UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries and Economics

Sublethal effects of environmental pollutant exposure in birds of prey

Evaluating biomarkers of health as indicators of contaminant-mediated effects in two sentinel raptor species

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"*A Who's Who of pesticides is therefore of concern to us all. If we are going to live so intimately with these chemicals eating and drinking them, taking them into the very marrow of our bones - we had better know something about their nature and their power*."

— Rachel Carson, **Silent spring** (1962)

Cover image: adult white-tailed eagle (*Haliaeetus albicilla*). Photo by Elisabeth Hansen.

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Table of Contents

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List of abbreviations

BFRs - Brominated flame retardants

DDE - Dichlorodiphenyldichloroethylene

DDT - Dichlorodiphenyltrichloroethane

fCORT - Feather corticosterone

HCB - Hexachlorobenzene

HCH - Hexachlorocyclohexane

LCC - Leukocyte coping capacity

LC-PFCAs – Long-chained perfluoroalkyl carboxylic acids

OHCs - Organo-halogenated contaminants

PBDEs - Polybrominated diphenyl ethers

PCBs - Polychlorinated biphenyls

PFAS - Per- and polyfluoroalkyl substances

PFHxS – Perfluorohexane sulfonate

PMA – Phorbol-myristate-acetate

POPs - Persistent organic pollutants

RBCs - Red blood cells

RLUs – Relative light units

ROS - Reactive oxygen species

WTE - White-tailed eagle (*Haliaeetus albicilla*)

List of papers

I: Hansen E, Huber N, Bustnes JO, Herzke D, Bardsen BJ, Eulaers I, Johnsen TV, Bourgeon S. 2020. A novel use of the leukocyte coping capacity assay to assess the immunomodulatory effects of organohalogenated contaminants in avian wildlife. Environment International. 142: 105861.

II: Hansen E, Skotnes T, Bustnes JO, Helander B, Eulaers I, Sun JC, Covaci A, Bardsen BJ, Zahn S, Criscuolo F et al. 2022. Telomere length in relation to persistent organic pollutant exposure in white-tailed eagle (*Haliaeetus albicilla*) nestlings from sweden sampled in 1995-2013. Environmental Research 208:112712.

III: Hansen E, Sun J, Helander B, Bustnes JO, Eulaers I, Jaspers VLB, Covaci A, Eens M, Bourgeon S. 2023. A retrospective investigation of feather corticosterone in a highly contaminated white-tailed eagle (*Haliaeetus albicilla*) population. Environmental Research 228:115923.

IV: Hansen E, Bustnes JO, Eulaers I, Herzke D, Bårdsen B-J, Bangjord G, Ballesteros M, Bourgeon S. Feathers as temporal archives of environmental stress: a multi-decadal study on metal exposure, dietary proxies and physiological stress in a terrestrial raptor. Advanced manuscript.

Conference presentations

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Preface

Coming from a small Island in Northern Norway, I have always been fascinated by the fantastic wildlife we are fortunate to be surrounded by. During my bachelor's degree, I wrote a thesis on the migration patterns and biology of the humpback whales and orcas that visit our fjords every autumn. As I continued my master's degree, I sought new challenges. Besides being interested in the biology of wild animals, I have also been particularly concerned about human impact on nature through pollution. Pursuing my curiosity, I embarked on a project studying the impact of environmental pollutants on the largest raptor in northern Europe: the white-tailed eagle. Following the completion of my master's degree, I was motivated to continue further along this path and began as a doctoral student, focusing on researching how organic pollutants and toxic metals affect the health of wild birds of prey.

Throughout my studies, I have had the unique opportunity to closely observe both white-tailed eagles and tawny owls. For two summers, I worked alongside experienced field personnel, spending weeks collecting blood samples from white-tailed eagle chicks in Northern Norway that I subsequently processed in the lab for contaminant analysis. Later, I also participated in fieldwork with tawny owls, where the collection of feather samples was central. These field experiences have provided me with a broader appreciation and a better understanding of the biology of white-tailed eagles and tawny owls and the environment they inhabit. This, in turn, has provided important context and allowed me to gain a deeper insight into the empirical data I have worked with in this project.

During my doctoral research, I have worked with large datasets based on observations and sampling of white-tailed eagles in Sweden and tawny owls in Trondheim over several decades. These impressive long-term data series on wild raptors are a result of the hard work and dedication of passionate individuals who collected blood and/or feather samples over many years, even decades before I was even born. In all research on wild animals, and especially in ecotoxicology, obtaining long-term series covering periods with significant variations in the levels of environmental contaminants in top predators is rare. It requires logistics, time, funding, and cooperation. The large number of co-authors in the articles included in my doctoral thesis reflects the collaborative effort required to orchestrate both the collection and analysis of this data. I was fortunate to have the opportunity to contribute to data collection and laboratory work, as well as benefit from data gathered and analysed by many of my co-authors. With this doctoral thesis, I hope that we can contribute to the research field with new knowledge on how exposure to environmental pollutants can impact the health of birds of prey in the natural environment.

Abstract

Environmental pollution emerged as a major concern in the mid-20th century primarily due to the unrestricted release of industrial chemicals and pesticides. Among wildlife species, apex predators, like raptors, have been heavily exposed to environmental pollutants such as organo-halogenated contaminants (OHCs) and metals (e.g., mercury). Due to their high trophic position, harmful levels of these pollutants ensued through biomagnification and resulted in drastic population declines during the 1950s/60s. Following a series of regulative efforts that began in the 1970s, exposure to acutely toxic and lethal doses of OHCs and metals has become rare. However, chronic exposure to lower levels of contaminants can also be harmful by triggering sublethal health effects that may ultimately affect individuals' survival and fitness. In this context, biological endpoints that act as early warning indicators of adverse health effects, that is, biomarkers, can prove useful. This thesis aimed to study contaminant-mediated health effects in two wild raptor species, the white-tailed eagle *Haliaeetus albicilla* (WTE) and the tawny owl *Strix aluco*, over interannual to decadal time series. The selected biomarkers were leukocyte coping capacity (LCC; indicator of immunocapacity), telomere length (proxy of genotoxicity), and feather corticosterone (fCORT; measure of physiological stress). We observed that higher OHC concentrations in Norwegian WTE nestlings were associated with lower LCC, suggesting a reduced immune system capacity following OHC exposure. By contrast, telomere lengths in Swedish nestlings did not correlate with OHC concentrations. Our findings therefore suggest that telomere length is not a useful proxy for contaminant-mediated effects in WTE nestlings. Additionally, no links were found between fCORT and OHCs in adult Swedish WTEs, despite the population experiencing contrasting OHC levels throughout the study period. In Norwegian adult tawny owls, a positive relationship between fCORT and aluminium and cadmium was observed, along with a negative association with mercury, suggesting that these metals may influence long-term stress physiology. This thesis provides further evidence that the relationships between biomarkers and contaminants can be complex and species-specific. A strength of this thesis was the inclusion of long-term studies, which can help minimize the influence of confounding environmental stressors and thus reduce the chance of reporting spurious relationships. In summary, this thesis demonstrates that some biomarkers can offer valuable insights into potential physiological consequences of contamination in wild animals in their natural environment. Namely, LCC stands out as a potential new tool to evaluate contaminant-mediated health effects in wildlife ecotoxicology. Future research should continue to prioritize non-destructive and preferably non-invasive biomarkers, which are invaluable when working with protected species such as the WTE and the tawny owl.

1 Introduction

1.1 Anthropogenic pollution: A brief historical perspective

Human activities have been impacting the environment for thousands of years. The impact escalated significantly during the Industrial Revolution in the late 18th century due to the combustion of fossil fuels and large-scale industrial processes (Newman 2020). However, environmental pollution first emerged as a major concern in the decades following World War II, primarily due to the unrestricted release of organochlorine (OC) pesticides (Rattner et al. 2011). Such chemicals were unequivocally linked to adverse effects in wildlife in the book "Silent Spring" by Rachel Carson (1962), which brought the detrimental impacts of OCs and related chemicals to widespread public attention (Dunn 2012).

The wildlife most notably affected by anthropogenic pollution have been top predatory species, such as birds of prey (Rattner 2009). During the 1950s and 60s, alarming reports surfaced about the declining populations of various raptor species in Europe and North America (Shore and Taggart 2019). Subsequent investigations revealed that these raptors had accumulated high levels of OC pesticides such as dichlorodiphenyltrichloroethane (DDT) and dieldrin (Shore and Taggart 2019). Consequently, raptors experienced acute and reproductive toxicity causing local extinctions and/or rapid population declines (Newton 2017). As a response to the observed catastrophic impact of contaminants on raptor and wildlife health, the field of ecotoxicology emerged in the 1970s aiming to study the effects of contaminants on ecosystems, organisms, and human health and to inform environmental management and policy decisions (Newman 2020).

Despite various regulatory measures to curtail the introduction of harmful chemicals like DDT into the environment (Kessler 2013; Lallas 2001), many contaminants are still present in biota due to their ubiquitous distribution and long degradation time in the environment (Ashraf 2017), leading to ongoing universal exposure in both humans and wildlife. Additionally, many of the now banned contaminants have been replaced with new chemicals, mirroring the potential detrimental effects of their predecessors on living organisms (Landrigan et al. 2018). Furthermore, a historical increase in fossil fuel combustion and mining activities have led to widespread distribution and increased concentrations of toxic metals like mercury beyond natural levels (Tchounwou et al. 2012). Continued monitoring of the harmful effects of contaminants on living organisms is crucial, given their pervasive abundance in the environment and the persistent and serious environmental management challenge they pose.

1.2 Contaminants of environmental concern

In avian ecotoxicology, the most extensively studied contaminant classes are organo-halogenated contaminants (OHCs), which include the above-mentioned OC pesticides, and metal and metalloid elements. While OHCs are categorized as organic contaminants (i.e., chemicals based on compounds with carbon-hydrogen bonds), metal(loid)s are categorized as inorganic contaminants (i.e., a compound that has no carbon-hydrogen bonds) (Newman 2020). Many OHCs and metal(loid)s exhibit similar physicochemical properties that make them persistent, bioaccumulative, and toxic (Abelkop et al. 2018). Consequently, these two groups of contaminants have drawn significant attention from ecotoxicologists (Newman 2020). In addition, both OHCs and metal(loid)s represent substantial contributors to environmental pollution on a global scale, and their presence is primarily attributed to human activities (Landrigan et al. 2018).

1.2.1 Organo-halogenated contaminants (OHCs)

Organo-halogenated contaminants (OHCs) are carbon-based substances that incorporate halogen elements, such as chlorine, bromine, or fluorine. These compounds found widespread use in industry, agriculture, and consumer products, either intentionally as pesticides or industrial chemicals, or unintentionally as by-products (Harrad 2009). Once released, OHCs display persistence in the environment, resisting degradation through chemical, biological, and photolytic processes (Harrad 2009). They can be widely distributed through various pathways, including the atmosphere, oceans, rivers, and migrating biota, travelling far from their emission sources (Ashraf 2017). OHCs also have the capacity to bioaccumulate in living organisms and biomagnify along the food chain (Fig. 1), with their physicochemical properties influencing their preferential accumulation in specific tissues and transfer between trophic levels (Kelly et al. 2007).

OHCs can be divided into different groups based on their chemical properties. The most well-known group are the OCs, which have mainly been produced as pesticides such as DDT (Walker 2009). Other infamous examples of OC pesticides are hexachlorobenzene (HCB), chlordane and dieldrin. OCs also include polychlorinated biphenyls (PCBs), which are a group of industrial chemicals previously used as coolants and insulating agents. Further, brominated flame retardants (BFRs), such as polybrominated biphenyl ethers (PBDEs), represent another important group of OHCs. Both OCs and PBDEs are lipophilic, implying that they have a high probability of accumulating in the adipose tissues of organisms (Walker 2009). Lastly, we have

the perfluoroalkyl substances (PFAS), which have been mainly used in the production of fire-fighting foams, stain-repelling agents, and lubricants (Buck et al. 2011). In contrast to OCs and BFRs, PFAS are lipophobic and have a high affinity towards protein-rich tissues such as the liver and blood (Buck et al. 2011).

In the 1970s/80s, many countries prohibited or restricted the production and use of various OCs including DDT and PCB (Newman 2020). BFRs and PFAS, however, remained largely unregulated until the 1990s and later (Newman 2020). In 2004, the Stockholm Convention on Persistent Organic Pollutants (POPs) was initiated as an international environmental treaty that aims to protect human and wildlife health and the environment from detrimental organic contaminants (Hagen and Walls 2005). Initially, twelve POPs were listed under the convention, also known as the "Dirty Dozen", of which all were OCs (Lallas 2001). Since 2004, several other compounds have been added to the list, including many BFRs and a selection of PFAS (Sheriff et al. 2022).

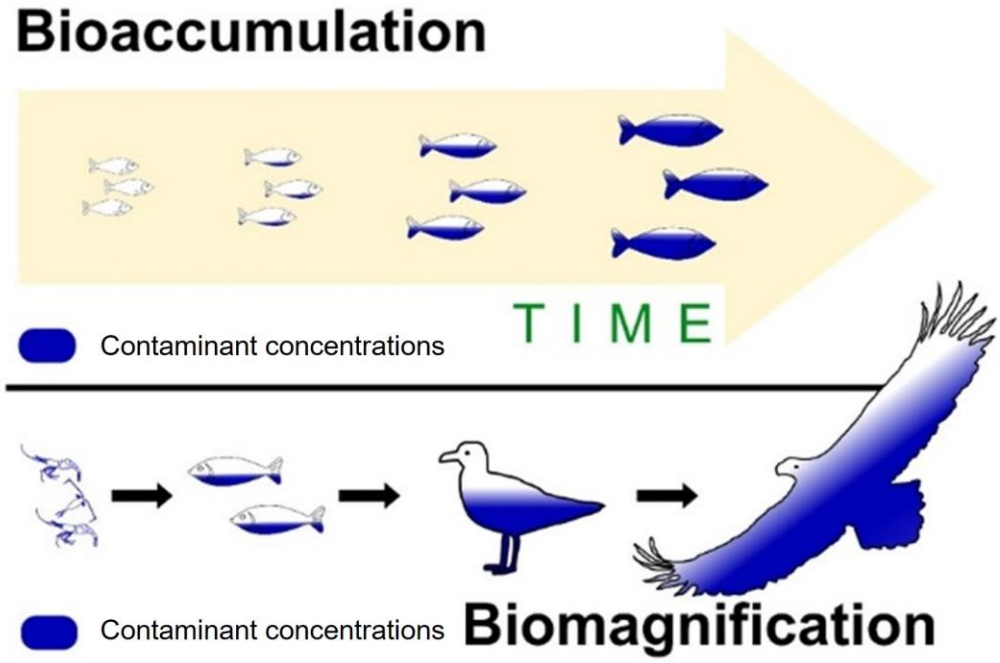


Figure 1 Bioaccumulation of contaminants in individuals over time and biomagnification of contaminants through food webs. Illustration adapted from figure in Øygarden (2015).

1.2.2 Metal and metalloid elements

Metals and metalloids (hereafter referred to as metals for simplicity) constitute the largest group of elements in the periodic table. Metals occur naturally in the environment, but human activities such as mining, smelting and coal burning have significantly contributed to their redistribution and release into the environment (Fig. 2), resulting in elevated concentrations beyond natural levels (Tchounwou et al. 2012). Metals are extensively used in their pure state

or in compounds in various products due to their excellent electrical and thermal conductivity, including electronics, batteries, piping, paints, and ammunition. Several metals have also been employed as pesticides, such as arsenic (As), cadmium (Cd), and lead (Pb), while mercury (Hg) has been widely used as a biocide in seed treatment and electronics (Tchounwou et al. 2012).

Some metals, such as copper, iron, and zinc, are essential for life and play crucial roles in biological processes (Zalups and Koropatnick 2000). However, excessive, or insufficient concentrations of these essential elements can be harmful (Goldhaber 2003). Non-essential metals, like As (metalloid), Pb, and Hg, can be toxic even at trace levels (Zalups and Koropatnick 2000). Among these, Hg, particularly in its organic form methylmercury, stands out as the most significant in terms of environmental contamination due to its high toxicity and bioaccumulation potential (Rattner et al. 2011).

Much like the measures taken to regulate the release of OHCs, there are also various global regulations and mechanisms in place to curb anthropogenic emissions of harmful metals. In 2013, the Minamata Convention on Mercury was established to combat the harmful effects of Hg pollution on human health and the environment (Kessler 2013). Named after the city of Minamata in Japan, where severe Hg poisoning occurred due to industrial wastewater discharges in the mid-20th century, the convention's primary objective is to reduce and ultimately eliminate anthropogenic emissions and releases of Hg into the environment (Kessler 2013).

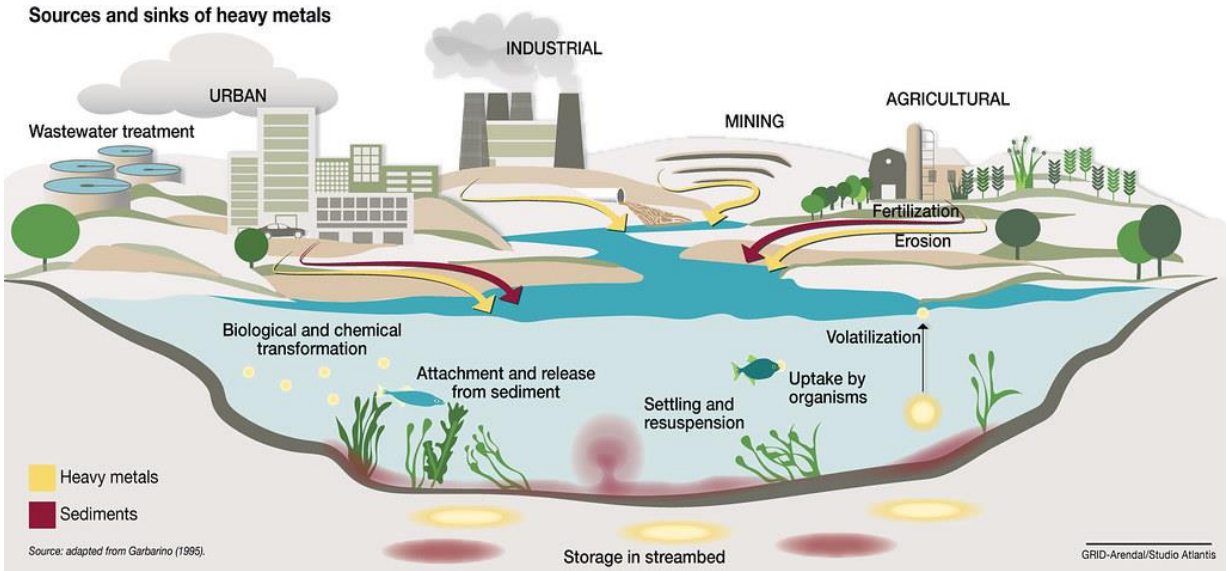


Figure 2 Illustration of sources and sinks of toxic metal(loid)s (here referred to as heavy metals) in the environment. Source: GRID-Arendal/Studio Atlantis (2020), retrieved the 19.07.2023 from https://www.grida.no/resources/13718.

1.3 Raptors as biomonitors of environmental pollution

Since the 1950s/60s, birds of prey (interchangeably referred to as raptors) have been widely used as biomonitors of environmental pollution (Shore and Taggart 2019). Due to their upper trophic position, raptors are characterized by relatively high concentrations of contaminants such as OHCs and toxic metals and are therefore prone to suffer from adverse health effects (Newton 2017; Shore and Taggart 2019). Furthermore, raptors, and birds in general, are particularly well-suited as biomonitors for several reasons. First, they can be relatively easily monitored in the field during their breeding season, particularly around the nesting period. Second, their cosmopolitan distribution renders them excellent indicators of environmental pollution across various habitats and ecosystems (Badry et al. 2020). Third, non-destructive sampling methods, such as collecting eggs, feathers, and blood, provide viable alternatives for contaminant analyses, which in an ethical perspective is important when dealing with protected species, as is the case with many raptors. These matrices have been found to correlate well with internal body burdens, allowing for effective assessment of pollutant exposure levels in avian species (Berglund 2018; Eulaers et al. 2011a; Eulaers et al. 2011b; Jaspers et al. 2019; Jaspers et al. 2013; Lodenius and Solonen 2013).

The primary route of exposure to contaminants in birds of prey is through their diet (Espín et al. 2016). Hence, dietary patterns, including trophic levels (where higher trophic species show higher contamination) and the prey habitat (e.g., marine animals being more contaminated than terrestrial ones), play significant roles in explaining interspecies and interindividual variation in contaminant levels (Elliott et al. 2009; Eulaers et al. 2014; Ratajc et al. 2022). However, collecting information about the diet of wild raptors requires the analysis of e.g., regurgitated pellets or monitoring of prey brought to the nest (Jordan 2005), both of which can be challenging and time-consuming. To address this challenge, researchers often employ the analysis of bulk stable isotopes of nitrogen ($\delta^{15}N$ - a proxy for prey trophic level) and carbon ($\delta^{13}C$ - a proxy for dietary source/habitat of prey) in samples such as blood and feathers, as an alternative approach to investigate the feeding ecology of wild birds (Inger and Bearhop 2008). In biomonitoring programmes, integrating variations in dietary patterns and plasticity improves accuracy when examining levels and trends in exposure to contaminants in wild birds (Elliott et al. 2021; Ratajc et al. 2022). The analysis of stable isotopes in eggs, feathers or blood facilitates the assessment of dietary patterns in a non-destructive manner.

The white-tailed eagle (hereafter WTE; *Haliaeetus albicilla*) and the tawny owl (*Strix aluco*) are common birds of prey in Europe (Fig. 3). While the WTE is an apex predator associated with marine or freshwater environments (Cramp 1980), the tawny owl is a terrestrial predator inhabiting the interface between urban areas and agricultural land (Cramp 1985). Therefore, it is likely that WTEs and tawny owls are exposed to different contaminant levels since animals associated with marine food webs typically exhibit higher contaminant concentrations than terrestrial species (Bustnes et al. 2013a; Chetelat et al. 2020; Gómez-Ramírez et al. 2023). Nonetheless, the WTE and the tawny owl share many biological and ecological characteristics that make them valuable biomonitors in their respective habitats. Both are non-migratory (i.e., resident) and territorial species with strong nest site fidelity (Cramp 1980; 1985). This means that they represent local contamination in a relatively small geographical area. Additionally, they have long lifespans (10+ years) and consequently many years to accumulate high levels of contaminants. Indeed, long-term studies analysing contaminants in feathers and eggs from these species have proven to be highly reflective of the historical use and release of several OHCs and toxic metals into the environment (Ahrens et al. 2011; Bustnes et al. 2022; Devalloir et al. 2023; Helander et al. 2008; Sun et al. 2019a; Sun et al. 2019b; Sun et al. 2020). Accordingly, both species serve as exceptional biomonitors within their respective ecosystems and are also widely recognized as sentinel species for environmental pollution in monitoring programs throughout Europe (Dietz et al. 2021; Ratajc et al. 2022).

Figure 3 Adult white-tailed eagle Haliaeetus albicilla *(left) and adult tawny owl* Strix aluco *(right). Photos by Elisabeth Hansen.*

The WTE has, however, received more extensive research attention than the tawny owl when it comes to evaluating ecotoxicological effects. The WTE is the largest eagle in Europe and its distribution ranges along coastlines and lakes, where it mainly feeds on fish, seabirds, and carrion (Cramp 1980). In the early 20th century, WTEs faced critical population declines and even extinction in many European countries due to human persecution (Hailer et al. 2006). In Sweden, the WTE population was saved from extinction and began to recover after receiving national protection in 1924 (Roos et al. 2012). During the 1950s, concerning reports of declining WTE numbers emerged, mirroring a similar trend observed among other raptor populations across Europe (Helander 1985). In response, Sweden established a national monitoring program for WTEs in 1964 (Helander 2003).

Long-term studies through the Swedish monitoring program clearly showed that levels of DDT and PCB were negatively correlated with productivity in the WTE (Fig. 4; Bignert and Helander 2015). The main driver for reproductive failure was a decrease in eggshell thickness due to the use of DDT (Ratcliffe 1967). DDT is not especially toxic to birds, but after a bird is exposed, it is readily converted to dichlorodiphenyldichloroethylene (DDE), a much more stable metabolite (Newton 2017). In some species, DDE causes eggshell thinning by reducing the availability of calcium carbonate during eggshell formation, leading to the breakage of thin-shelled eggs during incubation (Lundholm 1997). DDE-induced eggshell thinning, combined with increased offspring mortality following PCB exposure, led to a significant reduction in reproductive output in the Swedish WTE (Helander et al. 2002). Only following the regulations and bans of DDT, PCBs, and dieldrin did the WTE population in Sweden recover (Fig. 4; Bignert and Helander 2015).

The large-scale impacts on the reproduction of WTEs and other raptor species around Europe demonstrate how serious anthropogenic pollution can affect wildlife and highlight the importance of detecting and regulating harmful contaminants in the environment before population-level impacts occur. The WTE has also been monitored in other Nordic and Baltic countries (Dietz et al. 2021; Kenntner et al. 2003; Scharenberg and Struwe-Juhl 2006; Sun et al. 2020), although not to the same extent as in Sweden. In Norway, the WTE was not protected from hunting before 1968 (Hailer et al. 2006), resulting in a relatively small population during the period of severe pollution. As human persecution was a significant driver of population decline, it would have been challenging to accurately assess the impact of pollution during that time. Only in the mid-2000s and beyond did ecotoxicological studies start investigating the

levels and effects of contaminants on Norwegian WTEs (Bustnes et al. 2013a; Eulaers et al. 2011a; Jouanneau 2019; Løseth et al. 2019a; Løseth et al. 2019b; Sletten et al. 2016; Sonne et al. 2012). Studies on the Norwegian WTEs also revealed significantly lower levels of pollutants compared to Swedish WTEs during the same time periods (Sun et al. 2019b, Sun et al. 2020). This difference is likely due to the less polluted environment of the Norwegian coast in contrast to the Baltic Sea. The Baltic Sea is often regarded as one of the most polluted seas globally, primarily due to agricultural runoff and wastewater discharge from surrounding industrialized regions (Leppäranta and Myrberg 2009). In addition, limited convective mixing with the North Atlantic exacerbates pollution levels in the Baltic Sea (Leppäranta and Myrberg 2009).

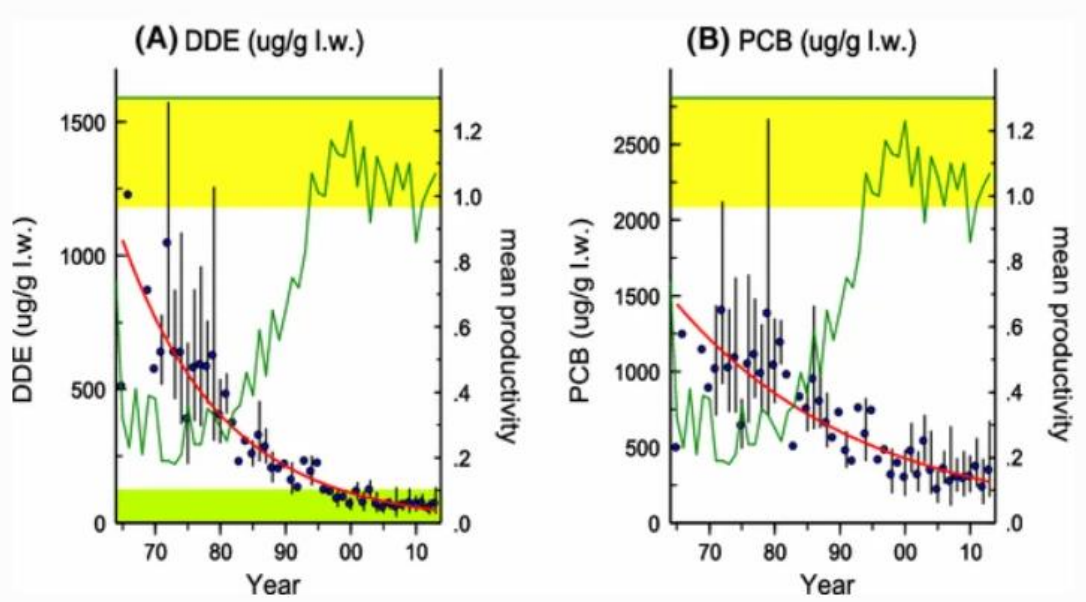


Figure 4 Temporal changes in concentrations of A) DDE and B) PCB in egg contents in relation to productivity in White-tailed eagles on the Swedish Baltic coast, 1965–2013. The black dots indicate mean concentrations of DDE with 95 % confidence intervals; the jagged line shows the annual productivity. The green upper line indicates the estimated pre-1950s reference level for productivity of Baltic white-tailed eagles, and the yellow area indicates its lower 95 % confidence limit. Retrieved from Bignert and Helander (2015).

The habitat and proximity to contaminant sources both play a crucial role in determining the magnitude of exposure to pollutants (Devalloir et al. 2023; Donaldson et al. 1999), making urban-dwelling birds like the tawny owl susceptible to contaminants associated with urban and industrialized environments (Kekkonen 2017). The tawny owl, being a generalist predator that primarily feeds on small mammals and birds, with occasional consumption of insects and amphibians (Zalewski 1994), is likely exposed to varying levels of contaminants based on the dietary composition (e.g., trophic level) of its prey. In Central Norway, a resident nest box population of tawny owls breeding at their northern ecological limit has been supplying egg and feather samples for several decades, dating back to the early 1980s (Bustnes et al. 2022; Devalloir et al. 2023). Similar to the WTEs in Sweden, long-term data series on tawny owls in

Norway have offered a unique opportunity to analyse multi-decadal levels of OHCs (Ahrens et al. 2011; Bustnes et al. 2015a; Bustnes et al. 2022; Bustnes et al. 2011) and metal concentrations (Bustnes et al. 2013b; Devalloir et al. 2023) in this key terrestrial raptor. To date, few studies have explored ecotoxicological effects in tawny owls (Ratajc et al. 2022; Yoccoz et al. 2009), leaving much unknown about the physiological consequences of contaminant exposure in individuals of this species.

1.4 Biomarkers as indicators of contaminant-mediated effects

Many OHCs and metals can be lethal at high doses causing acute or reproductive toxicity, but wildlife rarely encounter such magnitudes of exposure at present. On the other hand, since wildlife is exposed to a mixture of different pollutants rather than single compounds, it is also important to consider the possibility of a chemical cocktail effect (García-Fernández et al. 2020). Moreover, chronic exposure to contaminants at concentrations below their toxicity threshold can also be problematic by triggering sublethal health effects that may ultimately affect individual survival and fitness (Michelangeli et al. 2022; Whitney and Cristol 2018). Evaluating such sublethal effects in wild animals is however challenging because we often have limited information available about their natural environment or the level of exposure to contaminants. To address this challenge, scientists have widely used biomarkers of health in ecotoxicological studies since the late 20th century (Rattner 2009). These biomarkers serve the purpose of identifying potential sublethal contaminant-related effects in wild species (Amiard-Triquet et al. 2012). In essence, biomarkers are measurable biological indicators that act as early warning signals, alerting to potential harm to the well-being and health of animals (Amiard-Triquet et al. 2012). These indicators are typically associated with various physiological functions, such as the endocrine or immune system, that are vital for maintaining the health of individuals (Dietz et al. 2019; Harris and Elliott 2011). Specifically, previous effect studies on wild avian populations have documented a range of adverse effects from their exposure to OHCs and metals at different levels of biological organizations (Fig. 5), as succinctly exemplified below:

- **Endocrine disruption:** refers to the interference or alteration of the normal functioning of the endocrine system (Monneret 2017). The underlying mechanisms are often linked to the ability of contaminants to interfere with hormone synthesis, receptor binding, or metabolism (Vandenberg et al. 2012). Endocrine disruption following pollutant exposure can encompass several hormones such as:

- *Stress hormones.* Exposure to OCs and Hg have been linked with disruption of the hypothalamic-pituitary-adrenal axis, which modulates the production and release of stress-related hormones like glucocorticoids, in passerine birds (Mayne et al. 2004; Wada et al. 2009) and the black-legged kittiwake *Rissa tridactyla* (Tartu et al. 2015c). Furthermore, OCs and PBDEs have been linked to elevated baseline stress hormone levels in several seabird species (Tartu et al. 2015a; Verboven et al. 2010). Elevated stress hormone levels for a prolonged period can lead to chronic stress that can weaken the immune system, reduce reproductive success, and ultimately lower an individual's survival and fitness (McEwen and Wingfield 2003). In addition, OCs and Hg have been shown to interfere with the stress-induced response, as found in white storks *Ciconia ciconia* (Baos et al. 2006), black-legged kittiwakes (Tartu et al. 2014), and common loons *Gavin imtner* (Franceschini et al. 2017), potentially diminishing these birds' ability to effectively manage stressors (Romero and Beattie 2021).
- *Sex hormones*. OHCs and Hg have been shown to disrupt the delicate balance of sex hormones like prolactin in the glaucous gull *Larus hyperboreus* (Verreault et al. 2008), snow petrels *Pagodroma nivea* (Tartu et al. 2015b), and black-legged kittiwakes (Tartu et al. 2016), which could ultimately affect parental care and reproductive behaviour in these species. Further, changes in oestradiol and testosterone levels following Hg, OC or PBDE exposure have been documented in white ibises *Eudocimus albus* (Heath and Frederick 2005) and glaucous gulls (Verboven et al. 2008), potentially impacting their reproductive success by affecting nesting, courtship, and mating behaviours.
- *Thyroid hormones*. Thyroid hormones, including thyroxine (T4) and triiodothyronine (T3), are crucial for regulating metabolism, growth, and development (Boas et al. 2012). Correlations between thyroid hormones and OCs, PBDEs, PFAS, and Hg have been reported in multiple bird groups and species, including waders (Champoux et al. 2017), raptors (Brogan et al. 2017; Cesh et al. 2010), passerines (Morrissey et al. 2014; Wada et al. 2009) and seabirds (Blevin et al. 2017b; Sebastiano et al. 2023; Svendsen et al. 2018; Techer et al. 2018). A contaminant-induced change in thyroid hormones may consequently lead to metabolic imbalances and altered growth rates (Hao et al. 2021).

- **Immune system impairment:** leaves birds vulnerable to a higher risk of infections, reduced ability to combat diseases, and overall decreased fitness (Grasman 2002). The underlying mechanisms of immune impairment by pollutants can, among others, involve disruption of immune cell function, oxidative stress, and alterations in cytokine production, which can negatively impact immune responses (Grasman 2002). Impairment by pollutants encompasses various immune parameters that serve as crucial indicators of immunocompetence, such as:
    - *Immunoglobulins and antibodies.* These are proteins produced by the immune system that play essential roles in defending the body against infections and invading pathogens (i.e., antigens). For example, measurement of antibody levels in response to specific antigens can assess the ability of an organism's immune system to mount an immune response (Bustnes et al. 2004). Changes in antibody levels have been found in glaucous gulls in relation to OC exposure (Bustnes et al. 2004), in great tits *Parus major* nesting close to a metallurgic smelter (Snoeijs et al. 2004), in different waterfowl species in relation to Hg exposure (Teitelbaum et al. 2022), and in black-headed gulls *Chroicocephalus ridibundus* in relation to Pb exposure (Ushine et al. 2023). In addition, changes in immunoglobulin levels have been associated with exposure to PBDEs in Great skua *Stercorarius skua* (Bourgeon et al. 2012), exposure to PCBs in chinstrap penguins *Pygoscelis antarctica* (Jara et al. 2018), and exposure to toxic metals in Eurasian tree sparrows *Passer montanus* (Li et al. 2021), indicating contaminant-induced changes in the humoral immune response.
    - *White blood cell counts.* White blood cells (i.e., leukocytes) play a fundamental role in immune defence. Altered white blood cell levels can indicate an immune system suppression or overstimulation, and alterations have been detected in relation to OC exposure in several seabird species (Bustnes et al. 2004; Grasman et al. 1996; Jara-Carrasco et al. 2015) and the pied flycatcther *Ficedula hypoleuca* nesting in the vicinity of a metal-polluted area (i.e., copper smelter; Eeva et al. 2005).
    - *Lymphocyte proliferation.* Lymphocyte proliferation refers to the process by which lymphocytes, a type of white blood cell involved in the immune response, increase in number in response to various stimuli, particularly antigens or immune challenges (Lewis et al. 2013). A study on the black-footed albatross *Phoebastria nigripes* found that lymphocyte proliferation was positively related

to OC exposure, which may suggest misregulation of the immune function, possibly in the direction of hypersensitivity (Finkelstein et al. 2007).

- **Oxidative stress:** is the result of an imbalance between the production of reactive oxygen species (ROS) and the organism's ability to neutralize them with antioxidants (Sies 2020). OHC and/or metal exposure have been shown to induce oxidative stress in birds by increasing the formation of ROS (e.g., Isaksson 2010, Koivula and Eeva 2010) and/or through observed changes in the antioxidant capacity or activity as shown in several species of passerines (Berglund et al. 2007; Kanwal et al. 2020; Lopez-Antia et al. 2019), raptors (Abbasi et al. 2017; Espín et al. 2014; Kanwal et al. 2020; Sletten et al. 2016), seabirds and waders (Costantini et al. 2014; Fenstad et al. 2016; Hegseth et al. 2011; Wayland et al. 2010).

- **Genotoxicity:** refers to the property of certain agents or substances to cause damage to an organism's genetic material, which includes DNA and, in some cases, RNA (Costa 2022). Pollutants, including OHCs and metals, can act as genotoxic agents and potentially induce various types of genetic damage, including reduced DNA integrity (Louzon et al. 2019), and DNA strand breaks as reported in studies on common eiders *Somateria mollissima* (Fenstad et al. 2014) and American kestrels *Falco sparverius* (Frixione and Rodriguez-Estrella 2020) in relation to OC exposure. In addition, OHCs and metals can indirectly damage DNA through the production of harmful ROS secondary to oxidative stress (Barzilai and Yamamoto 2004).

The above selected examples illustrate how contaminants can alter essential physiological functions in wild birds, of which seabirds have been the most studied group of birds. The consequences of these detrimental physiological effects can be multifaceted, including physiological and behavioural alterations that eventually may reduce the reproductive success or survival of individuals (Saaristo et al. 2018). This emphasizes the significance of using biomarkers such as hormones, immune parameters, or genetic material to effectively detect the effects of OHCs and metals on wild birds at different levels of biological organizations. From a conservation and management standpoint, biomarkers offer promising tools to proactively mitigate large-scale population impacts, by identifying and addressing problematic contamination before it becomes irreversible.

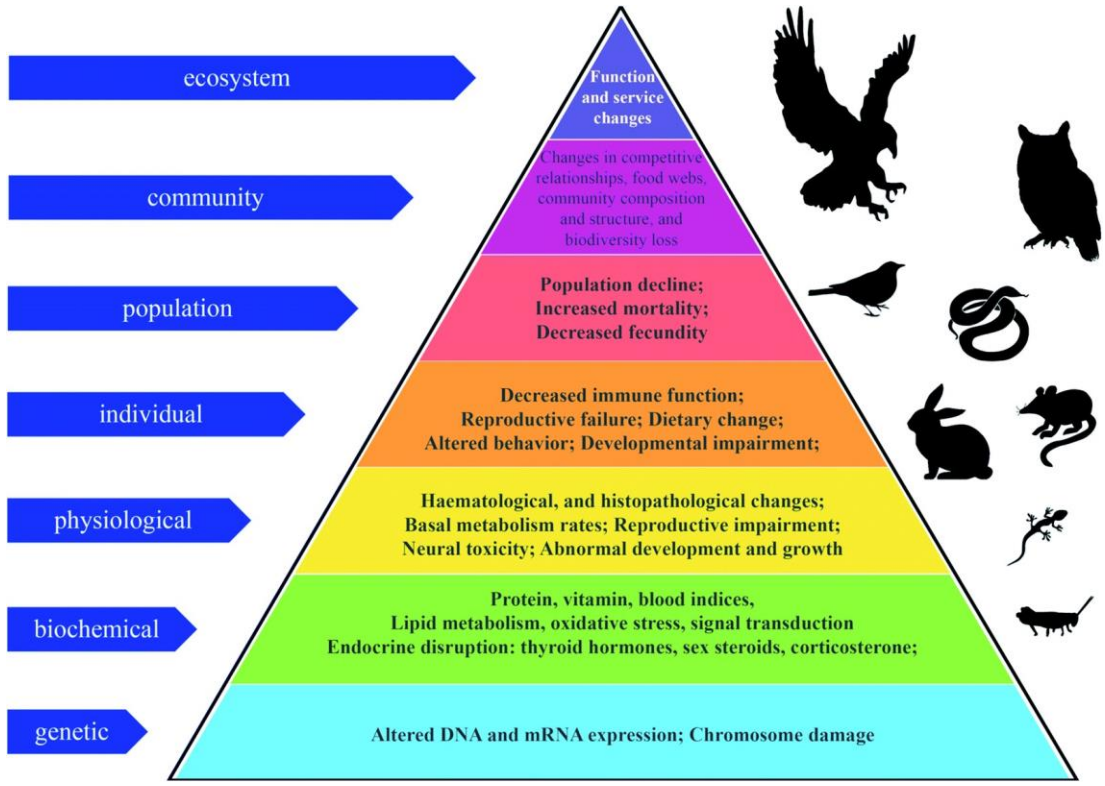


Figure 5 Direct and possibly indirect adverse effects from contaminant exposure on avian species at different levels of biological organizations. Reproduced from Hao et al. (2021) with permission from the Royal Society of Chemistry.

However, using biomarkers as health indicators of pollution exposure should be exercised with caution. One major challenge is the lack of a baseline for these biomarker profiles in nature, which forces us to depend on relative comparisons among individuals and evaluations of their contaminant levels (Whitehead and Dunphy 2022). Additionally, most wildlife studies are observational and correlative, meaning that they cannot establish causal relationships between the biomarkers and contaminant concentrations. Moreover, these studies often struggle to account for confounding factors, such as age, reproductive or nutritional status, and environmental variables like temperature, wind, and precipitation, all of which can influence the biomarker and contaminant profiles of animals (Ratajc et al. 2022; Whitehead and Dunphy 2022). As a result, correlative studies rely on a weight-of-evidence approach, where similar findings from different studies reinforce the evidence of contaminant-related impacts (Dietz et al. 2019).

Acknowledging potential drawbacks with using biomarkers in wildlife research is crucial. However, there are methods available to address these limitations when planning and conducting wildlife studies in ecotoxicology. Firstly, collecting larger sample sizes can provide more robust statistics (Bissonette 1999). Sample sizes in avian studies vary depending on the study species and accessibility. Generally, obtaining larger numbers of non-destructive tissue

samples such as eggs, blood, and feathers is more feasible compared to collecting samples from dead birds (either deceased or sacrificed), adipose tissue, or other organs through lethal methods, due to ethical and conservation considerations. Secondly, utilizing long-term data series covering fluctuating environmental conditions helps reduce the influence of confounding factors that could otherwise lead to spurious apparent relationships between biomarker and contaminant levels.

Conducting biomarker analysis in long-term studies does, however, raise some additional challenges. For example, when using blood sampling to analyse biomarkers and contaminants, conducting regular blood sampling from numerous individuals over several years necessitates capturing the birds (hence the prevalence of studies on nestlings) and skilled personnel to perform the blood draws. This approach involves performing numerous chemical analyses on many individuals every year, which can be time-consuming and expensive, making it challenging to sustain such a research project. Some assays require larger blood samples to cover both the biomarker and contaminant analyses, which means only birds above a certain size may be utilized. Additionally, accumulating blood samples over several years and analysing them simultaneously is only feasible for specific biomarkers, as some, like ROS and antioxidants (oxidative stress markers), degrade in blood samples over time (Bortolin et al. 2017). For long-term retrospective studies, it is advantageous to use biomarkers that remain stable over time.

Certain biomarkers are particularly well suited to long-term retrospective analyses in avian ecotoxicology. Corticosterone (CORT), the primary glucocorticoid and stress-associated hormone in birds, stands out as a well-established biomarker for assessing health in avian conservation research and ecotoxicology (Ganz et al. 2018; Whitehead and Dunphy 2022). CORT can be measured in blood, reflecting immediate levels, or in feathers, which integrate CORT levels during the feather growth period (lasting from days to weeks) (Bortolotti et al. 2008). A crucial benefit of feather CORT is its stability after incorporation, making it an ideal biomarker to assess CORT physiology retrospectively over a long timescale (Bortolotti et al. 2008). Another relevant biomarker for long-term effect studies is telomere length. Telomeres are repetitive sequences of non-coding DNA that cap the ends of chromosomes, playing a crucial role in maintaining DNA and chromosome stability (Blackburn 1991). In birds, DNA can be extracted from red blood cells, allowing for the analysis and measurement of telomere length (Criscuolo et al. 2009). A notable property is that the DNA remains stable in blood samples over time, making telomere length an ideal biomarker for long-term retrospective

studies (Criscuolo et al. 2009). In recent years, telomere length has gained popularity as a biomarker in ecology, particularly associated with age and fitness-related traits (Tobler et al. 2022). Moreover, telomeres have recently garnered increasing interest as potential proxies of contaminant-mediated effects including genotoxicity (Louzon et al. 2019), highlighting their relevance in ecotoxicological research. In summary, both feather CORT and telomeres represent useful non-destructive biomarkers that have been widely applied in ecology and increasingly in wildlife ecotoxicology.

