Metal concentrations in blood of white-tailed eagle (*Haliaeetus albicilla*) nestlings and potential effects on metallothionein induction

Paula Marcinekova
Master student of Environmental Toxicology and Chemistry

Norwegian University of Science and Technology

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Supervisors

Assoc. Prof. Veerle Jaspers
Environmental Toxicology
Dept. of Biology, NTNU

Dr. Mari Engvig Løseth
Environmental Toxicology
Dept. of Biology, NTNU

Abstract

Environmental pollution with metals in large marine raptors is not a well-studied area, besides for lead poisoning from ammunition. There is a great need for monitoring the evolving trends after several international environmental treaties and restrictions on metal production have been enacted. In this study, two sampling locations (Smøla archipelago and Steigen archipelago) and two sampling years (2015 and 2016) provided samples for analyzing internal exposure of white-tailed eagle (*Haliaeetus albicilla*) nestlings to 13 essential and non-essential metals. Arsenic (As), calcium (Ca), cadmium (Cd), copper (Cu), iron (Fe), mercury (Hg), potassium (K), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), selenium (Se) and zinc (Zn) were analyzed in the whole blood of 47 nestlings using inductively coupled plasma mass spectrometer (ICP-MS). The differences between the two study sites and the two years were analyzed using principal component analysis (PCA) and (generalized) linear models. The results show that three metals (Hg, Mg and Mo) were significantly different between Smøla and Steigen when the two years were pooled together. In all cases, the levels were higher in Smøla compared to Steigen. Only Na showed significant differences between 2015 and 2016 when the data from the two locations were pooled together. The effect of diet, trophic level and sex on metal concentrations were also studied. There was no significant difference in any of the 13 metals between sexes. Diet and/or trophic level were found to be significant factors influencing the concentrations of As, Fe and Se. The potential effect of five metals (As, Cd, Cu, Hg and Zn) on metallothionein (MT) induction was also studied using a commercial ELISA assay. The MT concentrations ranged from 4.98 to 10.78 ng/ml plasma with a mean concentration of 7.73 ± 0.27 ng/ml and were not significantly influenced by any of the five metals. In addition, MT induction was investigated for potential influence by biological factors (sex and age). However, no significant influence was found. Overall, the present study showed that concentrations of essential and non-essential metals in Norwegian white-tailed eagle nestlings are mostly influenced by location (industrialized vs. isolated area), diet (marine vs. terrestrial based) and trophic level.

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Abbreviations:

AICc – Akaike's Information Criteria

Al – Aluminum

AMAP – Arctic Monitoring and Assessment Programme

As – Arsenic

BCI – Body Condition Index

Ca – Calcium

CAT – Catalase

Cd – Cadmium

Co – Cobalt

Cr – Chromium

Cu – Copper

DDT – Dichlorodiphenyltrichloroethane

DF – Detection Frequency

EEA – European Environmental Agency

Fe – Iron

GPx – Gluthation Peroxidase

Hg – Mercury

ICP-MS – Inductively coupled plasma mass spectrometer

IDL – Instrumental Detection Limit

K – Potassium

LOD – Limit of Detection

MeHg – Methylmercury

Mg – Magnesium

Mn – Manganese

Mo – Molybdenum

MT – Metallothionein

Na – Sodium

Ni – Nickel

NIVA – Norwegian Institute for Water Research

NOAEL – No Observed Adverse Effect Level

OCP – Organochlorine Pesticides

PBDE – Polybrominated diphenyl ether

PCA – Principal Component Analysis

PCB – Polychlorinated Biphenyl

Sb – Antimony

Se – selenium

SMA – Standardized Major Axis

SOD – Superoxide Dismutase

U.S. EPA – United States Environmental Protection Agency

V – Vanadium

Zn – Zinc

1 Introduction

1.1 Metals

Metals are naturally occurring elements present in our environment since the formation of Earth. They come from various geological processes such as rock and soil formation and weathering and are also present in ground and surface waters and the atmosphere. The main natural sources of metals in the environment are magmatic, sedimentary and metamorphic rocks (Bradl 2005). In addition, direct processes such as the escape of gases and fluids along major fractures in the Earth's crust, forest fires and volcanic activity can provide significant sources of metals to the atmosphere and the sea floor (Garrett 2000).

