et al., 2013). Ample background information is available on the breeding bird populations in this area (Eeva and Lehikoinen, 2013, 2015; Sanderfoot and Holloway, 2017).

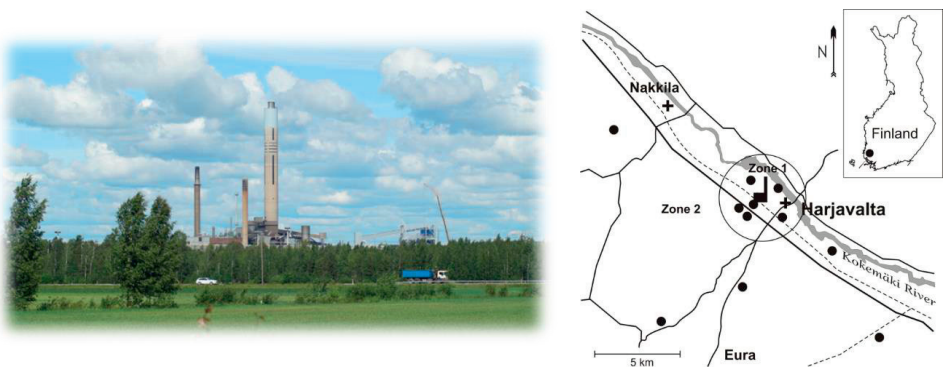

Figure 5. Picture of the copper-nickel smelter in Harjavalta (Finland) and map of the study area, showing the study sites around the smelter (in the middle). Sites within the circle (radius of 2.5 km) are considered highly polluted.

Some passerine birds (e.g. great tit) are considered good bioindicators of metal pollution because they fulfill different criteria: (i) they are ubiquitous, living in different habitats and frequently in high densities; (ii) they are resident in many populations and are territorial during breeding, and forage in small home ranges, so they reflect local contamination; (iii) they are mainly insectivorous during the breeding season and they are situated high in the food web; and (iv) they readily nest in human-made nest boxes, so they are easily monitored (Figure 6). As well, ecological and behavioral information on some passerine species is abundant (Cramp and Perrins, 1993; Dauwe et al., 2000; Eens et al., 1999). All these characteristics and the fact that most studies evaluating As exposure and effects in passerines have been done in great tit (Sánchez-Virosta et al., 2015), led us to believe that great tit could be a good model species to perform this thesis.

Figure 6. Monitoring nest boxes and 14-day old great tit nestling.

2.3. Calcium and arsenic experiments (articles II and III, and manuscript IV)

During the breeding seasons of 2014 and 2015, we developed a Ca and As manipulation experiment in great tit, respectively (Figure 7). There are ca. 500 nest boxes in the study area that we regularly monitored to record basic breeding parameters, including laying date of the first egg, clutch size, hatching date and fledging number.

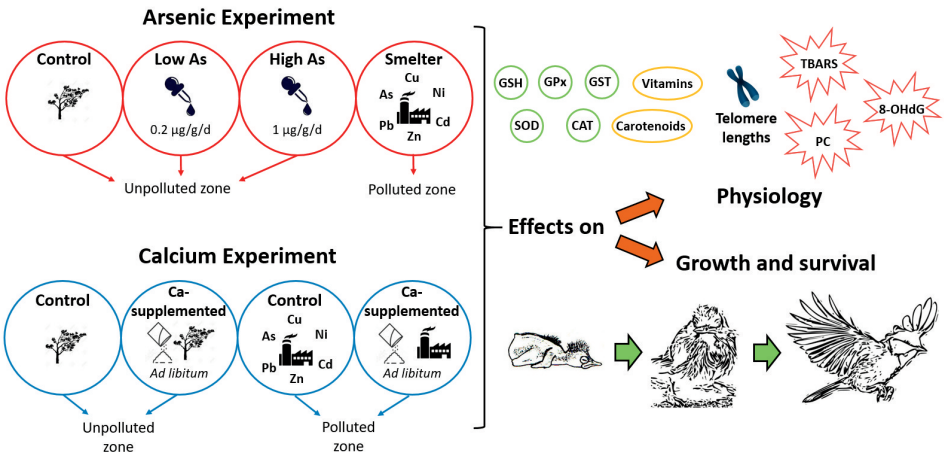

Figure 7. Scheme of the As and Ca experiments performed in wild great tit nestlings.

During the Ca experiment in 2014, we randomly assigned nests to the Ca-supplemented group (*ad libitum* Ca supplementation in feeders) or to the control group, while during the As experiment in 2015 we assigned nests to different doses of As (1 µg g^{-1} d^{-1} or 0.2 µg g^{-1} $d^{-1}$of liquid sodium arsenite orally dosed with pipette) or control treatment (Figure 8). Different criteria were evaluated to select the As dosing levels (see Supplementary material, document S2, in Sánchez-Virosta et al., 2018). Some nests were located in the vicinity of the smelter, where there is a long-term exposure of toxic metals, while other were located in the unpolluted zone (Figure 5 and 7). Licenses from the Centre for Economic Development, Transport and the Environment, ELY Centre Southwest Finland (VARELY/319/07.01/2014 and VARELY/593/2015) and the Animal Experiment Committee of the State Provincial Office of Southern Finland (ESAVI/1650/04.10.03/2012 and ESAVI/11579/04.10.07/2014) were obtained to perform the experiments.

Figure 8. Ca-supplemented nest (*ad libitum* Ca supplementation in feeder) and control nest (empty feeder) during the Ca experiment in 2014, and dosing of a great tit nestling during the As experiment in 2015.

2.4. Sampling and measurements (articles II and III, and manuscript IV)

On day 7 after hatching (d7), nestlings were ringed and combined excrements of several siblings from the same brood were collected (on d7 in the Ca experiment and d8 in the As experiment) and stored at −20°C for metal analysis. On d7/d8 and d14, nestlings were weighed using a Pesola spring balance to the nearest 0.1 g. The wing length was measured with a precision of 0.5 mm using a ruler, and total head (bill+head) and tarsus lengths were measured with a digital caliper to the nearest 0.01 mm (Figure 9). On d9 (Ca experiment)/d8 (As experiment) and d14, ca. 75 µl of blood was collected by venipuncture of the brachial vein using a needle and sodium-heparinized capillary tubes (80 iu/ml, Marienfeld) (Figure 9). Blood was centrifuged (4400 g, 5 min) in the field and hematocrit (HCT, % of red blood cells, RBC, from total sample volume) was measured. Plasma and RBC were split in different tubes and kept in liquid nitrogen and then stored at −80°C in the lab. These samples were typically collected from four nestlings per brood. On d9/d8, plasma was pooled by brood for vitamin and carotenoid analysis, while plasma from d14 was collected individually for biochemical analysis (Ca, uric acid, UA, creatine kinase, CK and alkaline phosphatase, ALP). Red cells, collected at d14 from two randomly selected nestlings per brood, were used to measure antioxidants and oxidative damage biomarkers. On d14, an additional small blood sample was collected using non-heparinized microhematocrit capillary tubes (one nestling per brood) for DNA-related analyses in the As experiment. No heparin was used in those samples since it may interfere with PCR analysis (Espín et al., 2016a).

Figure 9. Measuring great tit (7-day old nestling) and collecting blood samples (14-day old nestling).

Dead nestlings found in the nests were collected and frozen at −20°C until necropsies could be performed. During necropsies, we took liver, bone and feathers for As and metal determination.