While conventional biomarkers have proven useful, there is also a drive to expand the available toolbox by exploring the use of new biomarkers in an ecotoxicological context, such as leukocyte coping capacity (LCC). In brief, the LCC assay is a real-time measure of the leukocytes' ability to release ROS following a secondary stressor, indicating their potential to produce a respiratory burst, thereby enhancing individuals' capacity to respond to bacterial challenges (McLaren et al. 2003). LCC has been used as an integrated measure of immunity following stress exposure (Huber et al. 2019), which is pertinent considering the immunotoxicity caused by environmental contaminants (Desforges et al. 2016; DeWitt et al. 2012). The analysis of LCC is carried out *in situ*, utilizing just a droplet of blood (Huber et al. 2017b). Consequently, the method is considered minimally invasive, and there is no requirement for storage to conduct subsequent biomarker analysis, except for the contaminant analysis. In summary, it is important to study both established and to pioneer novel biomarkers, in order to gain the best possible insights into the effects of contaminants on avian wildlife, and thereby better inform conservation and management efforts.

Despite the widespread use of biomarkers in avian ecotoxicological research over the past three decades, there remains a critical need to deepen our understanding of the physiological consequences of contaminant exposure. Most biomarker studies on wild birds have been limited to short-term assessments, typically lasting no more than 1-2 years. Furthermore, there is a lack of long-term studies that encompass the large variations in environmental concentrations of OHCs and metals before and after regulations were implemented. This thesis aims to fill this knowledge gap by utilizing long-term data series from two sentinel raptor species, providing further insights into the use of biomarkers to detect sublethal effects caused by contaminant exposure. Additionally, we seek to expand the ecotoxicological toolbox by introducing the novel use of LCC as a biomarker of effect related to contaminant exposure in avian wildlife. By employing these approaches, we hope to better assess and understand the consequences of contaminant exposure on wild birds of prey.

1.5 Thesis objectives

The overall objective of this thesis is to investigate sublethal health effects from exposure to environmental contaminants in wild birds of prey, based on case studies on the WTE in Norway and Sweden and the tawny owl in Norway. These long-lived species are upper trophic level predators subject to heightened contaminant exposure via biomagnification and are therefore considered good candidates for investigating environmental contaminant effects. Contaminant-mediated effects were studied in the two raptor species by using selected biomarkers of health and measures of contaminant concentrations (OHCs or metals), in combination with relevant biological and ecological data over interannual to decadal time series. Within the overall scope of this thesis, the following sub-objectives were defined:

1) To examine concentrations, composition, and trends in environmental contaminants in the two raptor species (OHCs in WTEs and metals in tawny owls).
2) To investigate biomarker profiles in the WTE and tawny owl and their relationship with biological and ecological variables, of which the latter were based on dietary variability proxied by stable isotopes of carbon (habitat of prey) and nitrogen (trophic level). Dietary proxies are important covariates in relation to contaminant concentrations and may induce direct effects on biomarkers.
3) To investigate relationships between selected biomarkers of health and contaminant concentrations, in order to assess their relevance as indicators of sublethal health effects.

In this study, the choice of biomarkers was based on their minimal invasiveness, suitability for retrospective analyses, and their potential as indicators of health effects caused by exposure to contaminants. The selected biomarkers included:

- leukocyte coping capacity (LCC) as an indicator of immunocapacity,
- telomere length as a proxy of genotoxicity,
- and feather corticosterone (fCORT) as a measure of long-term stress physiology.

2 Methods

This thesis includes studies that were conducted within the Ecostress (Papers I-III) and Envistress (Paper IV) research projects, funded by the Norwegian Research Council. All papers in this thesis are based on observational field studies involving either blood or feather samples from nestling and adult white-tailed eagles *Haliaeetus albicilla* (referred to as WTE) or adult female tawny owls *Strix aluco*. All the studies share the common approach of analysing samples for organic (OHCs) or inorganic (metals) contaminants, as well as physiological biomarkers. This section gives an overview summarizing the sampling protocols and methods, while the reader should refer to the included papers (I-IV) for complete detailed descriptions.

2.1 Field sampling

Field protocols including capturing, handling and blood sampling live birds were carried out under approved permits and by highly experienced field personnel.

2.1.1 WTEs (papers I-III)

Sampling of nestling and adult WTEs (Fig. 6) was conducted in two subpopulations in Norway and Sweden. In Norway, only nestlings were sampled (Paper I). In Sweden, both nestlings and adults of the same subpopulation were sampled (Papers II and III). Sampling procedures for nestling and adult WTEs are provided in the sections below.

Figure 6 A white-tailed eagle nestling (left) a few weeks before fledging, and an adult white-tailed eagle (right; age 6 years + based on adult plumage which is most notably characterized by a complete white tail and a yellow beak). Photos by Elisabeth Hansen in Norway.

2.1.1.1 Nestlings

WTE nestlings were sampled from two different geographical regions (northern Norway and the Baltic coast of Sweden) but with similar sampling protocols. In Sweden, nestlings were sampled annually in 1995-2013 on the Swedish Baltic coast (58.5-61.5 °N, 17-19 °E). In

Norway, nestlings were sampled in 2017 and 2018 in Troms and Nordland counties (65.5-70 °N, 12.5-22 °E), northern Norway.

WTE nests were visited prior to sampling between late March and mid-May to record breeding activity and egg-laying. To avoid disturbing the nesting eagles, field observations of nests were carried out from a distance using binoculars and a spotting scope. Sampling took place as close to fledging as possible, i.e., when the chicks were approximately 8 weeks of age. Nestlings (1-3 per nest) were captured at their nest, accessed from the land or the sea, and removed from the nest during sampling to collect data. The following morphological measurements were collected: body mass (g) with a spring balance; wing length (mm) with a ruler; skull (mm), tarsus (mm) and hind claw (mm) with a sliding calliper.

Blood samples (5-10 ml) were collected from all nestlings of the same nest in northern Norway, while only one specimen per nest was randomly blood-sampled in Sweden. Detailed descriptions of blood sampling, treatment and storing procedures are provided in papers I and II for the Norwegian and Swedish nestlings, respectively. In summary, 84 samples of whole blood, blood plasma and red blood cells (RBCs) from the Norwegian nestlings were obtained and used for chemical analyses. From the Swedish nestlings, 71 samples of whole blood and 81 samples of serum and RBCs were obtained and used for chemical analyses.

2.1.1.2 Adults

In 1967-2012, shed feathers of adult WTEs were opportunistically collected at nests in different breeding territories (58.5-61.5 °N, 17-19 °E) on the Swedish Baltic coast. In total, 135 feather pool samples were used for chemical analysis, of which each pool (ca. 10 feathers) represented one territory per year. Feather pools were stored at room temperature at the Swedish Museum of Natural History, from where they were later retrieved for laboratory analysis.

2.1.2 Tawny owls (Paper IV)

Tawny owls were sampled in the area surrounding Trondheim (63.4 °N, 10.4 ºE), central Norway in 1986-2019 (Fig. 7). There, nest boxes were utilized to annually capture female tawny owls during their breeding season (March-May) to collect data on reproductive traits (clutch size, egg size, and fledgling success), body condition (morphometrics: body weight [g] using a spring balance and wing length [mm] using a ruler) and survival (individual owls identified by a unique ring ID number). In addition, one tail feather was pulled from the female at each capture and in total 1202 feathers have been sampled over the study period. As tawny owls may

breed for up to 10 years (Georg Bangjord, pers. com.), some females were repeatedly sampled over the study period. Approximately 20-70 females were captured each year for data collection and the number of individual owls that were sampled was 487. Feathers were stored in envelopes marked with year and ring ID numbers at ambient temperature until chemical analysis.

Figure 7 Breeding female tawny owl (age: 1 year +) captured in the next box during fieldwork in Trondheim. Photos by Elisabeth Hansen.

2.2 Chemical analyses

In this thesis, all chemical analyses followed established protocols, which ensures the reliability and comparability of results across studies. Detailed analytical methods are described in the material and methods section in papers I-IV.

2.2.1 Contaminants

2.2.1.1 Blood

Different groups of OHCs were analysed in plasma from WTE nestlings in northern Norway (OCs, PFAS; Paper I) and whole blood or serum in WTE nestlings from the Swedish Baltic coast (OCs, PBDEs; Paper II).

Using plasma samples, OCs and PFAS were analysed at the Norwegian Institute for Air Research, Tromsø, Norway. OCs were analysed following the protocol by Herzke et al. (2009) and PFAS were analysed using the protocol by Sletten et al. (2016). In brief, OCs were extracted with *n*-hexane and quantitively analysed by gas chromatography coupled to mass spectrometry (GS-MS), while PFAS were extracted using methanol and the analysis of compounds was

performed by ultrahigh-pressure liquid chromatography triple-quadrupole mass spectrometry (UHPLC-MS/MS). Quantification of OCs and PFAS was done by using the internal standard method using isotopically labelled compounds. Reference materials and blanks were prepared and treated in the same way as the plasma samples to ensure the quality of the method. The concentration of OHCs in plasma was expressed in ng g^{-1} in wet weight (*ww*).

Using whole blood or serum samples, OCs and PBDEs were analysed at the Toxicological Centre, University of Antwerp, Belgium. Analysis of compounds was conducted following the protocols described in Eulaers et al. (2011b) and Covaci and Voorspoels (2005). In brief, compounds were extracted using methanol, and detection and quantification of OCs and PBDEs were performed using GS-MS. Analytical quality assessment and control were supported by the analysis of procedural blanks and the addition of internal standard solutions to each sample. The concentration of OHCs in whole blood and serum was expressed in ng mL^{-1} *ww*.

2.2.1.2 Feather

OHCs were analysed in body feathers of adult WTEs (Paper III), while metals were analysed in tail feathers of female tawny owls (Paper IV).

Feather OHCs (OCs and PBDEs) were analysed at the University of Antwerp, Belgium, following the protocols described in Sun et al. (2020) and Hansen et al. (2023). OCs and PBDEs were extracted using a mixture of hexane:dichloromethane and HCl, and quantified using GS-MS negative ionization or in electron impact mode. The analytical quality assurance and control were evaluated based on the analysis of procedural blanks (concurrently run every 11 samples) and internal standards. The concentration of OHCs in feathers was expressed in ng g^{-1} in dry weight (*dw*).

Metal and metalloid elements were analysed at the heavy metal laboratory at the Norwegian Institute for Nature Research in Trondheim, Norway, as described in Devalloir et al. (2023). In brief, following acid digestion, chemical analysis of metals was carried out using high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS). Measurement accuracy, uncertainty and reproducibility were checked to ensure quality control as explained in Devalloir et al. (2023). The concentration of metals in feathers is expressed in µg g^{-1} *dw*.

2.2.2 Biomarkers

2.2.2.1 Leukocyte coping capacity (LCC)

LCC was measured *in situ* in WTE nestlings using the procedure described by Huber et al. (2017a) (Paper I). LCC involves measuring the ability of leukocytes to produce an oxidative burst following a chemical challenge (here, phorbol-myristate-acetate [PMA]) assayed in terms of reactive oxygen species (ROS) and calibrated through the emission of photons via their interaction with a luminogenic substrate. In brief, two tubes with 10 µl of whole blood were spiked with lucigenin and either PMA (the chemical challenge inducing the release of ROS) or phosphate-buffered saline (PBS; the control representing baseline ROS levels). Blood chemiluminescence for each tube was then assessed for 30 s every 5 minutes for 60 minutes using a chemiluminometer and expressed in relative light units (RLUs). To express the unadulterated LCC response, the control sample score was subtracted from the PMA-challenged sample to correct for baseline leukocyte activity and potential background noise. In addition, total leukocyte counts from blood smears were used to correct for a potential mass effect of leukocytes on the measured LCC response. In Paper I, the amplitude of the maximum LCC score (called LCC-peak) was statistically measured and subsequently used to represent the entire LCC response.

2.2.2.2 Telomere length (Paper II)

Telomere length was analysed in WTE nestlings (Paper II) at IPHC-DEPE, CNRS, Strasbourg, France. Telomere length was analysed using the qPCR procedure described by Criscuolo et al. (2009). Briefly, genomic DNA was extracted from frozen RBCs, using an adapted protocol of a commercial kit (NucleoSpin® Blood Quickpure, Macherey Nagel, Germany). Because of the duration of the study (nearly 20 years), we made sure that prior to the measurement of relative telomere length, there was no efficiency variation during the sampling years for the telomeric and control genes. Relative telomere length (T/S ratio) was obtained using a ratio of the number of amplification cycles between a genomic control gene (S), and the telomeric sequences (T).

2.2.2.3 Feather corticosterone (Papers III-IV)

Corticosterone (CORT) was measured in feathers of adult WTEs (Paper III) and tawny owls (Paper IV) at UiT – The Arctic University of Norway, Tromsø. Following the protocol by Bortolotti et al. (2008), CORT was extracted from a single body feather in WTEs (calamus removed) and the distal part of the tail feather in tawny owls. In short, following a methanol-based extraction, feather extracts were assessed for CORT using an enzyme immunoassay kit

(901-097, Assay Designs Inc., USA) per the instruction of the manufacturer. Feather CORT concentrations are expressed in pg g^{-1} mm.

2.2.3 Sex determination

Molecular sexing of WTE nestlings was performed at IPHC-DEPE, CNRS, Strasbourg, France, using the PCR and gel protocols adapted from Helander et al. (2007). In brief, DNA was extracted from whole blood using a commercial kit (NucleoSpin® Blood QuickPure, Macherey Nagel, Germany) and the sections of the sex-linked chromo-helicase-DNA-binding gene (CHD-Z and CHD-W) were amplified by PCR using primers 2550 F and 2718R (Fridolfsson and Ellegren 1999).

The sex of adult WTEs was not known due to the use of archived feathers, but the feather pools likely consisted of moulted feathers from both the female and the male of the pair as they are both present at the nest during the breeding season. With regards to the tawny owl, only females incubate and occupy the nest box (Hirons 1985). Therefore, molecular sex determination of the tawny owls was unnecessary.

2.2.4 Stable isotope analysis

Body feathers from adult WTEs (Paper III) and tail feathers from tawny owls (Paper IV) were analysed for bulk carbon and nitrogen stable isotopes at the Laboratory of Oceanology (University of Liège, Belgium: WTEs) and the Stable Isotope Lab (University of Koblenz-Landau, Germany: tawny owls). Feather material from WTEs and tawny owls was analysed using an Isoprime 100 isotopic ratio mass spectrometry (Isoprime, UK) and a Delta V Advantage isotope ratio mass spectrometry (Thermo Scientific, Germany), respectively. Quality assurance and control were assessed from the concurrent analysis of internal reference materials. The stable isotope ratios for carbon and nitrogen are expressed as δ values (‰) relative to their respective international measurement standards.

2.2.5 Data treatment and statistical analyses

Data processing and statistical analyses were performed using Microsoft Excel and R software (2023). Overall, different statistical analyses have been performed by using general/generalized linear models or their mixed-effect counterparts (Zuur et al. 2009). Before applying any statistical models, thorough data exploration was carried out following the protocol by Zuur et al. (2010) to ensure that model assumptions of linearity, homoscedasticity, independence and

normality were fulfilled. In most cases, predictor (x) and response (y) variables were transformed by the natural log (ln) to meet the assumption of homoscedasticity and normality.

We analysed a wide range of OHCs and up to 50 compounds per tissue (Papers I-III). However, to ensure robust statistical analyses, we decided to reduce the number of compounds due to collinearity. We focused on grouping compounds with similar physicochemical properties (e.g., the sum of PCB and PBDE congeners, respectively) while separately examining OC pesticides such as DDE and HCB (Papers I and II). Additionally, we employed principal component analysis (PCA) as an alternative approach to capture variations in contaminants, as detailed in Paper III. Metal(loid)s on the other hand, were not grouped because we specifically selected only five different elements for our statistical analyses, as further described in Paper IV.

3 Results

This chapter summarizes key findings on contaminant concentrations (patterns and trends), biomarker profiles and biomarker-contaminant relationships in white-tailed eagles (WTEs) and tawny owls. Findings are based on results reported in Papers I-IV (Table 1).

Table 1 List of Papers in chronological order (publication year or in prep.) summarizing key methodological details. Abbreviations: organo-halogenated compounds (OHCs), leukocyte coping capacity (LCC) and feather corticosterone (fCORT). Sample size corresponds to the number of tissue samples.

Paper (pub. year)	Species	Life-stage	Location	Period	Tissue	Sample size	Contaminant class	Biomarker of health
I (2020)	WTE	Nestlings	Northern Norway	2017-2018	Blood[a]	84	OHCs (OCs, PFAS)	LCC
II (2022)	WTE	Nestlings	Sweden, Baltic coast	1995-2013	Blood[a]	168	OHCs (OCs, PBDEs)	Telomere length
III (2023)	WTE	Adults	Sweden, Baltic coast	1968-2012	Feather	135[b]	OHCs (OCs, PBDEs)	fCORT
IV (in prep.)	Tawny owl	Adults	Central Norway	1986-2019	Feather	1202	Metal(loid)s	fCORT

[a] Biomarkers were analysed in whole blood, while contaminants were analysed in different blood matrices depending on the study as described in section 3.2.1.1 and figure 1.
[b] Not a single feather, but the number of feather pools.

3.1 Contaminant concentrations

3.1.1 Patterns in composition

In WTEs, three groups of OHCs were examined: OCs (i.e., PCBs and pesticides; Papers I-III), BFRs represented by PBDEs (Papers II and III), and PFAS (Paper I). The composition of the targeted OCs in all matrices exhibited similar trends in their relative contributions to the targeted OHC load (Fig. 8). Across all matrices, ΣPCBs accounted for the majority of the analysed OCs (Fig. 8). After PCBs, DDE contributed the second-highest proportion to the total targeted OCs (Fig. 8). Other OC pesticides (including HCB, chlordanes, and hexaclorocyclohexane [HCH]) comprised less than one-third of the total targeted OCs in all matrices (Fig. 8). ΣPBDEs, analysed in all matrices except plasma, represented less than 1% of the total targeted OHC load (Fig. 8). PFAS, measured in plasma only, accounted for 52%, surpassing the contribution of OCs to the targeted plasma OHC load (Fig. 8).

In tawny owls, five non-essential toxic metals were analysed in feathers: aluminium (Al), arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) (Paper IV). Among these, Al contributed the most with 58.6%, followed by Hg (36.8%), while Cd contributed the least (0.1%) to the total targeted metal load (Fig. 9).

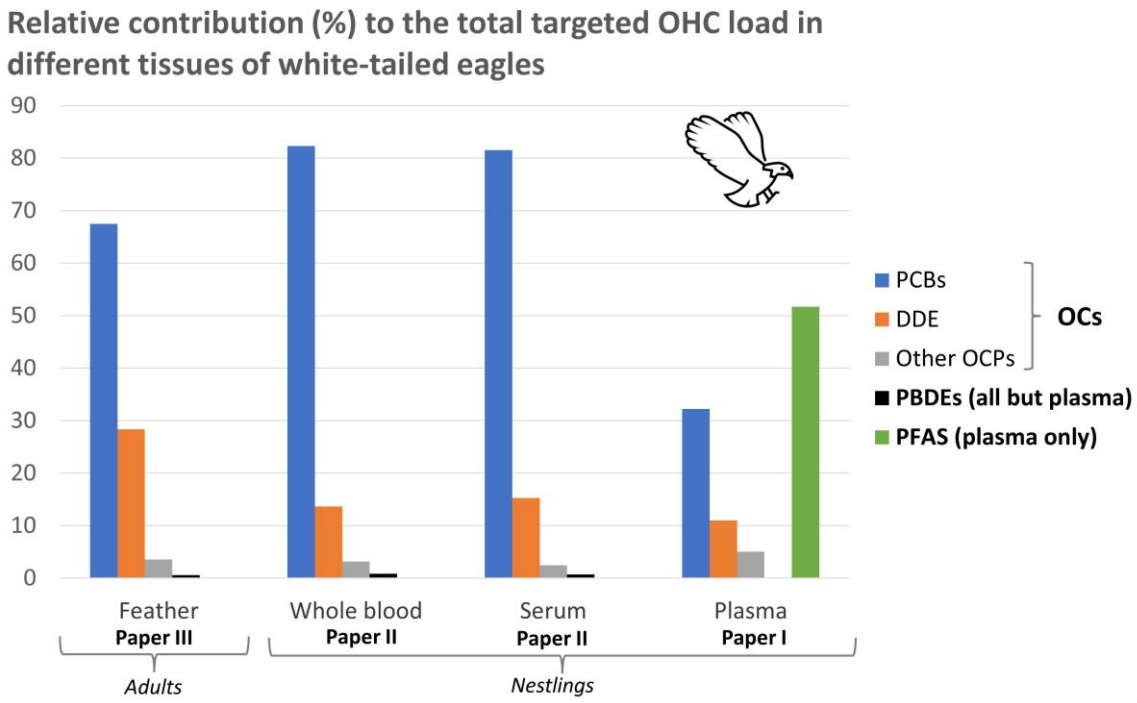


Figure 8 Overview of the relative contribution of different organo-halogenated contaminant (OHC) groups and selected compounds to the total targeted OHC load in different matrices of adult and nestling white-tailed eagles. The contaminant load is calculated based on the average concentration across the respective study locations and periods ranging from 2-44 years (Papers I-III).

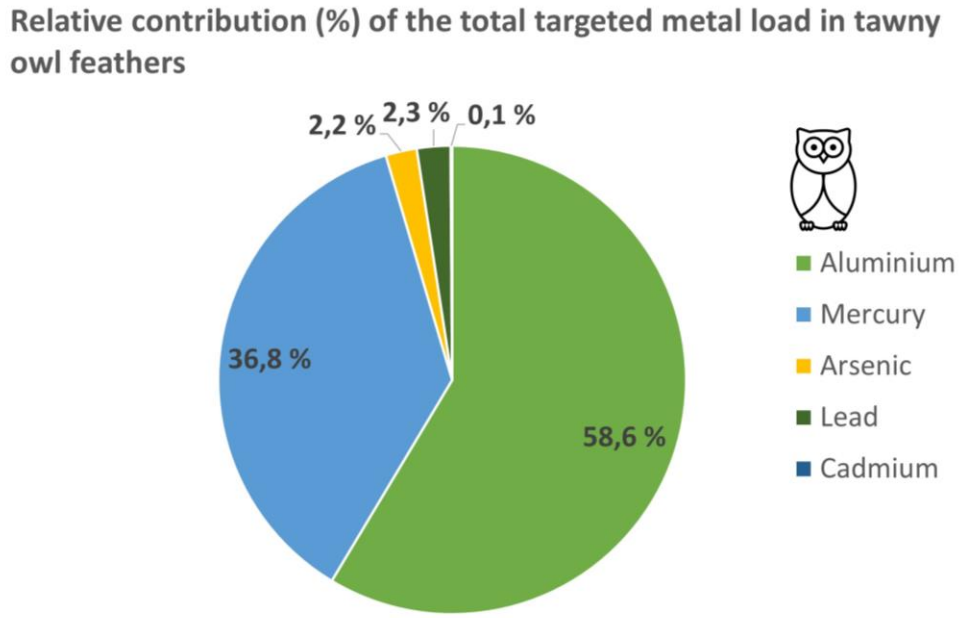


Figure 9 Overview of the relative contribution of different non-essential toxic metal(loid)s to the total targeted contaminant load in adult tawny owls. The contaminant load is calculated based on the average concentration per element across the study period 1986-2019 (Paper IV).

3.1.2 Temporal variations

In the two long-term studies of this thesis (Papers III and IV), we investigated the temporal variations of contaminant concentrations in adult WTEs and tawny owls across their respective study periods. In the Swedish adult WTEs, there were clear trends in feather OHC concentrations over time (Paper III). OCs demonstrated a decreasing linear trend with an average annual decline of 5 % in the period 1968 to 2012 (Fig. 10). PBDE concentrations, on the other hand, displayed a different pattern, characterized by a bell-shaped curve with peak concentrations in the 1990s, followed by relatively stable concentrations that showed a declining trend (Fig. 10). In the tawny owls, feather concentrations of all metals demonstrated an average annual decline in the period 1986 to 2019 by 1, 2, 3, 8, and 11 % for Hg, Al, As, Cd, and Pb, respectively (Paper IV).

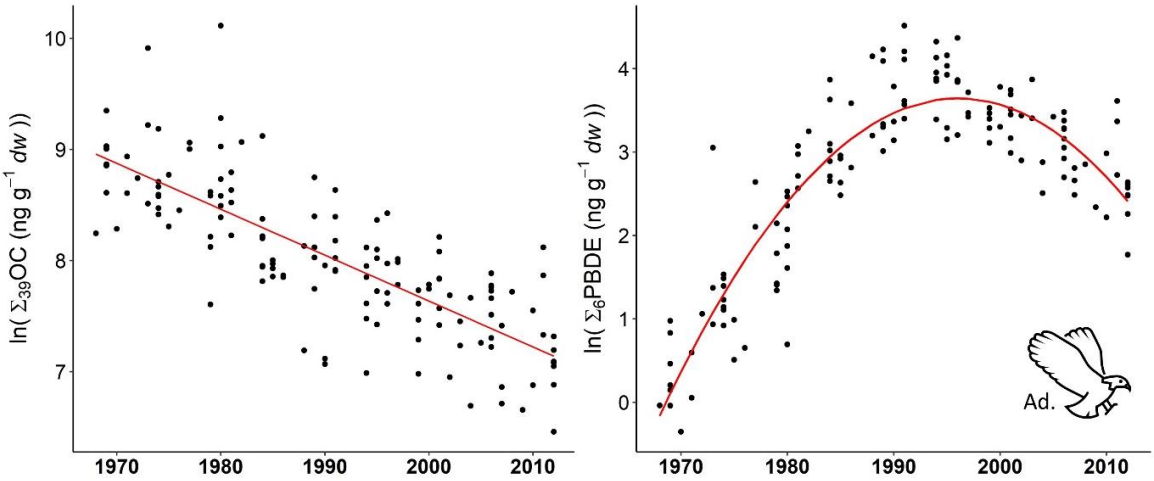


Figure 10 Annual concentrations (log transformed) of organochlorines (OCs; left) and polybrominated diphenyl ethers (PBDEs; right) in feathers of adult white-tailed eagles in Sweden. Red lines indicate significant linear ($p < 0.01$) and non-linear effects ($p < 0.01$) of year on OC and PBDE concentrations. Adapted from figures in Paper III.

3.1.3 Relationships with biological and ecological variables

In Norwegian WTE nestlings, we examined variations in OHC concentrations in relation to biological variables, namely body condition, sex, and brood size. We found a significant negative effect of body condition on contaminant concentrations, that is, nestlings in poorer condition exhibited higher concentrations of most tested OHCs, except PFHxS and long-chained perfluoroalkyl carboxylic acids (LC-PFCAs; Paper I). In contrast, sex or brood size did not explain variations in OHC concentration in the WTE nestlings of the same population (Paper I).

In adult WTEs and tawny owls, we investigated temporal variation in stable isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) (i.e., ecological variables) and their relationship with contaminant concentrations. Ranges in stable isotope signatures did not overlap between the two species where tawny owls exhibited lower $\delta^{15}N$ and $\delta^{13}C$ values than WTEs (Fig. 11). This contrast in isotopic values reflects their respective associations to terrestrial (tawny owl) and marine (WTE) ecosystems. In WTEs, there was a positive linear effect of year on $\delta^{15}N$, but no effect on $\delta^{13}C$ (Paper III). However, although we did not directly test the effect of $\delta^{15}N$ and $\delta^{13}C$ on OHC concentrations in WTEs, $\delta^{15}N$ and $\delta^{13}C$ were included as covariates in the models investigating the biomarker-contaminant relationships (Paper III). In Tawny owls, there was a significant negative effect of year on $\delta^{13}C$, but no temporal trend in $\delta^{15}N$ (Paper IV). Furthermore, there was a significant negative and positive effect of $\delta^{15}N$ and $\delta^{13}C$, respectively, on Hg in the tawny owls (Fig. 12).

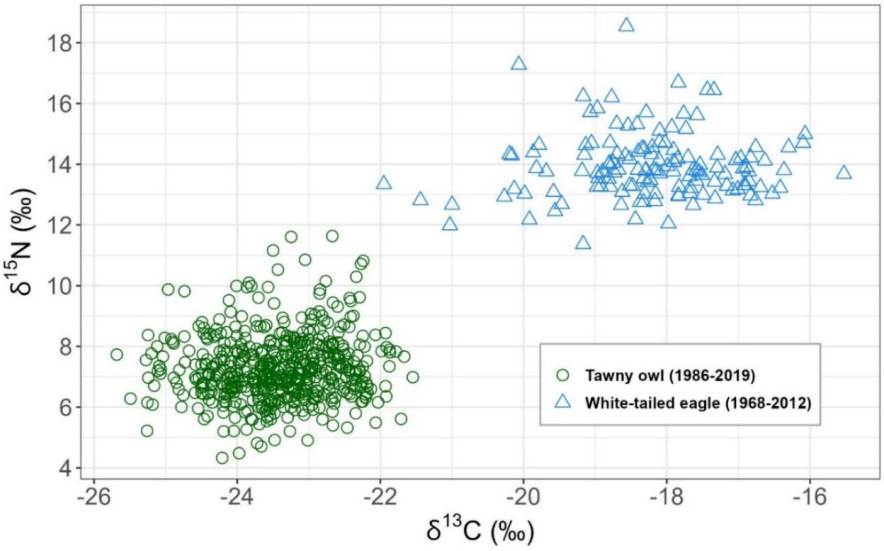


Figure 11 Isotopic ranges of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in feathers of adult tawny owls (green circles) and adult white-tailed eagles (blue triangles).

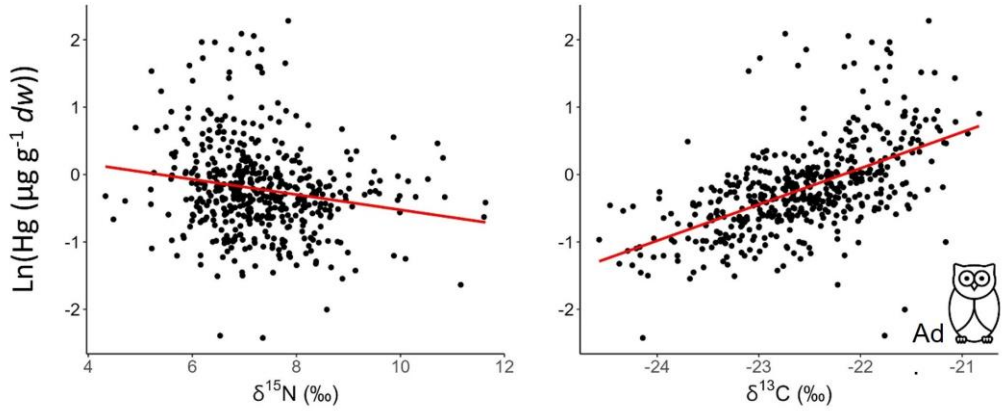
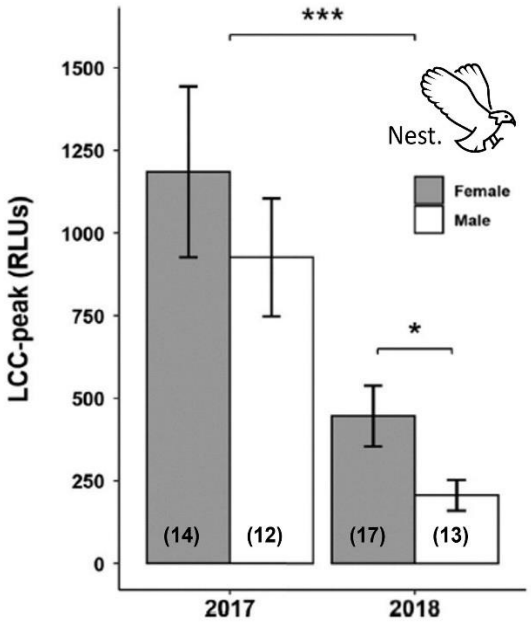


Figure 12 Feather mercury (Hg) concentrations in relation to $\delta^{15}N$ (left) and $\delta^{13}C$ (right) in the tawny owls sampled between 1986-2019. Red lines indicate significant linear relationships (both $p < 0.01$), and the figure is adapted from Paper IV.

3.2 Biomarkers of health



3.2.1 LCC (Paper I)

LCC-peak amplitudes in WTE nestlings ranged from 19.6 to 4368.7 RLUs, with a mean of 678 RLUs. LCC-peaks differed between the two study years, with the mean amplitude of LCC-peaks approximately 5 times higher in 2017 compared to 2018 (Fig. 13). The amplitude of LCC-peaks was higher in females than in males, but only significantly so in 2018 (Fig. 13). LCC-peaks were, however, not related to either body condition or brood size.

*Figure 13 Mean ± SE intensity of the leukocyte coping capacity (LCC) peak amplitudes in white-tailed eagle nestlings sampled in northern Norway. Asterisks (*) indicate statistically significant differences between the variables (*p < 0.05, ***p < 0.001) and the bracketed numbers represent sample size for each group. Figure adapted from Paper I.*

3.2.2 Telomere length (Paper II)

Mean telomere lengths (T/S ratio; unitless) in the WTE nestlings were 1.03 and the T/S ratio ranged from 0.16 to 2.30 (Fig. 14). Telomere lengths of nestlings varied on a year-to-year basis. Variations in telomere lengths were however not related to sex, brood size, or wing length.

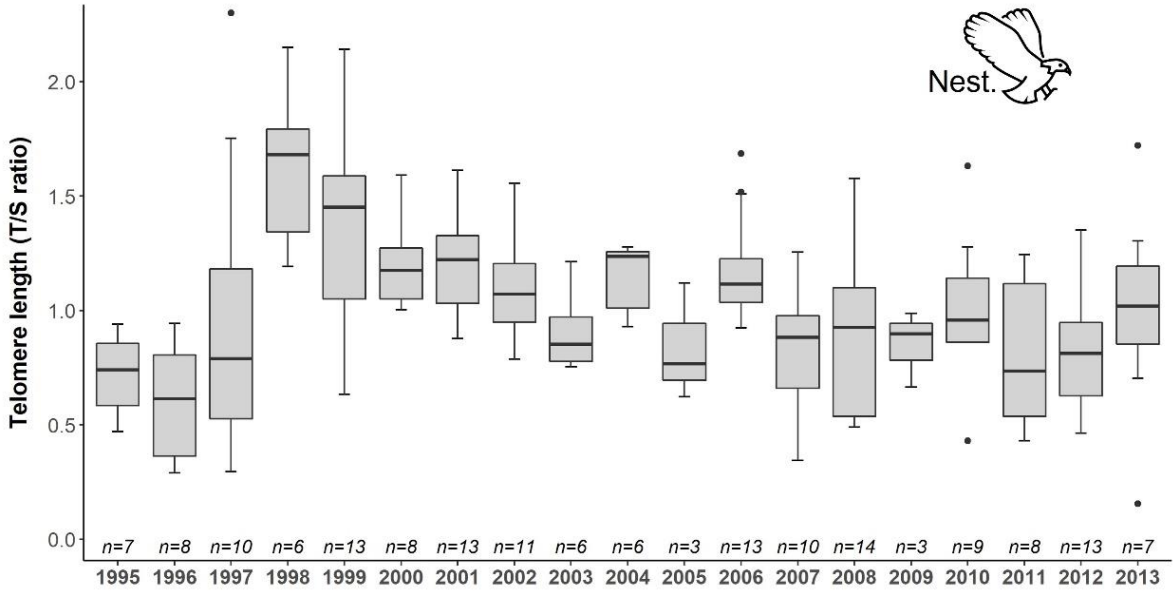


Figure 14 Boxplots showing the median, 25th-50th quartiles (boxes) and range (whiskers) in the observed telomere lengths among white-tailed eagle nestling cohorts sampled at the Swedish Baltic coast during 1995–2013. Annual sample size (n) is indicated above the respective year on the graph. Figure adapted from Paper II.

3.2.3 fCORT (Papers III and IV)

In adult WTEs, the median concentration of fCORT was 18 pg mm^{-1} with an order of magnitude difference in range between the lowest (8 pg. mm^{-1}) and highest (94 pg mm^{-1}) concentrations (Fig. 15). We did not detect any linear effect of year on fCORT concentrations over the 44-year study period (Paper III). In tawny owls, median concentrations of fCORT were 18 pg mm^{-1} and ranged between 3 and 327 pg mm^{-1} (Fig. 16). There was a significant linear increase in average fCORT concentrations from 1986 to 2019 (Paper IV). In the WTEs, fCORT was not related to δ^{15}N or δ^{13}C values (Paper III), while in the tawny owls, fCORT was negatively and positively associated with δ^{15}N and δ^{13}C values, respectively (Paper IV).

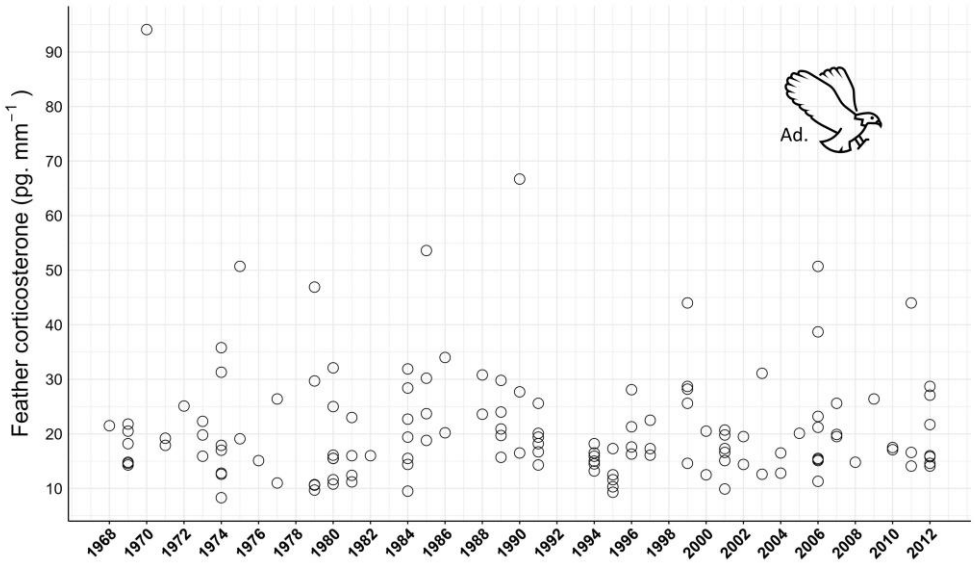


Figure 15 Corticosterone concentrations in feathers of adult white-tailed eagles in Sweden in 1968-2012. Figure adapted from Paper III. Sample size ranged from 1-9 feathers per sampling year.

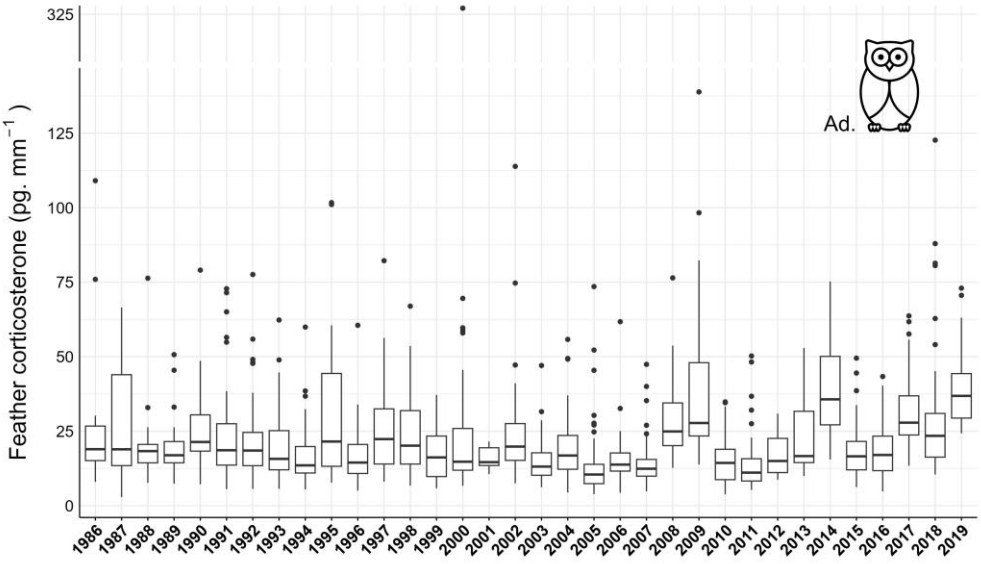


Figure 16 Boxplots showing the median, 25th-50th quartiles (boxes) and range (whiskers) in the observed corticosterone concentrations in feathers of adult tawny owls in Norway in 1986-2019. Sample size ranged from 20-70 feathers per year.

3.3 Relationships between biomarkers and contaminants

A schematic overview of the biomarker-contaminant relationships observed in the different studies is provided in Figure 17. In summary, in Norwegian WTE nestlings, all nine OHCs, except *cis*-chlordane, showed negative relationships with the amplitudes of LCC-peaks (Paper I). However, these negative relationships were only significant for ΣPCBs, DDE, LC-PFCAs, and PFHxS (Paper I). In contrast, no significant relationships were found between telomere length and OHC concentrations in whole blood or serum for Swedish WTE nestlings (Paper II). Additionally, there were no associations between fCORT and OHCs in adult WTEs (Paper III). Conversely, fCORT showed a positive relationship with Al and Cd, and a negative relationship with Hg in adult tawny owls (Paper IV).

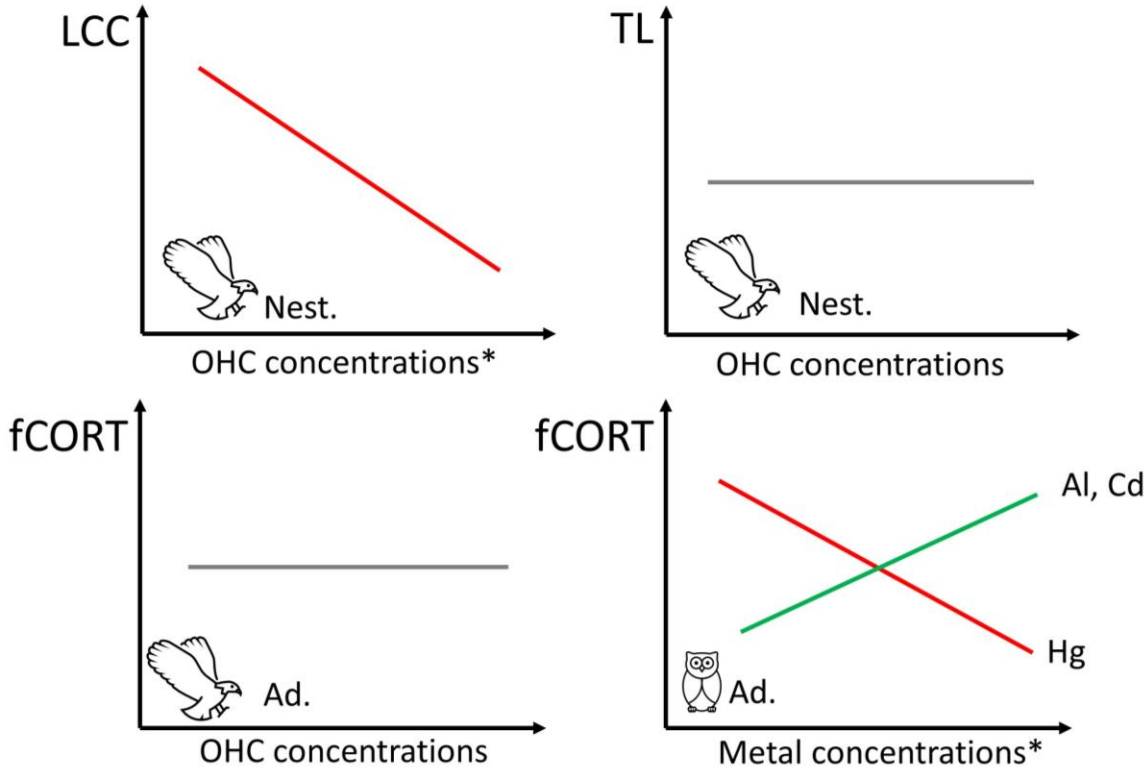


*Figure 17 Schematic overview of biomarker-contaminant relationships in white-tailed eagles (eagle icon) and tawny owls (owl icon). Plotted lines indicate the following detected relationships (based on linear models): red = negative and significant relationship, grey = no relationship, and green = positive and significant relationship. Abbreviations: Leukocyte coping capacity (LCC), telomere length (TL), feather corticosterone (fCORT), organo-halogenated compounds (OHC), and metal and metalloid elements (ME). *Signifies that not all OHCs or metals tested showed significant negative relationships with the investigated biomarker.*

4 Discussion

4.1 Biomarker-contaminant relationships in the WTE and the tawny owl

4.1.1 LCC in nestling WTEs (Paper I)

We found that the nestlings in Norway sampled in 2017-2018 with higher OHC concentrations (i.e., OCs and PFAS) also typically had lower LCC, indicating a potential negative effect of OHCs on the immune capacity of WTE nestlings. LCC has not been studied in an ecotoxicological context before, but it is well documented that exposure to OHCs, and PFAS in particular, is associated with negative impacts on the immune system in wildlife (Desforges et al. 2016; DeWitt et al. 2012) including seabirds and raptors (Bustnes et al. 2004; Jara-Carrasco et al. 2015; Jara et al. 2018; Sagerup et al. 2009; Smits et al. 2002; Smits and Bortolotti 2001).

Based on our findings, LCC appears to be a useful tool to assess sublethal effects from OHC exposure on immune capacity in avian wildlife. However, to assess if a reduced LCC affects the long-term health or reproductive success (i.e., fitness costs) of birds, further studies are needed involving recapture. Recapturing fledged WTEs, for example, proves exceptionally challenging, given their tendency to disperse far from their natal environment in the first few years after fledging (Engler and Krone 2022; Whitfield et al. 2009). In addition, tracking with e.g., GPS technology, or recapturing is also costly, requires special permits, and demands substantial time and effort be devoted to operating e.g., baited traps. Therefore, future research applying the LCC assay should target species that can be more readily recaptured. Birds that are territorial year-round and use nest boxes to breed, like tawny owls, could be a useful species to target in order to investigate the potential long-term fitness costs of a reduced LCC.