Apart from the natural processes, metals are introduced to the environment via anthropogenic activities. Over decades, fossil fuel burning, coal combustion, mining and smelting processes, traffic and industrial activities have increased metal concentrations in the environment (Alyemeni and Almohisen 2014; Tchounwou et al. 2012). Metal pollution has been detected in terrestrial and aquatic environments, including soils, wastewater treatment plant effluents, rivers, lakes, oceans and the atmosphere (Tchounwou et al. 2012). Both in densely populated areas and at remote sites, concentrations of many metals are generally much higher than expected based on their natural occurrence. The emitted metals are often deposited back into the terrestrial or aquatic ecosystems, where they can be taken up by living organisms (Pacyna and Pacyna 2001). Lead (Pb), mercury (Hg) and cadmium (Cd) pose the largest threat to the biota due to their profound toxicity even in very small concentrations (AMAP 2005). These metals are found in high concentrations close to the source of pollution, but the most volatile ones, e.g. Hg, can be also transported through the atmosphere to remote sites and induce adverse health effects to organisms far away from the point source of pollution (AMAP 2011). It is therefore important to identify the major emission sources and regions. It is also necessary to establish bio-monitoring programs that can assess the current contaminant levels in the environment and determine the level of exposure in different ecosystems (Bustnes et al. 2013; AMAP 2005).

1.1.1 Emissions and sources of metals in the environment

In the past three decades, a great effort has been made by national and international authorities to decrease the European emissions of metals. A 2017 report from the European Environmental Agency (EEA) states that emissions of Pb, Hg and Cd decreased by 92%, 81% and 73%

respectively between 1990 and 2015 (European Environmental Agency 2017). However, these three metals together with other trace elements (copper (Cu), chromium (Cr), manganese (Mn), nickel (Ni), antimony (Sb), selenium (Se), vanadium(V), and zinc(Zn)) and a metalloid arsenic (As) are still being released in great amounts, in particular by industries located in Central and Eastern European countries (European Environmental Agency 2018). An EEA report from 2018 states that in 2016, 978 European facilities have been releasing metals into the air with 18 of them responsible for more than 50% of the associated environmental pressure (Figure 1A, European Environmental Agency 2018). Additionally, another 2585 facilities were reported to have released metals to water bodies across Europe in 2016 (Figure 1B, European Environmental Agency 2018). On a global scale, the major anthropogenic sources of Cr, Hg, Mn, Sb, Se, Sn, Ni and V are the fossil fuel combustion with a respect to coal and oil combustion and non-ferrous metal production. Vehicular traffic and the combustion of leaded and low-leaded gasoline still heavily contributes to atmospheric Pb emissions worldwide (van der Gon and Appelman 2009; European Environmental Agency 2017).

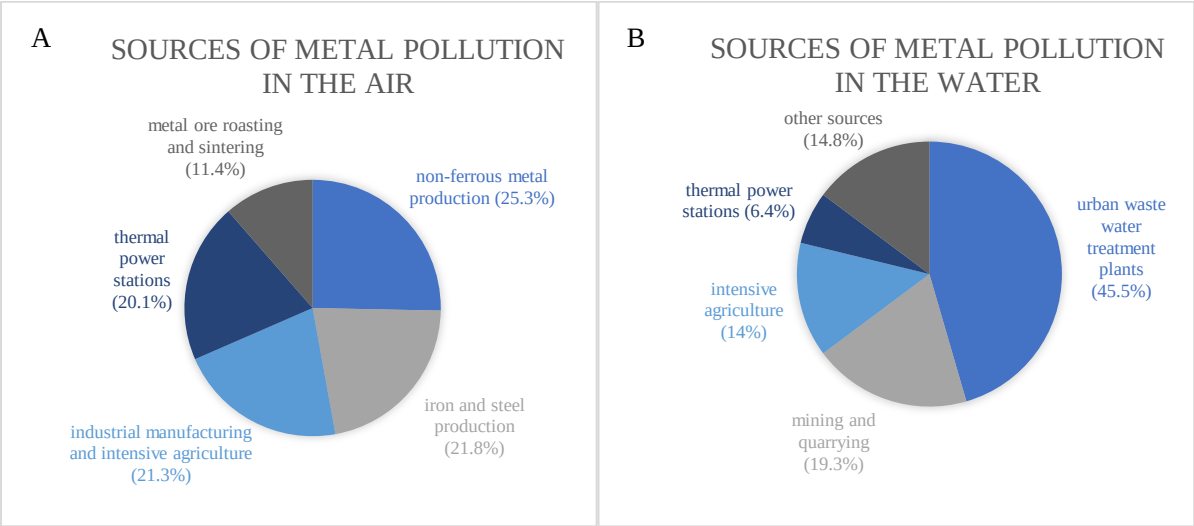

Figure 1: Sources of metal pollution released into the air (A) and the water (B) in 2016 by 978 and 2585 European facilities, respectively (European Environmental Agency 2018).