2.5. Laboratory analyses (articles II and III, and manuscript IV)

Feces from d7/d8 nestlings, and liver, bone and feathers from dead nestlings in the As experiment, were dried at 45 °C for 72 h. Fecal samples were combined per brood, while

liver, bones and feathers were measured individually. Feces, liver and bones were ground into a fine powder using a tissue lyzer. Arsenic and other element concentrations (Ca, Cu, Ni, Pb, Cd, Zn, and selenium, Se) were determined using an inductively coupled plasma optical emission spectrometer (ICP-OES). The quantification limit was 1 ppm for Ca and 0.01 ppm for the other elements. The samples were submitted to a microwave acid digestion. Samples were placed in digestion tubes with 5 ml of a mixture of HNO_3 (70%) and H_2O_2 (33%) 4:1. The digested samples were then diluted in ultrapure water. Total Hg concentration in feces (from the Ca experiment) was measured by atomic absorption spectrophotometry using a Milestone Direct Mercury Analyzer DMA-80. The limit of detection was 0.005 ng. Precision and accuracy of the methods were tested by analyzing certified reference material (CRM) (TORT-2, lobster hepatopancreas reference material for trace metals; National Research Council Canada). Recovery of metals ranged 77-118%. Metal concentrations were expressed in dry weight (d.w.).
The vitamin analysis was based on the technique described by Priego Capote et al. (2007). Briefly, 90 µl of plasma was vortex-mixed with 1 ml methanol, centrifuged (4500 rpm, 5 min, 4°C) and the supernatant was transferred into a new tube. The vitamins were extracted with n-hexane three times. The solvent was evaporated and the extract was reconstituted with 75 µl of methanol, vortex-mixed and filtered. The vitamin composition of the extracts was analyzed by ultra-performance liquid chromatography tandem-mass spectrometry (UPLC-MS/MS) on the Acquity UPLC system (Waters Corp., Milford, MA, USA), interfaced to a Xevo TQ triple-quadrupole mass spectrometer with electrospray ionization (ESI) (Waters Corp., Milford, MA, USA). Carotenoids were measured at 450 nm by UPLC with diode array detection (DAD).

The enzyme activities of ALP and CK, and the plasma constituents UA and Ca were measured using a microplate reader (EnSpire, Perkin-Elmer). A reduction of reagent volumes was required as compared to the method instructions of the commercial kits from BioSystems S.A. Antioxidants (tGSH, GSH:GSSG ratio, SOD, CAT, GST, GPx), thiobarbituric acid reactive substances (TBARS) and protein carbonylation (PC) were measured from RBC. Red blood cells were diluted with 0.9% NaCl. The measurement of proteins (mg/ml) was done with the BioRad protein assay (BioRad, Espoo, Finland) and bovine serum albumin (Sigma, USA) was used as a standard according to the Bradford method (Bradford, 1976), using a microplate reader (Rainio et al., 2015). The activities of GPx, CAT and GST were measured using Sigma kits (Sigma Chemicals, St. Louis, USA), and a Fluka kit was used for SOD activity (Fluka, Buchs, Germany; Rainio et al., 2015). Catalase, GST and GPx activities are expressed in µmol min^{-1} mg^{-1} of protein, while SOD is referred to inhibition percentage. The ThioStar® glutathione detection reagent (Arbor Assays, Michigan, USA) was used to measure tGSH (nmol/mg), as well as the ratio of reduced to oxidized glutathione (GSH:GSSG ratio; Rainio et al., 2015). Reduced glutathione was used as standard (Sigma Chemicals, Missouri, USA). Protein carbonylation was measured with a colorimetric assay and results are expressed as nmol/mg of protein (Rainio et al., 2015). Thiobarbituric acid reactive substances were analyzed fluorometrically following the technique described by Espín et al. (2017b) to estimate lipid peroxidation expressed as

nmol/mg. Antioxidants, TBARS and PC were analyzed using a microplate reader (each method was done either with EnSpire or Envision, Perkin-Elmer). All samples were measured in triplicates (intra-assay coefficient of variability < 15%). To minimize the sample volume needed, 96- or 384-well microplates were used, reducing reagent volumes accordingly as required. Inter-assay variation was corrected using three control samples with the ratio specific to each particular plate (range 0.8-1.2).

Total genomic DNA from blood was extracted using salt extraction method (Aljanabi and Martinez, 1997) to measure relative telomere length and 8-hydroxy-2'-deoxyguanosine (8-OHdG). Relative telomere length was measured using a real-time polymerase chain reaction (qPCR) method adapted to great tit (Stauffer et al., 2017). DNA damage was assessed through the measurement of 8-OHdG in whole blood using the DetectX® DNA Damage ELISA kit (Arbor Assays). Prior to DNA damage measurement, extracted DNA was normalized to 5 µg/75 µL and digested with Nuclease P1 (Sigma-Aldrich) and alkaline phosphatase (NEB) as described in Rattner et al. (2013). Absorbance was read on a FluoroStar Omega (BMG LabTech, Cary, NC).

2.6. Statistical analyses (articles II and III, and manuscript IV)

Statistical analyses were carried out using the statistical package SAS 9.4. Linear mixed models (LMMs) were run to test the effect of the experiments on the different response variables: survival and growth parameters, biochemical parameters, vitamins/carotenoids and oxidative stress biomarkers. Tukey's test (Ca experiment) or Dunnett's test (As experiment) were used to make pairwise comparisons between treatment groups or between treatment groups with the control. The site was included as a random factor in the models.

In a second set of LMMs, the effects of Ca levels in plasma (Ca experiment) or the As levels in feces (As experiment) and fecal metal concentrations (as a proxy of dietary exposure) on the response variables were also evaluated, including hatching date and brood size at the age of three days as variables, and sampling site as a random factor. In order to reduce the number of inter-correlated explanatory variables, Principal Components Analysis (PCA) was carried out for metals after checking that their levels in feces were positively correlated. For all the parameters individually measured, the mean value per brood was considered in the models because of the non-independence of measurements from nestlings of the same brood and because not all parameters could always be measured from the same nestlings. Metal concentrations below the detection limit were substituted with a value equal to limit of quantitation (LOQ)/√2.

The residuals of the model were used to check the normality of variables and variables were $\log_e$-transformed for normalization when needed. The Kenward-Roger method was used to adjust the degrees of freedom according to the recommendation of Littell et al. (2006). Explanatory variables were retained when statistically significant ($p < 0.05$), while

non-significant variables were dropped one by one from the model starting from interactions.

For the Ca experiment, we also analyzed associations of the biomarkers of oxidative status with growth and size (LMMs), number of fledglings (generalized linear mixed model, GLMM, using Poisson error distribution) and probability of a hatchling to fledge (fledging success; GLMM using binary error distribution).

Pearson (r_p) or Spearman's (r_s) correlation coefficients were studied to check the correlations between response variables depending on the normality of data (checked by the Kolmogorov-Smirnov test). These correlations were mainly tested to inspect associations among our variables before formulating more sophisticated models. Therefore, Bonferroni adjustment for multiple testing was not applied since this approach may be too conservative and important biological knowledge can be missed. The level of significance was set at $p < 0.05$.