4.1.2 Telomere length in nestling WTEs (Paper II)

Although we detected significant year-to-year variations in telomere lengths among the Swedish WTE nestlings in 1995-2013, telomere lengths did not correlate with any of the investigated OHC groups or compounds. Previous research has explored telomere length in wild birds (Louzon et al. 2019), including the WTE (Sletten et al. 2016). Specifically, Sletten et al. (2016) examined Norwegian WTE nestlings and found no correlation between telomere length and OHC concentrations. Both Sletten et al. (2016) and our findings suggest that telomere length is not a useful proxy for OHC-mediated effects in nestling WTEs. However,

studies on various seabird species have reported associations between telomere length and exposure to OCs (Blevin et al. 2016) and PFAS (Blevin et al. 2017a; Sebastiano et al. 2020; Sebastiano et al. 2023). This implies that telomere length in other species might be more sensitive to OHC exposure. Indeed, the biology and ecology of seabirds like black-legged kittiwakes *Rissa tridactyla* and glaucous gulls *Larus hyperboreus* in the above-mentioned studies differ significantly from those of territorial and non-migratory raptors such as the WTE. For instance, both kittiwakes and glaucous gulls are colony breeders that undergo long-range migrations each year between their breeding and wintering grounds (Furness 2012). Such biological and ecological differences among species may contribute to the observed discrepancies in studies using telomere length as a health biomarker. Telomere length may therefore be more applicable in assessing such effects in other bird taxa and species than the WTE.

4.1.3 fCORT in adult WTEs (Paper III) and tawny owls (Paper IV)

We found no significant associations between fCORT and OHCs in the adult Swedish WTE in 1968-2012, despite this population being exposed to contrasting levels of OCs and PBDEs during the study period (Paper III). In the tawny owl, however, we found that fCORT was positively related to Al and Cd, and negatively related to Hg (Paper IV), indicating that these toxic metals could modulate long-term stress physiology of the tawny owl. In total seven studies have previously investigated fCORT-metal relationships in wild birds, and five reported on the fCORT-OHC relationship. Among the studies on metals, three reported positive relationships (Meillere et al. 2016; Powolny et al. 2020; Strong et al. 2015) and four detected no relationships between fCORT and metal exposure (Branco et al. 2022; Ganz et al. 2018; Heath and Frederick 2005; Mancuso et al. 2022). Among the studies on OHCs, two reported positive (Monclus et al. 2018; Monclus et al. 2019), two negative (Bourgeon et al. 2012; Løseth et al. 2019a) and one detected no relationship between fCORT and OHCs (Randulff et al. 2022).

There is an overall lack of consistency in the outcomes across studies on the fCORT-contaminant relationship, regardless of whether they investigate metals or OHCs. Unravelling the impact of contaminants on long-term stress physiology (as measured by fCORT) in wild birds is an important research challenge in this field. Based on our studies, fCORT may be more sensitive to metals (Paper IV) than to OHCs (Paper III; i.e., OCs and PBDEs). However, the discrepant result may also stem from species-specific differences between the WTE and the tawny owl. For example, factors such as phenotype (e.g., biochemical processes), biology (e.g.,

life-history traits: tawny owls are first-time breeders at 1 year old, while WTEs are first-time breeders from 5-6 years on), or ecology (terrestrial vs. aquatic species) could all play a role in explaining the observed differences in our findings on fCORT as a biomarker of health in relation to contaminant exposure.

4.2 Biomarkers to assess sublethal effects of contaminants: what are the alternatives?

Despite extensive study of health biomarkers in ecotoxicology over many decades (Amiard-Triquet et al. 2012; Dietz et al. 2019; Hao et al. 2021), including this thesis, the variability in reported correlations has hindered the identification of specific biomarkers as definitive indicators ("golden proxies") of sublethal effects resulting from contaminant exposure. This variability means it remains difficult to answer the simple question of whether a given biomarker can reliably serve as a proxy for contaminant exposure. However, what could be better alternatives to evaluate sublethal effects in wildlife? Examples of the main possible alternatives are outlined and discussed below:

- **Laboratory experiments on captive-bred animals.** Laboratory experiments involving animals bred in captivity offer advantages in terms of controlled environmental conditions and precise management of contaminant exposure levels (Tveden-Nyborg and Lykkesfeldt 2021). This allows for a more rigorous assessment of health effects based on varying doses, with comparisons against a control group. Nevertheless, translating toxicological findings from laboratory studies to wild animals is challenging (Köhler and Triebskorn 2013). Firstly, the species typically studied in captivity are model species with specific characteristics and attributes that make them suitable for studying e.g., particular biological processes or diseases (Ankeny and Leonelli 2011). Traits that make model species like mice, rats and zebrafish suitable could be simple husbandry requirements, short reproductive cycles, and cost-effectiveness (Ankeny and Leonelli 2011). Consequently, contaminant-related effects observed in model species are not necessarily applicable to wild species (Calisi and Bentley 2009; Griffith et al. 2021). Secondly, captive-bred animals reside in controlled environments with specified stressors allowing potentially subtle effects to be observed (Relyea and Hoverman 2006). In contrast, wild animals face the possibility of exposure to multiple, unknown stressors, which may obscure, or mask the hypothesised contaminant-mediated effects making them undetectable (Calisi and Bentley 2009). Consequently, there is no

guarantee that an observable effect in a laboratory is translatable to wild animals (Beaulieu 2016; Calisi and Bentley 2009).

- **Experiments on captive wildlife.** Conducting experimental studies on wild animals in captivity offers certain benefits, such as the ability to manage exposure doses and exert some control over the environment. For wild avian species, this might involve maintaining them within outdoor enclosed facilities (e.g., Monclus et al. 2018; Sagerup et al. 2009). However, the process of capturing and confining wild animals can induce significant stress, potentially leading to biased results (Fischer and Romero 2019). Moreover, obtaining permits for such studies can be challenging or not allowed (e.g., for protected species in Norway; Norecopa 2023), and from a conservation standpoint, these practices can be deemed highly invasive (Robinson et al. 2019). An acute ethical dilemma arises when exposed animals cannot be safely released back into the wild and must be sacrificed at the study's conclusion (Rai and Kaushik 2018). Ethical concerns are also of particular importance when dealing with vulnerable species (Mason 2010).

- **The use of *ex vivo* techniques.** Recent ecotoxicological and ecological research has demonstrated an increasing interest in employing *in vitro* techniques such as the use of cell lines (Castano-Ortiz et al. 2019; Kroglund et al. 2022) or omics (Ebner 2021) to detect contaminant-mediated effects in wild animals. Such *ex vivo* techniques can be used to identify changes in for instance cellular responses or gene expressions (e.g., transcript- or proteomics) in response to contaminant exposure (Martyniuk and Simmons 2016; Wagner et al. 2017). *Ex vivo* techniques also offer several advantages, such as controlled experimental conditions and contaminant doses, reduced ethical concerns, and cost-effectiveness (Athira et al. 2022). In birds, applying *ex vivo* tools can be done minimally or non-invasively by using blood or feather samples (Kroglund et al. 2022). However, *ex vivo* techniques also have limitations, as they may not fully replicate the complexity and interactions present in living organisms (Nesslany 2017). Therefore, findings from *ex vivo* studies often need to be validated and further explored using *in vivo* experiments before they can be directly applied to real-life biological scenarios. One potential avenue for further exploration is establishing pathways between modifications in gene expression, for instance, and specific biological endpoints, such as biomarkers (Zhang et al. 2018).

Nevertheless, to comprehensively evaluate the long-term consequences on survival and fitness, it is essential to complement the above-mentioned controlled studies with field research, observing the animals in their natural habitats. I would therefore argue that biomarkers, at present, remain the best option to evaluate contaminant-mediated health effects in wildlife in terms of ethical considerations and our current lack of understanding of the implications of changes in e.g., omics on the health of wild animals (Rattner et al. 2023). Importantly, many biomarkers like LCC, telomere length and fCORT can be applied non-destructively, minimally, or non-invasively, which is essential from an ethical and conservation perspective when studying protected species like the WTE and tawny owl (Chaousis et al. 2018).

4.3 Future suggestions for implications of biomarkers in ecotoxicology

Wild birds in their natural environment encounter a variety of stressors, which, whether acting individually or in combination, can result in physiological changes during stressful events (McEwen and Wingfield 2003). Subsequently, these physiological changes can reduce an animal's ability to allocate energy towards biochemical processes (e.g., antioxidant defence, DNA repair or detoxification pathways) adapted to mitigating the effects of contaminants (Wallig et al. 2017). For instance, during periods of sufficient food availability and favourable climatic conditions, an animal might effectively counteract contaminant-induced effects (Bustnes et al. 2015b). Conversely, during challenging periods e.g., inclement weather, low food availability, or high disease prevalence, animals may struggle to mitigate pollutant effects (Bårdsen et al. 2018; Smith et al. 2022; Tartu et al. 2017). Pollutants can therefore interact with other biological and environmental stressors cumulatively, influencing the well-being of wild animals collectively (Rattner et al. 2023). Consequently, conducting studies within a single year might inadvertently place the studied species in either favourable or unfavourable conditions, which could, in turn, influence the observed biomarker-contaminant relationship. In the worst-case scenario, spurious relationships may consequently be reported in short-term studies unable to control for all latent variables. Conducting long-term studies, as in this thesis (Papers II-IV), can help minimize the influence of latent variables, including other environmental stressors, particularly if their variation can be approximated as random in nature over the study period (Likens 2012).

Conducting long-term studies can be challenging in wildlife research for practical reasons such as logistics, funding and access to study species or field sites (Lindenmayer et al. 2012). Collaboration between multiple field biologists who have collected samples such as eggs,

blood, or feathers, can facilitate this effort, as evidenced by the work conducted on the Swedish WTEs (Papers II and III) and Norwegian tawny owls (Paper IV). Furthermore, utilizing biomarkers amenable to retrospective analysis, such as fCORT and telomere length, enhances the feasibility of conducting long-term effect studies. Within Europe, notable collaborative initiatives have been initiated toward pan-European biomonitoring efforts for raptors (Badry et al. 2020; Gómez-Ramírez et al. 2014; Movalli et al. 2022). Establishing such platforms fosters cooperation and access to data series from wild animals, like raptors, for application in long-term effect studies.

In addition to long-term data series, increasing the sample size when evaluating biomarkers as indicators of contaminant effects is advantageous. Larger sample sizes enhance the statistical robustness of our results, leading to more confident conclusions (Bissonette 1999). The number of samples collected depends on several factors, like the species being studied, its habitat, and the time spent in the field (Albert et al. 2010). Selecting species that are abundant, such as WTEs and tawny owls, facilitates the acquisition of larger sample sizes. Tawny owls are more readily accessible for sampling than WTEs due to their nest boxes, which can be conveniently placed near roads. Conversely, WTEs nest on cliffs or in trees, making their habitat a determining factor in sample accessibility. Furthermore, the implementation of long-term studies and collaborative efforts significantly contributes to the augmentation of the sample size. In recent years, data repositories have gained popularity among scientists, providing yet another opportunity to expand the sample size for researchers studying similar topics (Michener 2015; Powers and Hampton 2019). Lastly, non-invasive techniques, like collecting shed feathers or addled eggs, offer easier and more ethical means to gather numerous samples. By utilising these strategies, we can increase our sample size, making our research more robust and ultimately drawing stronger, more meaningful conclusions from our observations.

Lastly, continuing the development of novel tools for assessing sublethal effects from contaminant exposure also remains of paramount importance in ecotoxicological research (Artigas et al. 2012). Drawing inspiration from advancements in related fields such as ecology and conservation research, we have the potential to identify biomarkers that can be applied in an ecotoxicological context (Whitehead and Dunphy 2022). Our ongoing focus should prioritize the search for non-destructive and preferably non-invasive biomarkers, with the overarching goal of minimizing undue stress on wild animals. The integration of these new tools into the ecotoxicological toolbox holds great promise, as in the case of LCC (Paper I) and may significantly advance our understanding of sublethal effects of contaminant exposure.

5 Conclusion

This thesis has provided new insights into biomarker-contaminant relationships in two sentinel raptor species: the white-tailed eagle (WTE) and the tawny owl. Both species have been exposed to contrasting levels of environmental contaminants over the past decades as documented in this thesis. Our findings contribute to the understanding of how environmental contaminants, specifically organo-halogenated compounds (OHCs) and toxic metals, can induce physiological effects in avian wildlife, which ultimately may affect individual survival or fitness over time. More specifically, this thesis demonstrates that certain biomarkers, such as leukocyte coping capacity (LCC) and feather corticosterone (fCORT), can be related to sublethal effects resulting from exposure to environmental contaminants in wild raptors. On the other hand, telomere length did not appear to be a good biomarker for contaminant-mediated effects in the WTE. Furthermore, a novel result of this thesis was that LCC appears to be a useful tool that can expand the ecotoxicological toolbox to assess sublethal effects in wildlife.

While the relationships between biomarkers and contaminants are complex and species-specific, they offer valuable insights into potential physiological consequences of contamination in wild animals not readily studied in their natural environment. Although alternatives like laboratory experiments, studies on captive wildlife, and *ex vivo* investigations offer benefits in terms of additional experimental control, they are constrained by other challenges. These include the potential non-transferability of findings to wild animals and the ethical concerns associated with conducting contamination experiments on live animals. On balance, biomarkers, despite their complexity and variability, remain a useful tool for assessing contaminant-mediated health effects under real-world conditions. In a multiple-stressor context, long-term studies can help tease apart the influence of various environmental stressors (including climate change). In this way, we can work towards a more comprehensive understanding of the complex dynamics governing contaminant-mediated physiological responses, as evaluated from biomarkers, over time.

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Paper I

A novel use of the leukocyte coping capacity assay to assess the immunomodulatory effects of organohalogenated contaminants in avian wildlife

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A novel use of the leukocyte coping capacity assay to assess the immunomodulatory effects of organohalogenated contaminants in avian wildlife

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ABSTRACT

Apex predators are characterized by high levels of biomagnifying organohalogenated contaminants (OHCs) which have been found to induce detrimental health effects in wildlife, such as immune system impairment. The leukocyte coping capacity (LCC) assay is a functional real-time measure of an innate immune response essential in pathogen resistance, known as the respiratory burst. The current study suggests the novel use of this tool to test whether OHCs impair the innate immune system of a sentinel top predator, the white-tailed eagle (*Haliaeetus albicilla*; WTE). The LCC analysis was performed in the field on WTE nestlings (n = 84) from northern Norway over two breeding seasons. Poly- and perfluoroalkyl substances (PFAS) dominated the total OHC load, surpassing the levels of legacy organochlorines. In addition, we detected significant negative correlations between concentrations of all polychlorinated biphenyls, *p,p′*-dichlorodiphenyldichloroethylene, perfluorohexane sulfonic acid and long-chain perfluorocarboxylic acids and the LCC of WTE nestlings. Based on our current findings reflecting a potential negative effect of both emerging and legacy OHCs on innate immune capacity, we suggest LCC to be a relevant and accessible test expanding the ecotoxicological toolbox to assess sub-lethal effects of OHCs in apex avian wildlife.

1. Introduction

Organohalogenated contaminants (OHCs), such as organochlorines (OCs) and poly- and perfluoroalkyl substances (PFAS), are anthropogenic chemicals utilized for a wide array of industrial and commercial purposes (Buck et al., 2011; Walker, 2001). OHCs are of concern because of their persistence, ubiquitous distribution through long-range transport, capacity to bioaccumulate in organisms and biomagnify through food chains, and reported toxic effects in both humans and wildlife (Jones and de Voogt, 1999). Consequently, apex predatory species are characterized by high exposure and are therefore more prone to suffer from severe health effects (Letcher et al., 2010). High exposure to OHCs has been associated with detrimental effects in wildlife through reproductive impairment in females, lower survival rates or increased mortality of offspring (Letcher et al., 2010; Rattner, 2009) as seen in the white-tailed eagle (*Haliaeetus albicilla*; hereafter WTE) on the Swedish coast of the Baltic Sea (Helander et al., 2002). Due to its apex position, the WTE functions as a sentinel species to monitor environmental pollution (Helander et al., 2008).

Immune system impairment is one potential sub-lethal effect induced by OHC exposure (Desforges et al., 2016). Immune impairment is manifested either directly (Bustnes et al., 2004; Jara-Carrasco et al., 2015; Sagerup et al., 2009) or indirectly through endocrine disruption (DeWitt and Patisaul, 2018; Kuo et al., 2012), and may potentially lead to increased susceptibility to diseases and pathogens (Badry et al., 2020; Jepson et al., 1999). In addition to the physiological impact from OHC exposure, stress (manifested by elevated corticosterone levels, i.e. the major stress hormone in birds; Romero and Romero, 2002) can have pervasive effects on both immunity (Bourgeon and Raclot, 2006; McEwen and Wingfield, 2003) and oxidative stress (i.e. the imbalance

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between free radicals and antioxidants; Costantini et al., 2008; Stier et al., 2009). Simultaneously, the induction of an immune response can lead to higher oxidative stress (Costantini and Moller, 2009) which can induce damage to biomolecules such as lipids, proteins and DNA, and increase the rate of telomere erosion (Houben et al., 2008; Watson et al., 2015; Young et al., 2016). The modes of action of biomarkers of physiological stress, oxidative stress and immunocompetence are therefore highly inter-connected and should ideally be assessed simultaneously and/or through an integrated measure.

The leukocyte coping capacity (LCC) assay represents an integrated measure of the patho-physiological state of the organism (Huber et al., 2019; Lilius and Marnila, 1992) making it a potential biomarker for ecotoxicological studies. Polymorphonuclear leukocytes (PMNLs), i.e. heterophil granulocytes in birds (Harmon, 1998), constitute one of the first lines of innate immune defense against invading pathogens and represent the most abundant cellular component of the immune system (Abbas et al., 2014; Rungelrath et al., 2020). Activated PMNLs release an array of factors and mediators, including reactive oxygen species (ROS; Quinn and Gauss, 2004), which are produced in an anti-pathogenic defense cascade known as the respiratory burst (Nathan, 2006; Shelton-Rayner et al., 2010). In the LCC analysis, the respiratory burst is stimulated *in vitro* by a potent PMNL activator, phorbol myristate acetate (PMA; McLaren et al., 2003). Several studies show that PMNLs of individuals previously exposed to environmental (external) or organismal (internal) stressors have a reduced capacity to produce ROS in response to a PMA challenge (Huber et al., 2017a, b; McLaren et al., 2003; Moorhouse et al., 2007; Shelton-Rayner et al., 2010, 2012). Lower LCC scores indicate a lower potential to produce a respiratory burst and individuals with a decreased LCC response are therefore physiologically less responsive towards invading pathogens (McLaren et al., 2003; Shelton-Rayner et al., 2012). Simultaneously, studies on the immunomodulatory properties of OHCs in birds have found impairments of the innate and the adaptive arm of the immune system such as altered leukocyte composition (Bustnes et al., 2004; Jara-Carrasco et al., 2015), lowered ability of lymphocytes to proliferate *in vitro* in response to antigens (Sagerup et al., 2009), and abnormal antibody function (Sagerup et al., 2009).

The present study aimed to test the novel use of the LCC as a measure of early innate immunocompetence in relation to OHC exposure in WTE nestlings. We predicted that nestlings with higher OHC exposure have a reduced LCC, i.e. a lower early innate immune defense. Prior to evaluating whether OHC exposure in WTE nestlings can affect the leukocytes' ability to produce a respiratory burst (LCC-method), we identified potential ecological and environmental drivers of plasma LCC and OHC levels in nestlings.

2. Material and methods

2.1. Field sampling

Fieldwork was conducted between the 17th of June and 6th of July 2017 and the 24th of June and 6th of July 2018 in different geographical areas of northern Norway: Steigen (67°44′ N 14°48′ E), Harstad (68°48′ N 16°32′ E) and Tromsø (69°39′ N 18°57′ E). Morphological measurements (body mass [kg], wing and tail length [both measured in mm]), body feathers and a blood sample were collected from 84 WTE nestlings at approximately 5–9 weeks since hatching, before fledging. All nestlings in a brood were captured on their nest, accessed from the land or the sea. Brood size ranged between 1 and 3 chicks per nest. The field protocol was approved by the Norwegian Food Safety Authority.

A lithium-heparinized syringe with needle was used to puncture the brachial vein and approximately 5–10 mL of blood was drawn. After aliquoting 20 μL of whole blood for immediate LCC measurements, the remaining blood was stored under dark and cold conditions until centrifugation (8000 rpm for 10 min) within 12 h. Plasma and red blood cells (RBCs) were separated after centrifugation and kept frozen at −20 °C until chemical analysis.

2.2. LCC-analysis

Out of 84 sampled WTE nestlings, 78 were successfully assayed for their LCC. Measurement of LCC in the nestlings was initiated within 30 min of blood sampling. Based on the description by Huber et al. (2017b), 10 μL of heparinized whole blood and 90 μL of 10^{-4} mol L^{-1} lucigenin (bis-N-methylacridinium nitrate; Sigma Aldrich, Vienna, Austria) dissolved in dimethyl sulfoxide (VWR International, Stockholm, Sweden) and diluted with phosphate-buffered saline (PBS, pH 7.4), were transferred into two silicon anti-reflective tubes, respectively (Lumivial, EG & G Berthold, Germany). Into the first tube, we added 10 μL of PBS to measure unstimulated blood chemiluminescence levels, which provides information on individual baseline levels of superoxide anion and acts as a control. In the second tube, we added 10 μL of 10^{-5} mol l^{-1} phorbol-myristate-acetate (PMA; Sigma Aldrich, Vienna, Austria) to assess whole blood chemiluminescence in response to this secondary chemical challenge (the first constant challenge occurring in the circulation in vivo). The tubes were swiveled gently to mix the solutes. Blood chemiluminescence for each tube was assessed for 30 s every 5 min for a period of 60 min using a portable high sensitivity chemiluminometer (Junior LB 9509, E G & G Berthold, Germany) and expressed in relative light units (RLUs) whose values equal to total photon count divided by 10 (Huber et al., 2017a). Between single measurements, the tubes were kept under a constant temperature of 39 °C in a light-proof metal bead bath (Grant Instruments, JB Nova5, UK).

2.3. Leukocyte differentiation

Blood smears were prepared from whole blood in the field using the standard two-side wedge procedure (Houwen, 2000). Total and differential leukocyte counts were performed at the INVITRO Labor für Veterinärmedizin, Diagnostik und Hygiene (Vienna, Austria; Appendix A, Table A.1). Heterophil numbers from the individual total leukocyte counts were used to correct for a potential mass effect of leukocytes on the measured LCC response in the WTE nestlings.

2.4. Molecular sexing

The sex of nestlings was genetically determined using RBCs at the Institut Pluridisciplinaire Hubert Curien – Département Ecologie, Physiologie et Ethologie (IPHC-DEPE) in Strasbourg, France. Molecular sexing analysis followed the protocol described by Helander et al. (2007). In summary, DNA was extracted from RBCs using a commercial kit (NucleoSpin® Blood QuickPure, Macherey Nagel, Germany) and the sections of the sex-linked chromo-helicase-DNA-binding gene (CHD-Z and CHD-W) were amplified by polymerase chain reaction using primers 2550F and 2718R (Sletten et al., 2016).

2.5. Contaminant analyses

Chemical analyses of organochlorines (OCs) and poly- and perfluoroalkyl substances (PFAS) in blood plasma were carried out at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. All solvents used were of Suprasolv® grade and purchased from Merck-Schuchardt (Hohenbrunn, Germany). The concentration of compounds are expressed in ng g^{-1} wet weight (*ww*) and a full list of the targeted compounds are given in Table A.2.

2.5.1. OC analysis

The method used has previously been described in detail by Herzke et al. (2009). In brief, 0.98–1.02 g of plasma was spiked with internal standard solutions containing all compounds of interest in their isotopic labelled version, before being denatured with ethanol and further

treatment with saturated ammonium sulphate solution. Next, the samples were extracted twice with *n*-hexane before volume reduction, and finally re-dissolved in hexane and cleaned up by a Florisil column to remove biological matrix. A recovery standard (^{13}C-PCB-159) was added to all samples prior to analysis. The extracts were quantitatively analyzed by gas chromatography coupled to mass spectrometry by using the internal standard dilution method. All samples were analyzed for 12 polychlorinated biphenyl congeners (PCB-28/31, 52, 99, 101, 105, 118, 138, 153, 180, 183, 187 and 194), dichlorodiphenyltrichloroethanes (*p,p′*-DDT and *o,p′*-DDT) and their metabolites (*p,p′*-DDE, *o,p′*-DDE, *p,p′*-DDD and *o,p′*-DDD), hexachlorobenzene (HCB), hexachlorocyclohexanes (*α*-, *β*- and γ-HCH), chlordanes (*cis*-chlordane, *trans*-chlordane, *trans*-nonachlor, oxychlordane), and Mirex.

2.5.2. PFAS analysis

The method used is reported in more detail by Sletten et al. (2016). First, 200 μL of plasma sample was spiked with an isotopic labelled internal standard solution ($^{13}C_4$-PFBA, $^{13}C_5$-PFPA, $^{13}C_5$-PFHxA, $^{13}C_4$-PFHpA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_6$-PFDA, $^{13}C_7$-PFUnDA, $^{13}C_2$-PFDoDA, $^{13}C_2$-PFTeDA, $^{13}C_3$-PFHxS, $^{13}C_4$-PFOS, $^{13}C_8$-FOSA, and $^{13}C_2$-6:2 FTS; Wellington Laboratories, Ontario, Canada), then extracted with methanol by repeated sonication in an ultrasonic bath and vortexing. Next, the samples were centrifuged until sedimentation, and the supernatant was purified using ENVI-Carb 120/400 (Supelco 57210-U) and glacial acetic acid as described by Powley et al. (2005). Finally, 0.5 mL of supernatant solution was spiked with 3,7-diMeo-PFDcA recovery standard, and the sample was vortexed. The analysis of compounds was performed by ultrahigh-pressure liquid chromatography triple-quadrupole mass spectrometry and quantification was done by internal standard method using the isotopically labelled PFAS.

2.5.3. Quality assurance and quality control

Reference materials (OCs: human serum, NIST SRM 1958; PFAS: AM-S-Y 1804) and blanks were prepared and treated in the same way as the plasma samples to assure the quality of the method.

The limit of detection (LOD) was defined as the average signal to noise ratio for the analyzed matrix, or average concentration of the procedural blanks (if a signal was detected), plus 3 times the standard deviation. The limit of quantification (LOQ) was set to 3 times the average signal to noise ratio in plasma concentrations, or average concentration of the procedural blanks plus 10 times the standard deviation.

2.6. Data analysis

We performed statistical analyses and plotting of results in R (R Development Core Team, 2019). All tests were two-tailed and the null-hypotheses were rejected at an α-level of 0.05. Contaminant concentrations and LCC scores were $\log_e$-transformed prior to analyses in order to meet the assumption of normality. All the selected models were visually examined through standard diagnostics plots (e.g. visual examination of residual plots in order to assess the normality assumption).

Since nestlings were sampled at different developmental stages, an index for body condition (hereafter BC) was calculated based on the residuals from the regression between body mass and wing length (Helander et al., 2002; Stevenson and Woods, 2006). Sex was included as an additive predictor in the regression since body mass significantly differed between sexes (Welch's two-sample *t*-test, $P < 0.01$; Table A.3).

To express the unadulterated LCC response, the control sample score was subtracted from the PMA-challenged sample in order to correct for baseline leukocyte activity and potential background noise. Subsequently, the cleansed LCC response curve was fitted with a piecewise linear regression (PLR) model using the *seg.mod* function in the Segmented package (Fig. A.1; Muggeo, 2008). A positive slope of the first segment and a negative slope of the second segment were set as our *a priori* selection criteria for the LCC response (supplementary material including all curves that show both the raw data and fitted lines; Fig. A.1. for curve explanation). These selection criteria were fulfilled for 62 out of 78 fitted LCC curves, highlighting that the majority of the nestlings responded to the PMA challenge following a similar pattern. Only the latter 62 selected LCC curves were used in further statistical analyses.

Using Pearson's Product-Moment correlation (*r*), we documented that the slope of segments and the peak of LCC (i.e. the amplitude of the maximum LCC, hereafter LCC-peak) were linearly related to each other (Table A.4). Therefore, the extracted LCC-peak was used as a variable for all subsequent analyses as it is highly representative for the entire LCC response curve. Finally, the LCC-peak was corrected for the total number of heterophils (LCC-peak divided on the number of heterophils for each individual nestling) to exclude a potential mass-effect and resulted in a final sample size of 56.

Only OHCs detected in more than 75% of the samples were statistically analyzed, after substitution of non-detects by half the compound-specific LOQ-value. Based on their degree of correlation (Table A.5a–c), their physio-chemical properties, relative contribution to the total contaminant load, including visual examination of potential clusters revealing correlated compounds through Principal Component Analysis biplots (*prcomp* function; Fig. A.2a–c; Zuur et al., 2007), we selected the final compounds in the present study to compose: Σ_{12}PCBs, HCB, *cis*-chlordane, *trans*-nonachlor, *p,p′*-DDE, PFHxS, PFOS (branched and linear isomers), PFOA and long-chained perfluorocarboxylic acids (LC-PFCAs; PFDoDA, PFUnDA and PFTrDA).

The majority of the nests, i.e. 25 out of 39 nests (65%), contained only one nestling, and this prevented us from estimating inter- and intra-nest variability in our analyses using Linear Mixed-Effect Models (LMEs: Pinheiro and Bates, 2000; Zuur et al., 2009). Bustnes et al. (2013) outline the rationale for this where they chose to apply LME in statistical analyses of goshawks (*Accipiter gentilis*), a species that commonly produce more chicks per nest, but not for WTEs (since 60% of the nests in their study contained single chicks). Consequently, we follow Bustnes et al.'s (2013) assessment and decided to apply standard linear regression models (LMs) in our analyses. Nonetheless, we fitted and compared LMEs (using the *nlme*-library: Pinheiro et al., 2017) and LMs, and documented that our biological conclusions were not affected by the choice of modelling approach. Even though the estimated parameters slightly differed, both the estimates ($r = 0.95$, $P < 0.01$) and the predictions ($r = 1$, $P < 0.01$) from the models were highly correlated.

A set of *a priori* candidate linear regression models using *lm* function were created to explain variation in 1) OHC levels (each compound tested separately) and 2) LCC-peaks (Table A.6). Predictor variables for the models were chosen based on their biological relevance, i.e. year, BC, brood size and sex while avoiding collinearity (Zuur et al., 2009), by not including correlated variables in the same models (tested by using the *ancova* function in the HH package; Heiberger and Holland, 2004). A model selection of the set of candidate models was performed using the *aictab* function in the AICcmodavg package (Mazzerolle, 2019), and the most parsimonious model was defined as the one with the lowest second-order Akaike Information Criterion (AIC_c; Burnham et al., 2002; Table A.7a-b). Finally, a linear model was tested to investigate the relationship between the LCC-peak and the different OHC concentrations.

3. Results and discussion

3.1. Variation in OHC levels

ΣOCs and ΣPFAS represented 48.3% and 51.7%, respectively, of the total targeted plasma contaminant load of the WTE nestlings in northern Norway in 2017 and 2018 (Table 1). PFOS was detected in

Table 1
Mean ± standard error (*SE*), median and range (min–max) of the plasma concentrations (in ng g^{-1} wet weight) of the targeted organohalogenated compounds detected in at least 75% of white-tailed eagle nestlings sampled in northern Norway during 2017 and 2018. Compounds are categorized as organochlorines (OCs) and poly- and perfluoroalkyl substances (PFAS).

	Mean ± *SE*	Median	Min-max
OCs			
HCB	1.46 ± 0.10	1.11	0.37–5.40
cis-chlordane	0.26 ± 0.02	0.22	0.04–1.06
trans-chlordane	0.19 ± 0.01	0.16	0.04–0.55
cis-nonachlor	0.33 ± 0.02	0.28	0.08–1.17
trans-nonachlor	0.90 ± 0.07	0.71	0.19–4.10
Mirex	0.44 ± 0.08	0.23	0.04–4.56
p,p′-DDD	0.15 ± 0.01	0.13	0.00–0.60
p,p′-DDE	7.68 ± 0.84	5.46	0.94–46.47
PCB-28/31	0.19 ± 0.01	0.18	0.06–0.47
PCB-52	0.13 ± 0.01	0.12	0.02–0.37
PCB-99	1.04 ± 0.12	0.67	0.12–4.80
PCB-101	0.24 ± 0.02	0.19	0.07–0.82
PCB-105	0.5 ± 0.05	0.38	0.08–2.60
PCB-118	1.97 ± 0.21	1.31	0.31–9.96
PCB-138	4.85 ± 0.54	3.04	0.57–22.53
PCB-153	7.90 ± 0.98	4.97	0.54–51.05
PCB-180	3.36 ± 0.54	1.97	0.39–33.06
PCB-183	0.55 ± 0.08	0.33	0.03–4.65
PCB-187	1.33 ± 0.15	0.86	0.15–7.97
PCB-194	0.42 ± 0.09	0.22	0.04–5.73
Σ_{12}PCBs	22.50 ± 2.70	14.12	3.00–141.76
ΣOCs	33.77 ± 3.70	22.77	4.77–199.58
PFAS			
PFHxS	0.69 ± 0.07	0.59	0.09–3.52
PFOS	24.26 ± 1.82	19.86	1.22–99.60
ΣPFSAs	*24.94 ± 1.87*	*20.44*	*1.53–103.12*
PFNA	3.63 ± 0.30	2.76	0.99–15.82
PFOA	0.76 ± 0.05	0.65	0.05–1.98
PFDA	1.67 ± 0.11	1.40	0.45–6.81
PFUnDA	3.51 ± 0.18	3.21	1.09–8.49
PFDoDA	0.50 ± 0.04	0.39	0.03–1.67
PFTrDA	1.12 ± 0.09	0.99	0.06–3.71
ΣPFCAs	*11.19 ± 0.62*	*10.05*	*3.35–33.60*
LC-PFCAs[1]	5.13 ± 0.30	4.56	1.18–13.42
ΣPFAS	36.13 ± 2.38	30.08	5.53–119.86

Complete list of contaminants and their abbreviations are given in Table A.2.
[1] The combined concentrations of the long-chain perfluorinated carboxylic acids PFUnDA, PFDoDA and PFTrDA.

overall the highest concentration of all targeted compounds (Table 1), which is consistent with previous observations on WTE nestlings in northern Norway (Loseth et al., 2019; Sletten et al., 2016) and bald eagle (*Haliaeetus leucocephalus*) nestlings in North America (Elliott et al., 2019). Fluorinated sulfonates (PFSAs), such as PFOS, have been found to be the dominating fluorinated compounds in wildlife species although fluorinated carboxylates (PFCAs) are found to have comparable concentrations (Butt et al., 2010). In the present study, PFSAs represented approximately 70% of the total PFAS load in the WTE nestlings and PFCAs the remaining 30% (Table 1). Concentrations of PFOS in avian and mammalian wildlife have shown decreasing trends over the last decades (Ahrens et al., 2011; Riget et al., 2019; Sun et al., 2019), most likely as a result of large reductions in its production and utilization in the early 2000s (Buck et al., 2011). On the contrary, PFCAs have shown increasing trends (AMAP, 2016; Sun et al., 2019; Vorkamp et al., 2019). Decreasing and increasing trends of fluorinated contaminants reflect a change in use of such compounds, with certain compounds being phased out following restrictions while being substituted with other compounds sharing similar properties.

Further, PCBs still constitute a major group of compounds detected in the WTE nestlings (Bustnes et al., 2013; Sletten et al., 2016), with PCB-153 displaying the highest concentrations among the Σ_{12}PCBs (Table 1). PCB-153 is found in relatively high levels in many avian apex predators due to its high persistence and bioaccumulation potential (Bustnes et al., 2004; Bustnes et al., 2005; Leat et al., 2019). Concurrently, *p,p′*-DDE displayed similar high concentrations as PCB-153 in the nestlings (Table 1). Plasma concentrations of *p,p′*-DDE in northern Norwegian WTE nestlings in 2017 and 2018 were found to be higher compared to the same population in 2011–2012 and 2015–2016 (Loseth et al., 2019; Sletten et al., 2016), but lower compared to 2008–2010 (Bustnes et al., 2013). Plasma concentrations are considered a short-term measure of contaminant levels in the nestlings that mainly reflect recent dietary exposure and are therefore highly dependent on the time of sampling (e.g. right before/just after feeding; Espín et al., 2016). Nevertheless, temporal trends of legacy organochlorines such as PCBs and *p,p′*-DDE show significant decreasing trends in biota, reflecting the ban of these compounds 20–30 years ago (AMAP, 2016).

Elevated plasma levels of Σ_{12}PCBs, HCB, *cis*-chlordane, *trans*-nonachlor, *p,p′*-DDE and PFOA were significantly associated with a lower BC in the WTE nestlings ($P < 0.05$; Table 2). Maternal transfer is an important source of contaminants in nestlings, but as the nestlings grow, such contaminants become diluted in their body tissues as long as the dietary intake is not compensating for this effect (Bustnes et al., 2013). As a result, nestlings in poor body condition often have higher contaminant levels than nestlings in good condition due to less dilution.

In 2018, plasma levels of LC-PFCAs and PFHxS were significantly elevated compared to plasma collected in 2017 ($P < 0.01$; Table 2), implying across-year variation in exposure of some contaminants. Variation in OHC levels between years is likely to be caused by a multitude of factors, e.g. location, diet and/or environmental fluctuations (Bustnes et al., 2015).

Although brood size was included in the most parsimonious model explaining variation in both PFOS and PFHxS concentrations, it was never found to have a significant effect ($P = 0.33$ and $P = 0.06$, $P = 0.22$ and $P = 0.09$, respectively; Table 2). Sex was the only predictor variable not selected in any of the retained candidate models explaining variation in OHC levels in the WTE nestlings.

3.2. Sex and inter-year differences in LCC-peak amplitudes

LCC-peak amplitudes ranged from 19.59 to 4368.74 RLUs, with a mean of 677.99 ± 93.99 RLUs (Fig. 1). Predominantly, there was a significant difference in the amplitude of the LCC-peak of WTE nestlings in northern Norway between the two sampling years, showing LCC-peaks to be approximately 5 times higher in 2017 compared to 2018 ($P < 0.01$; Fig. 1). Stressful conditions, such as lowered food availability, inclement weather or increased exposure to pathogens, are known to affect the immune system either by immunoenhancement or immunosuppression (McEwen and Wingfield, 2003). However, we observed no difference in BC between years (One-way ANOVA, $F_{1, 73} = 0.60$, $P = 0.44$) that could support between year differences in food availability, nor do we have information regarding pathogen exposure. Nevertheless, weather conditions appeared more challenging in 2018 compared to 2017 (data visually examined; Norsk Klimaservicesenter, n.d.) and we might speculate that less favorable weather conditions may have contributed to the large difference observed in LCC responses between years, regardless of pollution exposure.

Furthermore, the LCC-peak amplitude was generally found to be higher in females than in males, though only significantly so in 2018 ($P < 0.05$; Fig. 1), likely due to relatively low sample size and high inter-individual variation in LCC scores. Sex differences in LCC have previously been reported for captive house sparrows (*Passer domesticus*), with males having higher LCC-peak amplitudes than females (Huber et al., 2017b). Sex differences could be a result of sexual hormones, which have been found to contribute to the differential regulation of immune response between sexes (Klein and Flanagan, 2016).

Table 2
Parameter estimates from the most parsimonious linear models explaining variation in plasma contaminant levels (log_e-transformed) in white-tailed eagle nestlings sampled in northern Norway across two years (2017 [baseline] and 2018 acting as levels). The significant relationships are **bolded**.

Parameter	Estimate	Confidence Interval (95%)	*P*
Σ_{12}PCBs			
(F = 18.57, R^2 = 0.20, $P < 0.01$)			
Intercept	2.77	2.59–2.94	< 0.01
Body condition	**$-8.94e^{-04}$**	**$-1.30e^{-03}$ to ($-4.80e^{-04}$)**	**< 0.01**
HCB			
(F = 16.75, R^2 = 0.18, $P < 0.001$)			
Intercept	0.23	−5.35 to 0.96	0.17
Body condition	**$-5.85e^{-04}$**	**$-8.73e^{-04}$ to ($-2.96e^{-04}$)**	**< 0.01**
cis-chlordane			
(F = 7.46, R^2 = 0.09, $P < 0.01$)			
Intercept	−1.49	−1.63 to (−1.36)	< 0.01
Body condition	**$-4.30e^{-04}$**	**$-7.45e^{-04}$ to ($-1.16\ e^{-04}$)**	**< 0.01**
trans-nonachlor			
(F = 16.44, R^2 = 0.18, $P < 0.01$)			
Intercept	−0.25	−0.37 to (−0.13)	< 0.01
Body condition	**$-5.81e^{-04}$**	**$-8.67e^{-04}$ to ($-2.95e^{-04}$)**	**< 0.01**
p,p′-DDE			
(F = 14.92, R^2 = 0.16, $P < 0.01$)			
Intercept	1.76	1.59–1.92	< 0.01
Body condition	**$-7.51e^{-04}$**	**$-1.13e^{-03}$ to ($-3.63e^{-04}$)**	**< 0.01**
LC-PFCAs			
(F = 11.85, R^2 = 0.24, $P < 0.01$)			
Intercept	1.32	1.18–1.47	< 0.01
Year 2018	**0.44**	**0.24–0.65**	**< 0.01**
Body condition	$-2.30e^{-04}$	$-4.71e^{-04}$ to $63e^{-05}$	0.06
PFOA			
(F = 4.66, R^2 = 0.06, $P = 0.03$)			
Intercept	−0.47	−0.62 to (−3.16)	< 0.01
Body condition	**$-3.85e^{-04}$**	**$-7.41e^{-04}$ to ($-2.97e^{-05}$)**	**0.03**
PFOS			
(F = 2.90, R^2 = 0.07, $P = 0.06$)			
Intercept	3.09	2.89–3.28	< 0.01
Brood size (2)	−0.13	−0.39 to 0.14	0.34
Brood size (3)	0.45	−0.04 to 0.94	0.07
PFHxS			
(F = 5.60, R^2 = 0.19, $P < 0.01$)			
Intercept	−0.93	−1.30 to (−0.56)	< 0.01
Year 2018	**0.68**	**0.26–1.10**	**< 0.01**
Brood size (2)	−0.27	−0.70 to 0.17	0.22
Brood size (3)	0.69	−0.12 to 1.49	0.09

3.3. Amplitude of LCC-peaks in relation to OHC exposure

Negative relationships between OHC concentrations and the amplitudes of the LCC-peaks were found for all nine OHCs tested except *cis*-chlordane (Estimate < 0, Table 3). Yet, the latter negative relationships were only significant for Σ_{12}PCBs, *p,p′*-DDE, LC-PFCAs and PFHxS ($P < 0.05$; Table 3; Fig. 2), indicating elevated plasma concentrations to be associated with a reduced amplitude of the LCC-peak. These findings support our prediction that individuals exposed to higher OHC levels have a reduced LCC compared to less contaminated nestlings. Interestingly, contaminants detected in both relatively low and high concentrations in the WTE nestlings (Table 1) displayed the same lowering effect on the amplitude of the LCC-peak with increasing concentrations, indicating that some OHCs might be potent also at low levels. A reduced LCC, represented by a reduction in the amplitude of the LCC-peak, reflects a lowered innate immunity through a lowered potential of circulating leukocytes to release ROS in response to immune challenges. Since the heterophil respiratory burst represents an early innate immune defense to pathogen exposure, our results therefore suggest a possible immunomodulatory effect of Σ_{12}PCBs, *p,p′*-DDE, LC-PFCAs and PFHxS, which ultimately might be critical to the survival and fitness of WTE nestlings.

Exposure to PCBs and *p,p′*-DDE has been previously linked to immune impairment in several avian species (Grasman and Fox, 2001; Grasman et al., 1996; Jara et al., 2018; Mayne et al., 2004; Smits et al., 2002; Smits and Bortolotti, 2001), but studies on innate immune endpoints are scarce. Bustnes et al. (2004) and Jara-Carrasco et al. (2015) reported a positive relationship between PCB and *p,p′*-DDE concentrations and leukocyte indices in glaucous gulls (*Larus hyperboreus*) and chinstrap penguins (*Pygoscelis antarcticus*), respectively. The latter studies hypothesized these positive relationships to be a result of either a contaminant-mediated alteration of the leukocyte functions, or a contaminant-mediated immunosuppression contributing to infections, both resulting in a compensatory increased production of leukocyte numbers (Bustnes et al., 2004; Jara-Carrasco et al., 2015). Although our study reported negative relationships, it is noteworthy that the present and latter studies are based on different immunological endpoints, i.e. leukocyte responsiveness as opposed to absolute cell counts further explaining the discrepancies between studies. Nevertheless, either positive or negative, a significant association between contaminant exposure and immunological endpoints can lead to a suboptimal function of the immune system, potentially increasing the susceptibility to pathogens and diseases. In conclusion, legacy organochlorines such as PCBs and *p,p′*-DDE which still dominate the body burden of apex predators, keep acting as possible stressors on the immune system.