Although Scandinavia is considered to be one of the least polluted areas and the smallest contributor to European metal emissions, concentrations of some metals in the soils, water and air in this region is still higher than expected from the natural background levels (Harmens et al. 2004). This trend can be attributed to 1) local sources of pollution originating from past mining activities, 2) oil and fishing industry, 3) metal production in Kola peninsula, Russia (Koptsik et al. 2003). The Røros copper mine, although closed since 1997, is a good example of a local pollution source in Norway. Gundersen et al. (2001) found that concentrations of

dissolved Cu, Cd, Zn and aluminium (Al) in Naustebekken and Rugla streams, Røros still exceed the guidelines recommended by both Norwegian Institute for Water Research (NIVA) and United States Environmental Protection Agency (U.S. EPA), especially during episodes of high precipitations and floods (Gundersen et al. 2001). The northernmost border region between Norway and Russia is yet another example of a local pollution source in Norway. The cities of Nikel and Zapolyarny in Kola peninsula, Russia, are home to Severonikel Co., one of the largest Cu and Ni smelters in the world (Amundsen et al. 1997). The production of Ni and Cu in 2016 was 131 and 70 tons respectively, releasing several hundred tons of metals (Ni, Cu, Cd, Cr, cobalt (Co), As, Pb, Hg and Zn) into the environment (Nornickel 2016). These metals enter the local soils, streams and air and are transported to lakes and rivers in the Norwegian Arctic where the levels of these contaminants are often found to be higher than EU maximum levels suggested by EEA (Berglen et al. 2016; Hansen et al. 2017). Both European and global emissions as well as local sources contribute to metal pollution in Norway. Although the levels are often much lower compared to the rest of the world, the concentrations of metals in some areas still reach or exceed the guidelines proposed by environmental agencies.

1.1.2 Transport and deposition of metals in the environment

Long-range transport of volatile and semi-volatile pollutants, including metals, can result in high pollutant concentrations far from the source site. It is well documented that metal pollution in pristine northern Europe and Arctic regions is derived from distant sources brought to these regions by air currents (AMAP 2005; Harmens et al. 2004). Studies show that many metals (Pb, Zn, Cu, As, Sb, Se, Cd, Mn, Mo, V) have a potential for long-range transport and are deposited onto soils, plants and mosses, rivers and lakes (Steinnes et al. 1997; Steinnes 2001).

In Norway, mosses have been used as indicators for atmospheric pollution as they absorb nutrients from the air (Steinnes 1980). A study by Steinnes 1980 showed that a large part of metal deposition in Norwegian mosses is due to sources south and south-west of Scandinavia (Steinnes 1980). A study carried out 21 years later, however, indicates that while some metals (V, Zn, As, Se, Mo, Cd, Sn, Pb) are still transported to Norway from continental Europe, other (Cr, Ni and Cu) are derived from point sources in Norway and Russia (Steinnes 2001).

A monitoring study from Zeppelin atmospheric research station, Svalbard, between 1994 and 2003 showed that many metals, including Cd, Pb and Hg were detected in the same concentrations each year, without downward trend (Berg et al. 2004). However, the atmospheric measurements at Zeppelin started in 1994, long after the emission control had been introduced in Europe and may explanation for the lack of a further decrease of these

contaminants at this research station (Berg et al. 2004). Nevertheless, the concentrations are much lower compared to the concentrations registered at Zeppelin in the 1970s and 1980s (Maenhaut et al. 1989). In the period from 1994 to 2003, only Ni displayed a decreasing trend at Zeppelin, which may be due to the introduction of emission control in industrial processes and smelters, especially at the Kola peninsula, Russia, during this period (Berg et al. 2004).

Due to the distinctive behavior of Hg in the atmosphere, studies have focused on atmospheric transport and deposition of Hg separately from other metals. Hg can be transported over longer distances and may deposit at a higher rate than other metals. In its elemental form, Hg can stay in the atmosphere for up to one year before deposited onto land or oceans as oxidized Hg^{2+}. It can also travel much larger distances, even across the whole globe (Baya and Van Heyst 2010). In Norway, the atmospheric long-range transport of Hg is larger than the Norwegian Hg emissions. However, since Hg can be transported over great distances and stay in the atmosphere for a long time before deposited, it is often difficult to ascribe the emissions to a specific country (Berg et al. 2006). Nonetheless, Hg concentrations in Norway decreased from maximum levels in the 1980s by 30% in the late 1990s and then plateaued in the 2000s (Berg et al. 2006). Again, this is a result of the reduction in European and global Hg emissions. The Hg emissions have decreased from 630 t in 1990 to 340 t in 1995 and further down to 200 t in 2000 in the EU (EMEP MSC-W 2002). Globally, Hg emissions decreased from 3600 t/year during the 1980s to 2000 t/year in the second half of the 1990s (Pacyna and Pacyna 2002). It seems that the deposition of metals in Norway has been reduced significantly since the 1980s, which can be largely attributed to employment of regulations of metal emissions both in Europe and globally.