3. MAIN RESULTS AND DISCUSSION

3.1. Review on exposure and effects of As on passerine birds (article I)

The literature review presents a broad overview of the current status on As exposure and effects in birds, and especially in passerine birds, as a result of a thorough search of the publications available. Regarding the type of samples used for As measurement in passerines, internal tissues were the most frequently analyzed sample types (37.5% of the reviewed literature used internal tissues), followed by feathers and eggs or eggshells (32.5% each), feces (27.5%), and blood (15%). However, the tendency to the use of non-destructive matrices is evident in recent years. The suitability of the different matrix types, the information that each sample may provide and the As concentrations reported in the available literature are discussed in the review (see review article I, Sánchez-Virosta et al., 2015). In this sense, more studies are needed to evaluate potential differences in the As excretion efficiency by different avian species, so that fecal concentrations can be correctly interpreted. Similarly, further research should be performed to assess the effect of As external contamination in the surface of the feathers and the best washing method required; nestling feathers or recently grown feathers from adult individuals are recommended to reduce the effect of the external contamination. When available, internal tissues (i.e. liver, kidney, brain and muscle) are the best samples to determine As exposure and effects, while blood As levels show the most recent dietary exposure. Some authors have found that As is transferred into the eggshell. Additional studies are needed to evaluate if the structure and functioning of the eggshell could be affected by As exposure when formed, even if As is not transferred into the shell material.

Ecological factors, such as diet/trophic level and migratory status, are important sources of variation for differences in As exposure between species, which may differ in absorption, accumulation, metabolization and excretion capacity, and in resistance to As-related effects. Therefore, more studies on adult passerines would provide interesting additional data to evaluate potential differences between species. Further research is needed to evaluate if intraspecific factors (e.g. age, sex) affect As levels in different tissues.

Most studies on As concentrations in passerine birds have been done in great tit (50%) and pied flycatcher (*Ficedula hypoleuca,* 22.5%). Regarding the geographical ranges studied, most publications have been done in the United States (30%), Belgium (22.5%) and Finland (20%), while the lack of information in some countries is noticeable. Thus, additional studies are recommended to fill the data gap, particularly in the southern hemisphere.

Oxidative stress is one of the main mechanisms involved in As toxicity, and suitable biomarkers related to the antioxidant system and oxidative damage in passerines should be evaluated. Multiple effects on different organ systems have been found in humans and laboratory animals exposed to different forms of As by different routes. In experimental

studies on avian species, some authors have found reproductive, developmental, behavioral, biochemical and hepatic effects; but little information is available on the As-related effects on passerines in the field.

There are very few field studies on As exposure and effects in passerine birds and all of them are correlative so far. Experiments providing As to passerines at levels comparable to those happening in the environment are also needed to investigate the effects of As on physiology, growth and survival in free-living populations.

3.2. Effect of Ca supplementation on oxidative stress biomarkers in great tit nestlings inhabiting a metal-polluted area (article II)

In the Ca supplementation experiment, we found few negative effects of metal exposure on the oxidative stress biomarkers of great tit nestlings, mainly due to the relatively low metal levels observed (Berglund et al., 2012; Espín et al., 2016b). The activities of SOD and GPx (not statistically significant) were higher in the polluted area, probably because of a reaction of the organism to face the oxidative challenge (i.e. higher metal exposure, lower food quantity and quality, and consequent smaller nestling size and number of fledglings; Espín et al., 2016b).

The oxidative stress biomarkers evaluated showed a limited response to the Ca treatment, probably because nestlings received enough natural Ca-rich food items to achieve a proper antioxidant status. Similar results were found for growth, survival, plasma biochemistry and yolk and plasma vitamin levels in other publications from the same experiment (Espín et al., 2016b, 2016c; Ruiz et al., 2017). Only the CAT showed higher activity in Ca-supplemented nestlings. Higher Ca levels stimulate respiratory chain activity and lead to ROS generation as by-product (Görlach et al., 2015). Thus, increased levels of Ca could enhance the mitochondrial Ca cycling inducing ROS formation and stimulating CAT activity to scavenge ROS. In this sense, global CAT activity and plasma Ca concentrations were close to being positively associated. This association is especially evident in the polluted area (CAT activity and plasma Ca levels were positively correlated), where nestlings face an increased oxidative challenge.

In spite of the very limited effects of the Ca availability on the antioxidant status, blood antioxidant levels (tGSH levels, GSH:GSSG ratio, CAT, GPx and GST activities) changed over the range of metal concentrations depending on the Ca concentrations in plasma, suggesting that higher Ca levels stimulate antioxidants mitigating the effects of metals. Enhanced antioxidant levels were found with increasing fecal metal concentrations when plasma Ca levels were higher than ca. 10-14 mg/dl. This suggests that birds are able to enhance their antioxidant capacity to cope with metal exposure when Ca in plasma is adequate. However, decreased antioxidant levels were observed with increasing metals in feces when plasma Ca levels were lower than ca. 10-14 mg/dl, suggesting that metals are prone to inhibit the antioxidant system when Ca concentrations in plasma are low. An influence of dietary Ca on intestinal absorption could also partly explain these results,

since low Ca intake could lead to slightly higher metal absorption (Diamond et al., 1997), contributing to the partial consumption of the antioxidant defenses without reaching levels leading to oxidative stress. However, metal concentrations in feces did not significantly differ between Ca-supplemented and control nestlings, so the effect of Ca supplementation on metal absorption or excretion cannot be proved. Thus, adequate Ca levels in the organism possibly protect great tit nestlings against metal-induced oxidative stress by enhancing antioxidant capacity and/or reducing metal absorption (Jamakala and Rani, 2012; Prasanthi et al., 2010; Srivastava et al., 2010).

3.3. Experimental manipulation of dietary As levels in great tit nestlings: accumulation pattern and effects on growth, survival, plasma biochemistry and oxidative stress (article III and manuscript IV)

In the As manipulation experiment, the As accumulation pattern in liver, bone and feathers from dead nestlings showed that we successfully achieved relevant concentrations measured in polluted environments (Sánchez-Virosta et al., 2015). The experiment supports nestling feathers of passerines as an excellent sample type for As monitoring, as As external contamination should be negligible in the first plumage, not affected by migration or molting (Sánchez-Virosta et al., 2015). Therefore, prediction equations are provided to estimate As concentrations in liver and bone using the As concentrations in wing feathers from great tit nestlings. Additional studies would be needed to prove if these equations can be used for other bird species.

Nestlings from the High As group reached fecal As levels that could represent concentrations found in polluted sites, while nestlings in the Control group had levels similar to those found in unpolluted sites (Sánchez-Virosta et al., 2015). Interestingly, concentrations found in the Smelter group were significantly higher than those observed in nestlings in the Control and Low As groups. Feces represent non-absorbed As and As absorbed and excreted (Sánchez-Virosta et al., 2015), and possibly As is better absorbed from sodium arsenite provided in this experiment that from prey, which would result in higher fecal As from excretion in the Smelter group as shown in this study (Sánchez-Virosta et al., 2018). Moreover, in the polluted area there is constant exposure by polluted prey, while in the experiment As was dosed just once a day and 24 h before the fecal sampling (Sánchez-Virosta et al., 2018).