While immune system impairment is known to be associated with PFAS exposure in mammals, few studies have focused on immune endpoints and PFAS in birds (Castano-Ortiz et al., 2019; DeWitt et al., 2012). Experiments on chicken cells detected immunomodulation of PFOS on innate immunity signaling pathways (Castano-Ortiz et al., 2019). Additionally, negative effects of long-chained PFCAs have been

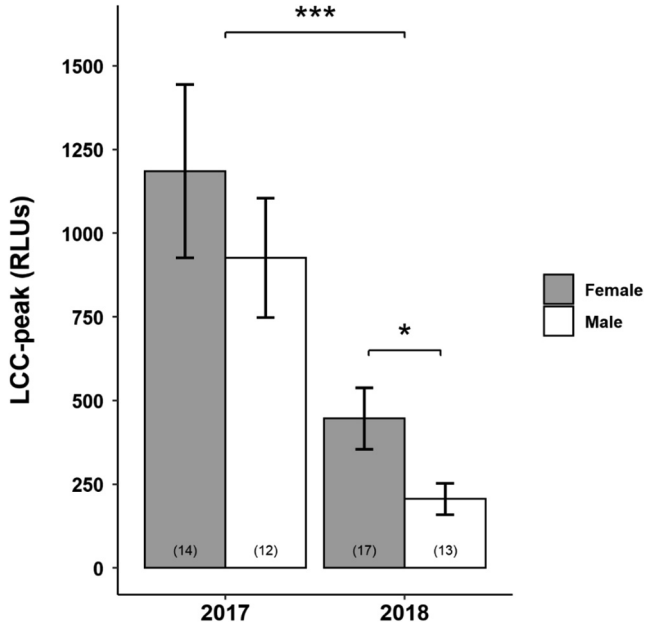

Fig. 1. Mean ± *SE* intensity of the leukocyte coping capacity (LCC) peak amplitudes between female and male white-tailed eagle nestlings sampled in northern Norway during 2017 and 2018. Asterisks (*) indicate statistical significant differences between the variables (*$P < 0.05$, ***$P < 0.01$) and the bracketed numbers represent sample size for each group.

Table 3
Parameter estimates for the linear models of the relationship between LCC-peak amplitude (response variable) and contaminant concentrations (predictor variables) in northern Norwegian white-tailed eagle nestling plasma sampled during 2017 and 2018. The LCC-peak and contaminant concentrations were $\log_e$-transformed prior to analysis, and the contaminants were tested separately. The significant relationships are **bolded**.

Predictor variable	Estimate	Confidence Interval (95%)	*P*
Σ_{12}PCBs	**−0.37**	**−0.69 to (−0.05)**	**0.02**
HCB	−0.31	−0.83 to 0.20	0.23
cis-chlordane	0.23	−0.35 to 0.82	0.43
trans-nonachlor	−0.19	−0.71 to 0.33	0.47
***p,p′*-DDE**	**−0.35**	**−0.69 to (−0.00)**	**< 0.05**
LC-PFCAs	**−0.91**	**−1.50 to (−0.32)**	**< 0.01**
PFOA	−0.17	−0.59 to 0.24	0.41
PFOS	−0.07	−0.64 to 0.49	0.80
PFHxS	**−0.43**	**−0.71 to (−0.15)**	**< 0.01**

reported on reproduction and physiological stress in black-legged kittiwakes (*Rissa tridactyla;* Tartu et al., 2014). Current and previous findings suggest that increasing trends of PFHxS and LC-PFCAs could be of concern to wildlife health, despite their relatively low concentrations.

Although our correlational study cannot reveal causal relationships, our results contribute to the weight of evidence suggesting immunomodulation of some OHCs in avian wildlife. Yet, our results do not help understand the underlying mechanisms of OHC exposure on the LCC of WTE nestlings. Further experimental studies are therefore needed to elucidate the possible proximate mechanisms causing contaminants to lower the immunological responses. Longitudinal studies should also examine the long-term effects of lowered innate immune response on survival and reproductive success, and ideally consider variables such as BC and annual variations in contaminant concentrations, as they might ultimately influence the relationships between the innate immune response (LCC) and contaminant exposure in WTE nestlings.

3.4. Relevance of the LCC in wildlife ecotoxicology

Based on our results, the LCC-method has clear methodological advantages in wildlife ecotoxicology as it is conducted almost instantly after blood sampling, allowing a real-time perspective of the leukocytes' ability to produce a respiratory burst. This *in vitro* method avoids both the potential impact of centrifugation and freezing on cell reactivity (Bortolin et al., 2017) as well as the stress related to any prior injection with an immune activator (e.g. antigens) or recapture. Finally, the method is cheap and requires only a droplet of blood for the analyses.

4. Conclusion

The present study reported significant negative associations between Σ_{12}PCBs, *p,p′*-DDE, LC-PFCAs and PFHxS and the LCC in nestlings of an established apex predatory bird species, the WTE. Our results contribute to the weight of evidence for immunomodulatory properties of OHCs in avian wildlife and suggest that LCC represents a relevant and accessible test to expand the toolbox of wildlife ecotoxicology. In the context of multiple stressors, future research should also consider environmental variables such as annual variations in OHC concentrations and/or food availability, as they might ultimately influence the relationships between the innate immune response (LCC) and contaminant exposure in WTE nestlings.

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CRediT authorship contribution statement

Elisabeth Hansen: Formal analysis, Investigation, Writing - original draft, Visualization. **Nikolaus Huber:** Methodology, Investigation, Validation, Writing - review & editing. **Jan O. Bustnes:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Dorte Herzke:** Formal analysis, Validation, Writing - review & editing. **Bård-Jørgen Bårdsen:** Formal analysis, Writing - review & editing. **Igor Eulaers:** Writing - review & editing. **Trond V. Johnsen:** Investigation, Resources. **Sophie Bourgeon:** Conceptualization, Investigation, Resources, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

The following files are available free of charge: Leukocyte data, targeted organohalogenated contaminants (OHCs), morphological data, correlation matrix of leukocyte coping capacity (LCC) curve estimates,

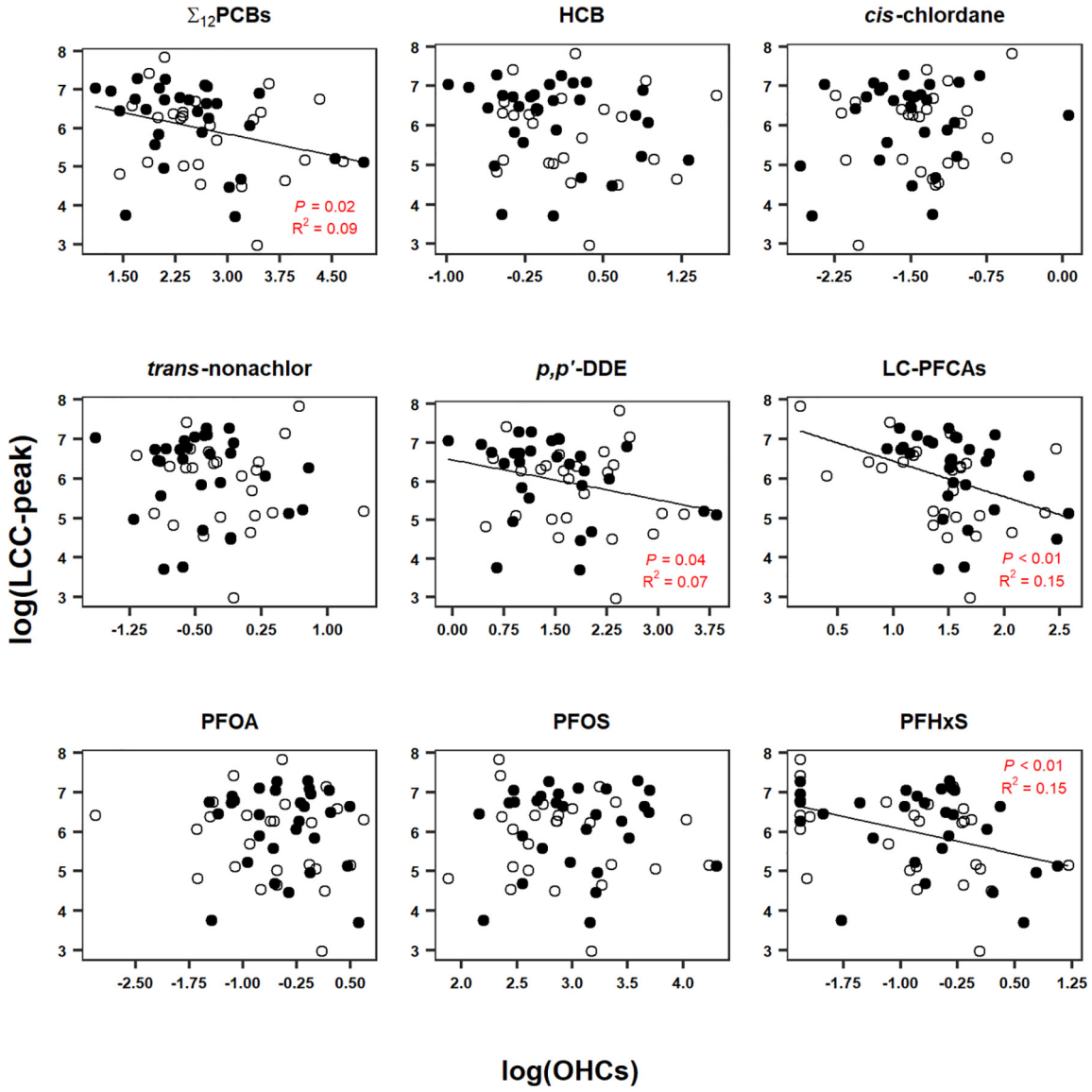

Fig. 2. Relationship between leukocyte coping capacity (LCC) peak amplitudes and organohalogenated contaminant (OHC) concentrations in female (filled circles) and male (open circles) white-tailed eagle nestlings in northern Norway in 2017 and 2018. Regression lines indicate a significant association. *P*-values and adjusted R^2 of the significant linear models are reported for each compound.

correlation matrices and principal component analysis (PCA) biplots of OHCs, candidate linear models, model selection output, and LCC response curve (with additional zip-file "LCC_curves" including all the individual LCC responses). Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105861.

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A novel use of the leukocyte coping capacity assay to assess the immunomodulatory effects of organohalogenated contaminants in avian wildlife

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APPENDIX A

TABLE OF CONTENT

List of tables:

List of figures:

Table A.1. Mean ± standard error (SE), median, and range (min-max) of total leukocyte concentration (g/L) and differential heterophil concentration (g/L) in the sampled white-tailed eagle nestlings in Northern Norway during 2017 and 2018.

	Mean ± SE	**Median**	**Min-max**
Leukocytes	14.99 **±** 0.85	12.81	6.25-46.00
Heterophils	13.53 **±** 0.79	11.93	4.63-41.40

Table A.2. List of targeted organohalogenated contaminants (OHCs, full names and abbreviations) with detection frequencies > 75%, < 75% and below the level of detection (< LOD), of the sampled white-tailed eagle (WTE) nestlings in Northern Norway during 2017 and 2018. OHCs detected in at least 75% of the sampled WTE nestlings were retained for statistical analysis (**bolded**).

	Detection		
OCs	> 75%	< 75%	< LOD
Hexachlorobenzene (HCB)	**X**		
α-Hexachlorocyclohexane (α-HCH)			X
β-Hexachlorocyclohexane (β-HCH)		X	
γ-Hexachlorocyclohexane (γ-HCH)			X
Oxychlordane (oxy-chl)		X	
***trans*-Chlordane (*t*-chl)**	**X**		
***cis*-Chlordane (*c*-chl)**	**X**		
***trans*-Nonachlor (*t*-Nonachl)**	**X**		
***cis*-Nonachlor (*c*-Nonachl)**	**X**		
Mirex	**X**		
o,p'-Dichloro-α,α-diphenyl-β,β,β-trichloroethane (o,p'-DDT)		X	
p,p'-Dichloro-α,α-diphenyl-β,β,β-trichloroethane (p,p'-DDT)		X	
o,p'-Dichlorodiphenyldichloroethane (o,p'-DDD)		X	
p,p'-Dichlorodiphenyldichloroethane (p,p'-DDD)		X	
o,p'-Dichlorodiphenyldichloroethylene (o,p'-DDE)		X	
p,p'-Dichlorodiphenyldichloroethylene (p,p'-DDE)	**X**		
Polychlorinated biphenyls (PCBs)			
PCB 28/31	**X**		
PCB 52	**X**		
PCB 99	**X**		
PCB 101	**X**		
PCB 105	**X**		
PCB 118	**X**		
PCB 138	**X**		
PCB 153	**X**		
PCB 180	**X**		
PCB 183	**X**		
PCB 187	**X**		
PCB 194	**X**		
PFAS			
4:2 Fluorotelomer sulfonic acid (4:2 FTS)			X
6:2 Fluorotelomer sulfonic acid (6:2 FTS)			X
8:2 Fluorotelomer sulfonic acid (8:2 FTS)			X
Perfluorobutane sulfonate (PFBS)			X
Perfluoropentane sulfonate (PFPS)			X
Perfluorohexane sulfonate (PFHxS)	**X**		
Perfluoroheptane sulfonate (PFHpS)		X	
Perfluorooctane sulfonate Branched (brPFOS)	**X**		

Perfluorooctane sulfonate Linear (PFOSlin)	**X**		
Perfluorononane sulfonate (PFNS)			X
Perfluorodecane sulfonate (PFDS)			X
Perfluorooctane sulfonamide (FOSA)			X
Perfluoroheptanoate (PFHpA)			X
Perfluorohexanoate (PFHxA)		X	
Perfluorooctanoate (PFOA)	**X**		
Perfluorononanoate (PFNA)	**X**		
Perfluorobutanoate (PFBA)			X
Perfluoropentanoate (PFPA)			X
Perfluorodecanoate (PFDA)	**X**		
Perfluoroundecanoate (PFUnDA)	**X**		
Perfluorododecanoate (PFDoDA)	**X**		
Perfluorotridecanoate (PFTrDA)	**X**		
Perfluorotetradecanoate (PFTeDA)		X	

Table A.3. Mean ± standard error (SE), median, range (min-max) and sample size (*n*) of morphological measurements from the sampled white-tailed eagle nestlings in Northern Norway during 2017 and 2018. Asteriks (* p-value < 0.001) indicate significant sex differences according to Welch's two sample t-test.

	Mean ± SE	**Median**	**Min-max**	***n***
Females				
Body mass (g)*	5281 ± 0.130	5425	2400-6480	36
Wing length (mm)	424.0 ± 13.89	436.0	186.0-560.0	37
Tail length (mm)	165.7±11.73	163.0	30.0-304.0	37
Tarsus width (mm)*	15.61 ± 0.11	15.60	14.20-16.90	37
Tarsus depth (mm)*	17.6 ± 0.15	17.50	15.5-19.70	37
Beak height (mm)*	35.36 ± 0.37	35.70	28.00-40.30	37
Beak length (mm)*	51.44 ± 0.62	52.20	38.20-57.20	37
Hindclaw (mm)*	39.77 ± 0.43	40.10	31.70-44.80	37
Males				
Body mass (kg)*	4.41 ± 0.85	4.50	2.10-5.22	44
Wing length (mm)	409.8 ± 11.70	432.0	168.0-570.0	47
Tail length (mm)	173.9 ± 9.77	194.0	35.0-305.0	47
Tarsus width (mm)*	13.98 ± 0.14	14.10	12.20-17.90	47
Tarsus Depth (mm)*	15.81 ± 0.13	15.90	12.40-18.00	47
Beak height (mm)*	32.20 ± 0.27	32.60	26.40-35.70	47
Beak length (mm)*	47.82 ± 0.47	48.60	38.50-52.80	47
Hindclaw (mm)*	36.97 ± 0.35	37.10	28.70-41.90	47

Table A.4. Correlation matrix between LCC estimates (Pearson's Product-Moment correlation test). Slope 1 is the estimate of the first segment, slope 2 is the estimate of the second segment, Intercept estimate, and LCC-peak. Numbers refer to *r*-values (Pearson's correlation coefficient) and colours indicate significance of test (set to α = 0.05): dark blue $P < 0.001$, white $P > 0.05$.

	Slope 2	**Intercept**	**LCC-peak**
Slope 1	-0,98	-0,94	0,99
Slope 2		0,97	-0,99
Intercept			-0,96

Table A.5. Correlation matrix based on Pearson's product moment correlation test for a) PCB congeners, b) organochloride pesticides and c) PFAS that were detected in >75% of the sampled nestlings. The contaminants were $\log_e$-transformed in the correlation tests. Numbers refer to *r*-values (Pearson's correlation coefficient) and colours indicate significance of test (set to $\alpha = 0.05$): dark blue $P < 0.001$, light blue $P < 0.05$, white $P > 0.05$.

a)

PCB-	28/31	101	99	105	118	138	153	180	183	187	194
52	0,33	0,75	0,06	0,06	0,07	0,06	0,08	0,12	0,12	0,12	0,08
28/31		0,52	0,73	0,72	0,72	0,69	0,71	0,72	0,72	0,72	0,71
101			0,30	0,31	0,32	0,32	0,32	0,40	0,39	0,37	0,40
99				0,98	0,98	0,98	0,94	0,92	0,95	0,93	0,89
105					1,00	0,98	0,96	0,94	0,95	0,92	0,90
118						0,99	0,96	0,95	0,96	0,93	0,91
138							0,97	0,96	0,98	0,95	0,92
153								0,94	0,95	0,92	0,89
180									0,98	0,92	0,97
183										0,96	0,96
187											0,89

b)

	***trans*-chlordane**	***cis*-chlordane**	***trans*-nonachlor**	***cis*-nonachlor**	**Mirex**	***p,p'*-DDD**	***p,p'*-DDE**
HCB	0,84	0,19	0,76	0,81	0,89	0,09	0,88
***trans*-chlordane**		0,58	0,91	0,92	0,77	0,45	0,80
***cis*-chlordane**			0,55	0,52	0,11	0,87	0,16
***trans*-nonachlor**				0,95	0,69	0,42	0,80
cis-Nonachlor					0,75	0,40	0,82
Mirex						0,01	0,88
***p,p'*-DDD**							0,08

c)

	brPFOS	**PFOSlin**	**PFOA**	**PFNA**	**PFDA**	**PFUnDA**	**PFDoDA**	**PFTrDA**
PFHxS	0,62	0,64	0,67	0,68	0,54	0,57	0,51	0,59
brPFOS		0,79	0,60	0,86	0,76	0,51	0,36	0,43
PFOSlin			0,55	0,74	0,76	0,57	0,41	0,47
PFOA				0,63	0,47	0,28	0,20	0,22
PFNA					0,84	0,55	0,46	0,49
PFDA						0,73	0,58	0,59
PFUnDA							0,81	0,88
PFDoDA								0,83

Table A.6. List of candidate linear models explaining the observed variation in response variables 1) Organohalogenated contaminants (OHCs; 9 different compounds/group of compounds) and 2) LCC-peak amplitudes. All contaminants and the LCC-peak were tested separately and $\log_e$-transformed prior to analysis.

*List of candidate models (response variables **bolded**)*	
OHC concentrations	**LCC-peak**
1. Null model	1. Null model
2. Year	2. Year
3. Sex	3. Sex
4. Brood size	4. Brood size
5. Body condition	5. Body condition
6. Brood size + Body condition	6. Brood size + Body condition
7. Year + Body condition	7. Year + Body condition
8. Year + Sex	8. Year + Sex
9. Year + Brood size	9. Year + Brood size
10. Year + Brood size + Body condition	10. Year + Brood size + Body condition

Table A.7. a) Model selection in search of the most parsimonious model explaining the observed variation in the 9 different OHCs (**bolded**). Predictor variables included the following variables: year (two-level factor: 2017 and 2018), sex (two-level factor: females and males), body condition (BC; continuous variable), and brood size (BS; continuous variable). The most parsimonious model is given on the top and presented the lowest AIC_c-value, w_i refers to the weight of the model, and K represents the number of parameters in the model.

	K	Δ_i	w_i		K	Δ_i	w_i		K	Δ_i	w_i
Σ_{12}PCB				***trans*-nonachlor**				**PFOA**			
BC	3	0.00	0.52	BC	3	0.00	0.61	BC	3	0.00	0.47
Year + BC	4	0.91	0.33	Year + BC	4	2.11	0.21	Year + BC	4	2.22	0.16
BC + BS	5	3.38	0.10	BC + BS	5	3.10	0.13	Null model	2	2.53	0.13
Year + BC + BS	6	4.41	0.06	Year + BC + BS	6	5.34	0.04	BC + BS	5	3.86	0.07
Year + Sex	4	15.18	0.00	Null model	2	13.78	0.00	Year	3	4.67	0.05
Sex	3	15.44	0.00	Sex	3	13.86	0.00	Sex	3	4.70	0.05
Null model	2	15.64	0.00	Year + Sex	4	15.44	0.00	BS	4	5.49	0.03
Year	3	16.01	0.00	Year	3	15.59	0.00	Year + BC + BS	6	6.21	0.02
BS	4	18.23	0.00	BS	4	15.66	0.00	Year + Sex	4	6.90	0.02
Year + BS	5	18.70	0.00	Year + BS	5	17.58	0.00	Year + BS	5	7.75	0.01
HCB				***p.p'*-DDE**				**PFOS**			
BC	3	0.00	0.52	BC	3	0.00	0.54	BS	4	0.00	0.24
Year + BC	4	0.91	0.33	Year + BC	4	1.00	0.33	BC + BS	5	0.61	0.18
BC + BS	5	3.38	0.10	BC + BS	5	3.74	0.08	BC	3	1.08	0.14
Year + BC + BS	6	4.41	0.06	Year + BC + BS	6	4.86	0.05	Year + BS	5	1.68	0.10
Year + Sex	4	15.18	0.00	Year + Sex	4	11.20	0.00	Null model	2	1.81	0.10
Sex	3	15.44	0.00	Sex	3	11.45	0.00	Year + BC + BS	6	2.17	0.08
Null model	2	15.64	0.00	Null model	2	12.52	0.00	Year + BS	4	2.52	0.07
Year	3	16.01	0.00	Year	3	13.00	0.00	Year	3	3.44	0.04
BS	4	18.23	0.00	BS	4	15.14	0.00	Sex	3	3.96	0.03
Year + BS	5	18.70	0.00	Year + BS	5	15.73	0.00	Year + Sex	4	5.66	0.01
***cis*-chlordane**				**LC-PFCAs**				**PFHxS**			
BC	3	0.00	0.26	Year + BC	4	0.00	0.56	Year + BS	5	0.00	0.43
BC + BS	5	0.12	0.25	Year	3	1.71	0.24	Year	3	1.78	0.18
Year + BC	4	0.47	0.21	Year + Sex	4	3.90	0.08	Year + BC + BS	6	2.10	0.15
Year + BC + BS	6	0.63	0.19	Year + BC + BS	6	3.98	0.08	Year + Sex	4	2.23	0.14
BS	4	5.30	0.02	Year + BS	5	5.25	0.04	Year + BC	4	3.09	0.09
Year + BS	5	5.32	0.02	BC	3	15.24	0.00	BS	4	8.90	0.00
Year	3	5.46	0.02	Null model	2	17.45	0.00	Null model	2	10.08	0.00
Null model	2	5.48	0.02	Sex	3	19.18	0.00	BC + BS	5	10.66	0.00
Sex	3	7.64	0.01	BC + BS	5	19.22	0.00	BC	3	10.93	0.00
Year + Sex	4	7.66	0.01	BS	4	21.01	0.00	Sex	3	11.55	0.00

Table A.7. b) Model selection in search of the most parsimonious model explaining the observed variation in the LCC-peak amplitude (**bolded**). Predictor variables included the following variables: year (two-level factor: 2017 and 2018), sex (two-level factor: females and males), body condition (BC; continuous variable), and brood size (BS; continuous variable). The most parsimonious model is given on the top and presented the lowest AIC_c-value, w_i refers to the weight of the model, and K represents the number of parameters in the model.

	K	Δ_i	w_i
LCC-peak			
Year + Sex	4	0.00	0.41
Year	3	1.14	0.23
Year + BS	5	1.28	0.21
Year + BC	4	3.00	0.09
Year + BC + BS	6	3.71	0.06
Null model	2	29.79	0.00
BS	4	29.97	0.00
Sex	3	30.17	0.00
BC	3	31.37	0.00
BC + BS	5	32.22	0.00

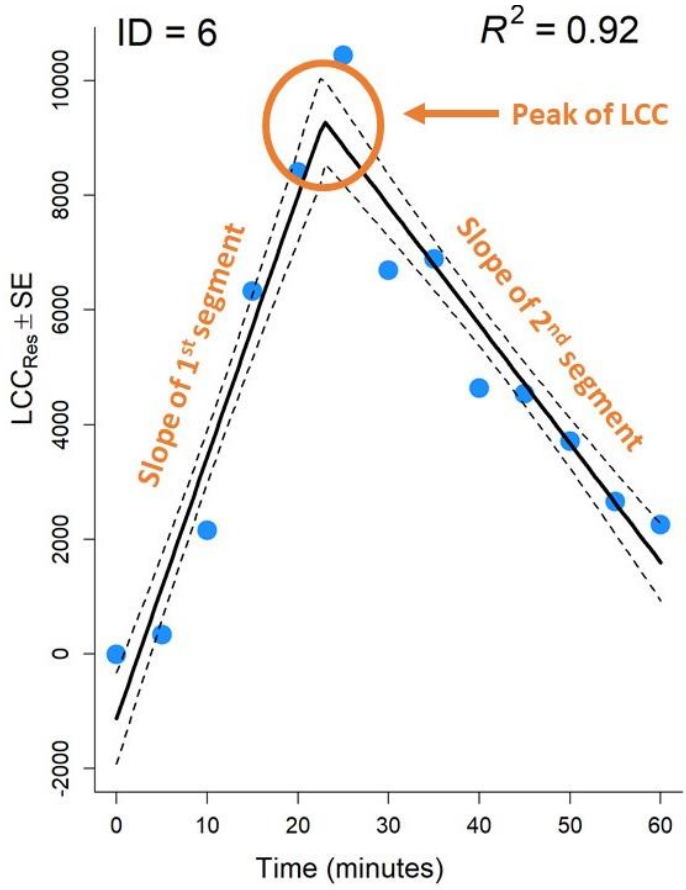

Figure A.1. Example of a fitted piecewise linear regression (PLR) model for the pure leukocyte coping capacity (LCC) response in one white-tailed eagle nestling. LCC estimates, ID of nestling and R^2 of model is given in the figure. The blue dots represent the raw LCC data, the black line is the fitted PLR model (*seg.mod* function using R Software; Muggeo, 2008), and the dashed lines represent the standard error of the fitted segments.

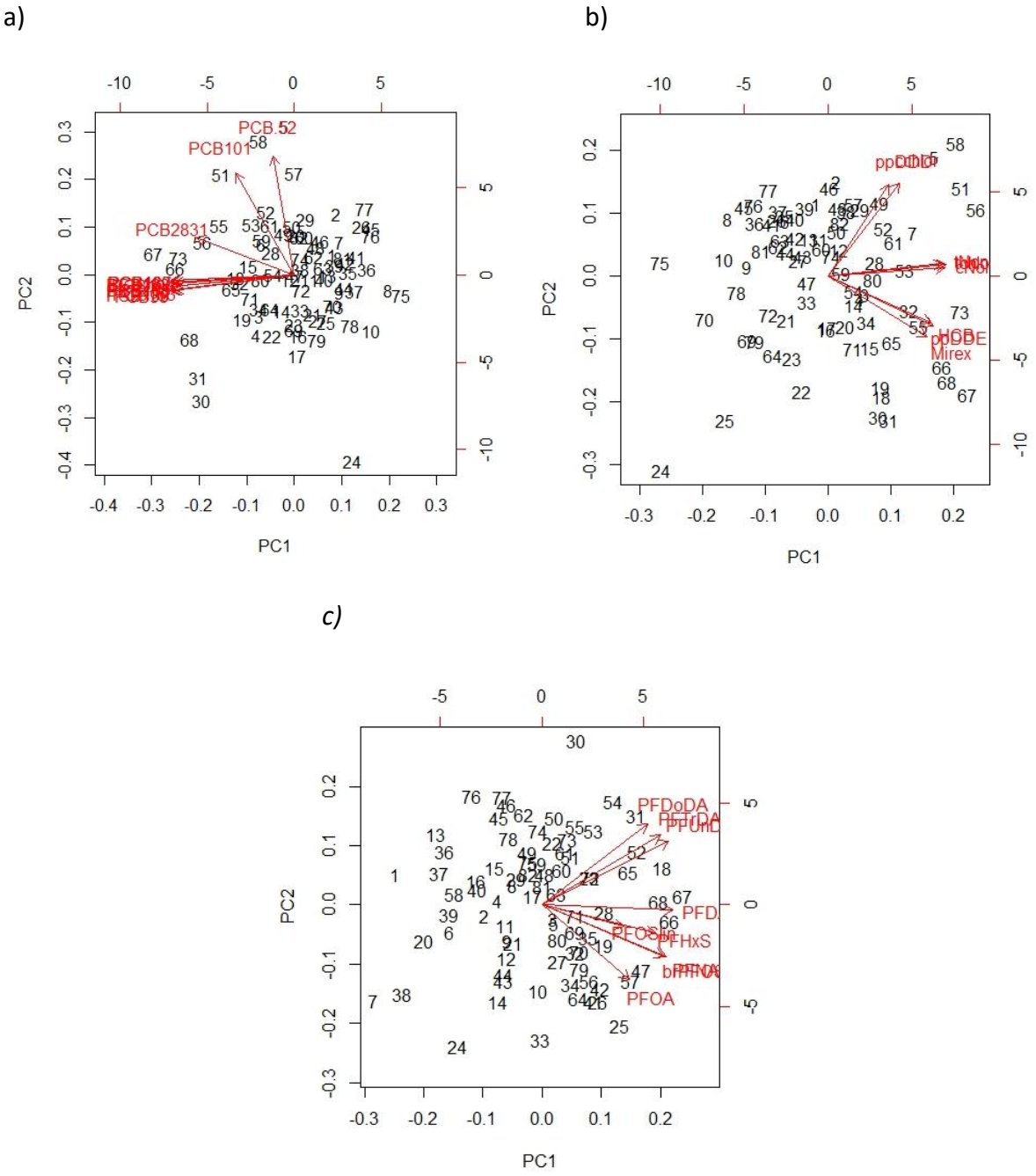

Figure A.2. Principal Component Analysis (PCA) of contaminants were performed using *prcomp* in R (R Development Core Team, 2019). Biplots (function *biplot*) were created to visualize clustering of a) PCB congeners (*12* in total), b) organochlorine pesticides (OCPs; *7*) and c) PFAS (*8*) that were detected in >75% of the sampled nestlings. a) PCA of PCBs: the first and second principal components (PC1 and PC2) explained 88.7% and 5%, respectively, i.e. a total of ca 94% of the variation in the original variables. (b) PCA of OCPs: PC1 and PC2 explained 68.4% and 21.6%, respectively, i.e. 90% of the variation in the original variables. (c) PCA of PFAS: PC1 and PC2 explained 65.4% and 13.8%, respectively, i.e. 79.2% of the variation in the original variables. The PCA thus revealed a high degree of correlation between the majority of the compounds (divided in each of the three groups) clustering along the PC1 axis, and some compounds along the PC2 axis of the PCA plot.

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Paper II

Telomere length in relation to persistent organic pollutant exposure in white-tailed eagle (*Haliaeetus albicilla*) nestlings from Sweden sampled in 1995-2013

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Telomere length in relation to persistent organic pollutant exposure in white-tailed eagle (*Haliaeetus albicilla*) nestlings from Sweden sampled in 1995–2013

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ABSTRACT

Telomeres are used as biomarkers of vertebrate health because of the link between their length, lifespan, and survival. Exposure to environmental stressors appears to alter telomere dynamics, but little is known about telomere length and persistent organic pollutant (POP) exposure in wildlife. The white-tailed eagle (WTE; *Haliaeetus albicilla*) is an avian top predator that accumulates high levels of POPs and may subsequently suffer adverse health effects. Here we study the Baltic WTE population that is well documented to have been exposed to large contaminant burdens, thereby making it a promising candidate species for analyzing pollutant-mediated effects on telomeres. We investigated telomere lengths in WTE nestlings (n = 168) over 19 years and examined legacy POP concentrations (organochlorines and polybrominated diphenyl ethers) in whole blood and serum as potential drivers of differences in telomere length. Although we detected significant year-to-year variations in telomere lengths among the WTE nestlings, telomere lengths did not correlate with any of the investigated POP concentrations of several classes. Given that telomere lengths did not associate with POP contamination in the Baltic WTE nestlings, we propose that other environmental and biological factors, which likely fluctuate on a year-to-year basis, could be more important drivers of telomere lengths in this population.

1. Introduction

Telomeres are repeated sequences of non-coding DNA that cap the ends of chromosomes and play an important role in stabilizing and protecting the coding sequences in eukaryote genomes (Blackburn, 1991). Telomeres shorten with every cell division, with the length eventually shortening to such an extent that the process triggers cell senescence (Angelier et al., 2018; Blackburn, 2000; Louzon et al., 2019). There are relationships between increased rates of telomere shortening and sub-optimal environmental conditions such as dramatic changes in climate (Mizutani et al., 2013), warmer and wetter springs (van Lieshout et al., 2021), unfavorable natal or wintering conditions (Angelier et al., 2013; Watson et al., 2015), and low adult habitat quality (Apfelbeck et al., 2019; Wilbourn et al., 2017), possibly mediated through increased oxidative stress (the imbalance within an organism between free radicals' production and antioxidants availability or activity) (Haussmann and Marchetto, 2010; von Zglinicki, 2002). Contrasting environmental conditions can therefore translate into inter-individual variability in telomeres (Ibanez-Alamo et al., 2018; Kärkkäinen et al., 2019) further influencing lifespan (Giraudeau et al., 2019; Monaghan and Haussmann,

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2006). Finally, as telomeres are robust, they allow a reliable retrospective analysis (Blackburn, 1991) to assess the impact of environmental stressors on wildlife.

The white-tailed eagle (hereafter referred to as WTE; *Haliaeetus albicilla*) is an avian top predator associated with aquatic habitats in many Eurasian countries (Cramp and Simmons, 1983). Due to its feeding habits and apex trophic position, the WTE accumulates high levels of persistent organic pollutants (POPs) and may therefore suffer detrimental health effects (Helander, 1985; Helander et al., 1982, 2002; Letcher et al., 2010; Sonne et al., 2020). For example, the Swedish WTE population on the Baltic coastline has suffered reproductive failure and population decline during the 1950s until the 1980s as a consequence of contamination by legacy POPs (Helander, 1985; Helander et al., 1982, 2002). Poor breeding success has also been reported for other WTE populations in the Baltic Sea region (Koivusaari et al., 1980; Scharenberg and Struwe-Juhl, 2006). Thus the WTE represents a species that is useful for examination of possible detrimental impacts of exposure to environmental POPs (Helander et al., 2008).

Although the production and use of many POPs (particularly organochlorine compounds) were banned or restricted decades ago, they remain highly present in the environment and biota (AMAP, 2016). Even low levels of POPs may induce sub-lethal health effects, such as endocrine disruption (Monclus et al., 2018; Nordstad et al., 2012; Verboven et al., 2010), immune system impairment (Bustnes et al., 2004; Hansen et al., 2020; Sagerup et al., 2009), and oxidative stress (Costantini et al., 2014; Fenstad et al., 2016; Sletten et al., 2016). While the relationship between POP exposure and telomere lengths has been investigated in some avian species (Blevin et al., 2016, 2017; Sebastiano et al., 2020; Sletten et al., 2016), findings are often inconsistent or inconclusive (Louzon et al., 2019), and wildlife ecotoxicology lacks long-term studies on how telomere lengths may fluctuate both over time and in relation to POP exposure.

In the present study, we examined telomere lengths and POP concentrations in WTE nestlings from the Swedish Baltic coast sampled across 19 years (1995–2013). In nestlings, telomere dynamics are less confounded by biological age and life-long exposure to other environmental factors, and are thus better suited than adults to examine the associations between telomere lengths and POP exposure (Boonekamp et al., 2014; Eastwood et al., 2019; Heidinger et al., 2012; Wood and Young, 2019). The current study aimed (1) to examine year-to-year variations in telomere lengths among the WTE nestling cohorts, and (2) to investigate the relationship between POP concentrations and telomere lengths.

2. Materials and methods

2.1. Field sampling

Sampling of WTE nestlings was carried out on the Baltic coast of Sweden at nine different locations (58°-61°N, 16°-19°E, Fig. 1) in the years 1995–2013 as part of the Swedish Environmental Monitoring Programme (Helander et al., 2008). During March–April, nests were inspected for breeding activity and from mid-May to mid-June, when the nestlings were 4–8 weeks post-hatching, all occupied nests were visited (Helander et al., 2008). There were 1–3 chicks per nest and all nestlings in a brood were ringed and measured for body mass [kg] and wing length [mm]. One specimen per nest was blood sampled (<10 mL) from the brachial vein using a 10 mL syringe with a 25 × 0.6 mm needle. From all samples, a small amount of whole blood (0.5–1.0 mL) was transferred directly to an EDTA-coated syringe and used in the present study for telomere length analysis and sexing. For samples from 1995 to 2002 the remaining blood sample was stored as whole blood, while for samples from 2003 to 2013 the remaining blood sample was centrifuged (2000 rpm for 10 min), separating serum from red blood cells. All blood

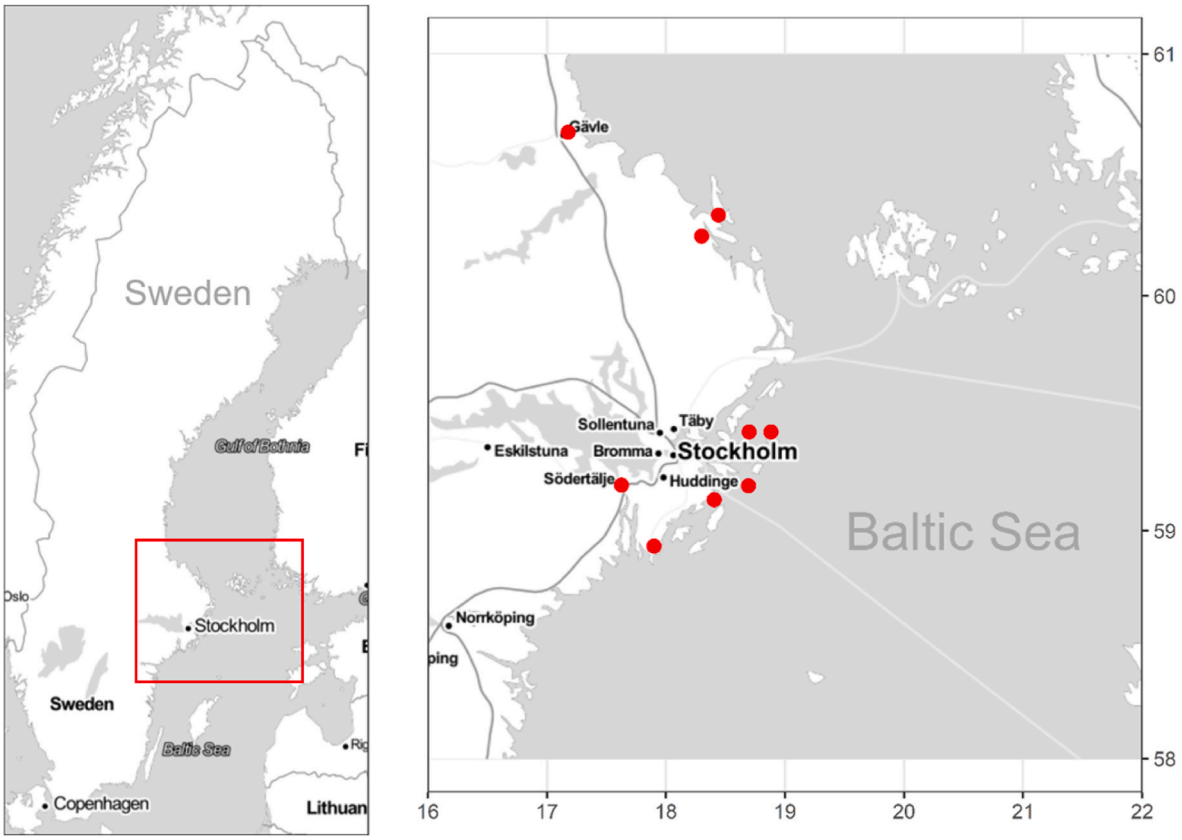

Fig. 1. Baltic coastline of Sweden indicating locations (red circles) where white-tailed eagle nestlings were sampled in 1995–2013. Labelled place names are included for regional context. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(fraction) samples were kept cool in an icebox for short transport in the field and transferred to a freezer (−20 °C) or liquid nitrogen the same day, and subsequently stored at −80 °C in the national specimen bank until laboratory analysis. In total, 184 WTE nestlings were blood sampled and morphometrically measured during the study period. Due to differing amounts of individual blood samples, 168 were analyzed for telomere lengths and molecular sexing. Of these, 152 were also analyzed for POPs (whole blood 1995–2002, $n = 71$; serum 2003–2013, $n = 81$).

2.2. Molecular sexing analysis

Sex determination of nestlings was performed at IPHC-DEPE (CNRS, France) using the PCR and gel electrophoresis protocols adapted from Helander et al. (2007) In brief, DNA was extracted from whole blood using a commercial kit (NucleoSpin® Blood QuickPure, Macherey Nagel, Germany) and the sections of the sex-linked chromo-helicase-DNA-binding gene (CHD-Z and CHD-W) were amplified by polymerase chain reaction using primers 2550 F and 2718R (Fridolfsson and Ellegren, 1999).

2.3. Telomere length analysis

Analysis of telomere length was performed at IPHC-DEPE (CNRS, France) using the qPCR procedure described by Criscuolo et al. (2009) adapted from a human-telomere qPCR methodology published by Cawthon (2002). The description of blood extraction and qPCR amplification conditions of WTE telomeres was previously described in further detail in Sletten et al. (2016) Briefly, genomic DNA was extracted from 5 μL of frozen red blood cells, using an adapted protocol of the commercial kit (NucleoSpin® Blood Quickpure, Macherey Nagel, Germany). Due to the age of the samples (over 19 years of storing) an extreme care was required to assess DNA quantity and quality (using Nanodrop, Thermo Fisher Scientific, USA; and fluorescence, Quanti-Fluor® ONE dsDNA System, Promega, USA) and no DNA degradation (using gel-migration). Relative telomere length (T/S ratio) were obtained using a ratio of the numbers of amplification cycles between a genomic control gene (S), and the telomeric sequences (T).

Because of the duration of this longitudinal study (nearly 20 years), we have, prior to the measurement of relative telomere length, checked if there is no efficiency variation during the sampling years for the telomeric and the control genes ($E_{Control} = 0{,}992 \pm 0{,}003$ and $E_{TEL} = 0{,}997 \pm 0{,}004$ (mean ± SE)). All samples were measured in one 384-well thermocycler (CFX-384 Touch Real-Time PCR Detection System, Biorad, USA) using the same amplification conditions for both the control gene and telomere sequences. All samples were used in duplicates, and the qPCR run contained a no template control (distillated water used for DNA dilution) and a calibrator sample dilution curve (issue from a pool of one sample, randomly chosen, per sampling years) to calculate the amplification efficiencies (0.998 for control and 0.996 for telomere amplifications). The qPCR run ended with a melting curve to control for unexpected non-specific amplification artefacts. Relative telomere lengths were calculated following Pfaffl (2001). Intra-run variation of T/S ratio was 0.792, as evaluated using intraclass correlation coefficient (Alyssa R. et al., 2021; Cicchetti, 1994).

2.4. POP analysis

POPs were analyzed at the Toxicological Centre, University of Antwerp (Belgium) using whole blood ($n = 71$; 1995–2002) or serum ($n = 81$; 2003–2013). The methods used to analyze organochlorines (polychlorinated biphenyls [PCB] and pesticides) or polybrominated diphenyl ethers (PBDEs) are described in further detail by Eulaers et al. (2011) and Covaci and Voorspoels (2005), respectively. Targeted compounds were 28 PCB congeners (28, 49, 52, 66, 74, 95, 99, 101, 105, 110, 118, 128, 138, 146, 149, 153, 156, 170,171, 177, 180, 183, 187, 194, 196/203, 199, 206, and 209), dichlorodiphenyltrichloroethane (*p,p'*-DDT) and its metabolites *p,p'*-DDE and *p,p'*-DDD, five chlordanes (*cis*- and *trans*-chlordane, *cis*- and *trans*-nonachlor, and oxychlordane), hexachlorobenzene (HCB), hexachlorocyclohexanes (HCHs; *α*-, *β*-, and *γ*-HCH), seven PBDE congeners (28, 47, 99, 100, 153, 154, and 183) and two PBDE metabolites (6-MeO-BDE-47 and 2-MeO-BDE-68). POP concentrations are expressed as ng mL^{-1} wet weight (*ww*) for whole blood and serum.

Analytical quality assessment and control was supported by the analysis of procedural blanks and addition of internal standard solutions (CB 143, *ε*-HCH, BDE 77 and 13C-HCB) to each sample. Every 21 samples, two procedural blanks and one standard reference material were analyzed. Regular analysis of plasma reference material (AM-S-W1705) under the inter-laboratory comparison exercises organized by the Arctic Monitoring and Assessment Programme reveal that the obtained precision is typically less than 20%. All contaminant concentrations were corrected for blank values, and the limit of quantification (LOQ) was set at 3 times the standard deviation of the blank concentrations or determined to be at a 10:1 signal to noise ratio for compounds not detectable in the blanks. The LOQs for the analyzed compounds ranged from 0.02 to 0.10 ng mL^{-1} *ww*. Mean internal standard recoveries of the internal standards PCB 143, *ε*-HCH, and BDE 77 were 86 ± 6%, 98 ± 8%, and 94 ± 8%, respectively.

2.5. Data analysis

Statistical analyses were performed using R version 3.6.1 (R Development Core Team, 2020). To determine whether the statistical models fulfilled their underlying assumptions, we conducted visual inspection of residual plots and normal QQ-plots (Zuur et al., 2010). All tests were two-tailed and the null hypothesis was rejected at an *α*-level of 0.05.