1.1.3 Essential and non-essential elements

Metals can be divided into two groups depending on whether or not they are crucial for a living organism to carry out its cellular processes. These groups are called the essential and non-essential elements. The essential metals include calcium (Ca), Co, Cu, Cr, iron (Fe), magnesium (Mg), Mn, molybdenum (Mo), sodium (Na), Ni, Se and Zn. These are elements that have an important biological role in organisms (Tchounwou et al. 2012, Prashanth et al. 2015). The essential metals can be further divided into two subgroups: macro and micro (or trace) elements. The macro elements include Ca, K, Mg and Na and are needed in larger quantities by the organism, generally > 100 mg/day. The trace elements include Co, Cu, Cr, Fe, Mn, Mo, Ni, Se and Zn and are needed in quantities of < 100 mg/day (Fraga 2005). Both macro and trace

elements are essential for enzymatic activities, redox reactions, immunological reactions and physiological mechanisms, but can be toxic if they exceed the required quantity for accomplishing their biological function (Fraga 2005; Van Gossum and Neve 1998). Similarly, a deficiency of these elements can stimulate an alternate pathway which might lead to the development of diseases on a cellular level. Fe is an essential component of heme-proteins, including hemoglobin. However, Fe levels above the threshold values, especially when chronic accumulation occurs, can lead to hepatic damage, diabetes, arthritis, etc. Chronic Fe deficiency, on the other hand, can lead to anemia and heart failure (Chitturi et al. 2015). The dose-response curve for essential elements follow a similar shape, with levels below low-critical concentration leading to deficiency while levels above upper-critical concentration leading to toxicity and subsequent adverse effects on health (Ross et al. 2009).

Separate from the essential metals, the non-essential metals have no demonstrated biological role in the organism and are toxic even in very small amounts. These include Hg, Pb and Cd and a metalloid As (Jaishankar et al. 2014). An acute exposure to low doses of these elements is often associated with headaches, dizziness and/or vomiting whereas acute exposure to high doses and a chronic exposure is associated with neurotoxicity, hepatotoxicity, renal toxicity, skeletal damage, various organ failures and interactions with essential elements (Goyer 1997). The dose-response curve of non-essential elements shows that increasing concentration of those elements will induce an increased adverse health effect to the organism (Ross et al. 2009).

1.1.4 Accumulation in biota

Bioaccumulation can be defined as "the accumulation and enrichment of contaminants in organisms, relative to that in the environment" (Borgå 2013). Bioaccumulation of metals has been studied and well documented in marine and freshwater invertebrates, fish and birds for many decades (Bocher et al. 2003; Burger 2008; Hobbelen et al. 2006; Vinodhini and Narayanan 2008). The diet is often the most important exposure route, as well as inhalation and absorption through skin or gills (Lehman-McKeeman 2013). Metals that are absorbed into the organism can accumulate in different tissues and induce toxic effects once they reach concentrations above no-observed adverse effect level (NOAEL) (Maedgen et al. 1982). The concentrations of metals are often low in the lower trophic levels and increase greatly with each trophic level. Thus, long-lived top predators often have high metal concentrations and are therefore most susceptible to their toxic effects (Bocher et al. 2003).

In birds, metal toxicity has been seen to influence reproduction, including reduced reproductive success and smaller clutch size. It has also been linked to reduced survival rate, decreased growth rate, and altered behavior such as delayed migration and alteration of feeding habits (De Francisco et al. 2003; Scheuhammer 1987). The non-essential metals, Cd, Pb and Hg, have received special attention as these metals are toxic even in very low concentrations. There is a strong evidence that they may also bioaccumulate in the food web (Burger 2008; Hahn et al. 1993; Maedgen et al. 1982). Hg is of particular concern as it is known to biomagnify with each trophic level when present in its organic form, methylmercury (MeHg) (Bocher et al. 2003; Hahn et al. 1993). MeHg exposure has also been linked to reduced reproductive success, decreased egg laying, impaired hatchability and decreased territorial fidelity at environmentally relevant concentrations (Bouton et al. 1999; Scheuhammer et al. 2007).

1.2 Antioxidant defenses

Oxidative stress is a disturbance in balance between free radicals and oxidative defenses leading to a possible tissue injury (Betteridge 2000). Free radicals are atoms or molecules with one or more unpaired electrons, enabling them to react more readily with other molecules compared to atoms or molecules without unpaired electrons (Halliwell 1994). These include for example hydrogen radical (•H), hydroxyl radical (•OH), superoxide anion ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) (Betteridge 2000; Halliwell 1994).

Free radicals can arise from endogenous cellular processes, such as mitochondrial oxidative phosphorylation, or from interactions with exogenous sources such as xenobiotics (Ray et al. 2012). Increased free radical formation can overpower the cellular antioxidant defense system in the body and lead to oxidative stress. Oxidative stress can then result in cellular damage (damage to lipids, nucleic acids or proteins) or cell death proceeded as necrosis or apoptosis. Such damages can increase a risk in developing carcinogenesis, neurodegeneration, atherosclerosis and diabetes or lead to aging (Halliwell 1994; Ray et al. 2012).