The As treatment had limited effects on growth, survival and biochemistry. Nestlings dosed with As showed slower wing growth rate compared to Control nestlings, only significant for the Low As group. An interaction between As and the mineral fraction of the bone, most likely with As substituting phosphate in the hydroxyapatite (Kretshmer et al., 2002), could explain that result. Therefore, free-living birds exposed to comparable doses could suffer skeletal growth problems, as it was observed in the beginning of the 1990's in pied flycatcher nestlings in the areas close to the Cu-Ni smelter (Eeva and Lehikoinen, 1996), which could have long-term consequences such as increased predation

risk after fledging. In addition, broods in the High As group showed lower number of fledglings per successful nest compared to the Low As group.

Some studies have reported hemolysis and reduced hematocrit in experimental animals exposed to As (Antonio-García et al., 2013; Hong et al., 1989). However, other authors found increased hematocrit after sodium arsenite administration in rats (Mitchell et al., 2000). Similarly, our As experiment showed that As concentrations in feces were positively related to hematocrit. This result could suggest a hormetic effect of As, in which exposure to relatively low doses of As would stimulate erythropoiesis, increasing hematocrit levels as a protective effect, but at certain As exposure, the hemolytic effect and consequent anemia would appear. In line with this, sodium arsenite has induced the expression of a mitochondrial porphyrin transporter essential for heme biosynthesis, ABCB6 (Krishnamurthy et al., 2006), in cells *in vitro* and in mice, which might be indicative of a hormetic mechanism triggered to protect cells against As-related oxidative stress (Chavan et al., 2011).

The typically higher plasma vitamin K1 concentrations in the Smelter group cannot be explained solely by metals, but maybe by a different diet composition (Basset et al., 2016; Eeva et al., 2005b) or a secondary effect of metals on the quality and quantity of the diet items, resulting in a mechanism to enhance vitamin K1 absorption. Additional studies on vitamin K1 levels in birds would be of interest to better understand the As/metal-related effects on its metabolism.

Arsenic treatment had no significant effect on most of the oxidative stress biomarkers measured in great tit nestlings: only the CAT activity was lower in the High As group and the GPx activity was enhanced in the Smelter group compared to the Control. Arsenic is known to produce H_2O_2 leading to cell damage and death due to the activation of oxidative sensitive signaling pathways (Flora et al., 2008). Thus, the lower CAT in the High As group may be related to its function catalyzing H_2O_2 to H_2O and oxygen (Koivula and Eeva, 2010). Moreover, recent results from other researchers seem to indicate that As might regulate the expression levels of CAT, since CAT is decreased in resistant cells (chronically exposed to an oxidant injury) incubated with As probably by a decreased transcriptional activity of the CAT promoter (Glorieux and Calderon, 2018). In light of the associations found in article II (Sánchez-Virosta et al., 2019) where significantly higher CAT activity was found in Ca-supplemented nestlings, and CAT activity and plasma Ca concentrations were positively correlated in the polluted area, the protective role of Ca against As-specific toxicity must be more thoroughly assessed in future studies.

Regarding the higher GPx activity in the Smelter group, as explained in the Ca experiment, it is likely a physiological reaction to the enhanced oxidative challenge due to the combination of increased As/metal exposure and poorer condition of birds growing in the polluted area (Espín et al., 2016b; Rainio et al., 2013).

Although no other statistically significant effects were found, nestlings tended to have higher levels of antioxidants and oxidative damage biomarkers (TBARS, PC, 8-OHdG and

relative telomere length) in the High As group and in the Smelter group compared to the Control. A more active antioxidant system would balance the increased ROS production and would protect against its effects on biomolecules in avian species exposed to As and metals (Espín et al., 2014; Martínez-Haro et al., 2011; Rainio et al., 2013). The inconsistency in the results for antioxidant molecules makes these biomarkers difficult to interpret. A combination of dose and time of exposure could be the key factor to explain the discrepancies in the literature (Espín et al., 2014; García-Fernández et al., 2002; Rana et al., 2012).

Lipid peroxides, protein carbonyls and 8-OHdG formation have been described as mechanisms of As toxicity, and elevated levels of these biomarkers, although not statistically higher, may suggest failure of the redox-defense system to prevent oxidative damage (Flora et al., 2008; Fujihara et al., 2009; Kharroubi et al., 2014; Ramos et al., 1995; Rana et al., 2012). Different researchers have shown that As up-regulated telomerase activity and elongated telomere length, which was also associated with increased cell proliferation in both in vivo and in vitro studies with human cells (Gao et al., 2015; Li et al., 2012; Zhang et al., 2003). Thus, the tendency found in the present study may be due to an As-related telomere elongation.

4. CONCLUSIONS

This thesis provides an extensive overview of As exposure and effects in birds, especially in passerines. Non-destructive matrices are increasingly used in the last years for As determination in passerines, and results support both feathers and excrements as good sample types for As monitoring. Further research is recommended to assess some potential constraints regarding those sample types (e.g. differences in As excretion efficiency by different species, As external contamination in feathers surface and the best washing method). Diet and migratory status are essential factors affecting As exposure between species, while intraspecific factors such as age or gender should be further studied to evaluate if they significantly affect As concentrations in different tissues. Multiple adverse effects on different organ systems have been reported on laboratory animals, humans and birds when they are exposed to As in its different forms (e.g. reproductive, developmental, behavioral, biochemical and oxidative status effects), and oxidative stress is proposed as one of the main mechanisms associated with As toxicity. The literature review pointed out that few studies have been done evaluating As exposure and effects in wild passerine birds, all of them correlative so far.

In the frame of this thesis, two unique field experiments providing Ca and As to wild great tit nestlings were performed, evaluating their effects on growth, survival and a wide range of physiological biomarkers. These experiments respond to the call of recent reviews encouraging further research investigating the impacts of urban and/or polluted environments on physiological traits (e.g. telomere dynamics, antioxidants and oxidative damage) in birds (Sánchez-Virosta et al., 2015; Sepp et al., 2018). It is worthwhile to emphasize the efforts of our research team to carry out these laborious experiments in a wide spatial scale and how valuable the data gathered is. Unlike laboratory experiments, field experiments include in their assessment the complexity of the countless factors wild animals have to cope with in nature. On the other hand, these experiments are a great tool to study in depth the individual effects of certain substances on birds as compared with correlative studies.

The Ca-supplementation experiment in great tit nestlings in a metal/As-polluted and a control zone in Finland, showed that nestling antioxidant capacity was not restrained by a deficiency in Ca availability in the studied population. Calcium levels in the organism are decisive in the ability of the individual to cope with metal oxidative stress (i.e. metals inhibit the antioxidant system when plasma Ca levels were below ca. 10-14 mg/dl, while enhanced antioxidant capacity to cope with higher metal exposure was observed when plasma Ca concentrations were higher than ca. 10-14 mg/dl). Therefore, future studies should consider Ca availability when assessing metal exposure and effects on free-living bird populations.

Great tit nestlings receiving environmentally relevant doses of sodium arsenite through the diet accumulate As in liver, bone and feathers in a dose-dependent manner. Nestlings in the High As and Control groups achieved fecal As levels that could represent

concentrations found in polluted and unpolluted sites, respectively. Broods in the High As group produced fewer fledglings per successful nest, while nestlings in the Low As group showed slower wing growth, which could increase post-fledging fitness costs. However, we did not find a dose-response relationship for this endpoint, and further research would be needed to support an effect on the wing growth.