Since the same WTE nests were sampled multiple times across the study period, we first chose linear mixed-effect (LME) models in our analyses with Nest ID as the random factor (a random intercepts only) to estimate and account for among-nest variability using the nlme-package (Pinheiro et al., 2017; Pinheiro and Bates, 2000). However, since the random factor in the LME revealed low among-nest variability (i.e., the random intercept variance; results not shown), we also fitted generalized least square (GLS; function '*gls*') models (i.e., without the random effect). We compared the model fit of the LME against the GLS based on the secondorder Aikake Information Criterion (AICc) (Anderson, 2008; Burnham et al., 2002). Namely, the final and selected LME models in our analyses were compared with their GLS equivalent, and the model with the lowest AICc (i.e. the most parsimonious model) was chosen for inference (Zuur et al., 2009).

First, we estimated differences in telomere length among WTE cohorts using a GLS with year (19-level factor and 1995 as baseline year) as a fixed effect. Next, we assessed POP concentrations as drivers of individual telomere length. To do so, we first divided the dataset into whole blood and serum POP concentration samples since these measures are not directly comparable (Batterman et al., 2016). Our statistical analyses only included the responses where POPs were present in >75% of the samples (Table S1), and levels below LOQ were arbitrarily replaced by half the compound-specific LOQ value (Hansen et al., 2020). To reduce the number of compounds used in the statistical analyses, their linear relationships were investigated using Pearson's product moment correlation tests (*r*; Figs. S1 and S2). Since many compounds were significantly correlated, we grouped them based on their commonness (i.e., belonging to the same chemical class) and physiochemical properties (Sletten et al., 2016) and summed the structurally similar compounds such as the PCBs, PBDEs, DDTs (*p,p'*-DDE and *p,p'*-DDD) and Nonachlors (CN and TN). Only HCB, *β*-HCH, and OxC were treated individually because of their particular physiochemical properties (Sletten et al., 2016). In total, seven groups of POPs were retained in the final analyses: the sum of 24 PCB congeners ($\sum_{24}$PCBs), the sum of five PBDE congeners ($\sum_{5}$PBDEs), the sum of two DDT metabolites ($\sum$DDT), the sum of two Nonachlors ($\sum$Nonachlor), HCB, *β*-HCH, and OxC. To attain the

assumption of normality, POP concentrations were ln-transformed prior to analyses (Zuur et al., 2007).

Thereafter, we created a set of *a priori* candidate LME models to investigate POPs as potential drivers of variation in telomere lengths among individual nestlings (Tables S2a and b) by using telomere length as the response variable and POP concentration (each group tested separately for whole blood and serum, respectively) as the explanatory variable. Wing length, sex (factor with female and male as levels) and brood size (factor with one, two and three chicks as levels) were included as additional predictors in the candidate models (Tables S2a and b). The latter three variables were included to control for important state variables expected to affect individual stress levels directly and telomere length indirectly. To avoid problems with multicollinearity, correlated variables were not placed in the same candidate models (Figs. S3a and b) (Zuur et al., 2009). A model selection of the set of candidate LME models was then performed using the *aictab* function in the AICcmodavg package (Mazzerolle, 2019) and the most parsimonious model explaining variation in telomere length was selected based on the lowest AICc ($\Delta_i \leq 2$; Tables S2a and b) (Anderson, 2008; Burnham et al., 2002). Finally, we compared the selected LME with a GLS (Tables S3a–c); in all cases, the GLS models had the lowest AICc and were therefore chosen for inference.

3. Results

3.1. Telomere lengths

The overall mean telomere length for all WTE nestling cohorts was 1.03 ± 0.03 (mean ± SE) and the individual values ranged from 0.16 to 2.30 (Fig. 2). There were differences in telomere lengths among annual cohorts with significantly longer telomeres being found in 1998–2002, 2004 and 2006 compared to the first year of sampling, 1995 (Fig. 2, Table S4, $p < 0.05$ in all cases). The shortest telomeres were recorded in 1996 (Fig. 2), but they did not differ significantly from 1995 (Fig. 2, Table S4, $p = 0.50$).

3.2. POP concentrations in whole blood and serum

Concentrations of POPs included in the statistical analyses are presented in Table 1. Σ_{24}PCBs made up the majority of the investigated POPs and represented 82% of the total concentration in both whole blood and serum (Table 1). Among the organochlorine pesticides (*p,p'*-DDD, *p,p'*-DDE, HCB, OxC, CN, TN, and *β*-HCH), *p,p'*-DDE contributed with the highest levels and represented 13.5% and 15% of the total whole blood and serum concentrations, respectively (Table 1). Σ_5PBDEs represented less than 1% of the total POP concentration in both matrices (Table 1).

3.3. Relationship between telomere lengths and POP concentrations

Overall, we detected no significant relationships between telomere length and POP concentrations in either whole blood or serum in the WTE nestlings (Fig. 3, Tables S5a and b: $p > 0.20$ in all cases). While wing length was the only additional predictor variable that was retained in some models (whole blood POP concentrations), it was never significantly correlated with telomere length (Table S5a: $p > 0.08$ in all cases). Sex and brood size were never retained in any of the models, which indicates that neither sex nor brood size were important predictor variables explaining variation in telomere lengths in the WTE nestlings.

4. Discussion

4.1. Telomere length in relation to POP concentrations

Based on the reported links between telomere lengths, fitness, longevity, and survival, telomeres could represent useful biomarkers of environmental stressors in wildlife research (Monaghan, 2014). While telomere erosion has been related to pollution exposure in humans (Barbullushi et al., 2009; De Felice et al., 2012; Hoxha et al., 2009; Zhang et al., 2013), only four studies have investigated the relationship between telomere length and POP concentrations in avian wildlife (Blevin et al., 2016, 2017; Sebastiano et al., 2020; Sletten et al., 2016). In the current study, we found no relationships between telomere lengths and POP concentrations in nestling WTEs, despite examining 152 individuals from a highly polluted population over a period of 19 years (1995–2013). Similar to our study, in a Norwegian population of WTE nestlings showing telomere lengths within the same range as ours but overall lower POP concentrations (on average twice lower for the dominating compounds), Sletten et al. (2016) found no association between telomere length and plasma POPs. In adult black-legged

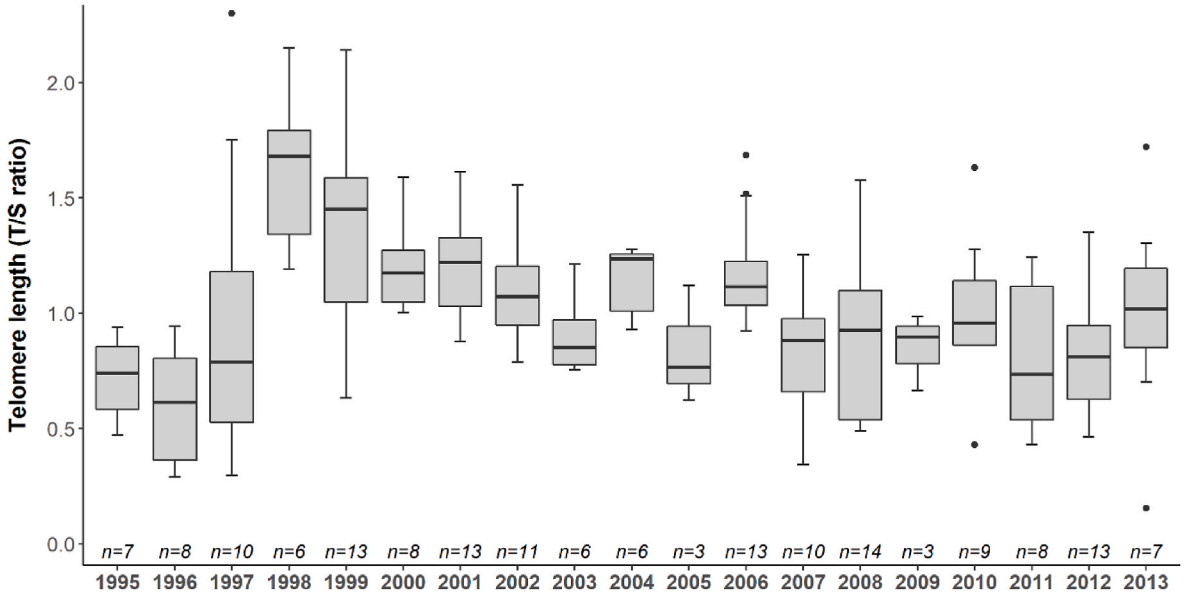

Fig. 2. Boxplots showing the median, 25th-50th quartiles (boxes) and range (whiskers) in the observed telomere lengths among white-tailed eagle nestling cohorts sampled at the Swedish Baltic coast during 1995–2013. Black dots represent extreme values and are plotted individually. Annual sample size (*n*) is indicated above the respective year on the graph.

Table 1
Descriptive statistics (mean ± SE, median, range [min-max]) for legacy persistent organic pollutant concentrations (ng mL^{-1} *ww*) in white-tailed eagle (*Haliaeetus albicilla*) nestlings sampled at the Swedish Baltic coast during 1995–2002 (whole blood; $n = 71$) and 2003–2013 (serum; $n = 81$).

	Whole blood			Serum		
	Mean	Median	Min-max	Mean	Median	Min-max
p,p'-DDD	0.33 ± 0.02	0.27	0.02–1.04	0.39 ± 0.04	0.27	0.05–3.10
p,p'-DDE	7.55 ± 0.48	6.56	1.02–20.5	14.2 ± 0.86	12.4	3.16–38.5
ΣDDT[a]	7.90 ± 0.48	6.96	1.07–20.9	14.6 ± 0.87	12.6	3.33–39.1
HCB	0.55 ± 0.05	0.49	0.09–3.10	0.64 ± 0.05	0.51	0.11–2.35
OxC	0.16 ± 0.01	0.12	0.03–0.44	0.23 ± 0.01	0.20	0.03–0.58
TN	0.23 ± 0.01	0.22	0.03–0.49	0.35 ± 0.02	0.30	0.12–1.19
CN	0.10 ± 0.01	0.10	0.03–0.22	0.16 ± 0.01	0.14	0.03–0.53
ΣNonachlor[b]	0.33 ± 0.02	0.32	0.05–0.69	0.51 ± 0.03	0.45	0.05–1.71
β-HCH	0.41 ± 0.03	0.38	0.03–1.01	0.53 ± 0.05	0.48	0.03–2.96
Σ_{24}PCBs[c]	45.7 ± 2.42	39.9	9.54–116	76.0 ± 4.96	66.5	21.4–241
Σ_5PBDEs[d]	0.48 ± 0.03	0.45	0.07–1.19	0.68 ± 0.11	0.49	0.19–4.94

[a] Sum of *p,p'*-DDD and *p,p'*-DDE (see Table S1 for full names and abbreviations).
[b] Sum of TN and CN (see Table S1 for full names and abbreviations).
[c] Sum of 24 congeners (see Table S1 for full names and abbreviations).
[d] Sum of 5 congeners (see Table S1 for full names and abbreviations).

kittiwakes (*Rissa tridactyla*), however, shorter telomeres were associated with higher whole blood OxC levels, but in females only (Blevin et al., 2016). Contrary to OxC, plasma concentrations of perfluoroalkyl substances (PFAS) were positively correlated to telomere lengths in both adult black-legged kittiwakes and glaucous gulls (*Larus hyperboreus*), which indicates that individuals with the highest PFAS concentrations also had longer telomeres (Blevin et al., 2017; Sebastiano et al., 2020). The seemingly disparate findings might be a result of measuring different POP classes (lipophilic organochlorines vs. proteinophilic PFAS) in various study species sampled at different life-history stages. The discrepancies between studies could also stem from different magnitude of POP concentrations detected in the birds (e.g., lower in this study compared to Blevin et al. (2016)).

In the studied population, levels of legacy POPs measured in eggs and feathers from adult WTEs have been steadily decreasing over the 20 years prior to our study period (Helander et al., 2008; Sun et al., 2020), leading to improved breeding success since the 1980s (Helander et al., 2002). In the current study, sampling started in 1995 when DDE and PCB levels were below the thresholds for effects on reproduction (Bignert and Helander, 2015), which could explain the lack of relationships between telomere length and POP concentrations in our study.

Despite not highlighting clear links between telomere length and different POP classes, our study has two major strengths. First, by investigating nestlings whose telomere attrition is greater compared to adults (Angelier et al., 2018; Boonekamp et al., 2014; Eastwood et al., 2019; Heidinger et al., 2012; Wood and Young, 2019), our study maximizes the potential of highlighting relationships while reducing the confounding effect of biological age and past exposure towards multiple environmental factors on telomere lengths (Dugdale and Richardson, 2018). Secondly, the long timeseries we analyze decreases the probability of reporting an apparent correlation with telomere length that is really due to coincidental inter annual variation between cohorts.

4.2. Year-to-year variation in telomere lengths

While telomere lengths did not vary with POP concentrations, they did differ on a year-to-year basis among the WTE nestling cohorts. Telomere length at a given time is a result of the initial telomere length (i.e., inherited from the parental gametes) combined with variable shortening from both cell divisions and exposure to stressors (Angelier et al., 2018; Blackburn, 2000; Houben et al., 2008). For example, environmental conditions (e.g. food availability and habitat quality) are suggested as important factors that drive telomere dynamics in wildlife (Foote et al., 2011; Hall et al., 2004; Heidinger et al., 2012; Spurgin et al., 2018). In a longitudinal study on Seychelles warblers (*Acrocephalus sechellensis*), Spurgin et al. (2018) found positive associations between telomere length and food availability (insect abundance) but not body mass. Conditions experienced during early life such as parental care (Viblanc et al., 2020) and sibling competition (Dupont et al., 2018; Nettle et al., 2013, 2015; Watson et al., 2015) also appear to determine telomere dynamics (Monaghan and Ozanne, 2018). We could not measure food availability in the current study, but we did not observe significant relationships between telomere lengths and brood size that could serve as a proxy of food provisioning and/or environmental conditions. Environmental and early life conditions likely fluctuate among years and may therefore give rise to year-to-year variations in telomere lengths in WTE nestlings. Among factors influencing within-species variations in telomere length, Monaghan and Ozanne (2018) pointed out early life conditions as having long-term consequences for health and longevity. Although telomere lengths ranged from 0.16 to 2.30 (T/S ratio) in WTE nestlings of the current study, it remains to be established whether these inter-individual differences can be biologically and ecologically meaningful.

4.3. Telomere length as a biomarker of health in a multiple stressor context

Conducting wildlife ecotoxicological studies in a multiple stressor context has recently gained significant momentum (Bardsen et al., 2018; Bustnes et al., 2015), and allows for a holistic examination of factors that could drive biomarkers of health in wildlife. Nestlings undergo energy-demanding periods of rapid growth and development and may be particularly vulnerable to multiple stress exposure that could have a profound influence on telomere length (Powolny et al., 2020). However, disentangling potential drivers of telomere length appear challenging (Angelier et al., 2018; Dugdale and Richardson, 2018; Monaghan and Ozanne, 2018). Despite studying free-ranging raptor nestlings from a highly polluted population over 19 years, we did not detect a relationship between telomere length and legacy POP contamination in Baltic WTE nestlings. Instead, year-to-year variability in telomere lengths among nestling cohorts suggests that environmental conditions and/or biological drivers as discussed in section 4.2, which likely vary between years, were more important drivers during the study period (Chatelain et al., 2020). Studies show potential indirect effects of environmental stressors on telomere dynamics, which are mediated through physiological mechanisms that are important in maintaining homeostasis (Angelier et al., 2018; Monaghan and Ozanne, 2018; Powolny et al., 2020). Namely, increased oxidative stress and glucocorticoid levels (the major stress hormone in vertebrates) are linked to telomere dynamics (Angelier et al., 2018; Kawanishi and Oikawa, 2004; Lemaitre et al.,

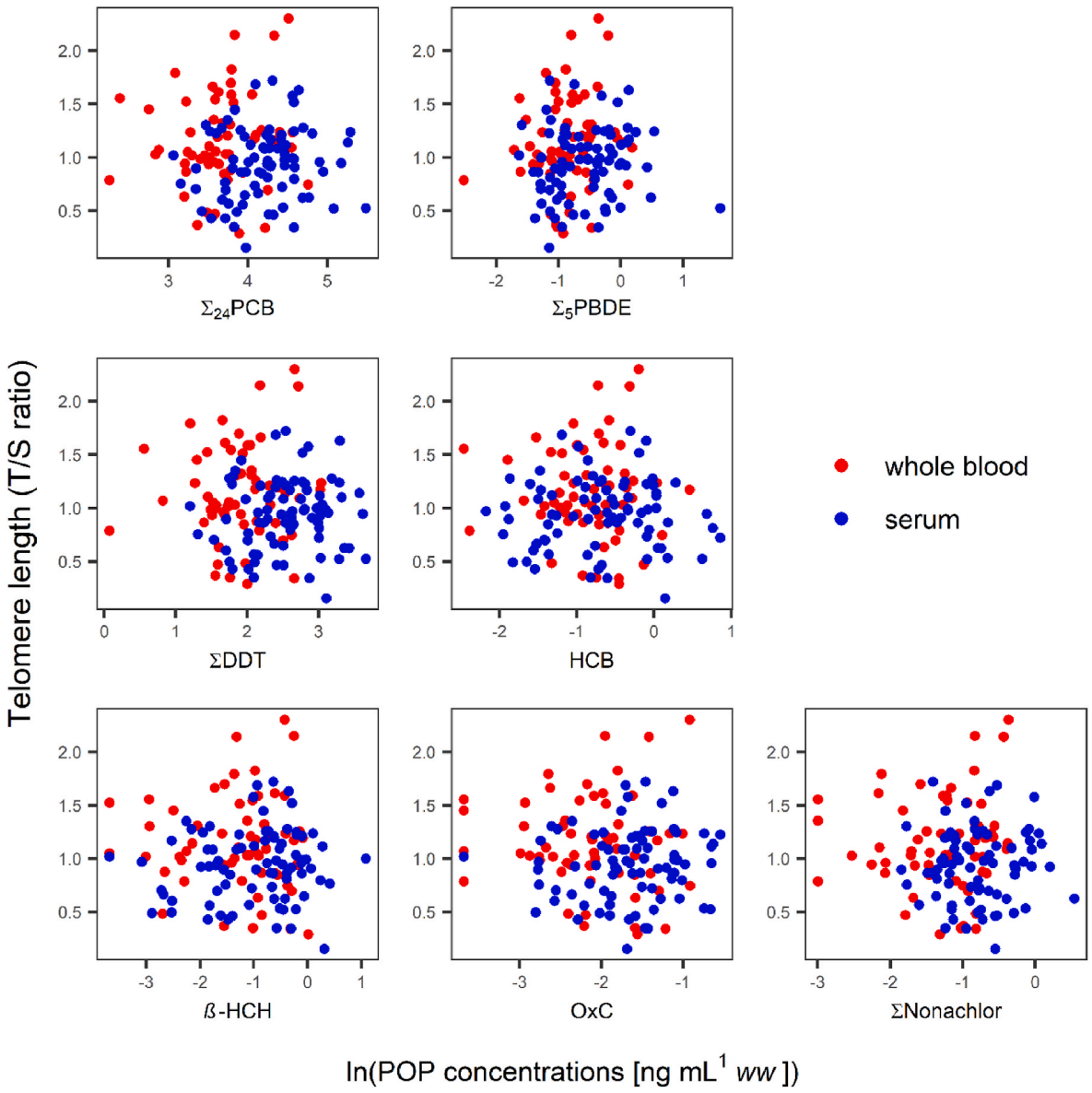

Fig. 3. Relationships between telomere lengths (T/S ratio) and persistent organic pollutant (POP) concentrations in white-tailed eagle (*Haliaeetus albicilla*) nestlings sampled at the Swedish Baltic coast (all P-values > 0.05). The investigated POP compounds were the sum of 24 polychlorinated biphenyl congeners ($\sum_{24}$PCBs), the sum of five polybrominated diphenyl ether congeners ($\sum_{5}$PBDEs), the sum of two dichlorodiphenyltrichloroethane metabolites (*p,p'*-DDE and *p,p'*-DDD: ∑DDT), hexachlorobenzene (HCB), *β*-hexachlorocyclohexane (*β*-HCH), *oxy*chlordane (OxC), and the sum of *trans*- and *cis*-Nonachlor (∑Nonachlor). Red dots represent whole blood POP concentrations and blue dots are serum POP concentrations. Estimates from linear-mixed effect models are given in Tables S5a and b. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2021), which are both also associated with POP and metal contamination (Dietz et al., 2019).

In conclusion, we did not detect clear associations between telomere length and concentrations of POPs of different classes in Swedish WTE nestlings, but telomere lengths did vary on a year-to-year basis. Analyzing additional physiological variables, such as oxidative stress parameters and glucocorticoid levels, could further explain variation in individual telomere length, and in combination with multiple environmental stressors, give more insight into the relationship between telomeres and environmental pollutants in avian wildlife.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.112712.

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Telomere length in relation to organic pollutant exposure in white-tailed eagle (*Haliaeetus albicilla*) nestlings from Sweden sampled in 1995-2013

SUPPORTING INFORMATION

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TABLE OF CONTENTS

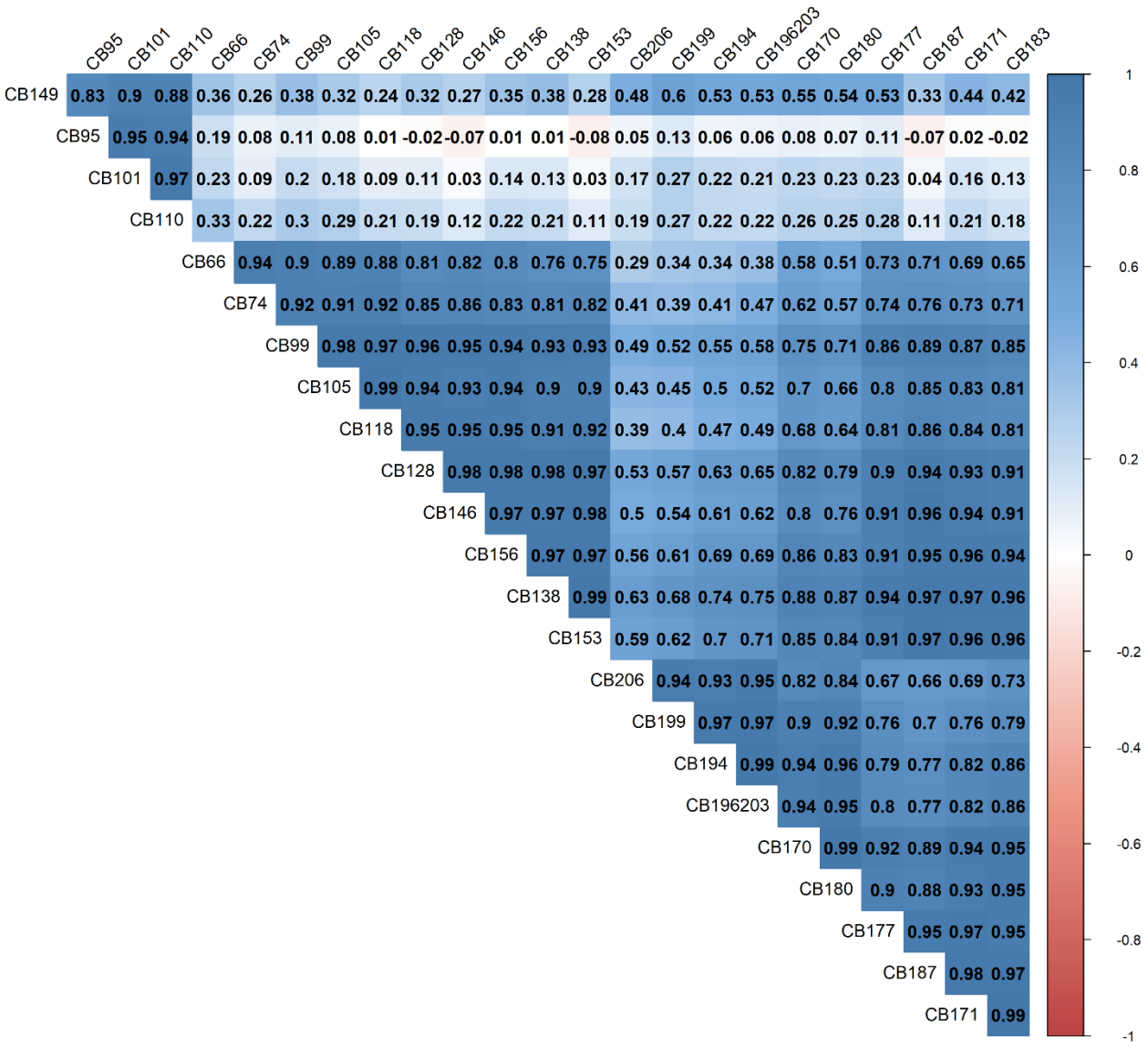
Table S1. List of targeted compounds (full names and abbreviations) with their limit of quantification (LOQ; compounds not detected are indicated as <LOQ) and their detection frequencies among individuals (> 75%, < 75%) in whole blood or serum of white-tailed eagle nestlings sampled at the Swedish Baltic coast during 1995-2013 (*n* = 152).

Organochlorine pesticides (OCPs)	LOQ	> 75%	< 75%	< LOQ
Hexachlorobenzene (HCB)	0.05	X		
α-Hexachlorocyclohexane (α-HCH)	0.05			X
β-Hexachlorocyclohexane (β-HCH)	0.05	X		
γ-Hexachlorocyclohexane (γ-HCH)	0.05			X
Oxychlordane (OxC)	0.05	X		
trans-Chlordane (TC)	0.05		X	
cis-Chlordane (CC)	0.05		X	
trans-Nonachlor (TN)	0.05	X		
cis-Nonachlor (CN)	0.05	X		
p,p'-Dichloro-α,α-diphenyl-β,β,β-trichloroethane (*p,p'*-DDT)	0.10		X	
p,p'-Dichlorodiphenyldichloroethane (*p,p'*-DDD)	0.10	X		
p,p'-Dichlorodiphenyldichloroethylene (*p,p'*-DDE)	0.10	X		

Polychlorinated biphenyls (PCBs)	LOQ	> 75%	< 75%	< LOQ
PCB 28	0.10		X	
PCB 49	0.10		X	
PCB 52	0.10		X	
PCB 66	0.10	X		
PCB 74	0.10	X		
PCB 95	0.10	X		
PCB 99	0.05	X		
PCB 101	0.05	X		
PCB 105	0.05	X		
PCB 110	0.05	X		
PCB 118	0.05	X		
PCB 128	0.05	X		
PCB 138	0.05	X		
PCB 146	0.05	X		
PCB 149	0.05	X		
PCB 153	0.05	X		
PCB 156	0.05	X		
PCB 170	0.05	X		
PCB 171	0.05	X		
PCB 177	0.05	X		
PCB 180	0.05	X		
PCB 183	0.05	X		
PCB 187	0.05	X		
PCB 194	0.05	X		
PCB 196/203	0.05	X		
PCB 199	0.05	X		
PCB 206	0.05	X		
PCB 209	0.05		X	

Polybrominated Diphenyl Ethers (PBDEs)	LOQ	> 75%	< 75%	< LOQ
PBDE 28	0.02		X	
PBDE 47	0.02	X		
PBDE 99	0.02	X		
PBDE 100	0.02	X		
PBDE 153	0.02	X		
PBDE 154	0.02	X		
PBDE 183	0.02		X	

a)


CB95 CB101 CB110 CB66 CB74 CB99 CB105 CB118 CB128 CB146 CB156 CB138 CB153 CB206 CB199 CB194 CB196203 CB170 CB180 CB177 CB187 CB171 CB183
CB149 0.83 0.9 0.88 0.36 0.26 0.38 0.32 0.24 0.32 0.27 0.35 0.38 0.28 0.48 0.6 0.53 0.53 0.55 0.54 0.53 0.33 0.44 0.42
CB95 0.95 0.94 0.19 0.08 0.11 0.08 0.01 -0.02 -0.07 0.01 0.01 -0.08 0.05 0.13 0.06 0.06 0.08 0.07 0.11 -0.07 0.02 -0.02
CB101 0.97 0.23 0.09 0.2 0.18 0.09 0.11 0.03 0.14 0.13 0.03 0.17 0.27 0.22 0.21 0.23 0.23 0.23 0.04 0.16 0.13
CB110 0.33 0.22 0.3 0.29 0.21 0.19 0.12 0.22 0.21 0.11 0.19 0.27 0.22 0.22 0.26 0.25 0.28 0.11 0.21 0.18
CB66 0.94 0.9 0.89 0.88 0.81 0.82 0.8 0.76 0.75 0.29 0.34 0.34 0.38 0.58 0.51 0.73 0.71 0.69 0.65
CB74 0.92 0.91 0.92 0.85 0.86 0.83 0.81 0.82 0.41 0.39 0.41 0.47 0.62 0.57 0.74 0.76 0.73 0.71
CB99 0.98 0.97 0.96 0.95 0.94 0.93 0.93 0.49 0.52 0.55 0.58 0.75 0.71 0.86 0.89 0.87 0.85
CB105 0.99 0.94 0.93 0.94 0.9 0.9 0.43 0.45 0.5 0.52 0.7 0.66 0.8 0.85 0.83 0.81
CB118 0.95 0.95 0.95 0.91 0.92 0.39 0.4 0.47 0.49 0.68 0.64 0.81 0.86 0.84 0.81
CB128 0.98 0.98 0.98 0.97 0.53 0.57 0.63 0.65 0.82 0.79 0.9 0.94 0.93 0.91
CB146 0.97 0.97 0.98 0.5 0.54 0.61 0.62 0.8 0.76 0.91 0.96 0.94 0.91
CB156 0.97 0.97 0.56 0.61 0.69 0.69 0.86 0.83 0.91 0.95 0.96 0.94
CB138 0.99 0.63 0.68 0.74 0.75 0.88 0.87 0.94 0.97 0.97 0.96
CB153 0.59 0.62 0.7 0.71 0.85 0.84 0.91 0.97 0.96 0.96
CB206 0.94 0.93 0.95 0.82 0.84 0.67 0.66 0.69 0.73
CB199 0.97 0.97 0.9 0.92 0.76 0.7 0.76 0.79
CB194 0.99 0.94 0.96 0.79 0.77 0.82 0.86
CB196203 0.94 0.95 0.8 0.77 0.82 0.86
CB170 0.99 0.92 0.89 0.94 0.95
CB180 0.9 0.88 0.93 0.95
CB177 0.95 0.97 0.95
CB187 0.98 0.97
CB171 0.99
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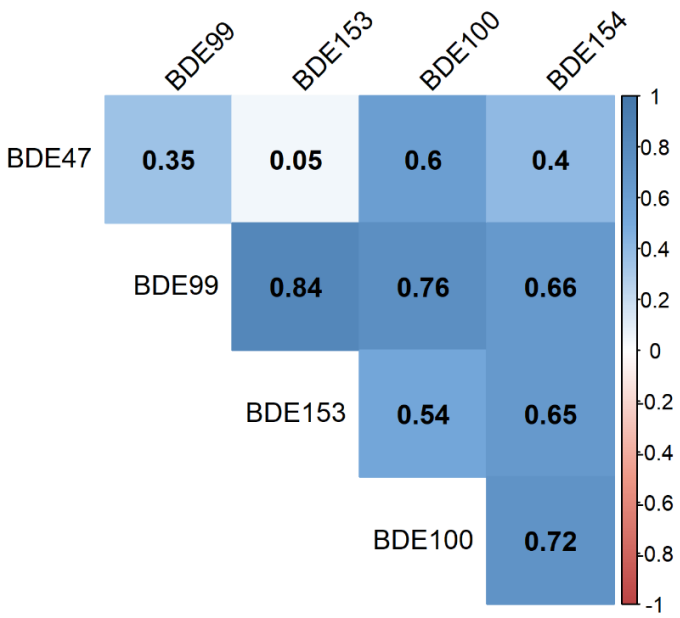
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

b)



c)

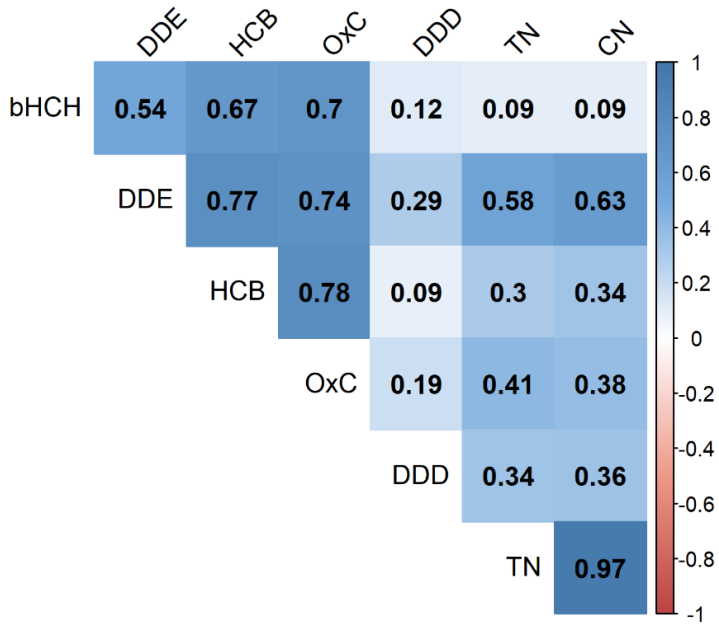
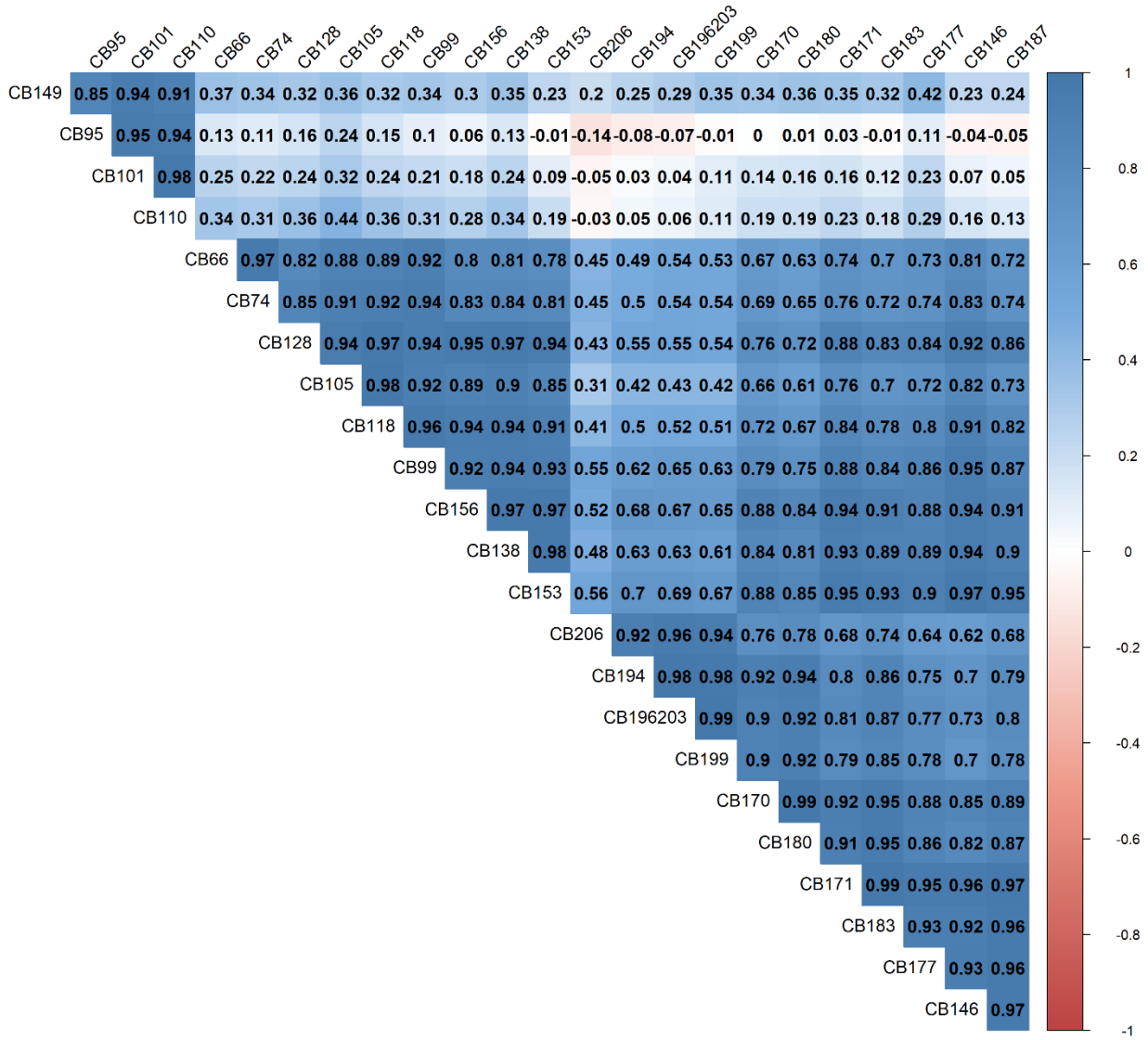
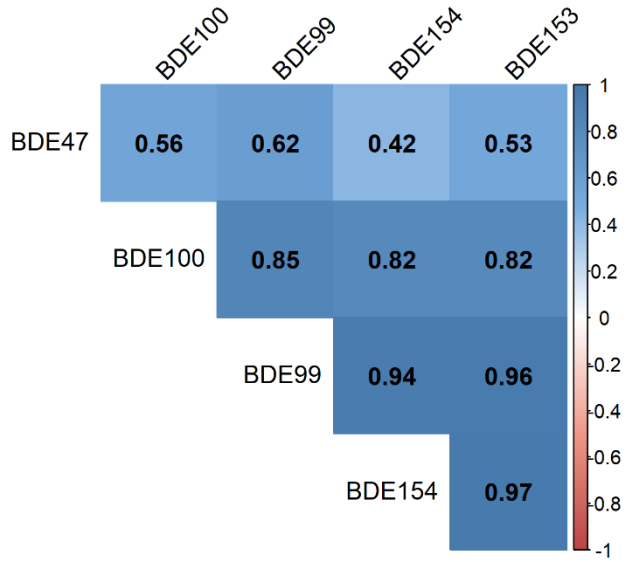


Figure S1. Pearson's product moment correlation's for concentrations of a) polychlorinated biphenyl (PCB) compounds, b) polybrominated diphenyl ether (PBDE) compounds, and c) organochlorine pesticides (OCP) in whole blood (n = 71; 1995-2002) of white-tailed eagle nestlings sampled at the Swedish Baltic coast.

a)


CB95 CB101 CB110 CB66 CB74 CB128 CB105 CB118 CB99 CB156 CB138 CB153 CB206 CB194 CB196203 CB199 CB170 CB180 CB171 CB183 CB177 CB146 CB187
CB149 0.85 0.94 0.91 0.37 0.34 0.32 0.36 0.32 0.34 0.3 0.35 0.23 0.2 0.25 0.29 0.35 0.34 0.36 0.35 0.32 0.42 0.23 0.24
CB95 0.95 0.94 0.13 0.11 0.16 0.24 0.15 0.1 0.06 0.13 -0.01 -0.14 -0.08 -0.07 -0.01 0 0.01 0.03 -0.01 0.11 -0.04 -0.05
CB101 0.98 0.25 0.22 0.24 0.32 0.24 0.21 0.18 0.24 0.09 -0.05 0.03 0.04 0.11 0.14 0.16 0.16 0.12 0.23 0.07 0.05
CB110 0.34 0.31 0.36 0.44 0.36 0.31 0.28 0.34 0.19 -0.03 0.05 0.06 0.11 0.19 0.19 0.23 0.18 0.29 0.16 0.13
CB66 0.97 0.82 0.88 0.89 0.92 0.8 0.81 0.78 0.45 0.49 0.54 0.53 0.67 0.63 0.74 0.7 0.73 0.81 0.72
CB74 0.85 0.91 0.92 0.94 0.83 0.84 0.81 0.45 0.5 0.54 0.54 0.69 0.65 0.76 0.72 0.74 0.83 0.74
CB128 0.94 0.97 0.94 0.95 0.97 0.94 0.43 0.55 0.55 0.54 0.76 0.72 0.88 0.83 0.84 0.92 0.86
CB105 0.98 0.92 0.89 0.9 0.85 0.31 0.42 0.43 0.42 0.66 0.61 0.76 0.7 0.72 0.82 0.73
CB118 0.96 0.94 0.94 0.91 0.41 0.5 0.52 0.51 0.72 0.67 0.84 0.78 0.8 0.91 0.82
CB99 0.92 0.94 0.93 0.55 0.62 0.65 0.63 0.79 0.75 0.88 0.84 0.86 0.95 0.87
CB156 0.97 0.97 0.52 0.68 0.67 0.65 0.88 0.84 0.94 0.91 0.88 0.94 0.91
CB138 0.98 0.48 0.63 0.63 0.61 0.84 0.81 0.93 0.89 0.89 0.94 0.9
CB153 0.56 0.7 0.69 0.67 0.88 0.85 0.95 0.93 0.9 0.97 0.95
CB206 0.92 0.96 0.94 0.76 0.78 0.68 0.74 0.64 0.62 0.68
CB194 0.98 0.98 0.92 0.94 0.8 0.86 0.75 0.7 0.79
CB196203 0.99 0.9 0.92 0.81 0.87 0.77 0.73 0.8
CB199 0.9 0.92 0.79 0.85 0.78 0.7 0.78
CB170 0.99 0.92 0.95 0.88 0.85 0.89
CB180 0.91 0.95 0.86 0.82 0.87
CB171 0.99 0.95 0.96 0.97
CB183 0.93 0.92 0.96
CB177 0.93 0.96
CB146 0.97
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

b)



c)

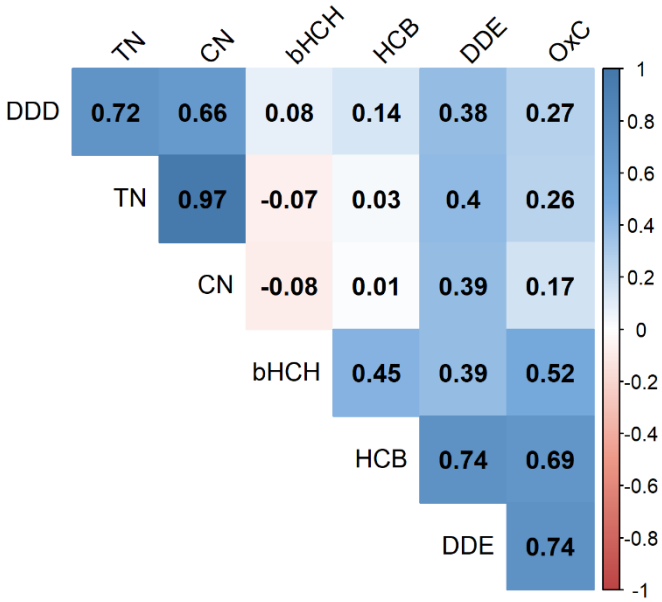
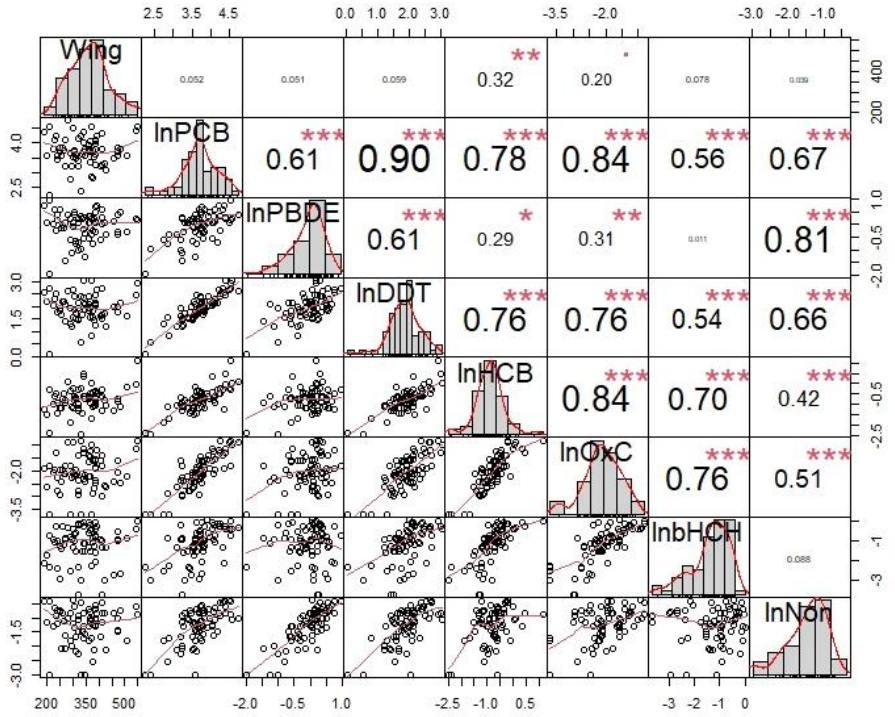


Figure S2. Pearson's product moment correlation's for concentrations of a) polychlorinated biphenyl (PCB) compounds, b) polybrominated diphenyl ether (PBDE) compounds, and c) organochlorine pesticides (OCP) in serum (n = 81; 2003-2013) of white-tailed eagle nestlings sampled at the Swedish Baltic coast.

a)



b)

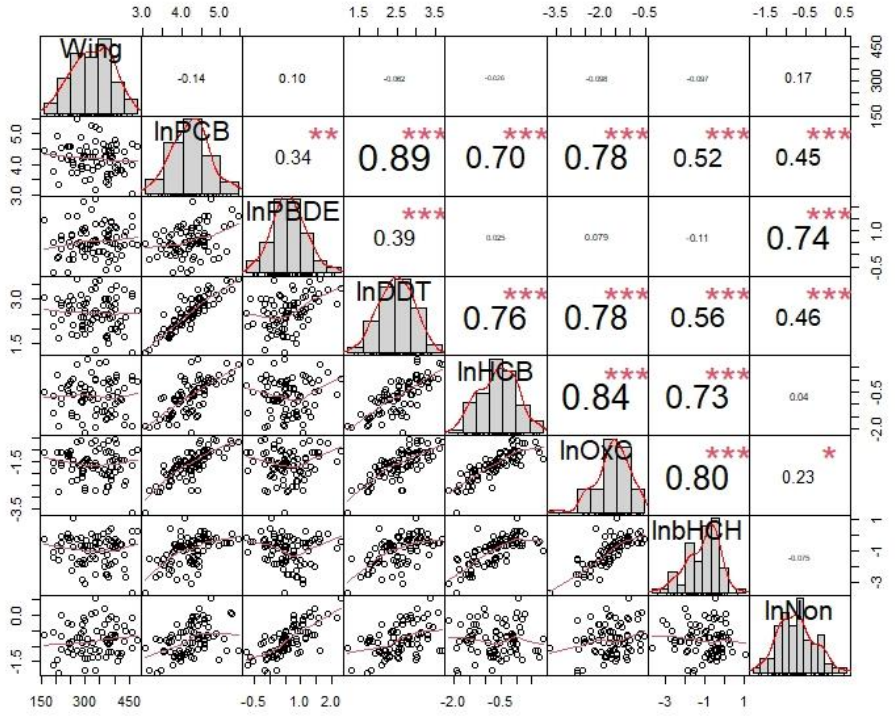


Figure S3. Pearson's product moment correlation's for wing length and concentrations of seven groups of persistent organic pollutants (POPs; see Tables 1 and S1 for details) measured in a) whole blood (n=71) and b) serum (n=81) of white-tailed eagle nestlings sampled at the Swedish Baltic coast. In addition, tests for multiple collinearity were used in the models where brood size and sex were included. These models were re-fitted without brood size and sex as predictors. Instead, we fitted model to data on each level of brood size and sex, but since the estimates and their level of statistical significance did not change neither across the models fitted to each level separately, nor to the original model containing brood size and/or sex (results not shown), we concluded that multiple collinearity did not affect our conclusions.