Free radicals can react either with other free radicals or other molecules like proteins or lipids. An example of two free radicals reacting together is a reaction between superoxide anion ($\bullet O_2^-$) and nitric oxide (•NO) producing a peroxynitrite ($ONOO^-$). Here, the two unpaired electrons – one from superoxide anion and one from nitric oxide – react to make a covalent bond between the two radicals. The product, a peroxynitrite, may damage proteins directly or decompose into toxic products including nitrogen dioxide gas, hydroxyl radical and nitronium ion. A free

radical reacting with amino acid guanine is an example of a reaction between a radical and other molecule. Guanine, for example, can react with hydroxyl radical (•OH), producing 8-hydroxyguanine radical that can further be oxidized to 8-hydroxyguanine or reduced to ring-opened guanine. These can cause mutagenic lesions and stop DNA replication, respectively (Halliwell 1994).

In order to protects themselves from free radical attack and the subsequent cellular damage, organisms have developed antioxidant defense mechanisms (Betteridge 2000). These can be either enzymatic or non-enzymatic and are vital for suppression of the oxidative stress (Betteridge 2000; Koivula and Eeva 2010). The major enzymatic antioxidant defense systems include the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) while the non-enzymatic systems include plasma proteins (such as ferritin, albumin etc.), metallothionein (MT), and dietary antioxidants (such as β-carotene, vitamin E and coenzyme Q) (Betteridge 2000; Koivula and Eeva 2010; Mirończuk-Chodakowska et al. 2018).

1.2.1 Metallothionein

One of the major antioxidant defense systems consists of metal binding proteins such as MTs (Betteridge 2000). These are cysteine-rich proteins that can protect a cell from metal-induced toxicity by binding metal atoms to sulfhydryl cysteine residues via the Fenton reaction (Ruttkay-Nedecky et al. 2013; Viarengo et al. 2000). MTs were discovered in 1957 as cadmium-binding proteins (Margoshes and Valiee 1957). Although they are involved in many cellular functions, their main role is to detoxify metals and maintain metal ion homeostasis in an organism (Ruttkay-Nedecky et al. 2013). MTs are phylogenically widespread and can be found among bacteria, fungi, plants and eukaryotes. MTs are highly conserved, sharing great structural homology between birds and mammals (Koivula and Eeva 2010). An MT molecule is composed of two binding domains, α and β, with an *N*-terminal part on the β-domain and *C*-terminal part on the α-domain (Ruttkay-Nedecky et al. 2013). The synthesis of metallothioneins is induced by metals, cytokines and hormones, but also by different oxidants (Viarengo et al. 2000). In mammals and birds, MTs bind to Cd but also to Cu and Zn if these are in excess (Ruttkay-Nedecky et al. 2013).

MTs have a great potential to be used in environmental monitoring programs (Amiard et al. 2006) and can be used as biomarkers of metal contamination in the environment (Cosson 2000). This is especially the case for areas where Cd and Cu contamination is prevalent. The levels of

MTs, related to metal contamination, have been monitored among several taxa including marine invertebrates such zebra mussel (*Dreissena polymorpha*), Mediterranean mussel (*Mytilus galloprovincialis*) and grooved carpet shell (*Ruditapes decussatus*), terrestrial invertebrates such burgundy snail (*Helix pomatia*), white-lipped snail (*Cepaea hortensis*), fish such European flounder (*Pleuronectes flesus*) and birds such great tit (*Parus major)* (Dallinger et al. 2004; Ivanković et al. 2005; Rotchell et al. 2001; Smaoui-Damak et al. 2009; Vanparys et al. 2008).

1.3 Stable Isotopes

Stable isotope analysis is a useful tool in determining diet, characterizing trophic relationships and constructing food webs in the biology field (Boecklen et al. 2011). Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes are used to determine dietary source and trophic position of the species, respectively (Jaeger et al. 2010). Stable carbon isotope ratios ($^{13}C/^{12}C$) undergo little increase (generally 0–1‰) between trophic levels and are mostly used to distinguish between marine and terrestrial diet (Løseth et al. 2019a; Inger and Bearhop 2008). Marine primary producers have higher $\delta^{13}C$ values compared to the terrestrial ones (Løseth et al. 2019a). Stable nitrogen isotope ratio generally increases between 2 and 4‰ at each trophic level. This is because the light ^{14}N is lost in waste products while the heavier ^{15}N is incorporated into organism's tissue (Inger and Bearhop 2008; Løseth et al. 2019a). Many studies have previously used blood and tissues in order to establish dietary sources and trophic level of an organism. However, these matrices reflect only recent C and N uptake of an organism. To study diet and trophic level over longer time periods, keratinized matrices such as feathers and claws are often used (Inger and Bearhop 2008; Løseth et al. 2019a). These tissues are metabolically inert and can therefore preserve the isotopic record indefinitely. This means that we can investigate not only the diet and trophic relations of present species but also the extinct ones assembled in museums worldwide (Inger and Bearhop 2008).