In spite of the clear As accumulation produced by the experiment, limited effects on the plasma biochemistry and the RBC oxidative stress biomarkers were found. The dose and duration of the As exposure was not enough to induce oxidative damage in RBC of great tit nestlings. In spite of this, nestlings dosed with 1 μg g^{-1} d^{-1} of sodium arsenite showed non-significantly increased oxidative stress biomarker values compared to controls, suggesting that the exposure was close to the level altering reduction-oxidation balance. Oxidative effects at equivalent As levels combined with other stressors (e.g. other contaminants, food restrictions, low Ca availability, bad weather conditions, diseases) cannot be discarded.

Altogether, this thesis compiles the existing pool of knowledge regarding As exposure and effects in passerines, and it identifies and fills the main gaps, i.e. elucidates the limited physiological and developmental effects of environmentally relevant As levels on wild great tits, and the role of Ca as modulator of As and metal-related oxidative stress.

ACKNOWLEDGEMENTS

It's been fun. I wouldn't change it for anything, but it's also been tough at times. In any case, I have made it, so it's time to thank all those who made it possible.

First, I would like to thank my supervisor, Tapio Eeva. I feel so lucky Silvia read one of your articles and got the idea to contact you for a short stay for her thesis. How could I know that paper would change my life so deeply? You have been the best supervisor I could have had. You never said no when I came unannounced to your office with the silliest or the most complex question or idea. Besides, you were an impressive source of knowledge on everything related with birds or the study area. Thanks also for doing all you could to make our stay in your country so pleasant and for sharing with us your experience in Harjavalta. You made me fall in love with those never-ending working days with our little chicks.

Antonio J. García Fernández, my supervisor in Spain, has also been an essential support for me during these years. From its early planning and until the last day, you have been a massive source of toxicological knowledge for my development as a scientist. I know these have been extremely busy years for you, but you have always been there when I needed you. I also thank you for taking me into the impressive group you created more than two decades ago, and especially for having helped Silvia become the amazing scientist she is today. It's great to be part of it.

Thanks to the external reviewers of this thesis, Caroline Isaksson and Kim Fernie and to the opponent, Miguel Mora. I would also like to acknowledge all the anonymous reviewers that helped me improve my publications.

This thesis was supported by the Academy of Finland, University of Turku Graduate School, *Turun Yliopistosäätiö*, BGG travel grants, *Societas pro Fauna et Flora Fennica*, *Fundación Séneca*, and the U.S. Geological Survey. Thanks to all of them.

Thanks to Kai Norrdahl, my research director and Jon Brommer, the custos. Also to Jorma Nurmi, Miia Rainio, Sandra Ruiz, Lucy Winder and Hanna Holmström for their collaboration during the field work seasons, and Mirella Kanerva, Janina Stauffer, Mikko Nikinmaa, Kristiina Vuori, Juha-Pekka Salminen, Anne Koivuniemi, Bineet Panda, Petteri Ilmonen, Tuija Koivisto and Nina Vuori for their valuable help with the lab analyses.

I'm also grateful to other people in the Faculty and in Turku for their friendship and for sharing coffees and discussions: Barbara, Fabio, Lisa, Bea, Tuuli-Marjana, Suvi, Tom, Chiara, Julien, Sari, Matti, Kalle, Robert and Lidia.

Special thanks to Natalie Karouna-Renier and Sandy Schultz not only for letting me join their lab at the Patuxent Wildlife Research Center and for the much-appreciated opportunity to make the DNA damage measurements, but also for their warm welcome. You made me feel like at home. I am looking forward to collaborating with you again in

the future. Thanks also to Barnett Rattner, Tom Bean, Paula Henry, Jan-Michael Archer and Judit Smits.

Along these years, my Spanish friends and colleagues have made this journey really enjoyable. Thanks to all of you. The toxicology group at the University of Murcia: Pedro Jiménez, Emma, Pilar, Pedro María, Isabel, Pepe, Laura, Mari Paz, Aída, Alejandro, Silvia Fernández, Alessandro, Irene, Gabriel, Eduardo, María Yanneth, José Antonio, Carmen; also Mario and José Manuel (los Chotis), José Enrique, Rafa Mateo, Manuel Ortiz, Piet, Emi, Albert, Ana, Jose, Chus, Elena, Tomás, Rosa, Juli and Isaac. Selene, Simba, Luca, Audrey and Shiva, I owe you a big thank you. My love for animals has only grown by having you in my life.

Kiitos Suomi ja erityisesti Turku. Olette sydämessäni nyt ja aina. Thanks also to the great tits and the other species that we have messed with during these years. Perhaps you don't understand it, but I love you.

The Pelle (aka José Enrique Cano Bernal) deserves a special mention here. Our years in Finland would have been so different without you. I'm not saying worse, I'm saying different. I am very happy to have had the chance to become so close friends with you.

My family has always supported me. They have made this possible making the strongest efforts with a kid or a young man that wasn't always as responsible as he should. I think you are proud of me and that, together with your love, is all I could ask for. I love you and thank you all, the Sánchez and the Virostas but very especially mamá, papá, Álvaro, Daniel, Celia, Sara, Pablo and Mercedes. My in-laws are also acknowledged for letting me be a part of their family.

Finally, the biggest thank you in the world goes to my wife, Silvia. You are my life's supervisor, and have taught me more than anyone ever, not only in Science, but in life. I admire you in every single sense. There are no words to express how important you are for me and how much I love you. With you, I feel the luckiest man on Earth. *TQTQC*.

REFERENCES

Abbasi, N.A., Malik, R.N., Frantz, A., and Jaspers, V.L.B. (2016). A review on current knowledge and future prospects of organohalogen contaminants (OHCs) in Asian birds. Sci. Total Environ. *542*, 411–426.

Abdel-Hameid, N.-A.H. (2009). A Protective Effect of Calcium Carbonate Against Arsenic Toxicity of the Nile Catfish, *Clarias gariepinus*. Turk. J. Fish. Aquat. Sci. *9*, 191–200.

Aljanabi, S.M., and Martnez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res. *25*, 4692–4693.

Alonso-Álvarez, C., Bertrand, S., Faivre, B., and Sorci, G. (2007). Increased susceptibility to oxidative damage as a cost of accelerated somatic growth in zebra finches. Funct. Ecol. *21*, 873–879.

Antonio-García, M.T., Herrera-Dueñas, A., and Pineda-Pampliega, J. (2013). Hematological effects of arsenic in rats after subchronical exposure during pregnancy and lactation: the protective role of antioxidants. Exp. Toxicol. Pathol. Off. J. Ges. Toxikol. Pathol. *65*, 609–614.

ATSDR (2007). Toxicological profile for arsenic (Agency for Toxic Substances and Disease Registry, U.S. Public Health Service).

ATSDR (2017). The ATSDR 2017 Substance Priority List (Agency for Toxic Substances and Disease Registry, U.S. Public Health Service).

Basset, G.J., Latimer, S., Fatihi, A., Soubeyrand, E., and Block, A. (2016). Phylloquinone (Vitamin K1): Occurrence, Biosynthesis and Functions. Mini Rev. Med. Chem. *17*, 1028–1038.

Berglund, Å.M.M., Rainio, M.J., and Eeva, T. (2012). Decreased metal accumulation in passerines as a result of reduced emissions. Environ. Toxicol. Chem. *31*, 1317–1323.

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

Burger, J., and Gochfeld, M. (2000). Effects of lead on birds (*Laridae*): a review of laboratory and field studies. J. Toxicol. Environ. Health B Crit. Rev. *3*, 59–78.