Table S2a. List of candidate models to predict telomere lengths (T/S ratio, response variable) in relation to seven POP groups (predictor variables tested separately) measured in whole blood of Baltic white-tailed eagle nestlings during 1995-2002. In addition, the following predictor variables were included: wing length (Wing; continuous variable), sex (two-level factor: female, male), and brood size (three-level factor: 1, 2 and 3 chicks). The best model (in bold) was selected based on the lowest Δ_i, which measures the difference in AIC_c between each respective model and the model in the set with the lowest AIC_c value. The number of parameters (*K*) and the Aikaike's weights (w_i) are provided for all the candidate models.

Candidate model (*i*)	POP (whole blood)	Wing	Sex	Brood size	K	Δ_i	Δ_i weights
	ln($\sum_{24}$PCBs)[a]						
Mod2	**X**	**X**			**5**	**0.00**	**0.54**
Mod7	X	X		X	7	2.21	0.18
Mod4	X	X	X		6	2.48	0.16
Mod8	X	X	X	X	8	4.87	0.05
Mod1	X				4	4.95	0.05
Mod3	X		X		5	6.86	0.02
Mod5	X			X	6	7.15	0.02
Mod6	X		X	X	7	9.10	0.01
	ln($\sum_{5}$PBDEs)[b]						
Mod2	**X**	**X**			**5**	**0.00**	**0.58**
Mod4	X	X	X		6	2.48	0.17
Mod7	X	X		X	7	2.96	0.13
Mod1	X				4	4.93	0.05
Mod8	X	X	X	X	8	5.64	0.03
Mod3	X		X		5	6.90	0.02
Mod5	X			X	6	7.82	0.01
Mod6	X		X	X	7	9.83	0.00
	ln(∑DDT)[c]						
Mod2	**X**	**X**			**5**	**0.00**	**0.55**
Mod4	X	X	X		6	2.41	0.16
Mod7	X	X		X	7	2.48	0.16
Mod1	X				4	4.87	0.05
Mod8	X	X	X	X	8	5.08	0.04
Mod3	X		X		5	6.77	0.02
Mod5	X			X	6	7.17	0.02
Mod6	X		X	X	7	9.14	0.00
	ln(HCB)						
Mod2	**X**	**X**			**5**	**0.00**	**0.55**
Mod4	X	X	X		6	2.41	0.17
Mod7	X	X		X	7	2.48	0.16
Mod1	X				4	4.92	0.05
Mod8	X	X	X	X	8	5.08	0.04
Mod3	X		X		5	6.90	0.02
Mod5	X			X	6	7.94	0.01
Mod6	X		X	X	7	9.91	0.00
	ln(OxC)						
Mod2	**X**	**X**			**5**	**0.00**	**0.57**
Mod4	X	X	X		6	2.48	0.17
Mod7	X	X		X	7	2.93	0.13
Mod1	X				4	4.57	0.06
Mod8	X	X	X	X	8	5.60	0.03
Mod3	X		X		5	6.57	0.02
Mod5	X			X	6	7.82	0.01
Mod6	X		X	X	7	9.83	0.00
	ln(∑Nonachlor)[d]						
Mod2	**X**	**X**			**5**	**0.00**	**0.56**
Mod4	X	X	X		6	2.48	0.16
Mod7	X	X		X	7	2.59	0.15
Mod1	X				4	4.81	0.05
Mod8	X	X	X	X	8	5.27	0.04
Mod3	X		X		5	6.80	0.02
Mod5	X			X	6	7.30	0.01
Mod6	X		X	X	7	9.34	0.01

	ln(*β*-HCH)						
Mod2	**X**	**X**			**5**	**0.00**	**0.55**
Mod4	X	X	X		6	2.48	0.16
Mod7	X	X		X	7	2.87	0.16
Mod1	X				4	4.72	0.05
Mod8	X	X	X	X	8	5.55	0.04
Mod3	X		X		5	6.67	0.02
Mod5	X			X	6	7.86	0.02
Mod6	X		X	X	7	9.88	0.00

[a] Sum of 24 congeners (see table S1 for full names and abbreviations)
[b] Sum of 5 congeners (see table S1 for full names and abbreviations)
[c] Sum of DDT metabolites (*p,p'*-DDE and *p,p'*-DDD)
[d] Sum of *cis*- and *trans*-Nonachlor

Table S2b. List of candidate models to predict telomere lengths (T/S ratio, response variable) in relation to seven POP groups (predictor variables tested separately) measured in serum of Baltic white-tailed eagle nestlings during 2003-2013. In addition, the following predictor variables were included: wing length (Wing; continuous variable), sex (two-level factor: female, male), and brood size (three-level factor: 1, 2 and 3 chicks). The best model (in bold) was selected based on the lowest Δ_i, which measures the difference in AIC_c between each respective model and the model in the set with the lowest AIC_c value. The number of parameters (*K*) and the Aikaike's weights (w_i) are provided for all the candidate models.

Candidate model #	POP (serum)	Wing	Sex	Brood size	K	Δ_i	Δ_i weights
	ln($\sum_{24}$PCBs)[a]						
Mod1	**X**				**4**	**0.00**	**0.37**
Mod2	X	X			5	0.29	0.32
Mod3	X		X		5	2.28	0.12
Mod4	X	X	X		6	2.61	0.10
Mod5	X			X	6	4.52	0.04
Mod7	X	X		X	7	4.89	0.03
Mod6	X		X	X	7	6.93	0.01
Mod8	X	X	X	X	8	7.36	0.01
	ln($\sum_5$PBDEs)[b]						
Mod1	**X**				**4**	**0.00**	**0.42**
Mod2	X	X			5	0.91	0.27
Mod3	X		X		5	2.25	0.14
Mod4	X	X	X		6	3.18	0.09
Mod5	X			X	6	4.48	0.04
Mod7	X	X		X	7	5.50	0.03
Mod6	X		X	X	7	6.86	0.01
Mod8	X	X	X	X	8	7.93	0.01
	ln($\sum$DDT)[c]						
Mod1	**X**				**4**	**0.00**	**0.41**
Mod2	X	X			5	0.76	0.28
Mod3	X		X		5	2.25	0.13
Mod4	X	X	X		6	3.02	0.09
Mod5	X			X	6	4.48	0.04
Mod7	X	X		X	7	5.29	0.03
Mod6	X		X	X	7	6.87	0.01
Mod8	X	X	X	X	8	7.73	0.01
	ln(HCB)						
Mod1	**X**				**4**	**0.00**	**0.42**
Mod2	X	X			5	0.91	0.27
Mod3	X		X		5	2.25	0.14
Mod4	X	X	X		6	3.18	0.09
Mod5	X			X	6	4.48	0.04
Mod7	X	X		X	7	5.50	0.03
Mod6	X		X	X	7	6.86	0.01
Mod8	X	X	X	X	8	7.93	0.01
	ln(OxC)						
Mod1	**X**				**4**	**0.00**	**0.42**
Mod2	X	X			5	0.89	0.27
Mod3	X		X		5	2.26	0.13
Mod4	X	X	X		6	3.17	0.09
Mod5	X			X	6	4.45	0.05
Mod7	X	X		X	7	5.38	0.03
Mod6	X		X	X	7	6.86	0.01
Mod8	X	X	X	X	8	7.84	0.01
	ln($\sum$Nonachlor)[d]						
Mod1	**X**				**4**	**0.00**	**0.43**
Mod2	X	X			5	0.99	0.26
Mod3	X		X		5	2.25	0.14
Mod4	X	X	X		6	3.26	0.08
Mod5	X			X	6	4.50	0.05
Mod7	X	X		X	7	5.60	0.03
Mod6	X		X	X	7	6.88	0.01
Mod8	X	X	X	X	8	8.02	0.01
	ln(β-HCH)						
Mod1	**X**				**4**	**0.00**	**0.41**

Mod2	X	X			5	0.88	0.27
Mod3	X		X		5	2.26	0.13
Mod4	X	X	X		6	3.14	0.09
Mod5	X			X	6	4.38	0.05
Mod7	X	X		X	7	5.28	0.03
Mod6	X		X	X	7	6.78	0.01
Mod8	X	X	X	X	8	7.73	0.01

[a] Sum of 24 congeners (see table S1 for full names and abbreviations)
[b] Sum of 5 congeners (see table S1 for full names and abbreviations)
[c] Sum of DDT metabolites (*p,p'*-DDE and *p,p'*-DDD)
[d] Sum of *cis*- and *trans*-Nonachlor

Table S3a. Comparison of the linear mixed-effect model (fitted as *lme(Telomere length ~ Year + 1|Nest ID,…)*) with a generalized least square model without the random effect (fitted as *gls(Telomere length ~ Year,…)*) to assess model fit to our data. The most parsimonious model was selected based on the lowest second-order Aikaike Information Criterion (AICc) and subsequently used for inference (see Table S5).

	df	AICc
GLS	20	138.55
LME	21	141.16

Table S3b. Comparisons of the selected linear mixed-effect models (LMEs; see details in Table S2a) with a generalized least square model without the random effect (fitted as *gls(Telomere length ~ POP + Wing,…)*) to assess model performance when relating telomere length to (a) Σ_{24}PCBs, (b) Σ_5PBDEs, (c) ΣDDT, (d) HCB; (e) OxC, (f) ΣNonachlor, and (g) *β*-HCH. The most parsimonious model was selected based on the lowest second-order Aikaike Information Criterion (AICc) and subsequently used for inference (see Table S6a).

		df	AICc
a) Σ_{24}PCB[a]			
	GLS	4	90.39
	LME	5	92.78
b) Σ_5PBDE[b]			
	GLS	4	90.66
	LME	5	93.05
c) ΣDDT[c]			
	GLS	4	90.68
	LME	5	93.07
d) HCB			
	GLS	4	90.82
	LME	5	93.21
e) OxC			
	GLS	4	91.48
	LME	5	93.86
f) ΣNonachlor[d]			
	GLS	4	91.06
	LME	5	93.45
g) *β*-HCH			
	GLS	4	92.03
	LME	5	94.42

[a] Sum of 24 congeners (see table S1 for full names and abbreviations)
[b] Sum of 5 congeners (see table S1 for full names and abbreviations)
[c] Sum of DDT metabolites (*p,p'*-DDE and *p,p'*-DDD)
[d] Sum of *cis*- and *trans*-Nonachlor

Table S3c. Comparing the selected linear mixed-effect model (LME; see details in Table S2b) with a generalized least square model without the random effect (fitted as *gls(Telomere length ~ POP,…)*) to assess model performance when relating Telomere length to (a) Σ_{24}PCBs, (b) Σ_5PBDEs, (c) ΣDDT, (d) HCB; (e) OxC, (f) ΣNonachlor, and (g) *β*-HCH. The most parsimonious model was selected based on the lowest second-order Aikaike Information Criterion (AICc) and consequently used for inference (see Table S6b).

		df	AICc
a) Σ_{24}PCB[a]			
	GLS	3	62.66
	LME	4	64.88
b) Σ_5PBDE[b]			
	GLS	3	64.17
	LME	4	66.39
c) ΣDDT[c]			
	GLS	3	63.71
	LME	4	65.93
d) HCB			
	GLS	3	64.46
	LME	4	66.68
e) OxC			
	GLS	3	63.52
	LME	4	65.74
f) ΣNonachlor[d]			
	GLS	3	63.67
	LME	4	65.89
g) *β*-HCH			
	GLS	3	64.45
	LME	4	66.67

[a] Sum of 24 congeners (see table S1 for full names and abbreviations)
[b] Sum of 5 congeners (see table S1 for full names and abbreviations)
[c] Sum of DDT metabolites (*p,p'*-DDE and *p,p'*-DDD)
[d] Sum of *cis*- and *trans*-Nonachlor

Table S4. Parameter estimates for the generalized least squares model (fitted using '*gls*'; see table S3a for details) predicting telomere length in relation to sampling year (19-level factor: 1995 as reference year).

Parameter	Estimate	SE	*t*	*P*
Telomere length (T/S ratio)				
Fixed effects				
Intercept	0.72	0.13	5.61	< 0.01
1996	-0.12	0.17	-0.66	0.50
1997	0.25	0.16	1.50	0.14
1998	0.91	0.19	4.82	< 0.01
1999	0.65	0.16	4.13	< 0.01
2000	0.48	0.17	2.75	< 0.01
2001	0.52	0.16	3.28	< 0.01
2002	0.38	0.16	2.33	0.21
2003	0.19	0.19	0.99	0.32
2004	0.43	0.19	2.27	0.02
2005	0.12	0.23	0.50	0.61
2006	0.47	0.16	2.97	< 0.01
2007	0.09	0.17	0.54	0.58
2008	0.19	0.15	1.26	0.21
2009	0.13	0.23	0.56	0.57
2010	0.30	0.17	1.74	0.08
2011	0.09	0.17	0.51	0.60
2012	0.11	0.16	0.70	0.48
2013	0.28	0.18	1.54	0.12
			R^2 =	0.34
			$n_{Observations}$ =	168

Table S5a. Parameter estimates for the generalized least squares models relating telomere length to concentrations (log_e-transformed) of seven POP groups: (a) Σ_{24}PCBs, (b) Σ_5PBDEs, (c) ΣDDT, (d) HCB; (e) OxC, (f) ΣNonachlor, and (g) β-HCH measured in whole blood (n = 71; 1995-2002) of Baltic white-tailed eagle nestlings. The seven POP groups were kept as fixed effects in our models based on our *a priori* expectations whereas wing length, sex and brood size were included in the selected models if they improved the models using AICc as a model selection criterion (see Tables S2a and S3b for details).

Parameter	Estimate	SE	t	P
(a)				
Fixed effects				
Intercept	1.248	0.469	2.656	0.010
ln(Σ_{24}PCB)[a]	0.080	0.109	0.742	0.461
Wing length	-0.001	< 0.001	-1.689	0.096
			R^2 =	0.058
			$n_{Observations}$ =	59
(b)				
Fixed effects				
Intercept	1.558	0.231	6.721	< 0.001
ln(Σ_5PBDE)[b]	0.068	0.098	0.698	0.487
Wing length	-0.001	< 0.001	-1.173	0.089
			R^2 =	0.057
			$n_{Observations}$ =	59
(c)				
Fixed effects				
Intercept	1.420	0.304	4.666	< 0.001
ln(ΣDDT)[c]	0.066	0.099	0.664	0.509
Wing length	-0.001	< 0.001	-1.695	0.095
			R^2 =	0.057
			$n_{Observations}$ =	59
(d)				
Fixed effects				
Intercept	1.616	0.271	5.944	< 0.001
ln(HCB)	0.047	0.104	0.457	0.648
Wing length	-0.001	< 0.001	-1.774	0.081
			R^2 =	0.053
			$n_{Observations}$ =	59
(e)				
Fixed effects				
Intercept	1.566	0.312	5.005	< 0.001
ln(OxC)	0.005	0.083	0.069	0.945
Wing length	-0.001	< 0.001	-1.706	0.093
			R^2 =	0.049
			$n_{Observations}$ =	59
(f)				
Fixed effects				
Intercept	1.620	0.255	6.343	< 0.001
ln(ΣNonachlor)[d]	0.053	0.083	0.638	0.526
Wing length	-0.001	< 0.001	-1.716	0.091
			R^2 =	0.056
			$n_{Observations}$ =	59

Table S5a continued.

Parameter	Estimate	SE	*t*	*P*
(g)				
Fixed effects				
Intercept	1.571	0.247	6.351	< 0.001
ln(*β*-HCH)	0.014	0.061	0.231	0.817
Wing length	-0.001	< 0.001	-1.715	0.091
			R^2 =	0.050
			$n_{Observations}$ =	59

[a] Sum of 24 congeners (see table S1 for full names and abbreviations)
[b] Sum of 5 congeners (see table S1 for full names and abbreviations)
[c] Sum of *p,p'*-DDE and *p,p'*-DDD
[d] Sum of *cis*- and *trans*-Nonachlor

Table S5b. Parameter estimates for the generalized least squares models relating telomere length to concentrations (log_e-transformed) of seven POP groups: (a) Σ_{24}PCBs, (b) Σ_5PBDEs, (c) ΣDDT, (d) HCB; (e) OxC, (f) ΣNonachlor, and (g) β-HCH measured in serum (n = 81; 2003-2013) of Baltic white-tailed eagle nestlings. The seven POP groups were kept as fixed effects in our models based on our *a priori* expectations whereas wing length, sex and brood size were included in the selected models if they improved the models using AICc as a model selection criterion (see Tables S2a and S3b for details).

Parameter	Estimate	SE	t	P
(a)				
Fixed effects				
Intercept	0.603	0.321	1.880	0.063
ln(Σ_{24}PCB)	0.080	0.075	1.058	0.292
			R^2 =	0.014
			$n_{Observations}$ =	80
(b)				
Fixed effects				
Intercept	0.930	0.051	18.007	< 0.01
ln(Σ_5PBDE)	0.017	0.060	0.292	0.770
			R^2 =	0.001
			$n_{Observations}$ =	80
(c)				
Fixed effects				
Intercept	0.850	0.177	4.780	< 0.001
ln(ΣDDT)	0.035	0.068	0.523	0.601
			R^2 =	0.003
			$n_{Observations}$ =	80
(d)				
Fixed effects				
Intercept	0.942	0.052	17.931	< 0.001
ln(HCB)	0.001	0.054	0.029	0.976
			R^2 =	< 0.001
			$n_{Observations}$ =	80
(e)				
Fixed effects				
Intercept	1.026	0.108	9.439	< 0.001
ln(OxC)	0.050	0.061	0.828	0.409
			R^2 =	0.008
			$n_{Observations}$ =	80
(f)				
Fixed effects				
Intercept	0.962	0.071	13.476	< 0.001
ln(ΣNonachlor)	0.026	0.075	0.347	0.729
			R^2 =	0.001
			$n_{Observations}$ =	80
(g)				
Fixed effects				
Intercept	0.972	0.054	17.726	< 0.001
ln(β-HCH)	0.031	0.040	0.768	0.444
			R^2 =	0.007
			$n_{Observations}$ =	80

[a] Sum of 24 congeners (see table S1 for full names and abbreviations)
[b] Sum of 5 congeners (see table S1 for full names and abbreviations)
[c] Sum of *p,p'*-DDE and *p,p'*-DDD
[d] Sum of *cis*- and *trans*-Nonachlor

Paper III

A retrospective investigation of feather corticosterone in a highly contaminated white-tailed eagle (*Haliaeetus albicilla*) population

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A retrospective investigation of feather corticosterone in a highly contaminated white-tailed eagle (*Haliaeetus albicilla*) population

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ABSTRACT

Exposure to persistent organic pollutants (POPs), such as organochlorines (OCs) and polybrominated diphenyl ethers (PBDEs), is associated with adverse health effects in wildlife. Many POPs have been banned and consequently their environmental concentrations have declined. To assess both temporal trends of POPs and their detrimental impacts, raptors are extensively used as biomonitors due to their high food web position and high contaminant levels. White-tailed eagles (WTEs; *Haliaeetus albicilla*) in the Baltic ecosystem represent a sentinel species of environmental pollution, as they have suffered population declines due to reproductive failure caused by severe exposure to dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCB) during the 1960s through 1980s. However, there is a lack of long-term studies that cover a wide range of environmental contaminants and their effects at the individual level. In this study, we used 135 pooled samples of shed body feathers collected in 1968–2012 from breeding WTE pairs in Sweden. Feathers constitute a temporal archive for substances incorporated into the feather during growth, including corticosterone, which is the primary avian glucocorticoid and a stress-associated hormone. Here, we analysed the WTE feather pools to investigate annual variations in feather corticosterone (fCORT), POPs (OCs and PBDEs), and stable carbon and nitrogen isotopes (SIs; dietary proxies). We examined whether the expected fluctuations in POPs affected fCORT (8–94 pg. mm^{-1}) in the WTE pairs. Despite clear temporal declining trends in POP concentrations ($p < 0.01$), we found no significant associations between fCORT and POPs or SIs ($p > 0.05$ in all cases). Our results do not support fCORT as a relevant biomarker of contaminant-mediated effects in WTEs despite studying a highly contaminated population. However, although not detecting a relationship between fCORT, POP contamination and diet, fCORT represents a non-destructive and retrospective assessment of long-term stress physiology in wild raptors otherwise not readily available.

1. Introduction

Persistent Organic Pollutants (POPs), which comprise halogenated organic compounds such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and polybrominated diphenyl ethers (PBDEs), are anthropogenic contaminants that persist in the environment, are ubiquitously distributed, and have the capacity to bioaccumulate and biomagnify in living organisms (Harrad, 2009). Apex predators, susceptible of accumulating high levels of biomagnifying contaminants, have therefore been extensively used as biomonitors of environmental pollution (Rattner, 2009). The white-tailed eagle (WTE; *Haliaeetus albicilla*), the largest raptor in northern Europe, is a well-documented environmental sentinel species (Helander et al., 2008; Herrmann et al., 2011). On the Swedish coast of the Baltic Sea,

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WTE territories have been closely monitored since the 1960s following observed reproductive failures and population declines, mainly due to high POP contamination (Helander et al., 2002).

In avian ecotoxicology, the stress of being contaminated has been a subject of growing interest where corticosterone (CORT), the main glucocorticoid and stress-associated hormone in birds (Romero and Romero, 2002), has been proposed as a predictive tool for pollutant-mediated health effects in wild birds (e.g., Bourgeon et al. (2012); Nordstad et al. (2012); Strong et al. (2015); Tartu et al. (2014); Verboven et al. (2010)). Current findings on the relationship between CORT and anthropogenic contaminants are however ambiguous, as several studies on free-living birds report weak or no relationships (e.g., Bourgeon et al. (2012), Goutte et al. (2018), Monclus et al. (2019),Tartu et al. (2015c), and Randulff et al. (2022)). Discrepancies between previous studies can be due to CORT being influenced by additional environmental factors such as low or suboptimal food availability (Catitti et al., 2022; Patterson et al., 2015; Will et al., 2015) and inclement weather (de Bruijn and Romero, 2018; Kouwenberg et al., 2016). In addition, most effect studies investigating the influence of contaminants on CORT concentrations have been conducted within short time periods (maximum 1–2 sampling years except Goutte et al. (2018)), limiting the concentration range of the contaminants as well as the environmental stressors that individuals are exposed to. Many anthropogenic contaminants display clear declining temporal trends due to global regulations of compounds, and wild bird populations have thus experienced large variations in the magnitude of contaminant exposure over the last century (Sun et al., 2020).

Long-term studies on CORT-contaminant relationships in bird species are largely lacking, mainly pertaining to methodological limitations. Indeed, such retrospective studies can only be achieved by using a suitable matrix, which is not sensitive to time of conservation. As such, feathers allow the assessment of both POPs (Jaspers et al., 2019) and CORT concentrations (Bortolotti et al., 2008). While blood plasma represents immediate concentrations of circulating CORT (Romero and Romero, 2002), feather concentrations (hereafter fCORT) reflect the deposition of CORT during the time of feather growth (days to weeks; Bortolotti et al. (2008)) although studies report that fCORT can be used as a proxy of plasma CORT concentrations (Fairhurst et al., 2013a). Analysing CORT in feathers has therefore become increasingly popular since feathers can be collected from either live or dead birds (including museum specimens; Fairhurst et al. (2015)). In addition, sampling of shed feathers does not require capturing individuals (Sun et al., 2020) and allows investigation of fCORT also in non-breeding individuals (Fairhurst et al., 2015). Therefore, feather corticosterone represents a non-destructive and retrospective measure of stress physiology in wild avian species.

As part of a Swedish national WTE rescue project (Helander, 2003) and WTE monitoring program (Helander et al., 2008), shed feathers of territorial adults have been opportunistically collected during nest visits resulting in a collection of archived feathers that spans multiple decades. These archives create a unique opportunity to assess fCORT concentrations and POP contamination in adult pairs present at their nest (successful or unsuccessful breeders) of a sentinel raptor species exposed to a broad range of POP levels over many years. In total, 135 unique feather pools collected in the period 1968–2012 from pairs of WTEs were selected for this study. One pool consisted of 10 body feathers collected at the same nest, of which one was used to analyse fCORT (expressed as pg. per length unit) and the remaining 9 were homogenized and analysed for 47 POP compounds (39 organochlorines (OCs) and 6 polybrominated diphenyl ethers (PBDESs)) and stable isotope signatures (i.e., dietary proxies; δ^{15}N indicating trophic level and δ^{13}C indicating habitat of prey (Kelly, 2000)). Since WTEs are resident, monogamous birds that feed on similar prey and exhibit shared parental care during chick rearing, the use of pooled feathers can be considered a reliable proxy for pollution and dietary status within their territory. Previous studies have found no significant differences in POP concentrations between male and female WTEs (Jaspers, 2013; Krone, 2006), further supporting the use of pooled feathers as a representative sample. Further, as WTEs do not differ in feather plumage pigmentation and as sex was not shown to influence CORT levels in wild birds (Apfelbeck et al., 2017; Tartu et al., 2015a, 2015b; Verboven et al., 2010) including raptor species (Strong et al., 2015), fCORT was measured in a single feather (i.e., either male or female). This was done to minimize the high variations within and between individuals, as previously reported in poultry studies (Häffelin et al., 2021).

Our objectives were (1) to investigate the temporal variations in fCORT and POP contamination in the highly contaminated Baltic Sea WTE population over the study period 1968–2012, and (2) to examine whether the predicted strong fluctuations in contaminant levels affected fCORT. δ^{15}N and δ^{13}C isotopes were also tested as potential drivers since feeding ecology might influence fCORT concentrations (Fairhurst et al., 2015). The exploitation of this unique archive of WTE feathers allowed a retrospective long-term effect study in an avian apex predator.

2. Material and methods

2.1. Feather sampling

The present study used a collection of WTE body feathers comprising 135 samples from 25 breeding territories sampled at least once or multiple times in the period 1968–2012 (Table S1) and stored at the Swedish Museum of Natural History. The WTE population at the central Swedish Baltic coast (Fig. 1) has been monitored annually since 1968 and moulted body feathers of breeding eagle pairs were collected at individual nests in their specific territory (Helander et al., 2008). Feathers were stored in separate polypropylene bags upon collection and subsequently stored in dark polypropylene boxes at ambient temperature and humidity conditions. The feather collection has not been subjected to any preservative treatment before or during storage at the museum. Approximately ten body feathers per territory at a given year were extracted from the collection and stored in a similar fashion until chemical analysis.

2.2. Contaminant analysis

After reserving one body feather per territory per year for CORT measurement (see section 2.4), the remaining nine feathers were thoroughly cleaned using distilled water and dried overnight before they were cut into <1 mm pieces and homogenized to one pooled sample as described in Sun et al. (2020). The pooled sample was analysed for a wide range of POPs and stable carbon and nitrogen isotopes (section 2.3). By washing feathers in distilled water only, we were able to retain preen oil on the feather surface, as preen oil originates from internal sources of the bird and has been found to be significantly correlated with internal muscle concentrations (Jaspers et al., 2011, 2019). The analysis of contaminants was performed at the Toxicological Centre (University of Antwerp, Belgium). All feather pools were analysed for the following organochlorine (OC) compounds: 20 polychlorinated biphenyl (PCB) congeners (CB 49, 52, 74, 95, 99, 101, 105, 110, 118, 138, 149, 153, 156, 170, 171, 177, 180, 183, 187, and 194), *p,p'*- dichlorodiphenyltrichloroethane (*p,p'*-DDT) and its metabolites, *p,p'*- dichlorodiphenyldichloroethylene (*p,p'*-DDE) and *p,p'*-dichlorodiphenyl dichloroethane (*p,p'*-DDD), five chlordane-related compounds (CHLs: *cis*- and *trans*-nonachlor (CN and TN), *cis*- and *trans*-chlordane (CC and TC), and oxychlordane (OxC)),hexachlorobenzene (HCB), hexachlorocyclohexanes (HCHs; α-, β- and γ-HCH), seven polybrominated diphenyl ether (PBDE) congeners (28, 47, 99, 100, 153, 154, 183) and two PBDE metabolites (6-MeO-BDE-47 and 2-MeO-BDE-68).

The analytical methods for OCs have already been described in Sun et al. (2020). The analysis for PBDEs was conducted as following: each individual subsample (0.12–0.41 g) was spiked with internal standards (BDE 77 and BDE 128) and incubated overnight at 45 °C with hexane:

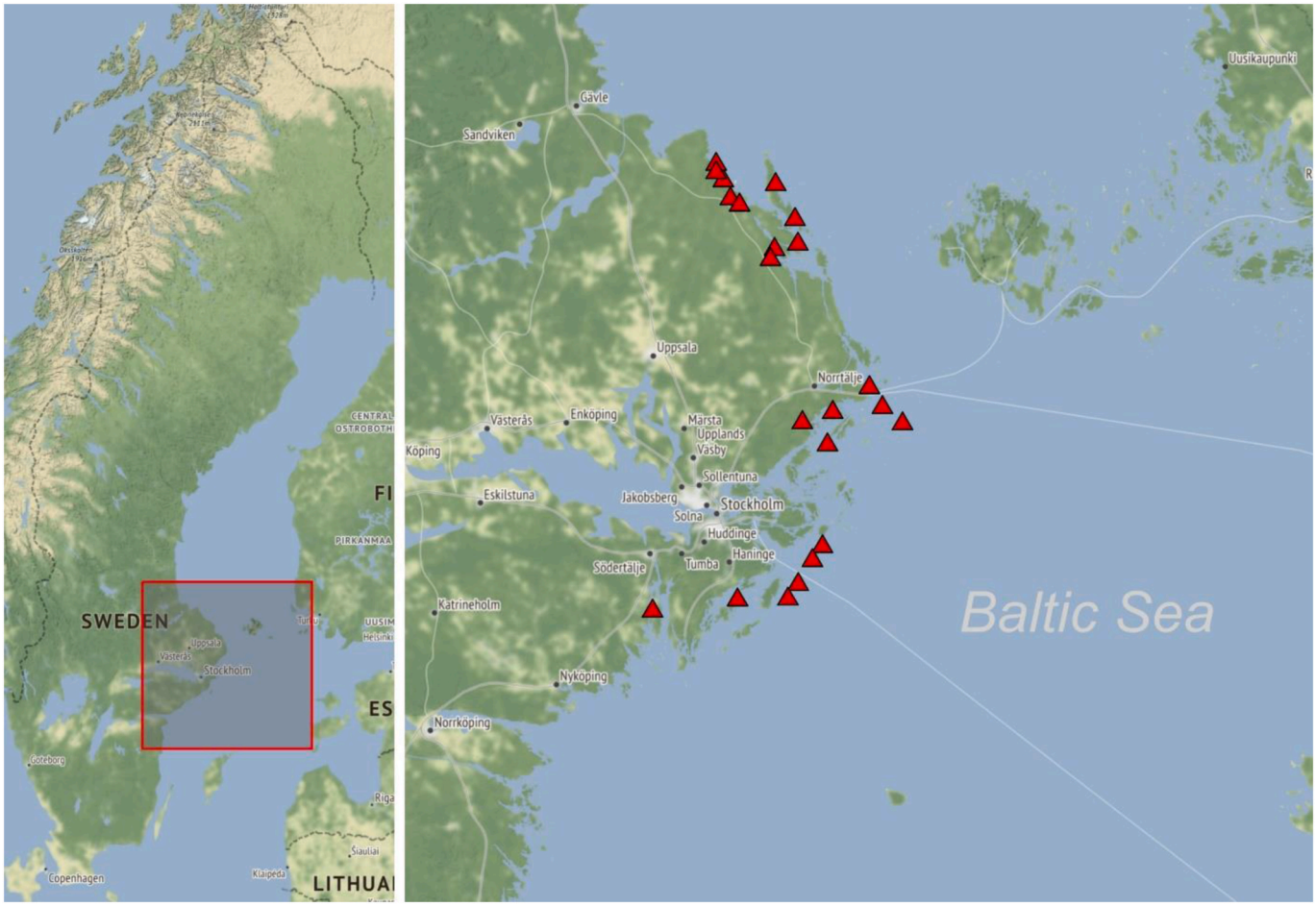

Fig. 1. Baltic coastline of Sweden indicating locations (red triangles) where shed body feathers in nests of breeding pairs of white-tailed eagles were collected in the period 1968–2012.

dichloromethane (4:1; v:v) and HCl (1 M). After liquid-liquid extraction, the extracts were fractionated on prepacked SupelcleanTM ENVITM--Florisil® SPE cartridges (3 mL; 500 mg; Supelco, Bellefonte, PA, USA) topped with anhydrous Na2SO4. A first fraction, containing PBDEs, was eluted with 10 mL of hexane and cleaned-up on acidified silica (10% H2SO4), while a second fraction, containing PFRs, was obtained in light of another study. The PBDE fraction was concentrated until dryness under a gentle nitrogen flow and reconstituted in iso-octane. All PBDEs were quantified using gas chromatography coupled to electron capture negative ionisation mass spectrometry.

Pesticide-grade solvents (Merck KGaA Chemicals, Darmstadt, Germany and Acros Organics, Geel, Belgium) were used throughout and every 23rd sample a procedural blank was analysed. Internal standard recoveries ranged between 86 and 106% and the obtained certified reference material concentrations where within 3.7 SD from the indicative values. All compounds were blank subtracted using average procedural blank values. The limit of quantification (LOQ) was set at 3*SD of the procedural blank values, or, for analytes not detectable in blanks, was calculated from a 10:1 signal to noise ratio.

2.3. Stable isotope analyses

The analysis was performed at the Laboratory of Oceanology of the University of Liège, Belgium. The stable isotope ratios and the analytical protocol and quality assurance were previously reported in Sun et al. (2019). The stable isotope ratios for carbon and nitrogen are expressed as δ values (‰) relative to their respective international measurement standards Vienna Pee Dee Belemnite and atmospheric N_2.

2.4. Corticosterone analysis

The analysis for feather corticosterone (fCORT) concentrations was performed on one whole body feather per sample at the Department of Arctic and Marine Biology at UiT - the Arctic University of Norway, Tromsø. Prior to CORT extraction, the length of each feather (from base to tip, without the calamus) was recorded to the nearest millimetre. Extraction of CORT followed the methanol-based extraction method reported earlier by Bortolotti et al. (2008). Final extracts were assessed for CORT with an enzyme immunoassay kit (901–097, Assay Designs Inc., USA). The quality of the assay was validated using serial dilutions of feather extracts (displacement curves), which confirmed the absence of interfering substances in the extract. Moreover, the cross-reactivity of the assay is reported to be high with CORT (100%) but low with related steroids (e.g., progesterone: 1.7%; cortisol: 0.05%; β-estradiol: 0.03%). Each feather extract was measured in duplicate, and for all four separate plates used in the present study we reported intra- and inter-assay variability of 2.6% ($n = 16$) and 0.8% ($n = 4$), respectively. The final concentration of CORT was calculated using a standard curve run on each plate and the unit is given as pg. mm^{-1}.

2.5. Statistical analyses

The statistical analyses were conducted using R version 4.2.1 (R Development Core Team, 2022). All tests performed were two-tailed and the null hypothesis was rejected at an alpha level of 0.05. To attain the assumptions of normality and homogeneity of variance, fCORT and POP concentrations were natural log-transformed (ln) prior to statistical

analyses. Since some WTE territories were sampled multiple times across the study period, we included territory as a random variable in a linear mixed effect model using function *lme* the *nlme*-package (Pinheiro et al., 2017). However, the *lme* revealed low among-territory variability (i.e., low random intercept variance; results not shown) and thus the random effect of territory in the models was negligible and therefore not included in the models. Model assumptions were visually assessed and validated through residual plots, quantile-quantile plots, and histograms of residuals following the protocol by Zuur et al. (2010).

We only included POP compounds with a detection frequency of more than 75% in the statistical analyses (Hansen et al., 2022; Sletten et al., 2016). The resulting compounds were 29 PCB congeners (28, 49, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138, 146, 149, 151, 153, 156, 170, 171, 174, 177, 180, 183, 187, 194, 196/203, 199, 206 and 209), four chlordane-related compounds (CN, TN, OxC and CC), *p,p'*-DDT and metabolites (*p,p'*-DDE and *p,p'*- DDD), HCB, β- and γ-HCH, and six PBDE congeners (28, 47, 99, 100, 153 and 154). Concentrations below the level of quantification were replaced with zero for the calculation of sums. Due to the high number of compounds and high correlations among the OCs and PBDEs, respectively, we conducted principal component analysis (PCA; function *prcomp* (Zuur et al., 2007)) on the 39 OC compounds and six PBDEs separately (PCA biplots, scree and correlational plots for OCs and PBDEs are presented in Figs. S1–S3). We then extracted values of the first and second components, which together explained >75% of the variation for OCs and PBDEs, respectively, and included them in the models as predictors.

Two sets of analyses were performed using linear regression models (function *lm*): one to investigate the temporal variation in fCORT, POPs (OCs and PBDEs separately), and SIs (δ^{13}C and δ^{15}N), and one to investigate the relationship between fCORT, POP contamination and feeding ecology (δ^{13}C and δ^{15}N) of the breeding pairs. In the temporal analyses, the main effect of year (a numeric variable) or its second-order polynomial (Year^2; to allow non-linearity) were used as predictors. δ^{13}C values were corrected for the oceanic Suess effect (as explained in Sun et al. (2019)), which is the temporal decline of atmospheric δ^{13}C and consequently oceanic organic baseline δ^{13}C values due to the large quantity of CO_2 released from fossil fuel burning in recent industrial times (Gruber et al., 1999). Next, In the relationship analyses (I.e., between fCORT, POPs and SIs), two models were performed in which principal components (PC) of OCs and PBDEs, respectively, were placed in separate models due to their expected differing temporal trends in concentrations. δ^{15}N (trophic position proxy) and δ^{13}C (habitat proxy) were also included in these models to assess whether fCORT concentrations were related to feeding ecology of the WTEs. To avoid problems with multicollinearity (Zuur et al., 2010), we assessed collinearity between predictor variables based on the variance inflation factor (VIF, *VIF* function in the package *regclass*; Pietre (2020)). All variables had a VIF <2, which indicated low collinearity among variables (Zuur et al., 2010).

3. Results and discussion

This study examined fCORT in feather samples ($N = 135$) from pairs of Baltic WTE adults sampled in 1968–2012 encompassing historical periods of peak exposures to OCs and PBDEs followed by decreases driven by mitigation measures (Bjurlid et al., 2018; Jinhui et al., 2017; Sun et al., 2020). Our original methodological approach exploiting archived feathers allowed a retrospective and long-term assessment of contamination stress in breeding pairs of birds of prey exposed to a 38- and 114-fold difference in ranges of OC and PBDE levels, respectively (Table S2: min-max concentrations and Sun et al., 2020 for OC data).

3.1. Temporal variation in POP and fCORT concentrations

As expected, feather concentrations of OCs and PBDEs changed over the study period but had distinct peak periods (Figs. 2 and 3). OC concentrations were highest in the 1960s followed by a steady linear decline (Fig. 2, Table S3; $F_{(1,\ 133)} = 210.5$, $p < 0.01$), while PBDE concentrations followed a bell-shaped curve with peak concentrations in the 1990s (Fig. 3, Table S3; $F_{(2,\ 132)} = 278.6$, $p < 0.01$). Our findings are in line with studies on trends in levels of POPs reported in aquatic wildlife in the Baltic ecosystem (Bignert and Helander, 2015; Bjurlid et al., 2018; Kierkegaard et al., 2004), where clear declines in concentrations matched the global regulations of OCs in the mid-seventies (Jones and de Voogt, 1999) and pentaBDEs (commercial mixture of PBDE congeners analysed in this study) in the 1990s (Jinhui et al., 2017). Importantly, our results provide evidence that the selected pools of feathers were a suitable and reliable matrix to measure temporal variation in environmental levels of lipophilic POPs such as OCs and PBDEs (Jaspers et al., 2019). Using shed feathers at the nest as passive monitors of environmental POP contamination is also particularly valuable since adult WTEs cannot be easily live captured and collecting eggs requires an invasive procedure that in addition does not provide a good proxy for measuring maternal CORT concentrations (Rettenbacher et al., 2005). Therefore, shed feathers at the nests were the only representative matrix available for analysis in the 1960s until 1980s when the reproductive output in the Swedish WTE population was at its lowest (Helander et al., 2002).

In contrast to the strong temporal trends in POP concentrations highlighted in the WTEs, we did not detect any clear temporal patterns in fCORT concentrations over the 44-year study period (Fig. 4, Table S3; $F_{(1,\ 133)} = 0.04$, $p = 0.84$). The lack of a temporal trend in fCORT is however in line with findings in a previous study that measured fCORT in a pelagic seabird over a 153-year period (Fairhurst et al., 2015), even though the WTEs displayed a relatively wide concentration range of fCORT. Median concentrations of fCORT in the present study were 18 pg. mm^{-1} and we reported a tenfold difference in range between the lowest (8 pg. mm−1) and highest (94 pg. mm^{-1}) concentrations (Fig. 3, Table S2). There are no previous records of fCORT in adult WTEs, but fCORT measured in 70 WTE nestlings sampled in two locations in Norway showed median concentrations of 3.26 and 2.62 pg. mm^{-1}, respectively (Loseth et al., 2019), which indicates that breeding WTE

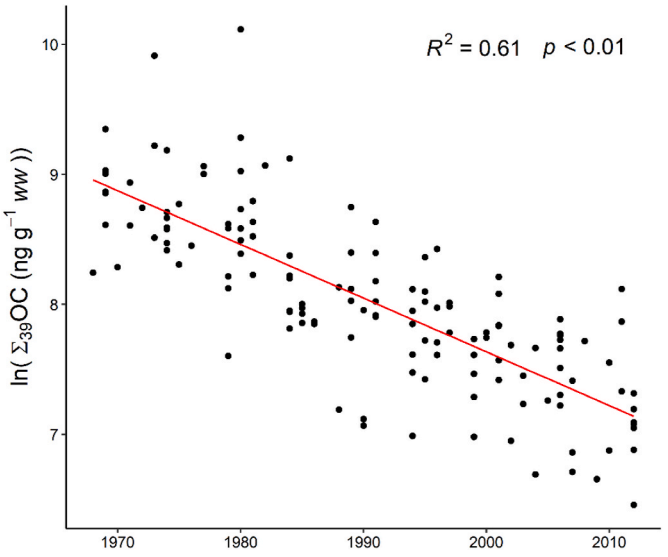

Fig. 2. Annual concentrations of the sum of 39 organochlorine (OC; list of compounds in section 2.5) in feather pools (N = 135) from breeding pairs of adult white-tailed eagles (Haliaeetus albicilla) in Sweden in 1968–2012. Concentrations are natural log (ln) transformed, and concentrations (filled dots) are calculated from a pool of feathers in which one pool represented a breeding pair. Number of feather pools analysed for pollutants were 1–9 per year, except in 1978, 1983, 1987, 1992–1993, and 1998, when feathers were not sampled. Estimate (red line) from the linear model investigating the temporal variations in OC concentrations is given in the supplementary information, Table S3.