1.4 Avian biomonitoring

Environmental contamination and the impact of xenobiotics on the environment has in recent years become a much-debated topic among researchers, politicians and the general public. Concerns about the present situation and the future are rising, leading the scientific community to evaluate the damage that has already been inflicted and to predict possible outcomes that the future can bring (Pérez-López et al. 2008). Biomonitoring is an important tool for assessing spatial and temporal trends of population density, reproductive success, disease levels and

contaminant effects on single organisms or on whole ecosystem (Burger and Gochfeld 1995). In addition, many biomonitoring programs cover larger geographical areas and regions, enabling researchers to look for patterns on a broader scale (Gómez-Ramírez et al. 2014). Biomonitoring programs are often carried out over the course of several years (Gómez-Ramírez et al. 2014; Espín et al. 2016). A proper choice of bioindicators, i.e. species that reflect contamination of their ecosystem, is crucial. Since the 1960s, birds have been recognized as attractive bioindicators of environmental pollution (Abdullah et al. 2015). Raptors and seabirds are considered to be well suited to this role as they are well studied, long-lived species that cover broad home ranges. They are often at the top of the food chain and are large enough to offer enough samples for adequate analysis without sacrificing the animal (Abdullah et al. 2015; Becker 2003; Hollamby et al. 2006). In addition, avian top predators are often very sensitive indicators, responding quickly to environmental changes in terms of physiology, reproduction and demography. Nowadays raptors are used in monitoring programs to study temporal and spatial pollution trends, oil pollution, environmental changes such as habitat alteration or fragmentation and the effect of climate change (Becker 2003; Espín et al. 2016; Gómez-Ramírez et al. 2014). Sampling of relatively large sized raptors allows for non-destructive, possibly non-invasive sampling yet still acquiring enough sample for chemical analysis. Blood, feathers, eggs/egg shells and preen oil are used as matrices for analysis of organic and inorganic pollutants, including metals (Abdullah et al. 2015; Eulaers et al. 2011a; Espín et al. 2016). Feathers usually have the highest concentrations of metals because birds excrete substantial amounts of metals, especially Hg, through feather molt (Abdullah et al. 2015). In addition, external contamination from ambient environment can largely contribute to the metal concentration in feathers (Espín et al. 2016). Eggs on the other hand represent contamination through maternal transfer while metal concentrations in blood reflect direct contamination from the diet (Becker 2003).

1.4.1 Non-destructive sampling

Compared to feathers, blood is a lesser used matrix for analyzing and monitoring metals in birds as it is less convenient to sample and may only reflect recent exposure mostly from dietary sources (Martínez-López et al. 2005). However, a study on great skua (*Stercorarius skua*) suggests that Hg levels in blood can reflect intake of Hg from current diet, intake over longer periods or a balance between recent Hg intake and Hg released into the blood stream that has been stored earlier in soft tissue (Bearhop et al. 2000). Once an individual is exposed to a contaminant via ingestion, inhalation or dermal contact, it will be absorbed into the blood

stream reflecting the current concentration of the contaminant in the ambient environment and/or the diet (Redig and Arent 2008). However, if the contaminant is effectively absorbed into the internal tissue, as in the case of Pb being absorbed into bones or Cd being absorbed in kidneys, the concentration of that contaminat in the blood might not be representative (Redig and Arent 2008). In addition, it is important to know in which blood fraction the particular contaminant is found. While Zn, Cu and Fe are found in plasma, Pb, Hg and Cd reside in the whole blood (Martínez-López et al. 2005; Osofsky et al. 2001). In the case of metal analysis it is therefore better to analyze the whole blood in order to ensure that all metals are included.

1.4.2 Nestlings in biomonitoring

When monitoring metals in birds, it is important to select species and individuals which are easily accessed and sampled. Using nestlings as biomonitors has several advantages. Firstly, the metal concentrations in their body closely reflect the concentrations in the diet, enabling the identification of pollution in the local environment/area (Espín et al. 2016). Secondly, as nestlings are still developing, their body condition can provide information regarding the effect of pollutants on their health. Third, the fact that they are unable to fly makes the capture process much easier and less stressful for the animal (Eulaers et al. 2011b). And finally, nestlings that stay in nests are less influenced by physiological and ecological cofounding factors such as migration, reproduction, foraging and molting, which all can significantly influence the variation of pollutant concentrations between individuals (Eulaers et al. 2011b). However, using nestlings as biomonitors has some disadvantages. Even small variations in food composition, feeding habits and age can strongly affect the level of contaminants in nestlings and therefore reflect the contamination loads in the sampling area inadequately (Eulaers et al. 2011b; Jaspers et al. 2006; Løseth et al. 2019a).