Chavan, H., Oruganti, M., and Krishnamurthy, P. (2011). The ATP-Binding Cassette Transporter ABCB6 Is Induced by Arsenic and Protects against Arsenic Cytotoxicity. Toxicol. Sci. *120*, 519–528.

Cramp, S., and Perrins, C.M. (1993). Handbook of the Birds of Europe, the Middle East and North Africa. The Birds of the Western Palearctic Vol 7: Flycatchers to Shrikes (London, U.K.: Oxford University Press).

Dauwe, T., Bervoets, L., Blust, R., Pinxten, R., and Eens, M. (2000). Can excrement and feathers of nestling songbirds be used as biomonitors for heavy metal pollution? Arch. Environ. Contam. Toxicol. *39*, 541–546.

Dauwe, T., Snoeijs, T., Bervoets, L., Blust, R., and Eens, M. (2006). Calcium availability influences lead accumulation in a passerine bird. Anim. Biol. *56*, 289–298.

Diamond, G.L., Goodrum, P.E., Felter, S.P., and Ruoff, W.L. (1997). Gastrointestinal absorption of metals. Drug Chem. Toxicol. *20*, 345–368.

Dowling, D.K., and Simmons, L.W. (2009). Reactive oxygen species as universal constraints in life-history evolution. Proc. Biol. Sci. *276*, 1737–1745.

Eens, M., Pinxten, R., Verheyen, R.F., Blust, R., and Bervoets, L. (1999). Great and blue tits as indicators of heavy metal contamination in terrestrial ecosystems. Ecotoxicol. Environ. Saf. *44*, 81–85.

Eeva, T., and Lehikoinen, E. (1996). Growth and Mortality of Nestling Great Tits (*Parus major*) and Pied Flycatchers (*Ficedula hypoleuca*) in a Heavy Metal Pollution Gradient. Oecologia *108*, 631–639.

Eeva, T., and Lehikoinen, E. (2013). Density effect on great tit (*Parus major*) clutch size intensifies in a polluted environment. Oecologia *173*, 1661–1668.

Eeva, T., and Lehikoinen, E. (2015). Long-term recovery of clutch size and egg shell quality of the pied flycatcher (*Ficedula hypoleuca*) in a metal polluted area. Environ. Pollut. *201*, 26–33.

Eeva, T., Lehikoinen, E., and Pohjalainen, T. (1997). Pollution-related variation in food supply and breeding success in two hole-nesting passerines. Ecology *78*, 1120–1131.

Eeva, T., Hasselquist, D., Langefors, Å., Tummeleht, L., Nikinmaa, M., and Ilmonen, P. (2005a). Pollution related effects on immune function and stress in a free-living population of pied flycatcher *Ficedula hypoleuca*. J. Avian Biol. *36*, 405–412.

Eeva, T., Ryömä, M., and Riihimäki, J. (2005b). Pollution-related changes in diets of two insectivorous passerines. Oecologia *145*, 629–639.

Eeva, T., Belskii, E., and Kuranov, B. (2006). Environmental pollution affects genetic diversity in wild bird populations. Mutat. Res. *608*, 8–15.

Eeva, T., Sillanpää, S., Salminen, J.-P., Nikkinen, L., Tuominen, A., Toivonen, E., Pihlaja, K., and Lehikoinen, E. (2008). Environmental pollution affects the plumage color of Great tit nestlings through carotenoid availability. EcoHealth *5*, 328–337.

Eeva, T., Rainio, K., and Suominen, O. (2010). Effects of pollution on land snail abundance, size and diversity as resources for pied flycatcher, *Ficedula hypoleuca*. Sci. Total Environ. *408*, 4165–4169.

Ercal, N., Gurer-Orhan, H., and Aykin-Burns, N. (2001). Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr. Top. Med. Chem. *1*, 529–539.

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

Espín, S., García-Fernández, A.J., Herzke, D., Shore, R.F., van Hattum, B., Martínez López, E., Coeurdassier, M., Eulaers, I., Fritsch, C., Gómez-Ramírez, P., et al. (2016a). Tracking pan-continental trends in environmental contamination using sentinel raptors — what types of samples should we use? Ecotoxicology *25*, 777–801.

Espín, S., Ruiz, S., Sánchez-Virosta, P., and Eeva, T. (2016b). Effects of calcium supplementation on growth and biochemistry in two passerine species breeding in a Ca-poor and metal-polluted area. Environ. Sci. Pollut. Res. Int. *23*, 9809–9821.

Espín, S., Ruiz, S., Sánchez-Virosta, P., Salminen, J.-P., and Eeva, T. (2016c). Effects of experimental calcium availability and anthropogenic metal pollution on eggshell characteristics and yolk carotenoid and vitamin levels in two passerine birds. Chemosphere *151*, 189–201.

Espín, S., Ruiz, S., Sánchez-Virosta, P., Lilley, T., and Eeva, T. (2017a). Oxidative status in relation to metal pollution and calcium availability in pied flycatcher nestlings - A calcium manipulation experiment. Environ. Pollut. *229*, 448–458.

Espín, S., Sánchez-Virosta, P., García-Fernández, A.J., and Eeva, T. (2017b). A microplate adaptation of the thiobarbituric acid reactive substances assay to determine lipid peroxidation fluorometrically in small sample volumes. Rev. Toxicol. *34*, 94–98.

Evans, H.L., Garman, R.H., and Laties, V.G. (1982). Neurotoxicity of methylmercury in the pigeon. Neurotoxicology *3*, 21–36.

Finkel, T., and Holbrook, N.J. (2000). Oxidants, oxidative stress and the biology of ageing. Nature *408*, 239–247.

Finley, M.T., and Stendell, R.C. (1978). Survival and reproductive success of black ducks fed methyl mercury. Environ. Pollut. *16*, 51–64.

Flora, S.J.S., Mittal, M., and Mehta, A. (2008). Heavy metal induced oxidative stress & its possible reversal by chelation therapy. Indian J. Med. Res. *128*, 501–523.

Florea, A.-M., and Büsselberg, D. (2009). Metallic compounds (arsenic trioxide and trimethyltin chloride) interact with calcium homeostasis and trigger cell death in “in vitro” systems. Mater. Werkst. *40*, 13–16.

Florea, A.-M., Yamoah, E.N., and Dopp, E. (2005). Intracellular Calcium Disturbances Induced by Arsenic and Its Methylated Derivatives in Relation to Genomic Damage and Apoptosis Induction. Environ. Health Perspect. *113*, 659–664.

Fujihara, J., Agusa, T., Tanaka, J., Fujii, Y., Moritani, T., Hasegawa, M., Iwata, H., Tanabe, S., and Takeshita, H. (2009). 8-Hydroxy-2’-deoxyguanosine (8-OHdG) as a possible marker of arsenic poisoning: a clinical case study on the relationship between concentrations of 8-OHdG and each arsenic compound in urine of an acute promyelocytic leukemia patient being treated with arsenic trioxide. Forensic Toxicol. *27*, 41–44.

Fullmer, C.S., Edelstein, S., and Wasserman, R.H. (1985). Lead-binding properties of intestinal calcium-binding proteins. J. Biol. Chem. *260*, 6816–6819.

Furness, R.W. (1993). Birds as monitors of pollutants. In Birds as Monitors of Environmental Change, (London, UK: Chapman and Hall), pp. 86–143.