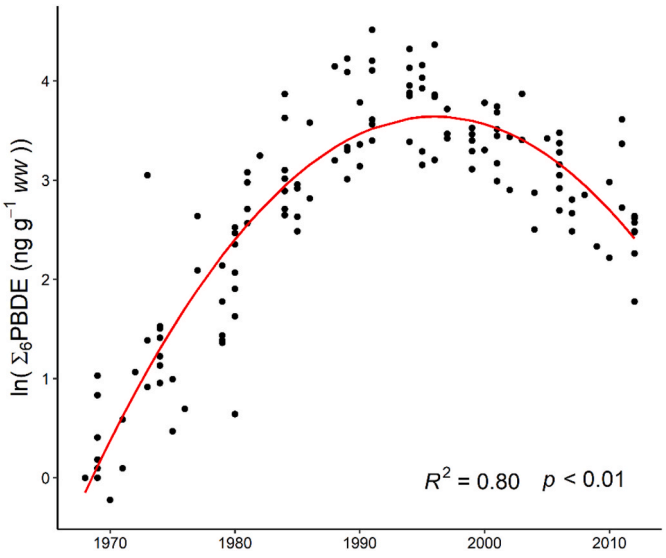

Fig. 3. Annual concentrations of the sum of 6 polybrominated diphenyl ether (PBDE; list of compounds in section 2.5) in feather pools (N = 135) from breeding pairs of adult white-tailed eagles (Haliaeetus albicilla) in Sweden, 1968–2012. Concentrations are natural log (ln) transformed, and concentrations (filled dots) are calculated from a pool of feathers in which one pool represented a breeding pair. Number of feather pools analysed for pollutants were 1–9 per year, except in 1978, 1983, 1987, 1992–1993, and 1998, when feathers were not sampled. Estimate (red line) from the linear model investigating the temporal variations in PBDE concentrations is given in the supplementary information, Table S3.

adults in the Baltics displayed 6 times higher fCORT concentrations than nestlings in Norway. In contradiction, a previous study investigating differences in fCORT among age classes of red kites (*Milvus*) within the same population showed that fCORT concentrations were 1.5 times higher at age 1 year compared to 7–11 year-old birds (López-Jiménez et al., 2017). Other studies have reported spatial differences in fCORT among populations of the same species (Bourgeon et al., 2012; Treen et al., 2015; Voit et al., 2021). Therefore, the difference in fCORT between Norwegian WTE nestlings (Loseth et al., 2019) and adult WTEs in the current study is likely a result of studying different age classes and populations in different environments.

3.2. *fCORT in relation to POP contamination and diet variability of breeding WTE pairs*

In general, stressful stimuli activate the hypothalamic-pituitary-adrenal (HPA) axis, which leads to the release of glucocorticoids (i.e., CORT) from adrenal tissue that mobilizes a suite of behavioural, physiological and endocrinological responses to maintain homeostasis (McEwen and Wingfield, 2003). While an acute activation of the HPA axis is crucial to survive a stressor, chronic stress is associated with deleterious health effects such as immunosuppression and reproductive impairment (McEwen and Wingfield, 2003). Direct negative population effects from POP exposure have been well documented in the Swedish WTEs (Helander et al., 2002, 2008; Sonne et al., 2020), but much less is known about potential health effects at the individual level. To fill this gap, we investigated whether the clear patterns in contaminants correlated with fCORT concentrations over the study period. We also included stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in the models due to the potential influence of feeding ecology on fCORT (Fairhurst et al., 2013b, 2015; Will et al., 2015). We did however not detect any significant relationships between fCORT and pollutants, i.e., OC-PCs (Table 1; $F_{(4,\ 110)} = 0.57$, $p > 0.005$ in all cases) nor PBDE-PCs

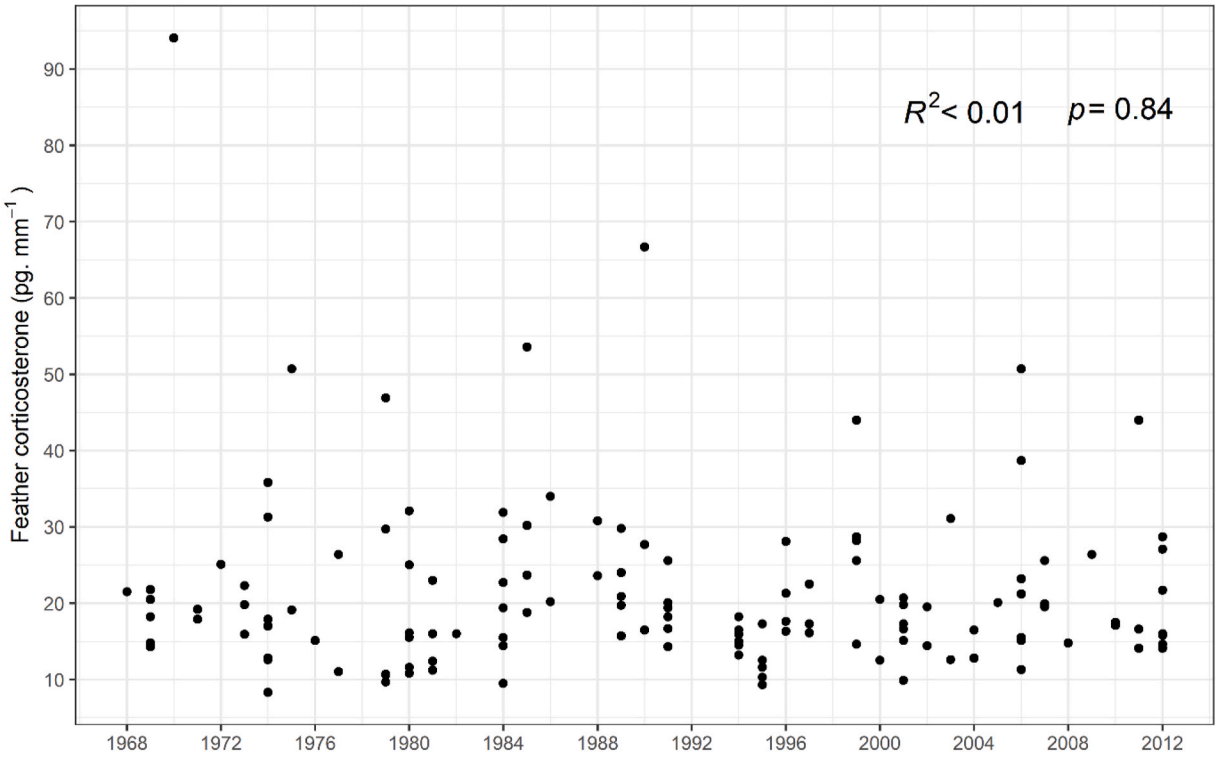

Fig. 4. Corticosterone concentrations (pg. mm^{-1}) in individual body feathers (N = 135) of adult white-tailed eagles (Haliaeetus albicilla) in Sweden, 1968–2012. The number of feathers analysed for corticosterone were 1–9 per year, except in 1978, 1983, 1987, 1992–1993, and 1998, when feathers were not sampled. One feather per nest was analysed. Estimate from the linear model investigating the temporal variations in feather corticosterone is given in the supplementary information, Table S3.

(Table 1; $F_{(4,\ 100)} = 1.08$, $p > 0.005$ in all cases). fCORT was also not significantly related to diet (proxied by δ^{15}N or δ^{13}C; Table 1, $p > 0.05$ in all cases).

The plethora of sampling procedures (mainly determined by the accessibility and biology of the investigated species) and methodological approaches available to researchers may contribute to discrepancies among studies. Similar to our study, Randulff et al. (2022) found no associations between fCORT and POP levels in Norwegian goshawk *(Accipiter gentilis)* nestlings, while two studies on red kites and cinereous vultures (*Aegypius monachus*) both reported positive relationships between fCORT and POP concentrations (Monclus et al. (2018) and Monclus et al. (2019), respectively). In contrast, Loseth et al. (2019) reported that fCORT decreased with increasing levels of perfluoralkyl substances (PFAS) in the Norwegian WTE nestlings although ranges in PFAS and fCORT levels encompassed different study locations. Thus, disentangling whether it was the direct effect of greater PFAS exposure that negatively affected fCORT in the Norwegian nestlings, or an effect of heterogenous habitats, is difficult. Spatial differences in the fCORT-contaminant relationship have been previously reported; for example, Bourgeon et al. (2012) compared Great Skuas (*Stercorarius skua*) from populations breeding at different locations (Iceland, Shetland, and Bjørnøya). They reported a negative relationship between fCORT and PBDE levels in the Iceland population only (i.e., the ecological context; Bourgeon et al. (2012)). As such, spatial variations in fCORT are more likely to be driven by heterogenous environmental conditions among locations/populations rather than pollutant exposure *per se*. Although anthropogenic pollutants have been proposed as a chronic stressor in wildlife (Ganz et al., 2018), findings in the current and above-mentioned studies by Loseth et al. (2019), Monclus et al. (2018, 2019), and Randulff et al. (2022) do not support that POPs influence CORT physiology. In addition, studies investigating other pollutants such as toxic metals (Strong et al., 2015) and other matrices (blood: e.g., Tartu et al. (2015a)) also reported contradicting findings on the CORT-contaminant relationship, further emphasizing the complexity behind establishing CORT as a proximal mechanism underlying the effects of contaminant exposure in avian wildlife.

To further investigate the impact of habitat on stress physiology, feathers do also allow for analysis of δ^{15}N and δ^{13}C isotopes that describe broad patterns in diet by indicating the trophic level and habitat at which an individual forage, respectively (Inger and Bearhop, 2008). Nutritional effects on CORT have previously been reported in studies on migrating seabirds (Fairhurst et al., 2015, 2017; Will et al., 2015), and Fairhurst et al. (2015) suggested a nutritional effect on fCORT in Leach Storm petrels (*Oceanodrama leucorhoa*), where higher δ^{15}N (i.e. trophic position of prey, of which also had higher food quality) were correlated with lower fCORT concentrations. We did however not report an effect of δ^{15}N or δ^{13}C on fCORT (Table 1), which corroborates previous findings on raptor nestlings (Loseth et al., 2019; Randulff et al., 2022). In addition, δ^{15}N and δ^{13}C were relatively stable across our study period (Figs. S3 and S4), which suggest that there were no substantial changes in diet over time. Year had however a weak positive effect on δ^{15}N (Table S3; $F_{(1,133)} = 5.48$, $p = 0.02$) but excluding potential extreme δ^{15}N-values (Fig. S5, values above >16) also removed the significant year-effect (results not shown). The outlier values did however not influence the estimates of the relationship models (Table 1) and therefore no δ^{15}N-values were excluded.

Overall, including ancillary information on biological and ecological variables appears essential to increase our understanding on how CORT responds to a defined stressor such as contaminant exposure in wildlife (Romero and Beattie, 2021). Namely, biological and environmental factors such as species differences (Cockrem, 2007; Strong et al., 2015), life-history stage (Boves et al., 2016; Goutte et al., 2010; López-Jiménez et al., 2017; Wilcoxen et al., 2011), nutrition (Fairhurst et al., 2015; Will et al., 2015, 2019) and climatic conditions (Hau et al., 2010; Treen et al., 2015) are likely more important variables than pollutants explaining variations in fCORT. In the Baltic WTE population, the most notable effect of pollution during the 1960s through 1980s was abnormal egg formation (eggshell thinning and egg desiccation), of which the latter was correlated with lowered productivity and subsequent population declines (Helander et al., 2002).

Table 1
Parameter estimates for the linear models of the relationship between feather corticosterone concentrations (pg. mm^{-1}) and the principal components (PCs) of organochlorines (OC-PC1 and OC-PC2) and polybrominated diphenyl ethers (PBDE-PC1 and PBDE-PC2), δ^{15}N (‰) and δ^{13}C (‰) in feather pools (N = 135) from breeding pairs of white-tailed eagles in Sweden between 1968 and 2012. Feather corticosterone and pollutant concentrations were ln-transformed prior to analysis, and calculations of the PCs of the OCs and the PBDEs are described in section 2.5 and PCA plots are provided in Figs. S1–S3.

Response variable	Coefficients	Estimate	Std. error	t-value	P-value
fCORT	*Intercept*	2.832	0.878	3.223	0.001
	OC-PC1	−0.008	0.007	−1.074	0.285
	OC-PC2	−0.010	0.019	−0.573	0.568
	δ^{15}N	0.022	0.036	0.619	0.537
	δ^{13}C	0.011	0.037	0.311	0.756
fCORT	*Intercept*	2.163	0.842	2.569	0.011
	PBDE-PC1	−0.022	0.021	−1.069	0.287
	PBDE-PC2	0.031	0.312	1.014	0.313
	δ^{15}N	0.005	0.376	0.158	0.874
	δ^{13}C	−0.041	0.037	−1.114	0.268

4. Conclusion

Despite the reported strong effects from pollutants on WTE reproduction during the study period, our results suggest that exposure to high POP levels did not impact the physiological stress of adults as measured by fCORT concentrations over the study period and the lack of association between POPs and fCORT. This study nonetheless provides novel information about fCORT concentrations in adult WTEs over a multi-decadal period covering periods during which this population suffered massive reproductive failure from being exposed to high POP contamination. Although we did not report significant relationships between fCORT, POP contamination or feeding ecology of the breeding pairs, analysis of fCORT represents a non-destructive and retrospective assessment of long-term stress in wild adult raptors otherwise not readily available. Since multiple biological and environmental factors likely affect CORT (e.g. Romero and Beattie (2021)), future studies using CORT as a biomarker of pollution exposure should consider the multiple stressor perspective, i.e., acknowledge that pollutants work in orchestra with other stressors when investigating the underlying mechanisms driving physiological stress in wild animals. In essence, feathers constitute a temporal archive for substances and remain the one matrix enabling a non-invasive and retrospective analysis of CORT physiology and POP exposure simultaneously in wild birds that have been exposed to harmful levels of environmental pollutants.

Credit author statement

Elisabeth Hansen: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Jiachen Sun**: Formal analysis, Investigation, Writing – review & editing. **Björn Helander**: Investigation, Writing – review & editing. **Jan Ove Bustnes**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Igor Eulaers**: Conceptualization, Resources, Writing – review & editing. **Veerle L. Jaspers**: Funding acquisition, Writing – review & editing. **Adrian Covaci**: Funding acquisition, Resources, Writing – review & editing. **Marcel Eens**: Funding acquisition, Resources, Writing – review & editing. **Sophie Bourgeon**: Conceptualization, Resources, Supervision, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.115923.

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A retrospective investigation of feather corticosterone in a highly contaminated white-tailed eagle (Haliaeetus albicilla) population

Supplementary information

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Table S1 Number of feather pools (n) by a) Year, and b) Territory with range of years.

a) Year	*n*	b) Territory	Time period	*n*
1968	1	B1	1972-2007	8
1969	6	B11	1974-2006	6
1970	1	B11b	2009	1
1971	2	B12	1973-2010	6
1972	1	B13	1969-2011	8
1973	3	B14	1969-2012	8
1974	7	B15	1969-2012	7
1975	2	B23	1990-2011	5
1976	1	B3	1969-2008	5
1977	2	B4	1974-1997	5
1979	5	B5	1984-2012	5
1980	7	B6	1973-1984	3
1981	4	B7	1971-2003	7
1982	1	C1	1971-2001	6
1984	7	C14	1979-2012	6
1985	4	C15	1974-2011	6
1986	2	C15b	2006	1
1988	2	C1b	2006	1
1989	5	C2	1970-2009	9
1990	3	C4	1969-2007	7
1991	6	C5	1969-2012	9
1994	6	C6	1973	1
1995	5	C7	1968-2012	8
1996	4	C9	1974-1996	4
1997	3	C9b	2001-2012	3
1999	5			
2000	2			
2001	6			
2002	2			
2003	2			
2004	2			
2005	1			
2006	8			
2007	3			
2008	1			
2009	1			
2010	2			
2011	3			
2012	7			

*Table S2 Descriptive statistics of feather corticosterone concentrations (fCORT), sum of organochlorine and polybrominated diphenyl ether concentrations (ΣOCs and ΣPBDEs), and stable isotope signatures of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) analyzed in feather pools (*N *= 135) from breeding pairs of white-tailed eagles.*

	Mean	Median	Sd	Min-max
fCORT (pg. mm^{-1})	21.2	17.9	11.43	8.30-94.1
Σ_{39}OCs[a] (ng g^{-1} dw)	2896.1	3878.5	3266.47	638.1- 24699.9
Σ_6PBDEs[b] (ng g^{-1} dw)	23.49	19.80	19.10	0.80- 91.3
$\delta^{15}N$ (‰)	13.93	13.79	1.07	11.37-18.54
$\delta^{13}C$ (‰)	-18.21	-18.18	1.12	-21.95-(-15.53)

[a] List the summed OCs: 29 polychlorinated biphenyl (PCB) congeners (28, 49, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138, 146, 149, 151, 153, 156, 170, 171, 174, 177, 180, 183, 187, 194, 196/203, 199, 206 and 209), four chlordane-related compounds (CN, TN, OxC and CC), *p,p'*-DDT and metabolites (*p,p'*-DDE and *p,p'*-DDD), HCB, *β*- and *γ*-HCH

[b] List the summed PBDEs: six PBDE congeners (28, 47, 99, 100, 153 and 154)

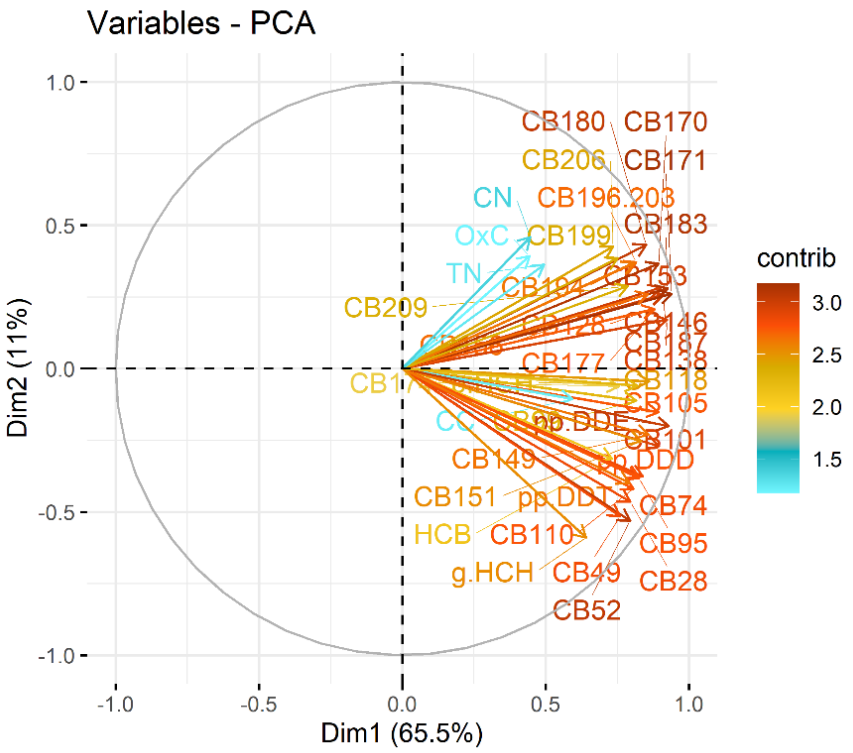
Table S3 Parameter estimates for the linear models of the annual variations in corticosterone concentrations (pg. mm^{-1}), organochlorines (OCs; ng g^{-1} dw), polybrominated diphenyl ethers (PBDEs; ng g^{-1} dw), $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) analyzed in 135 feather pools from breeding pairs of white-tailed eagles in Sweden between 1968-2012.

Response variable	Coefficients	Estimate	Std. error	t-value	P-value
fCORT	*Intercept*	4.035	5.525	0.730	0.466
	Year	-0.000	0.002	-0.195	0.845
Σ_{39}OCs[a]	*Intercept*	90.26	5.669	15.92	< 0.01
	Year	-0.041	0.002	-14.51	< 0.01
Σ_6PBDEs[b]	*Intercept*	-1.922e+04	1.099e+03	-17.49	< 0.01
	Year	1.926e+01	1.104e+00	17.45	< 0.01
	Year^2	-4.824e-03	2.773e-04	-17.40	< 0.01
$\delta^{15}N$	*Intercept*	-18.95	14.05	-1.349	0.179
	Year	0.016	0.007	2.341	0.020
$\delta^{13}C$	*Intercept*	-9.951	14.03	-0.709	0.480
	Year	-0.003	0.007	-0.521	0.603

[a] List the summed OCs: 29 polychlorinated biphenyl (PCB) congeners (28, 49, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138, 146, 149, 151, 153, 156, 170, 171, 174, 177, 180, 183, 187, 194, 196/203, 199, 206 and 209), four chlordane-related compounds (CN, TN, OxC and CC), *p,p'*-DDT and metabolites (*p,p'*-DDE and *p,p'*-DDD), HCB, *β*- and *γ*-HCH

[b] List the summed PBDEs: six PBDE congeners (28, 47, 99, 100, 153 and 154)

A)



B)

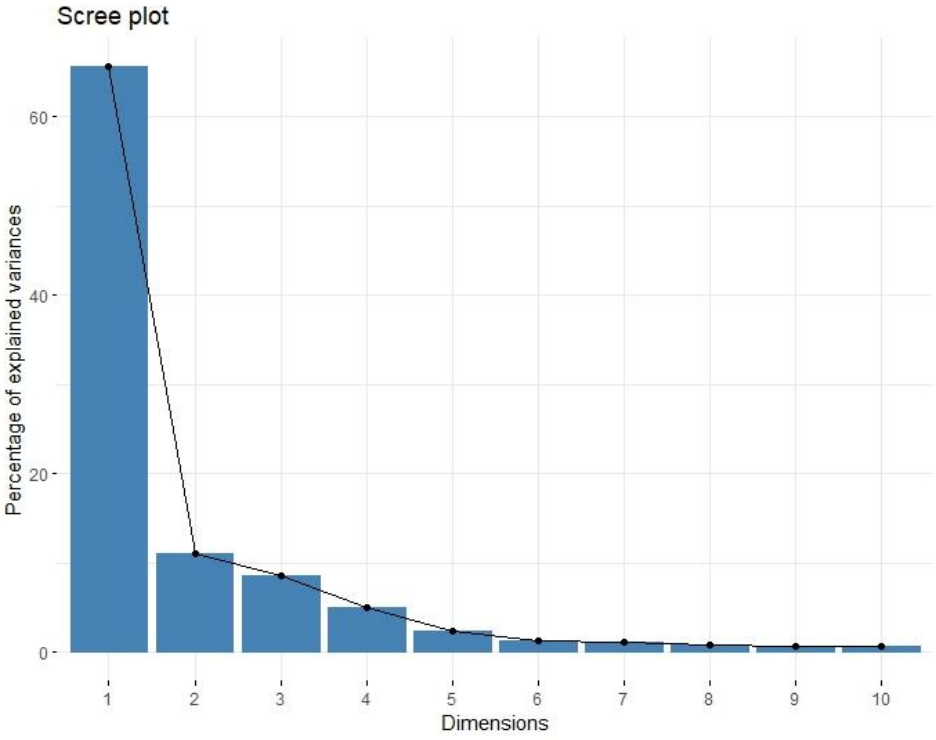
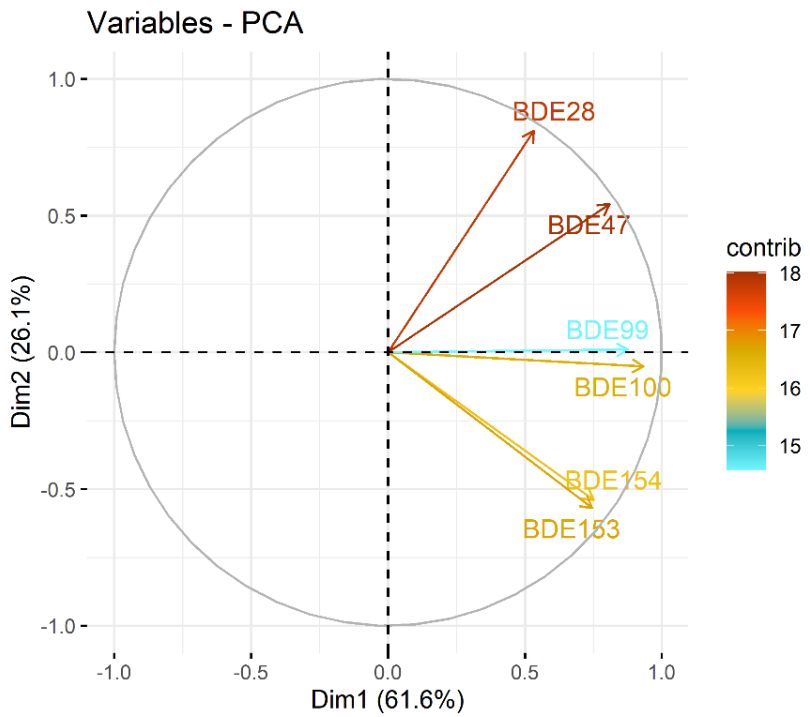


Figure S1. A) Principal component analysis (PCA) biplot of 39 organochlorine (OC) compounds. Dim1 and Dim2 represents the first and second principal components (PC1 and PC2) of the analysis. Contrib describes the contributions (in percentage) of the variables to the principal components. B) *Scree plot (function* fviz_eig *in package* factoextra*; Kassambara and Mundt, 2020) of eigenvalues of the dimensions from the results of the principal component analysis (PCA) of the organochlorine (OC) compounds (10 out of 39 dimensions shown for simplicity).*

A)



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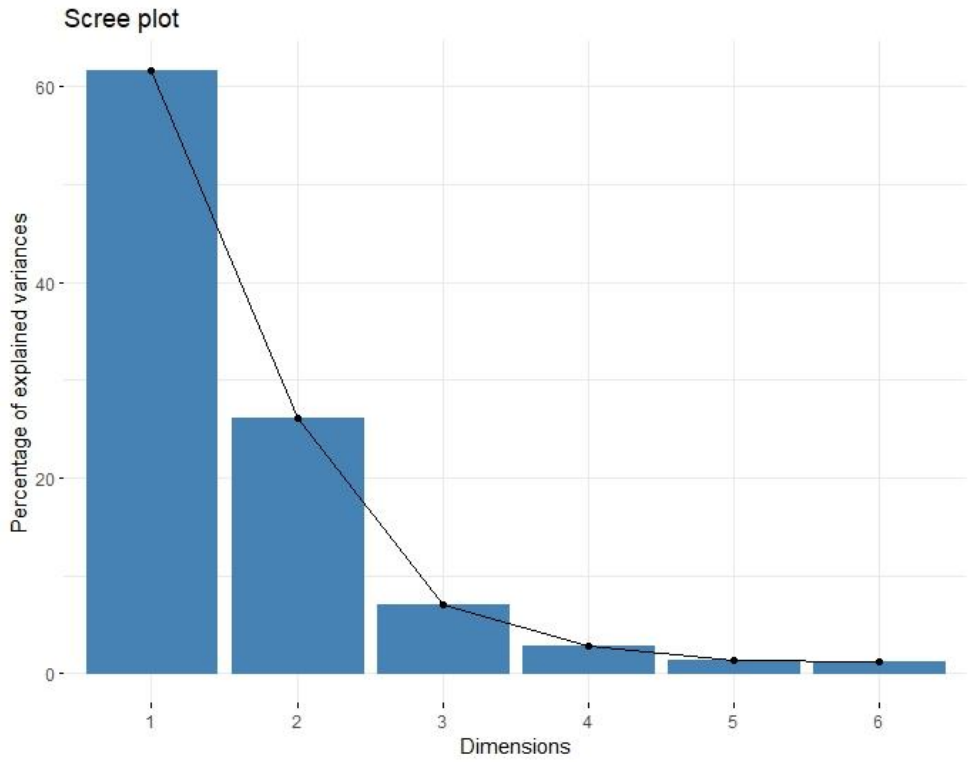
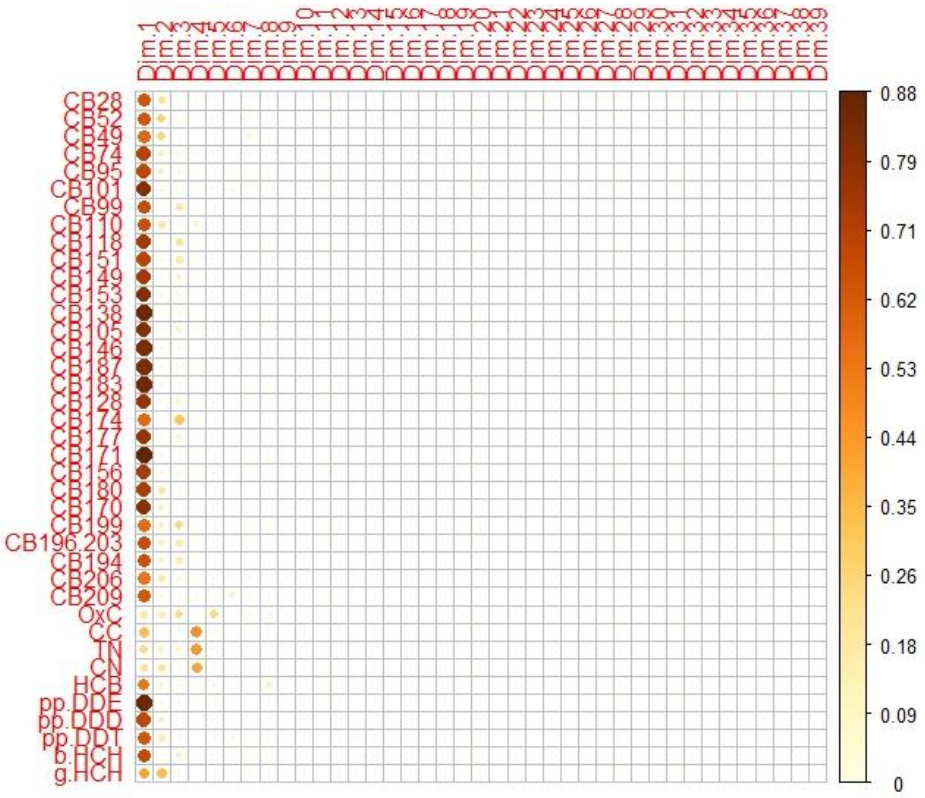


Figure S2. Principal component analysis (PCA) biplot of 6 polybrominated diphenyl ether (PBDE) compounds. Dim1 and Dim2 represents the first and second principal components (PC1 and PC2) of the analysis. Contrib describes the contributions (in percentage) of the variables to the principal components. *Scree plot (function* fviz_eig *in package* factoextra*; Kassambara and Mundt, 2020) of eigenvalues of the dimensions from the results of the principal component analysis (PCA) of the 6 polybrominated diphenyl ether (PBDE) compounds.*

A)



B)

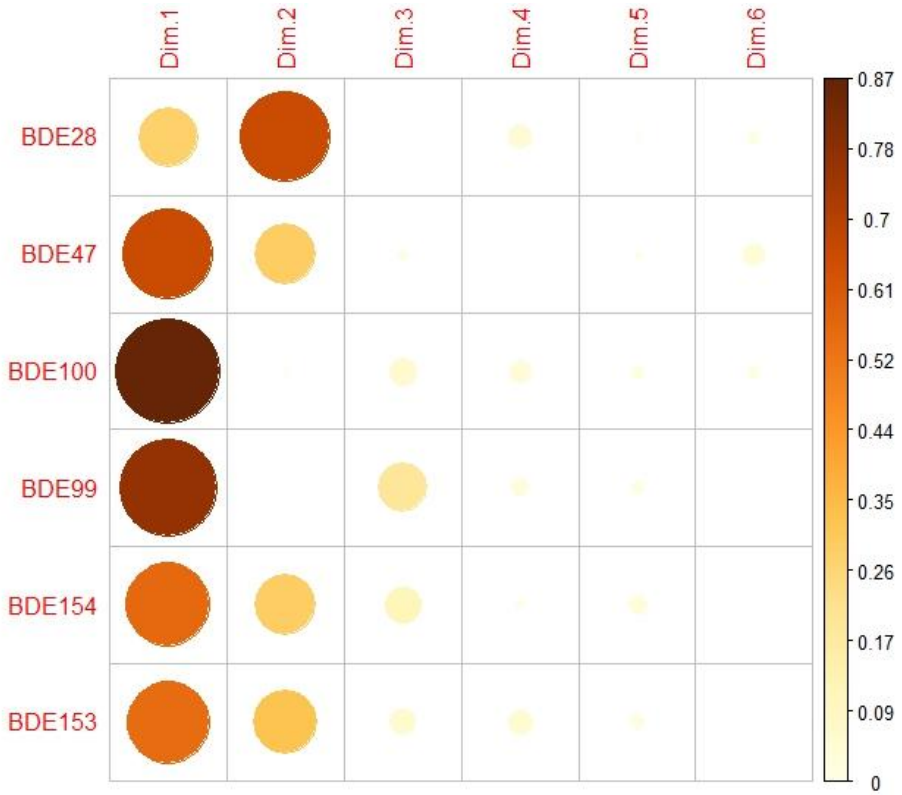


Figure S3 A) Correlational plot (function corrplot *in package* corrplot*; Wei and Simko, 2021) highlighting the most contributing variables (i.e., 39 organochlorine compounds) for each dimension. B) Correlational plot (function* corrplot *in package* corrplot*; Wei and Simko, 2021) highlighting the most contributing variables (i.e., 6 polybrominated diphenyl ether compounds) for each dimension.*

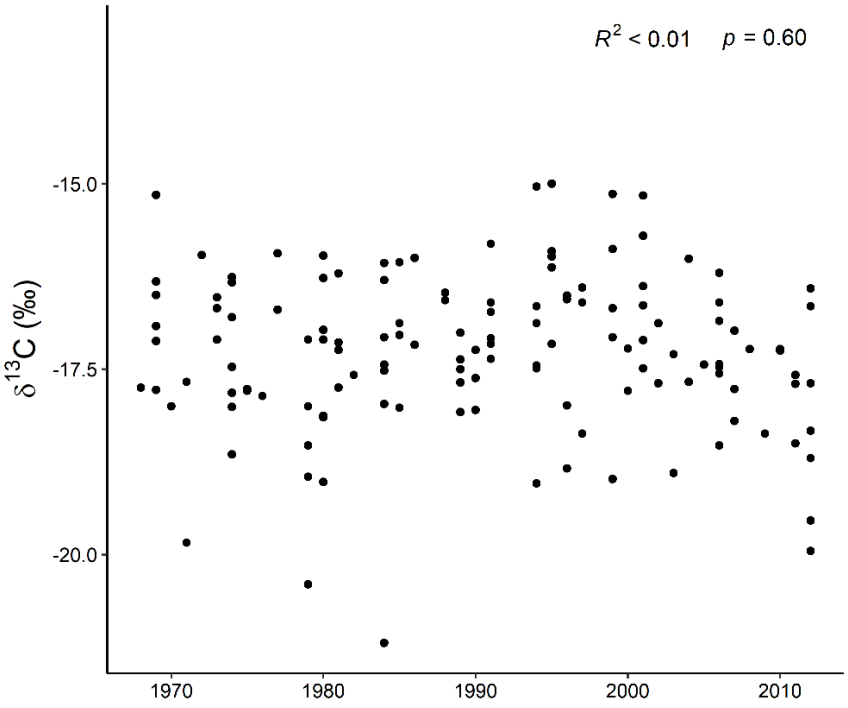


Figure S4. Levels of $\delta^{13}C$ analyzed in feather pools (N = 135) of adult white-tailed eagles in Sweden sampled during 1968-2012. $\delta^{13}C$ values were corrected for the oceanic Suess effect (as explained in Sun et al. (2019)), which is the temporal decline of atmospheric $\delta^{13}C$ values and consequently oceanic $\delta^{13}C$ values due to the large quantity of CO_2 released from fossil fuel burning in recent industrial times (Gruber et al., 1999). Estimates from linear models investigating the temporal variations in *$\delta^{13}C$* are given in Table S2.

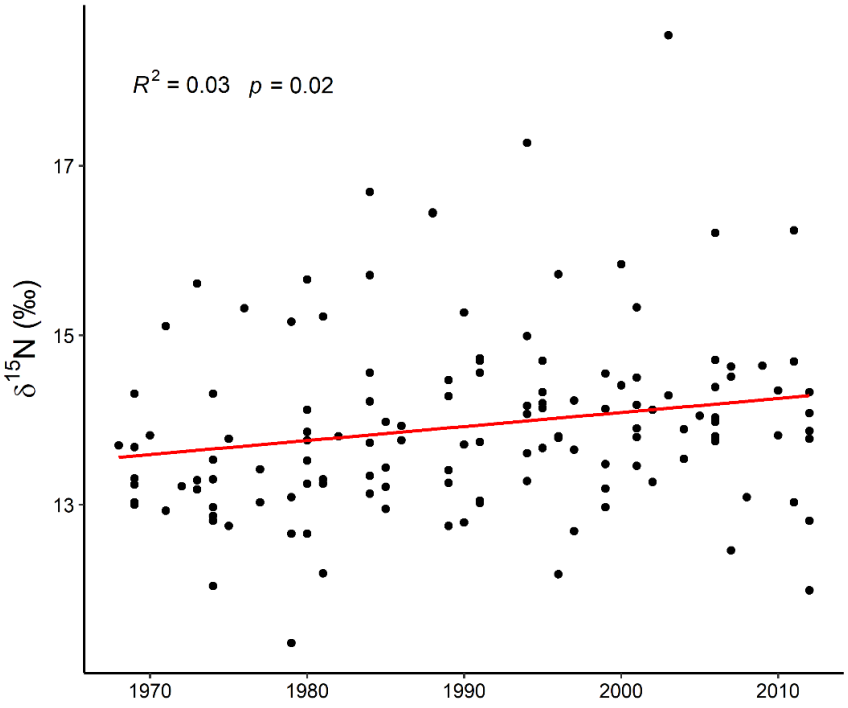


Figure S5. Levels of $\delta^{15}N$ analyzed in feather pools (N = 135) of adult white-tailed eagles in Sweden sampled during 1968-2012. Estimates from linear models investigating the temporal variations in *$\delta^{15}N$* are given in Table S2.

List of references:

Gruber, N., et al., 1999. Spatiotemporal patterns of carbon-13 in the global surface oceans and the oceanic Suess effect. Global biogeochemical cycles. 13**,** 307-335.

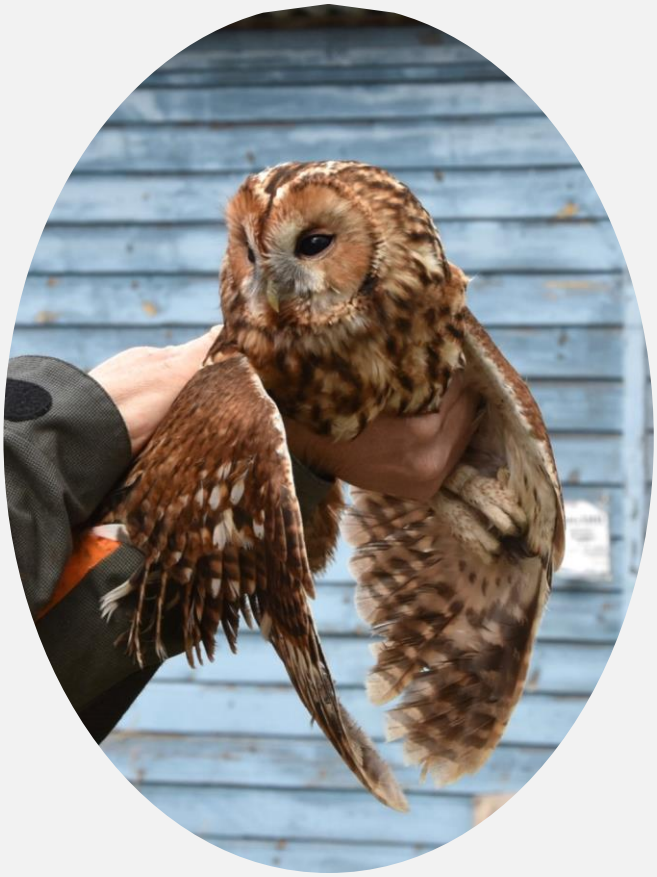
Kassambara, A., Mundt, F., Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R Package, 2020.

Sun, J. C., et al., 2019. Temporal trends of mercury differ across three northern white-tailed eagle (Haliaeetus albicilla) subpopulations. Science of the Total Environment. 687**,** 77-86.

Wei, T., Simko, V., R package 'corrplot': Visualization of a Correlation Matrix. 2021.

Paper IV

Feathers as temporal archives of environmental stress: a multi-decadal study on metal exposure, dietary proxies and physiological stress in a terrestrial raptor



This is an advanced manuscript that presents preliminary findings based on an incomplete dataset. The remaining data is anticipated to be analyzed and incorporated into the study during this autumn (2023). The ultimate goal is to submit the completed manuscript to the journal *Science of the Total Environment* for publication.

Feathers as temporal archives of environmental stress: a multi-decadal study on metal exposure, dietary proxies, and physiological stress in a terrestrial raptor

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Abstract

Pollution by various metals, primarily released by human activities like mining and smelting, is a global environmental issue. As exposure of wildlife to toxic metals such as mercury (Hg) has been associated with adverse biological effects, regulatory efforts have been implemented to mitigate the problem. Long-term studies spanning the large changes in metal emissions following legislative changes are therefore well suited to investigate adverse effects of toxic metals. Long-term studies also facilitate robust analysis by incorporating fluctuating environmental conditions (e.g., temperature, wind, precipitation) and food availability. However, long-term studies investigating the impacts of environmental stressors on wildlife are challenging and therefore scarce. To assess the impact of metal pollution on wildlife, raptors have often been used as biomonitors due to their high position in the food chain and high exposure to environmental contaminants, including several metals. Here, we studied a resident nest box population of tawny owls (*Strix aluco*) in Norway over 34 years. Between 1986 and 2019 tail feathers from females were collected annually, resulting in a long-term archive of over 1200 feathers in total. Each feather on its own represents an excellent archive of their local environmental conditions, including the presence of metals, and their dietary ecology, proxied in the present study by stable isotopes of nitrogen (δ^{15}N) and carbon (δ^{13}C). In addition, as the primary avian glucocorticoid, corticosterone levels in feathers (fCORT) provide a long-term integrated measurement of individual physiological stress. In this study, we examined how exposure to metals and metalloids (Al, As, Cd, Hg, Pb) and variability in dietary ecology, individually or jointly, modulate fCORT in a sentinel raptor. Using structural equation modelling, we found that higher Al and Cd were linked to higher fCORT (both $p < 0.02$), while higher Hg was associated to lower fCORT ($p = 0.01$). Further, higher δ^{15}N was related to higher fCORT, while δ^{13}C negatively related to fCORT. In addition, δ^{15}N and δ^{13}C were negatively and positively related (both $p < 0.01$), respectively, to Hg levels, suggesting that dietary patterns mediate the effect of Hg on fCORT. Our findings indicate complex interactions between dietary patterns, metal exposure, and CORT physiology in tawny owls, highlighting the importance of considering multiple variables to assess stress in wildlife ecotoxicology.

Keywords: Ecotoxicological effects; non-destructive; glucocorticoid hormone; stress physiology; adverse biological effects; avian biomonitoring; environmental contaminants

Introduction

Pollution by various metals, mostly due to anthropogenic activities such as mining and smelting, is a global environmental issue. While some metals (e.g., copper, iron, and zinc) are essential for life at trace levels and highly biologically regulated, non-essential metals can be toxic even at trace levels. Examples of non-essential toxic metals and metalloids (hereof metals for simplicity) include aluminium (Al), arsenic (As; metalloid), cadmium (Cd), mercury (Hg), and lead (Pb). As exposure of wildlife to high levels of toxic metals has been correlated with adverse biological effects (Dietz et al., 2019), regulatory efforts have been implemented to reduce their emissions worldwide (Harmens et al., 2015). Consequently, wildlife populations have been exposed to changing concentrations of metals in their environment over the last century. Long-term studies are therefore well suited to monitor exposure to metals and to subsequently investigate their detrimental effects by spanning temporal differences in environmental concentrations. However, long-term studies that investigate detrimental health effects of metal exposure in free-living birds are scarce.

Corticosterone (CORT) is the primary glucocorticoid and stress-associated hormone in birds (Romero and Romero, 2002) and is often used as a biomarker of health in avian ecology and conservation research (Romero and Beattie, 2021). When birds are exposed to stressors, CORT concentrations rise within 2 minutes from baseline to stress-induced levels (i.e., the stress-induced response), resulting in immediate physiological changes that maximize survival during stressful events including the activation of the fight or flight response (Romero and Romero, 2002). However, when birds are chronically exposed to stressors (e.g., contaminants), this can disrupt the stress response by maintaining elevated baseline CORT levels and/or by hindering a proper stress-induced response. Chronic stress and elevated CORT can have adverse health effects, including immunodeficiency and reproductive impairment (Chrousos, 2009) and were shown to affect individual fitness (Koren et al., 2012).

Obtaining both baseline and stress-induced plasma CORT concentrations in wild birds, particularly in long-term studies, is challenging. Feathers represent an alternative matrix to study CORT physiology, as they integrate both baseline and stress-induced CORT levels (Bortolotti et al., 2008) in addition to being easy to collect and store. When a feather grows and is connected to the bloodstream, CORT is deposited into the feather over a period of days to weeks (Bortolotti et al., 2009). Previous studies have shown that feather concentrations are well correlated with blood concentrations (Fairhurst et al., 2013a; Lattin et al., 2016). Importantly, CORT remains stable in the grown feather, which allows for retrospective studies. As such, feather CORT (hereof fCORT) provides a long-term integrated measurement of individual stress physiology (Bortolotti et al., 2008). Variations in fCORT

have been successfully used as a proxy of stress in relation to reproductive and fitness-related traits (Crossin et al., 2017; Crossin et al., 2013; Fairhurst et al., 2017; Monclús et al., 2020) or environmental stress linked to climate (Crino et al., 2020; Romero et al., 2000; Treen et al., 2015) and food availability (Catitti et al., 2022; Patterson et al., 2015; Will et al., 2015). In addition, the stress of being contaminated has been of recent growing interest in avian ecotoxicology, and several studies have reported links between metal exposure and CORT in wild birds (Baos et al., 2006; Eeva et al., 2005; Powolny et al., 2020; Strong et al., 2015; White et al., 2022).