1.5 White-tailed eagle

White-tailed eagles (*Haliaeetus albicilla*) are large and long-lived birds of prey residing in Eurasia, with more than half of the entire population living in Norway and Russia. They are marine species with hunting territories largely composed of coastal regions, although inland lakes (especially in Russia) constitute a minor part of their territories (Sulkava et al. 1997). As top predators, the white-tailed eagles are at the top of the food chain and therefore more susceptible to effects of biomagnifying compounds. The diet of white-tailed eagles is mostly composed of marine fish species such as trout, cod and salmon, making up to 90% of the total dietary intake in the coastal regions of Norway or Greenland (Wille and Kampp 1983). The remaining 10% is ascribed to various bird species, fox pups, lambs and lagomorphs (Whitfield

et al. 2013). The territory size of white-tailed eagles might vary from as few as 2 km^2 to several hundreds of square kilometers. The egg laying period starts in mid-March and finishes by the end of May in Norway with an average clutch size of 2 eggs per breeding season (Dahl et al. 2013). The nestlings are able to fly 12 weeks after hatching (Spence 2016), so the sampling of nestlings is preferred around 8-10 weeks of age, when the nestlings are still incapable of flying. This reduces the stress that can be induced by handling and trapping of the birds.

The population of white-tailed eagles is currently increasing, and it is estimated that around 3 600 pairs reside in Norway at present (Madsen 2015). However, as seen in the past, environmental pollutants pose a large threat to the eagle populations. In the mid-1950s to early 1980s the population of white-tailed eagles declined drastically in the Baltic Sea due to the harmful effects of dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs) and Hg (Herrmann et al. 2011). These birds may therefore be sensitive to environmental changes such as a sudden increase of environmental pollution or environmental changes. The current threats to white-tailed eagles also include continuing use of lead ammunition and with that associated occasional feeding on game meat (Nadjafzadeh et al. 2013).

1.6 Aim of the study

The aim of this master project was to investigate levels of essential (Ca, Cu, Fe, K, Mg, Mn, Mo, Na, Se and Zn) and non-essential (As, Cd, Hg) metals and elements in the blood of white-tailed eagle nestlings residing in coastal areas in Norway. Differences between two locations and years were investigated as well as potential differences between sexes. Blood samples were taken from white-tailed eagle nestlings at two locations in two sampling years. Additionally, MTs potential as a biomarker of metal exposure in these nestlings was studied.

The overall aim was further divided into five specific aims:

1) Is there a difference in metal concentrations in nestlings between the two locations, Smøla and Steigen? Here, I expect to see some differences, because Smøla is located close to Trondheim, which is a relatively large industrial city, while Steigen is located in the north of Norway in a more pristine environment.
2) Is there a difference in metal concentrations in nestlings between the two years, 2015 and 2016? Here, I only expect small variations in the metal concentrations between the two years, because one-year difference between the two sampling years might be too short for a significant change in environmental metal contamination.
3) Is there a difference in metal concentrations between male and female nestlings? I expect to see females having higher metal concentrations compared to the males. The females have a larger size and thus require greater food consumption compared to the males. This can potentially mean greater contamination loads from the diet.
4) Do diet and/or trophic level influence the metal concentrations in nestlings? Here I hypothesize that both diet and trophic level influence metal concentrations in blood, especially for metals that can biomagnify through the food chain.
5) Finally, does the level of metals (especially As, Cd, Cu, Hg and Zn) affect MT induction in white-tailed eagle nestlings? The hypothesis for this question is that MT induction will be affected by the level of metals and the higher the metal concentration the more MT will be induced.

2 Methods

2.1 Fieldwork and sampling

The fieldwork was carried out throughout two summer seasons in June-July in 2015 and 2016 at Smøla, Møre og Romsdal county, and Steigen, Nordland county in Norway. Blood, back feathers and preen oil were sampled from white-tailed eagle nestlings from Smøla 2015 (n_{total} = 13, n_{nest} = 10), Steigen 2015 (n_{total} = 12, n_{nest} = 8), Smøla 2016 (n_{total} = 22, n_{nest} = 14) and Steigen 2016 (n_{total} = 20, n_{nest} = 15). The number of nestlings per nest varied from 1 to 3 and all nestlings present in the nest were sampled (unless the nestlings were too young to be sampled). The GPS coordinates for each sampling site were noted and the sites are visualized in Figure 2.