Gao, J., Roy, S., Tong, L., Argos, M., Jasmine, F., Rahaman, R., Rakibuz-Zaman, M., Parvez, F., Ahmed, A., Hore, S.K., et al. (2015). Arsenic exposure, telomere length, and expression of telomere-related genes among Bangladeshi individuals. Environ. Res. *136*, 462–469.

García-Fernández, A.J. (2014). Ecotoxicology, Avian. In Encyclopedia of Toxicology, 3rd edition (P. Wexler, Elsevier Inc., Academic Press).

García-Fernández, A.J., Bayoumi, A.E., Pérez-Pertejo, Y., Motas, M., Reguera, R.M., Ordóñez, C., Balaña-Fouce, R., and Ordóñez, D. (2002). Alterations of the glutathione-redox balance induced by metals in CHO-K1 cells. Comp. Biochem. Physiol. Toxicol. Pharmacol. *132*, 365–373.

Glorieux, C., and Calderon, P.B. (2018). Catalase down-regulation in cancer cells exposed to arsenic trioxide is involved in their increased sensitivity to a pro-oxidant treatment. Cancer Cell Int. *18*, 24.

Görlach, A., Bertram, K., Hudecova, S., and Krizanova, O. (2015). Calcium and ROS: A mutual interplay. Redox Biol. *6*, 260–271.

Graveland, J. (1996). Avian eggshell formation in calcium-rich and calcium-poor habitats: importance of snail shells and anthropogenic calcium sources. Can. J. Zool. *74*, 1035–1044.

Graveland, J., and van Gijzen, T. (1994). Arthropods and seeds are not sufficient as calcium sources for shell formation and skeletal growth in passerines. Ardea *82*, 299–314.

Graveland, J., and van der Wal, R. (1996). Decline in snail abundance due to soil acidification causes eggshell defects in forest passerines. Oecologia *105*, 351–360.

Gurer, H., and Ercal, N. (2000). Can antioxidants be beneficial in the treatment of lead poisoning? Free Radic. Biol. Med. *29*, 927–945.

Halliwell, B., and Gutteridge, J. (2007). Free Radicals in Biology and Medicine (Oxford University Press, USA).

Hong, H.L., Fowler, B.A., and Boorman, G.A. (1989). Hematopoietic effects in mice exposed to arsine gas. Toxicol. Appl. Pharmacol. *97*, 173–182.

Isaksson, C., and Andersson, S. (2008). Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. Proc. Biol. Sci. *275*, 309–314.

Jamakala, O., and Rani, U.A. (2012). Protective role of trace elements against cadmium induced alterations in the selected oxidative stress enzymes in liver and kidney of fresh water teleost, *Oreochromis mossambicus* (Tilapia). Int J Pharm Pharm Sci *4*, 303–310.

Kharroubi, W., Dhibi, M., Mekni, M., Haouas, Z., Chreif, I., Neffati, F., Hammami, M., and Sakly, R. (2014). Sodium arsenate induce changes in fatty acids profiles and oxidative damage in kidney of rats. Environ. Sci. Pollut. Res. Int. *21*, 12040–12049.

Koivula, M.J., and Eeva, T. (2010). Metal-related oxidative stress in birds. Environ. Pollut. *158*, 2359–2370.

Kretshmer, X., Pingitore, N.E., and Cruz-Jiménez, G. (2002). Incorporation of arsenic in mammal bone: X-ray absorption spectroscopy. Am. Geophys. Union Fall Meet. 2002 Abstr. B51B-0728.

Krishnamurthy, P.C., Du, G., Fukuda, Y., Sun, D., Sampath, J., Mercer, K.E., Wang, J., Sosa-Pineda, B., Murti, K.G., and Schuetz, J.D. (2006). Identification of a mammalian mitochondrial porphyrin transporter. Nature *443*, 586–589.

Li, H., Engström, K., Vahter, M., and Broberg, K. (2012). Arsenic exposure through drinking water is associated with longer telomeres in peripheral blood. Chem. Res. Toxicol. *25*, 2333–2339.

Littell, R.C., Milligen, G.A., Stroup, W.W., Wolfinger, R.D., and Schabenberger, O. (2006). SAS for Mixed Models. SAS Inst. Inc.

Mänd, R., Tilgar, V., and Leivits, A. (2000). Reproductive response of Great Tits, *Parus major*, in a naturally base-poor forest habitat to calcium supplementation. Can. J. Zool. *78*, 689–695.

Marettová, E., Maretta, M., and Legáth, J. (2015). Toxic effects of cadmium on testis of birds and mammals: a review. Anim. Reprod. Sci. *155*, 1–10.

Martínez-Haro, M., Green, A.J., and Mateo, R. (2011). Effects of lead exposure on oxidative stress biomarkers and plasma biochemistry in waterbirds in the field. Environ. Res. *111*, 530–538.

Mateo, R.W., Beyer, W.N., Spann, J.W., Hoffman, D.J., and Ramis, A. (2003). Relationship between oxidative stress, pathology, and behavioral signs of lead poisoning in mallards. J. Toxicol. Environ. Health A *66*, 1371–1389.

de Matos, R. (2008). Calcium Metabolism in Birds. Veterinary Clin. North Am. Exot. Anim. Pract. *11*, 59–82.

Mitchell, R.D., Ayala-Fierro, F., and Carter, D.E. (2000). Systemic indicators of inorganic arsenic toxicity in four animal species. J. Toxicol. Environ. Health A *59*, 119–134.

Møller, A.P., Biard, C., Blount, J.D., Houston, D.C., Ninni, P., Saino, N., and Surai, P.F. (2000). Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? Avian Poult. Biol. Rev. *11*, 137–159.

Pacyna, J.M., and Pacyna, E.G. (2001). An assessment of global and regional emissions of trace metals to the atmosphere from anthropogenic sources worldwide. Environ. Rev. *9*, 269–298.

Pounds, J.G. (1984). Effect of lead intoxication on calcium homeostasis and calcium-mediated cell function: a review. Neurotoxicology *5*, 295–331.

Prasanthi, R.P.J., Devi, C.B., Basha, D.C., Reddy, N.S., and Reddy, G.R. (2010). Calcium and zinc supplementation protects lead (Pb)-induced perturbations in antioxidant enzymes and lipid peroxidation in developing mouse brain. Int. J. Dev. Neurosci. Off. J. Int. Soc. Dev. Neurosci. *28*, 161–167.

Priego Capote, F., Jiménez, J.R., Granados, J.M.M., and de Castro, M.D.L. (2007). Identification and determination of fat-soluble vitamins and metabolites in human serum by liquid chromatography/triple quadrupole mass spectrometry with multiple reaction monitoring. Rapid Commun. Mass Spectrom. RCM *21*, 1745–1754.

Rai, A.N., Srivastava, S., Paladi, R., and Suprasanna, P. (2012). Calcium supplementation modulates arsenic-induced alterations and augments arsenic accumulation in callus cultures of Indian mustard (*Brassica juncea* (L.) Czern.). Protoplasma *249*, 725–736.

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

Rainio, M.J., Eeva, T., Lilley, T., Stauffer, J., and Ruuskanen, S. (2015). Effects of early-life lead exposure on oxidative status and phagocytosis activity in great tits (*Parus major*). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. *167*, 24–34.

Ramos, O., Carrizales, L., Yáñez, L., Mejía, J., Batres, L., Ortíz, D., and Díaz-Barriga, F. (1995). Arsenic increased lipid peroxidation in rat tissues by a mechanism independent of glutathione levels. Environ. Health Perspect. *103*, 85–88.