To assess the impact of environmental contaminants on avian wildlife, birds of prey have been extensively used as biomonitors due to their high trophic position and high exposure to environmental contaminants (Badry et al., 2020; Helander et al., 2008; Shore and Taggart, 2019). Tawny owls (*Strix aluco*) are a common species of terrestrial birds of prey in Europe that are long-lived and resident (Millon et al., 2010). They mainly feed on small mammals and passerine birds depending on the seasonal variability of the different prey (Sunde et al., 2001). The tawny owl has recently been identified as one of the most suitable candidates for pan-European biomonitoring programs (Ratajc et al., 2022), and has been used in several long-term studies on environmental contaminants taking into account different matrices such as eggs (Bustnes et al., 2022) and feathers (Devalloir et al., 2023; Garcia-Seoane et al., 2017). The latter tissue represents an excellent non-destructive matrix to analyze metal exposure in the local environment (Lodenius and Solonen, 2013).

In the present study, tail feathers of tawny owls from a resident nest box population in Norway were sampled annually during breeding (i.e., April) in the period 1986-2019, resulting in a sample size of over 1200 feathers. Since tawny owls molt their tail feathers either annually or biannually after breeding (Petty, 1993), the collected feathers are assumed to encapsulate metabolites from the previous autumn (year-1) or the second previous autumn (year-2). Different sections of a single tail feather were used to analyze metals (lower shaft) and CORT (distal part). In addition, stable isotopes (middle shaft) of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were assessed to evaluate feeding sources and trophic levels (Inger and Bearhop, 2008), respectively. These factors have been shown to influence individual contaminant burden (Ratajc et al., 2022). Analyzing all metabolites in the same feather ensured that feather concentrations reflected the same overarching time period (i.e., last molt) regardless of the timing of molting. In addition, only females were sampled as only females incubate and occupy the nest box (Sunde et al., 2003), which eliminated possible sex-biased feather concentrations of metals and CORT in our study.

We explored the use of feathers as temporal archives, spanning a 34-year period, of physiological stress (fCORT) in relation to exposure to toxic metals and variability in dietary ecology

(proxied by $\delta^{15}N$ and $\delta^{13}C$) for free-ranging individuals of a terrestrial raptor. We hypothesized that toxic metals (Al, As, Cd, Hg, and Pb) and dietary proxies ($\delta^{15}N$ and $\delta^{13}C$), individually or jointly, modulated fCORT in the tawny owl. We investigated the relationships between all metabolites using structural equation modelling (Lefcheck et al., 2018). This approach allowed us to examine both direct and indirect effects of stable isotopes and metals on fCORT. For example, we expected that both diet and metals could negatively modulate fCORT concentrations as previously reported by e.g., Fairhurst et al. (2015), Fairhurst et al. (2013b) and Will et al. (2015) and e.g., Powolny et al. (2020), Strong et al. (2015) and White et al. (2022), respectively. Since we also expected that diet could influence metal exposure (Tasneem et al., 2020), we aimed to examine whether stable isotopes modulated the interaction between metal exposure and fCORT by mediating their effects on metal exposure.

2 Materials and methods

2.1 Field sampling and data collection

This study was carried out in the period 1986-2019 in the area surrounding Trondheim (63.42° N, 10.23° E), Norway, where more than 150 tawny owl nest boxes have been deployed (Devalloir et al., 2023). Each nest box was visited every year in late April or early May around hatching when the females were caught to collect one of the two mid-positioned tail feathers. The collected tail feathers were stored individually in paper envelopes (or taped to paper sheets) at room temperature until chemical analysis. One feather per female per year was used to analyze corticosterone, stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$), and metals (Fig.1). In total 487 unique individuals (identified by ring number) were sampled in this study and some females were repeatedly sampled in subsequent breeding seasons. The number of owls caught annually varied between 8-71, and the total number of sampled tail feathers across the 34-year study period was 1202.

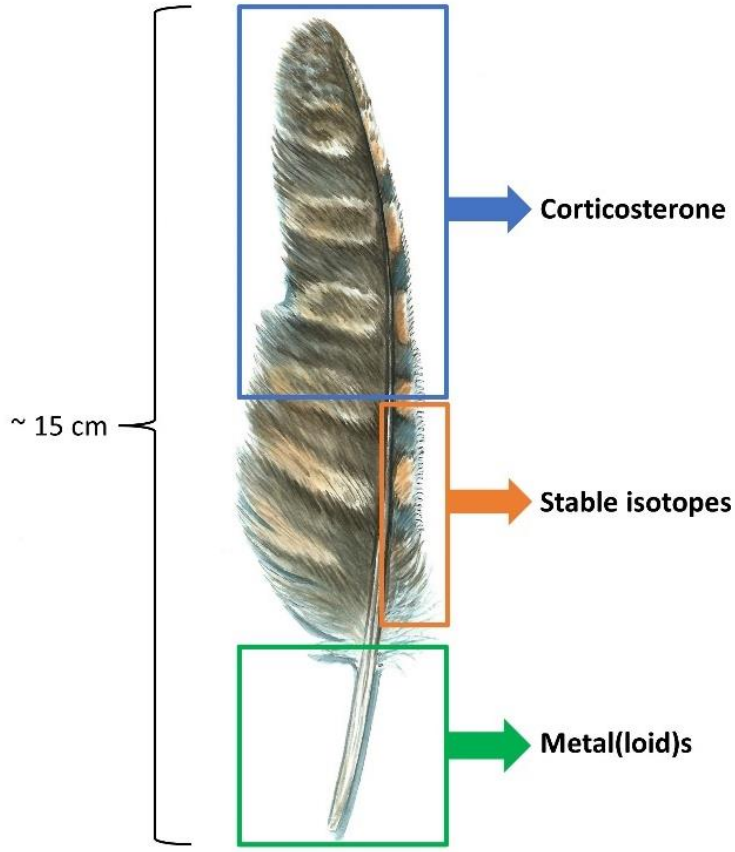

Figure 1 Illustration of the sections of tail feathers that were analyzed for corticosterone, stable isotopes, and metal(loid) concentrations.

2.2 Analysis of metal and metalloid elements

Analyses of metal and metalloid elements were carried out at the heavy metal laboratory at the Norwegian Institute for Nature Research in Trondheim. In the current study, feathers in the period 2017-2019 were analyzed following the same protocol as for metal measurements in tawny owl feathers from the same population for the period 1986-2016 already described in detail by Devalloir et al. (2023). Only the lower shaft of the feather was used in the analyses, as it is found to better represent the internal body burden of metals (Seoane et al., 2018). In brief, the shaft was acid digested in concentrated HNO_3 (ultra-pure grade) and maintained in a microwave oven (Milestone Mls Mega) at 180 °C for 45 min (from 20 °C to 180 °C to 20 °C). MilliQ-water was used for the dilution of the HNO_3 acid solution (q.s. 0.6 M). Chemical analyses were carried out using HR-ICP-MS Element 2 (Thermo Electronics). Among the 39 metallic elements that were analysed (Bustnes et al., 2013), the present study focuses on five non-essential metals based on their potential toxicity (Devalloir et al., 2023), namely Al, As (metalloid), Cd, Hg, and Pb. All elements could not be analyzed in all feather samples. Data were successfully acquired for 1168 samples for Al, 1165 for As, 1133 for Cd, 1169 for Hg and 1167 for Pb.

2.3 Feather corticosterone analysis

The analysis of feather CORT was performed at UiT, Tromsø, Norway, and the protocol is based on the description given by Bortolotti et al. (2008). A methanol-based extraction technique was used to extract CORT from feathers. First, the total weight and length of the feather (including the rachis) were measured before the distal 70 mm (cut straight across perpendicular to the rachis). Extraction of CORT followed the methanol-based extraction method reported earlier by Bortolotti et al. (2008). Final extracts were assessed for CORT with an enzyme immunoassay kit (901–097, Assay Designs Inc., USA). The quality of the assay was validated using serial dilutions of feather extracts (displacement curves), which confirmed the absence of interfering substances in the extract. Each feather extract was measured in duplicate in 36 separate plates, and for all separate plates used in the present study, we reported intra- and inter-assay variability of 14.97 % (n = 148) and 14.71 % (n = 164), respectively. In addition, a pooled calibrator feather extract (prepared from 70 random feather extracts) was assayed on each plate and showed an inter-assay variability of 13.05 % (n = 36). The final concentration of feather CORT (fCORT) was calculated using a standard curve run on each plate and the unit is given as pg mm^{-1}.

2.4 Stable isotope analysis

The analysis of stable carbon (^{13}C and ^{12}C) and nitrogen isotopes (^{15}N and ^{14}N) was carried out at the Stable Isotope Lab of the University of Koblenz-Landau, Germany. 500 feathers covering the entire

study period were randomly selected for stable isotope analysis. Feathers were washed in distilled water and dried overnight before the narrowest side of the vane (along the rachis) was cut off. The cut pieces from each feather were transferred to a Precellys tube and the feathers were ground to a fine powder in the Precellys 24. Finally, 1.00 ± 0.20 mg of ground feather sample were transferred to tin capsules ready for subsequent analysis. Stable isotope ratios of carbon (^{13}C:^{12}C) and nitrogen (^{15}N:^{14}N) were determined in bulk homogenised feather material using a Flash 2000 HT elemental analyser coupled via a ConFlo IV interface to a Delta V Advantage isotope ratio mass spectrometer (all Thermo Fisher Scientific, Bremen, Germany). The stable isotope ratios are expressed as δ values (‰) relative to their respective international measurement standards Vienna Pee Dee Belemnite and atmospheric N_2. For quality control, an internal reference material (i.e., casein) was measured in duplicate every ten samples revealing an imprecision (± *SD*) ≤0.06 ‰ for both $\delta^{13}C$ and $\delta^{15}N$.

Because atmospheric CO_2 has become progressively depleted in $\delta^{13}C$ during the last 150 years due to the burning of fossil fuels (known as the "Suess effect"; Keeling, 1979), $\delta^{13}C$ values were corrected accordingly. Using the SuessR package (Clark et al., 2021), $\delta^{13}C$ values in our dataset were corrected for the Suess effect by applying the *SuessR* function with the sub-arctic North Atlantic as the specified study region.

2.5 Statistical analysis

The statistical analyses were conducted using R version 4.2.1 (Team, 2022). All tests performed were two-tailed and the null hypothesis was rejected at an alpha level of 0.05. Model assumptions were visually assessed and validated through residual plots, quantile-quantile plots, and histograms of residuals following the protocol by Zuur et al. (2010). To attain the assumptions of normality and homogeneity of variance, fCORT and metal concentrations were log-transformed (ln) prior to statistical analyses. Furthermore, one fCORT value exceeding 10,000 pg mm^{-1} was considered an outlier and subsequently removed from the subsequent analysis.

We first used linear models to investigate possible temporal trends in fCORT, metals (Al, As, Cd, Hg, and Pb), and stable isotopes ($\delta^{13}C$ and $\delta^{15}N$). All variables displayed linear trends over time, except $\delta^{15}N$ (table S1; all but $\delta^{15}N$ showed $p < 0.05$). We then investigated the relationship between stable isotopes, metals, and fCORT, using linear mixed effect models (function *lme* in the nlme-package by Pinheiro et al. (2017)) with sampling year taken as a random factor. Although we had repeated measures for some owls, *lme* models investigating the random effect of Ring ID (i.e., individual owl) revealed large within-individual variability in fCORT compared among individual variability (i.e., low random intercept variance; results not shown). Therefore, including Ring ID was found negligible and superfluous in the models. We did however compare models with sampling year or Ring ID as the

random factor, of which sampling year performed better based on a lower Akaike information criterion (AIC).

Finally, we used structural equation modelling (SEM; Lefcheck et al. (2018)), to investigate both direct and indirect effects of stable isotopes and metals on fCORT. Since we need paired data for all variables, the SEMs were conducted on a subsample of 500 because we had stable isotope data for only 500 feathers. SEM allows including more than one response variable in models which can be beneficial when working with variables that can act as both predictor and response (Lefcheck et al., 2018), as in the case with stable isotopes and metals. Five different SEMs were fitted based on linear mixed effect models using the function *psem* (Lefcheck et al., 2018), of which each model included one metal (response or predictor), $\delta^{13}C$ and $\delta^{15}N$ (predictors) and fCORT (response) (Table S2). Correlated variables were not used in the same model to avoid problems with collinearity (Supplementary Material: fig. S1). We provided standardized path coefficients (i.e., partial regression parameters) to describe relationships between variables. Similar to linear models, a positive path coefficient (i.e., positive effect) implies an increase in the response variables (here, fCORT or metals), and a negative one (i.e., negative effect) implies a decrease.

3 Results and discussion

3.1 SEM results of direct and indirect effects

The SEMs applied in this study revealed both direct and indirect effects of dietary patterns ($\delta^{13}C$ and $\delta^{15}N$), and metal exposure (i.e., Al, Cd and Hg) on physiological stress (fCORT) in the tawny owl (fig. 2). Summary outputs of SEMs, including *p*-values, are reported in the Appendix Table S2. Path coefficients showed that Al (0.15) and Cd (0.13) had direct positive effects on fCORT ($p < 0.01$ and $p = 0.017$, respectively), while Hg showed a direct negative effect on fCORT (-0.11, $p < 0.01$). Further, $\delta^{13}C$ had a direct negative effect on fCORT (-0.09, $p = 0.049$) and a positive effect on Hg (0.56, $p < 0.01$). $\delta^{15}N$, on the other hand, had a direct positive effect on fCORT (0.14, $p < 0.01$) and a direct negative effect on Hg (-0.17, $p < 0.01$). In summary, our results imply that all variables except As and Pb modulate fCORT, and $\delta^{13}C$ and $\delta^{15}N$ mediate the effect of Hg on fCORT in the tawny owl.

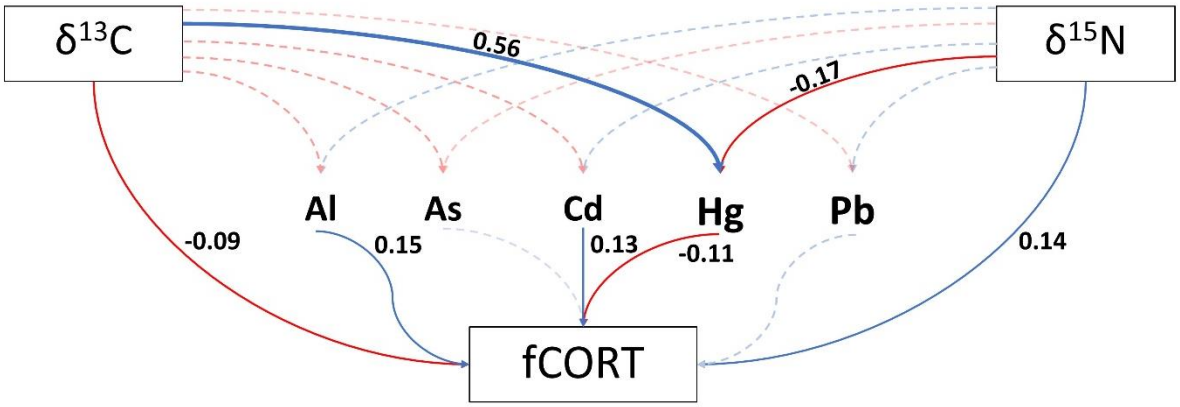

Figure 2 Path diagram from the structural equation models of the relations among dietary ecology(proxied by $\delta^{13}C$ and $\delta^{15}N$), metal exposure (Al, As, Cd, Hg, Pb), and physiological stress (feather corticosterone (fCORT)). Filled lines are significant relationships, and dashed transparent lines are not significant relationships. Blue lines indicate a positive effect and red lines indicate a negative effect. The width of the path lines is proportional to the strengths of the significant relationship (indicated by standardized path coefficients [Std. Estimate] in Table S2).

*Table 1 Descriptive statistics of feather concentrations (*dw*) of metal and metalloid elements (Aluminium [Al], Arsenic [As], Cadmium [Cd], Mercury [Hg], and Lead [Pb]), stable isotope values ($\delta^{13}C$ and $\delta^{15}N$), and corticosterone (CORT) in tawny owls from 1986-2019. Some metals exhibited concentrations below the level of detection (LOD), hence different sample sizes of metals. In addition, a subset of the total sample size was analyzed for stable isotopes.*

	n	Median	Mean (± SD)	Min	Max
Al (μg g^{-1})	1168	0.830	1.689 (± 5.144)	< 0.17 (LOD)	146.1
As (μg g^{-1})	1165	0.038	0.062 (± 0.141)	< 0.008 (LOD)	2.378
Cd (μg g^{-1})	1133	0.001	0.003 (± 0.011)	< 0.001 (LOD)	0.193
Hg (μg g^{-1})	1169	0.750	1.061 (± 1.256)	0.088	17.80
Pb (μg g^{-1})	1167	0.018	0.067 (± 0.199)	< 0.001 (LOD)	5.025
$\delta^{13}C$ (‰)	500	-22.54	-22.57 (± 0.700)	-24.57	-20.83
$\delta^{15}N$ (‰)	500	7.129	7.295 (± 1.083)	4.330	11.63
CORT (pg mm^{-1})	1199	17.68	23.37 (± 21.04)	2.915	327.0

3.2 Direct effects of metal exposure

In this study, we found a direct positive effect of Al and Cd on fCORT, suggesting that higher concentrations of these two metals elevate stress hormone levels in the tawny owl (Fig. 3a, b). This corroborates findings from previous studies reporting positive relationships between non-essential metals (including Cd and Hg) and fCORT in wild birds and raptors (Meillere et al., 2016; Powolny et al., 2020; Strong et al., 2015). In contrast, we found that Hg was negatively related to fCORT concentrations, suggesting that owls with higher Hg levels had lower fCORT concentrations (Fig. 3c).

Findings in the current and previous studies suggest that exposure to metals can modulate fCORT in wild birds, but determining whether elevated or lowered fCORT levels indicate negative effects on physiological stress is challenging. That is, higher fCORT could be a sign of chronic elevated CORT, or the accumulation of multiple stressful events during molt (Romero and Beattie, 2021). Also, lower CORT levels do not always indicate healthy individuals and may reflect difficulties in coping with stressors (Romero and Beattie, 2021) or suppressed baseline CORT levels, affecting various physiological functions detrimentally (Whitney and Cristol, 2018).

Various factors could account for the divergent fCORT-metal relationships. For example, wild birds like the tawny owl are exposed to a mixture of metals that enter their environment. Hence, the impact of metal exposure on physiological indicators such as fCORT might stem from the additive, synergistic, or antagonistic consequences arising from interplays among various metals (Lin et al., 2016; Powolny et al., 2020). Consequently, the opposite effects of Al, Cd, and Hg on fCORT could potentially arise from Al and Cd counteracting the actual impact of Hg. Alternatively, it could be the reverse scenario, where the impacts of Al and Cd are mitigated or masked by the presence of Hg.

Furthermore, all metals except Hg were positively correlated with each other (Fig. S1). Hg, on the other hand, was correlated with As only, and in a negative manner (Fig. S1), indicating that the presence of Hg in the tawny owl differs from the other metals.

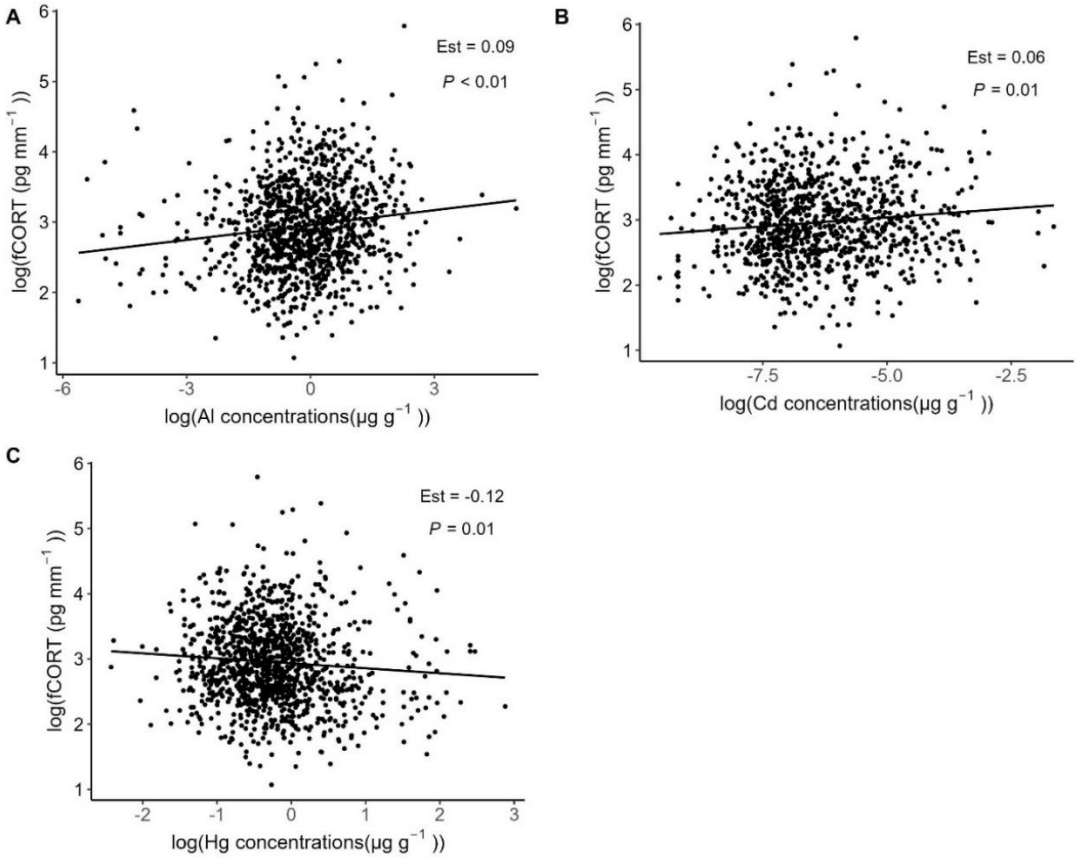

Figure 3 Feather corticosterone (fCORT) in relation to A) Aluminium (Al), B) Cadmium (Cd), and C) mercury (Hg) concentrations in tawny owls in Central Norway in the period 1986-2019. Parameter estimates (Est) and p-values are reported in Table S2.

3.3 Direct effects of dietary proxies

We found that higher $\delta^{15}N$ was associated with elevated fCORT levels (fig. 4a), while higher $\delta^{13}C$ was linked to lower fCORT levels (fig. 4b), suggesting that changes in dietary ecology may be able to modulate fCORT concentrations in the tawny owl, consistent with previous findings (Fairhurst et al., 2015; Fairhurst et al., 2013b; Randulff et al., 2022; Will et al., 2015). $\delta^{13}C$ values ranged from -20‰ to -24.4‰ (Table 1) and aligned with the typical range observed in predators that feed on the terrestrial ecosystem, that is, below -20 ‰ (Inger and Bearhop, 2008). Further, the $\delta^{15}N$ values observed in our study were highly variable, ranging from +4.3‰ to +11.6‰ (Table 1), suggesting that the prey items consumed by the tawny owls originated from various trophic levels (since each trophic level is successively enriched by ~3-4 ‰ (Kelly, 2000)). Notably, common prey of the tawny owl such as omnivorous and insectivorous animals including passerine birds and amphibians typically exhibit higher $\delta^{15}N$-values compared to herbivorous animals such as voles, as documented by e.g., Swan et al.

(2020). In contrast to $\delta^{15}N$, $\delta^{13}C$ is usually only marginally enriched between trophic levels (0-1‰) and therefore not deemed suitable to distinguish among trophic levels of prey (Inger and Bearhop, 2008). However, a study by Villegas et al. (2021) found that $\delta^{13}C$ in herbivorous finches was higher than in insectivorous finches, indicating that $\delta^{13}C$ might be useful for distinguishing among dietary guilds in free-living birds.

Tawny owls are known for their opportunistic feeding habits, adapting their prey preferences according to seasonal availability—favoring rodents in winter and birds in summer (Sunde et al., 2001), but feed mainly on rodents, particularly voles, when they are abundant (Solonen, 2009). Previous studies have demonstrated a positive correlation between the abundance of voles and the body condition of the owls (Solonen, 2009; Solonen, 2022), such that the presence of voles is also an important factor determining their breeding success (Millon et al., 2010). It is well established that small mammals, like voles, exhibit cyclical population fluctuations (Oli and Dobson, 2001). Long-term studies on small mammal populations in Europe and Fennoscandia have reported a dampening of population cycles since the 1980s (Cornulier et al., 2013; Hörnfeldt, 2004), which likely would cause alterations in the prey availability of small mammal predators like the tawny owl (Avotins et al., 2023; Millon et al., 2014). Interestingly, we detected a significant decline in $\delta^{13}C$ from 1986 to 2019 (Fig. S2; $p < 0.01$), after correcting for the Suess effect. The decline in $\delta^{13}C$ values indicates a shift in carbon sources from prey with higher $\delta^{13}C$ to prey having lower $\delta^{13}C$ across the study period. Based on the findings by Villegas et al. (2021)), decreasing $\delta^{13}C$ in the tawny owls might suggest that the proportion of insectivores in their diet, such as passerine birds or amphibians, have increased in the period 1986-2019. However, given the slight annual average decline of -0.01 ‰ and the fact that we do not have specific stable isotope values for the prey items, this interpretation should be treated with caution. We can therefore only speculate that the decrease in $\delta^{13}C$ detected in our study reflects a shift in prey composition with more insectivores (e.g., thrushes and frogs) than herbivores (e.g., voles). Further investigation is warranted to explore $\delta^{13}C$ in the prey of tawny owls to test this hypothesis.

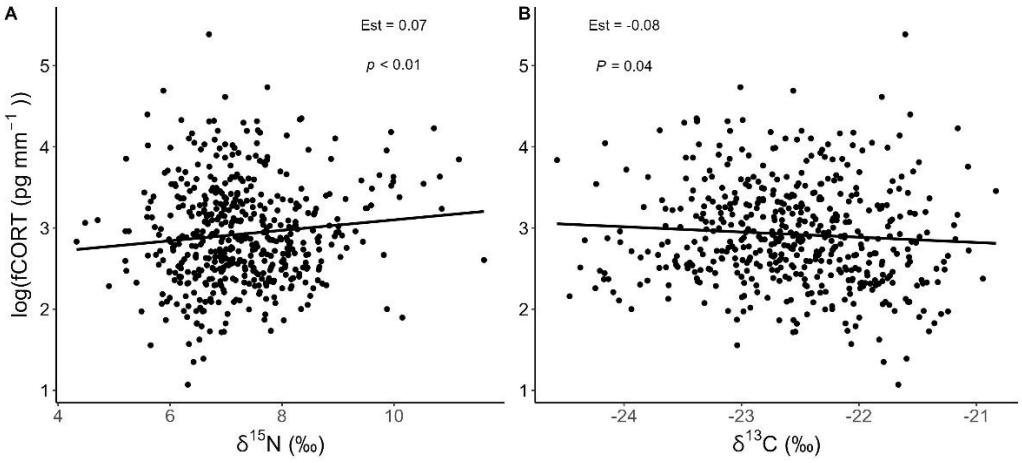

Figure 4 Feather concentrations of corticosterone (fCORT) in relation to A) $\delta^{15}N$, and B) $\delta^{13}C$. The black line indicates significant linear relationships (p-value) with coefficient estimates as reported in Table S2.

3.3 Indirect and direct effects of diet and Hg on fCORT

While our study revealed direct effects of metal exposure (i.e., Al, Cd, and Hg) and dietary proxies on fCORT in the tawny owl, we also reported indirect effects of dietary proxies. Namely, there was a direct negative effect of $\delta^{15}N$ (fig. 5a) and a direct positive effect of $\delta^{13}C$ (Fig. 5b) on Hg concentrations, suggesting that dietary patterns influence Hg exposure. Consequently, dietary ecology indirectly affects fCORT levels, by mediating through exposure to Hg. In addition, the effect of dietary proxies on Hg could explain why we observe discrepant effects of metals on fCORT in the tawny owl.

Compared to other metals, Hg exhibits higher bioavailability (especially in its organic methylated form) causing it to accumulate readily in biota (Chetelat et al., 2020). In marine ecosystems, Hg biomagnification is well-documented, such that higher trophic level species show elevated Hg levels (Chetelat et al., 2020). In the terrestrial ecosystems that tawny owls inhabit, Hg biomagnification is generally less pronounced compared to marine ecosystems(Dietz et al., 2022). The process still occurs, but the dynamics are influenced by differences in food chain structure, species composition, and environmental conditions (Douglas et al., 2012). In general, we do, however, expect a positive relationship between $\delta^{15}N$ and biomagnifying contaminants such as Hg (Gómez-Ramírez et al., 2023). Surprisingly, our study reported a negative relationship between $\delta^{15}N$ and Hg (Fig. 5a), contrary to the generally documented positive association (Badry et al., 2019; Carravieri et al., 2018; Chetelat et al., 2020). We did, however, find a relatively strong positive effect of $\delta^{13}C$ on Hg (fig. 5b, $p < 0.01$), suggesting that the source of carbon (e.g., prey types) is linked to Hg exposure in the tawny owl. Our findings therefore imply that exposure to higher levels of Hg in the tawny owl is linked to lower trophic prey with lower $\delta^{15}N$ and with higher $\delta^{13}C$.

In essence, dietary patterns influence exposure to, and therefore also the effect of, Hg on fCORT in the tawny owls. More specifically, the effect of Hg (fig.3; path coefficient: -0.11) on fCORT is reduced if the indirect effect of $\delta^{15}N$ (0.018; calculated by multiplying path coefficients of $\delta^{15}N$ and Hg: -0.17 x -0.11) or $\delta^{13}C$ (-0.06; $\delta^{13}C$ and Hg: 0.56 x -0.11) are considered. Overall, the SEM pathways in our study revealed diverse scenarios of how fCORT can be influenced and potentially mediated by dietary patterns and the magnitude of metal exposure (Fig. 3). It is, however, essential to consider the strength of these path coefficients (maximum possible range -1 to 1), as some factors exert stronger effects than others, such as $\delta^{13}C$ on Hg (0.56), while the direct impact of metals on fCORT appeared weaker (0.13, 0.15 and -0.11 for Al, Cd and Hg, respectively). In conclusion, although metals and stable isotopes appeared to influence fCORT levels, interpreting high vs. low fCORT remains challenging

(Romero and Beattie, 2021). Nevertheless, the present study underscores the complex interactions between metals and dietary patterns in response to fluctuating environmental conditions. Consequently, when assessing stress in wild animals, it is essential to consider multiple variables, as various factors can impact the stress response, making the process intricate.

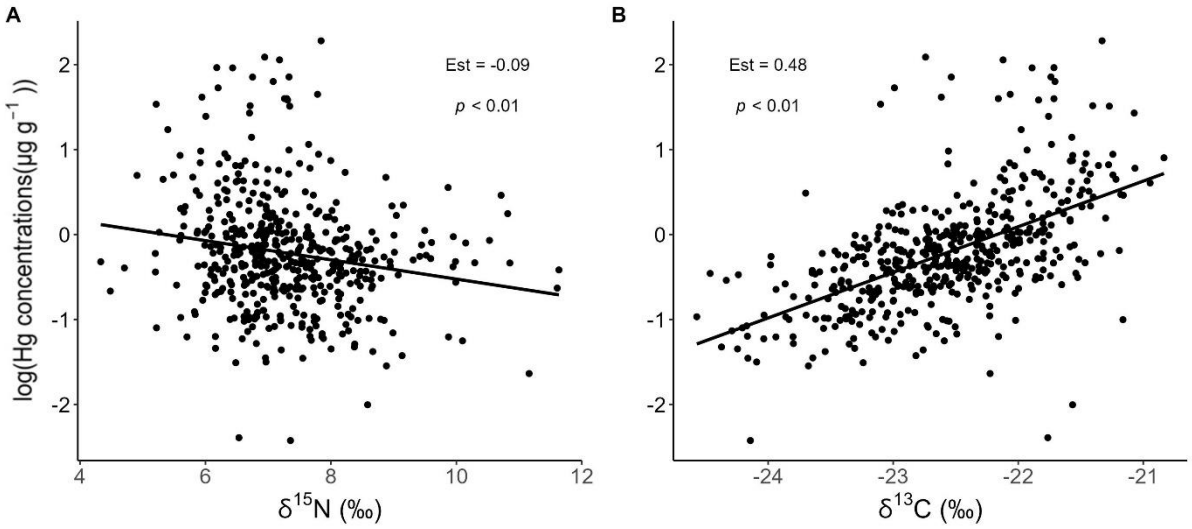

Figure 5 Feather concentrations of Hg in relation to A) $\delta^{15}N$, and B) $\delta^{13}C$. The black line indicates significant linear relationships (p-value) with coefficient estimates as reported in Table S2.

4 Conclusion

The use of feathers represents an excellent non-destructive integrated biomonitoring strategy for long-term retrospective analysis of stress physiology, toxic metal exposure and dietary patterns (proxied by stable isotopes) in avian wildlife. Indeed, such information is not easily accessible when studying wild birds using other methods. In the present study, we illustrated how feathers can be used to evaluate sublethal effects of environmental stress in wild birds by investigating the interrelationships between fCORT, metal exposure and stable isotopes over multiple decades in an established sentinel species, the tawny owl. Our study revealed both indirect and direct effects of $\delta^{13}C$, $\delta^{15}N$, and toxic metals (Al, Cd, and Hg) on fCORT, supporting our hypothesis that these factors may individually or jointly influence fCORT concentrations. Further research is required to fully comprehend the implications of these alterations on the tawny owl's health and fitness by investigating e.g., body condition and reproductive performance during and before molt, respectively. In addition, analyzing prey items and/or stable isotopes in the primary prey of tawny owls would offer valuable insights into how toxic metal exposure affects these owls in the context of a dynamic ecosystem.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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542

Feathers as temporal archives of environmental stress: a multi-decadal study on metal exposure, dietary proxies, and physiological stress in a terrestrial raptor

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Appendix: supplementary information

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*Table S1 Parameter estimates from linear models examining temporal trends in metal concentrations (ln-transformed; µg g^{-1} dry weight), $\delta^{15}N$ (‰), $\delta^{13}C$ (‰; corrected for the Suess effect), and feather corticosterone (fCORT, ln-transformed; pg mm^{-1}) analyzed in tail feathers of tawny owls (*Strix aluco*) sampled in 1986-2019.*

Response variable	Coefficient	Estimate	*SE*	*t*	*P*
Al	*Intercept*	37.39	7.822	4.780	0.000
	Year	-0.018	0.003	-4.803	0.000
			R^2	Multiple	0.020
				Adjusted	0.019
As	*Intercept*	47.17	6.867	6.870	0.000
	Year	-0.025	0.003	-7.364	0.000
			R^2	Multiple	0.045
				Adjusted	0.044
Cd	*Intercept*	159.7	16.28	9.806	0.000
	Year	-0.082	0.008	-10.18	0.000
			R^2	Multiple	0.507
				Adjusted	0.390
Hg	*Intercept*	8.796	4.345	2.024	0.231
	Year	-0.004	0.002	-2.078	0.038
			R^2	Multiple	0.004
				Adjusted	0.003
Pb	*Intercept*	224.4	6.933	32.37	0.000
	Year	-0.113	0.003	-32.92	0.000
			R^2	Multiple	0.490
				Adjusted	0.490
$\delta^{13}C$	*Intercept*	2.667	6.545	0.408	0.683
	Year	-0.012	0.003	-3.855	0.000
			R^2	Multiple	0.029
				Adjusted	0.027
$\delta^{15}N$	*Intercept*	25.67	10.24	2.506	0.012
	Year	-0.009	0.005	-1.794	0.073
			R^2	Multiple	0.006
				Adjusted	0.004
fCORT	*Intercept*	-10.38	3.997	-2.597	0.009
	Year	0.006	0.001	3.329	0.000
			R^2	Multiple	0.009
				Adjusted	0.008

Table S2 Coefficient estimates from linear mixed effect models and path coefficients (Std. Estimates) from structural equation models investigating the indirect and direct effects of stable isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$; corrected for the Suess effect) and metals (Al, As, Cd, Hg, Pb; all tested separately) on feather corticosterone (fCORT) in tawny owls sampled in Norway in 1986-2019. The sampling year was included as a random factor in all models. Text in bold indicates significant effects (P < 0.05).

Response	Predictor	Estimate	*SE*	*P*	Std. Estimate
Al	$\delta^{13}C$	-0.027	0.080	0.731	-0.018
Al	$\delta^{15}N$	0.079	0.048	0.102	0.082
fCORT	**Al**	**0.090**	**0.028**	**0.001**	**0.152**
fCORT	$\delta^{13}C$	-0.085	0.044	0.055	-0.094
			Individual R^2:		
			Response	Marginal	Conditional
			Al	0.006	0.119
			fCORT	0.034	0.206
As	$\delta^{13}C$	-0.145	0.084	0.084	-0.091
As	$\delta^{15}N$	0.068	0.051	0.182	0.065
fCORT	As	0.047	0.027	0.079	0.084
fCORT	**$\delta^{13}C$**	**-0.087**	**0.044**	**0.049**	**-0.097**
fCORT	**$\delta^{15}N$**	**0.079**	**0.026**	**0.002**	**0.140**
			Individual R^2:		
			Response	Marginal	Conditional
			As	0.012	0.089
			fCORT	0.038	0.228
Cd	$\delta^{13}C$	-0.009	0.079	0.908	-0.004
Cd	$\delta^{15}N$	-0.001	0.047	0.983	-0.000
fCORT	**Cd**	**0.064**	**0.027**	**0.017**	**0.139**
fCORT	**$\delta^{15}N$**	**0.077**	**0.026**	**0.003**	**0.137**
			Individual R^2:		
			Response	Marginal	Conditional
			Cd	0.000	0.520
			fCORT	0.042	0.249
Hg	**$\delta^{13}C$**	**0.480**	**0.035**	**0.000**	**0.569**
Hg	**$\delta^{15}N$**	**-0.092**	**0.021**	**0.000**	**-0.173**
fCORT	**Hg**	**-0.126**	**0.053**	**0.017**	**-0.118**
			Individual R^2:		
			Response	Marginal	Conditional
			Hg	0.352	0.400
			fCORT	0.014	0.193
Pb	$\delta^{13}C$	-0.008	0.078	0.913	-0.003
Pb	$\delta^{15}N$	0.051	0.046	0.261	0.036
fCORT	Pb	0.016	0.025	0.529	0.040
			Individual R^2:		
			Response	Marginal	Conditional
			Pb	0.001	0.664
			fCORT	0.001	0.195

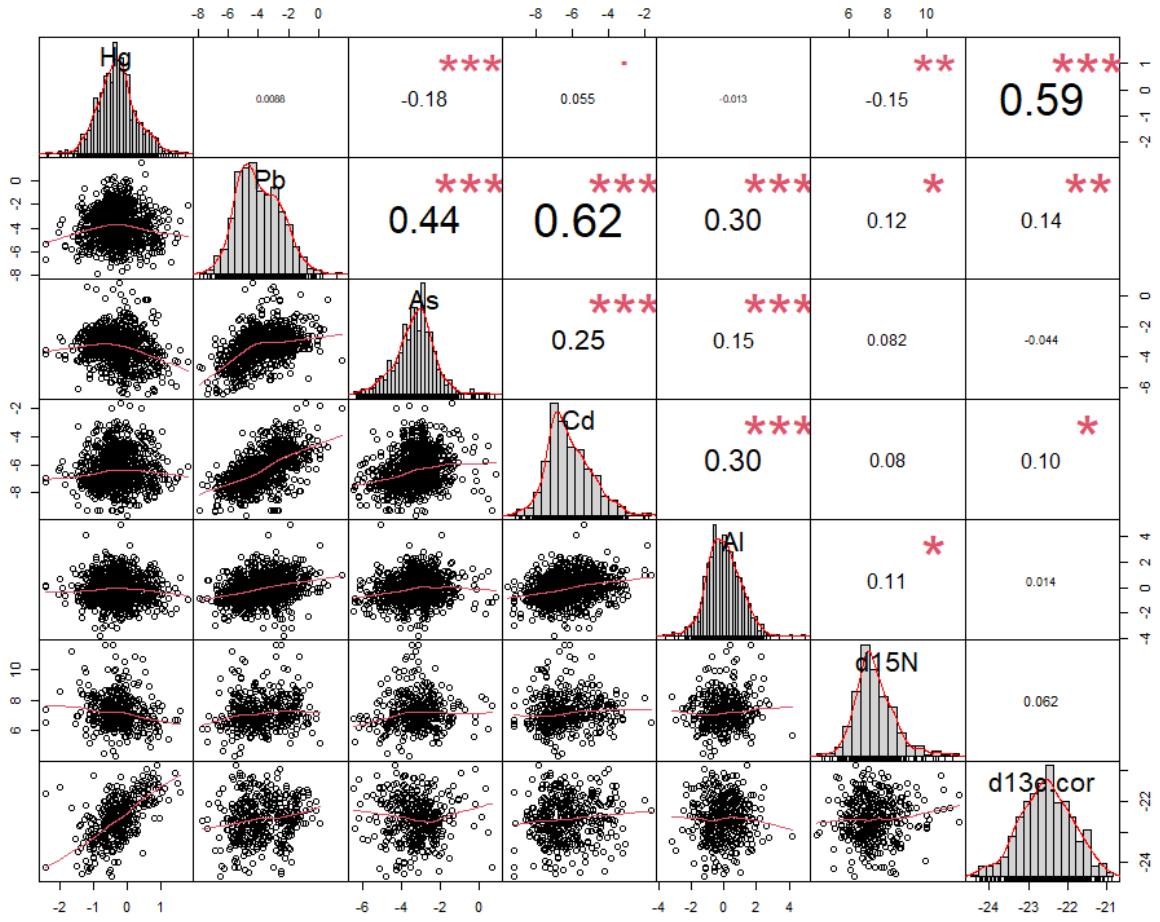

Figure S1 Correlation plot between metal concentrations (log-transformed) and stable isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C.cor$; corrected for the Suess effect) analyzed in tail feathers of tawny owls sampled in Norway in 1986-2019.

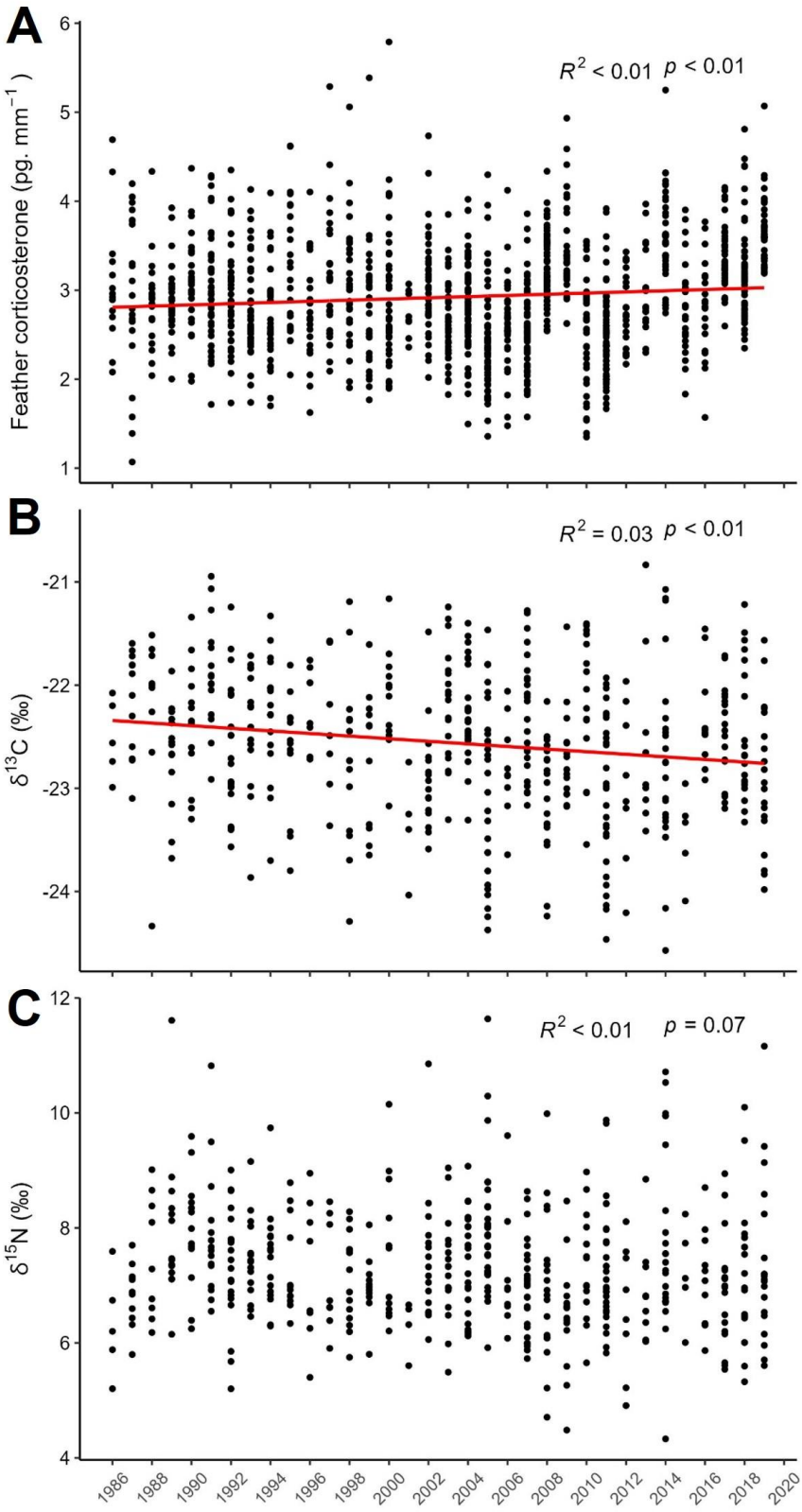

Figure S2 Temporal variations in A) feather corticosterone (ln-transformed concentrations), B) $\delta^{13}C$, and C) $\delta^{15}N$ analyzed in tail feathers of tawny owls sampled in Norway in 1986-2019. The red line indicates a significant effect of year based on parameter estimates from the linear models, including adjusted R^2 and p-values, reported in Table S1.