Rana, T., Bera, A.K., Das, S., Bhattacharya, D., Pan, D., and Das, S.K. (2012). Metabolic adaptations to arsenic-induced oxidative stress in male wistar rats. J. Biochem. Mol. Toxicol. *26*, 109–116.

Rattner, B.A., Lazarus, R.S., Heinz, G.H., Karouna-Renier, N.K., Schultz, S.L., and Hale, R.C. (2013). Comparative embryotoxicity of a pentabrominated diphenyl ether mixture to common terns (*Sterna hirundo*) and American kestrels (*Falco sparverius*). Chemosphere *93*, 441–447.

Reynolds, S.J., and Perrins, C.M. (2010). Dietary Calcium Availability and Reproduction in Birds. In Current Ornithology Volume 17, C.F. Thompson, ed. (Springer New York), pp. 31–74.

Reynolds, S.J., Mänd, R., and Tilgar, V. (2004). Calcium supplementation of breeding birds: directions for future research. Ibis *146*, 601–614.

Rotilio, G., Rossi, L., and de Martino, A. (1995). Free radicals, metal ions and oxidative stress: chemical mechanisms of damage and protection in living systems. J. Braz. Chem. Soc. *6 (3)*, 221–227.

Ruiz, S.R., Espín, S., Sánchez-Virosta, P., Salminen, J.-P., Lilley, T.M., and Eeva, T. (2017). Vitamin profiles in two free-living passerine birds under a metal pollution gradient – A calcium supplementation experiment. Ecotoxicol. Environ. Saf. *138*, 242–252.

Sánchez-Virosta, P., Espín, S., García-Fernández, A.J., and Eeva, T. (2015). A review on exposure and effects of arsenic in passerine birds. Sci. Total Environ. *512–513*, 506–525.

Sánchez-Virosta, P., Espín, S., Ruiz, S., Salminen, J.-P., García-Fernández, A.J., and Eeva, T. (2018). Experimental manipulation of dietary arsenic levels in great tit nestlings: Accumulation pattern and effects on growth, survival and plasma biochemistry. Environ. Pollut. *233*, 764–773.

Sánchez-Virosta, P., Espín, S., Ruiz, S., Stauffer, J., Kanerva, M., García-Fernández, A.J., and Eeva, T. (2019). Effects of calcium supplementation on oxidative status and oxidative damage in great tit nestlings inhabiting a metal-polluted area. Environ. Res. *171*, 484–492.

Sanderfoot, O.V., and Holloway, T. (2017). Air pollution impacts on avian species via inhalation exposure and associated outcomes. Environ. Res. Lett. *12*, 083002.

Scheuhammer, A.M. (1996). Influence of reduced dietary calcium on the accumulation and effects of lead, cadmium, and aluminum in birds. Environ. Pollut. *94*, 337–343.

Sepp, T., McGraw, K.J., Kaasik, A., and Giraudeau, M. (2018). A review of urban impacts on avian life-history evolution: Does city living lead to slower pace of life? Glob. Change Biol. *24*, 1452–1469.

Sharma, B., Singh, S., Siddiqi, N.J., Sharma, B., Singh, S., and Siddiqi, N.J. (2014). Biomedical Implications of Heavy Metals Induced Imbalances in Redox Systems. BioMed Res. Int. *2014*, e640754.

Snoeijs, T., Dauwe, T., Pinxten, R., Vandesande, F., and Eens, M. (2004). Heavy Metal Exposure Affects the Humoral Immune Response in a Free-Living Small Songbird, the Great Tit (*Parus major*). Arch. Environ. Contam. Toxicol. *46*, 399–404.

Snoeijs, T., Dauwe, T., Pinxten, R., Darras, V.M., Arckens, L., and Eens, M. (2005). The combined effect of lead exposure and high or low dietary calcium on health and immunocompetence in the zebra finch (*Taeniopygia guttata*). Environ. Pollut. *134*, 123–132.

Srivastava, D., Subramanian, R.B., Madamwar, D., and Flora, S.J.S. (2010). Protective effects of selenium, calcium, and magnesium against arsenic-induced oxidative stress in male rats. Arh. Hig. Rada Toksikol. *61*, 153–159.

Starck, J.M. (1998). Structural variants and invariants in avian embryonic and postnatal development. In Avian Growth and Development: Evolution Within the Altricial-Precocial Spectrum, (Oxford University Press), pp. 59–88.

Stauffer, J., Panda, B., Eeva, T., Rainio, M., and Ilmonen, P. (2017). Telomere damage and redox status alterations in free-living passerines exposed to metals. Sci. Total Environ. *575*, 841–848.

Stohs, S.J., and Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med. *18*, 321–336.

Surai, P.F., Speake, B.K., and Sparks, N.H.C. (2001). Carotenoids in Avian Nutrition and Embryonic Development. 1. Absorption, Availability and Levels in Plasma and Egg Yolk. J. Poult. Sci. *38*, 1–27.

Suzuki, N., Yamamoto, M., Watanabe, K., Kambegawa, A., and Hattori, A. (2004). Both mercury and cadmium directly influence calcium homeostasis resulting from the suppression of scale bone cells: the scale is a good model for the evaluation of heavy metals in bone metabolism. J. Bone Miner. Metab. *22*, 439–446.

Suzuki, Y., Chao, S.H., Zysk, J.R., and Cheung, W.Y. (1985). Stimulation of calmodulin by cadmium ion. Arch. Toxicol. *57*, 205–211.

Tilgar, V., Mänd, R., and Leivits, A. (1999). Effect of Calcium Availability and Habitat Quality on Reproduction in Pied Flycatcher *Ficedula hypoleuca* and Great Tit *Parus major*. J. Avian Biol. *30*, 383–391.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M., and Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact. *160*, 1–40.

Vizuete, J., Pérez-López, M., Míguez-Santiyán, M.P., and Hernández-Moreno, D. (2018). Mercury (Hg), Lead (Pb), Cadmium (Cd), Selenium (Se), and Arsenic (As) in Liver, Kidney, and Feathers of Gulls: A Review. Rev. Environ. Contam. Toxicol. First online 10 Nov 2018.

Whitney, M.C., and Cristol, D.A. (2018). Impacts of Sublethal Mercury Exposure on Birds: A Detailed Review. Rev. Environ. Contam. Toxicol. *244*, 113–163.

Williams, R.J., Holladay, S.D., Williams, S.M., and Gogal, R.M. (2018). Environmental Lead and Wild Birds: A Review. Rev. Environ. Contam. Toxicol. *245*, 157–180.

Wolfe, M.F., Schwarzbach, S., and Sulaiman, R.A. (1998). Effects of mercury on wildlife: A comprehensive review. Environ. Toxicol. Chem. *17*, 146–160.

Zhang, T.-C., Schmitt, M.T., and Mumford, J.L. (2003). Effects of arsenic on telomerase and telomeres in relation to cell proliferation and apoptosis in human keratinocytes and leukemia cells in vitro. Carcinogenesis *24*, 1811–1817.

Annales Universitatis Turkuensis

UNIVERSITY OF TURKU

ISBN 978-951-29-7670-6 (PRINT)
ISBN 978-951-29-7671-3 (PDF)
ISSN 0082-6979 (Print)
ISSN 2343-3183 (Online)

Grano Oy – Turku, Finland 2019