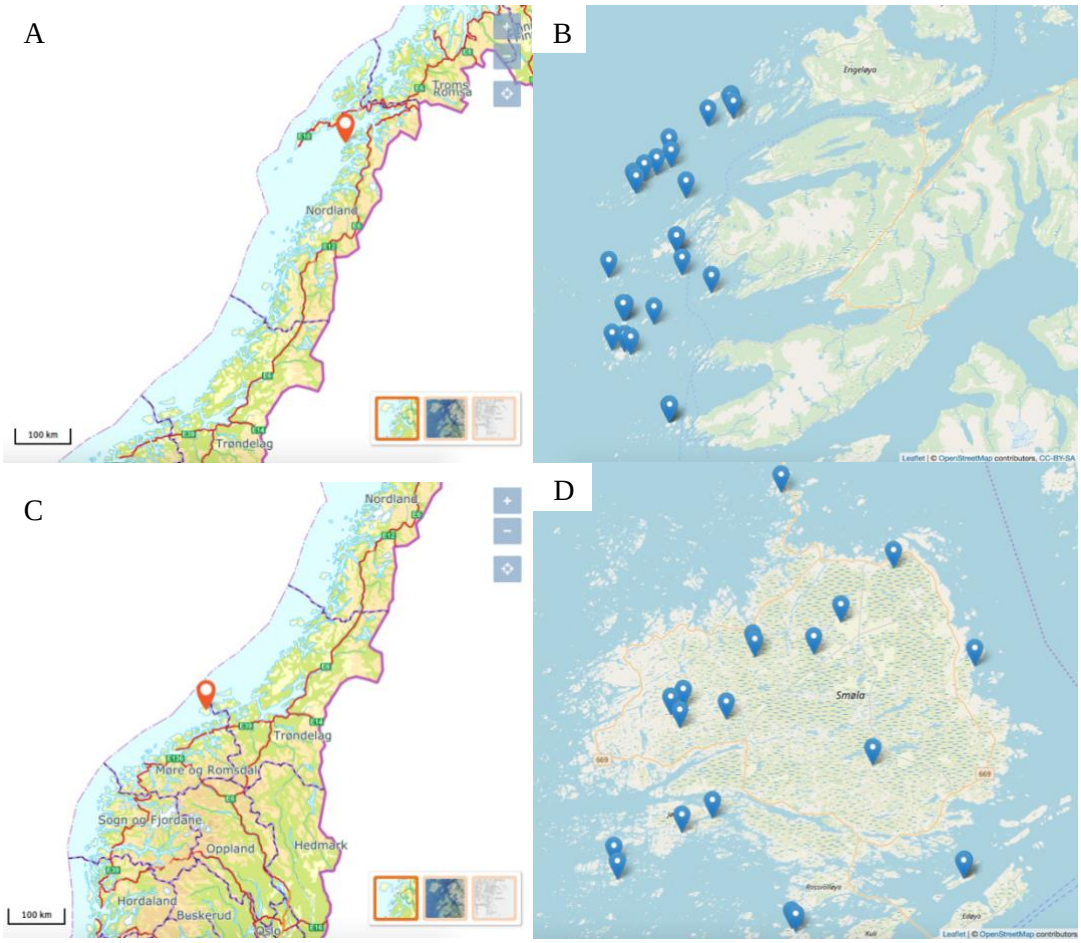

Figure 2: Map showing the sampling sites in Steigen archipelago, Nordland county (A,B) and Smøla archipelago, Møre og Romsdal county (C,D), Norway. The programs used to create the maps are Norgeskart.no (left) and OpenStreetMap.org (right).

2.1.1 Sampling techniques

The sampling sites were observed by local staff early in the breeding season to check for possible activity in the nests. The nests that were clearly inhabited by adult couples were revisited in June-July both in Smøla and Steigen archipelago and the nestlings were sampled.

Some nests were built high up in the trees, so the nestling had to be taken down in a large bag or backpack first. The measurements and sampling took approximately 10 - 15 minutes per bird. Afterwards, each bird was safely put back into its nest. Biometric measurements were recorded and blood, preen oil and back feathers were sampled. Using a heparinized syringe, 8 ml blood was sampled from a branchial vein into a 15 mL heparinized vacutainer. Eight to ten back feathers were pulled from the nestling's back and put into a ziplock bag. In addition, molted feathers from around the nest, prey remains from the nest and field blanks (one from the nest and one from its close proximity) were taken. All samples were then put into an insulated cooler bag for transportation. If the crop was full, the mass of the crop content was estimated and then subtracted from the bird's body mass.

The blood samples were processed at the end of each sampling day in a set up laboratory and placed into cryovials that were then frozen down to -80 °C. Back feathers, preen oil, prey remains, molted feathers and field blanks were all stored at -20 °C.

2.1.2 Biometric measurements

The biometric measurements including bill length, bill height, wing length, tail length, hallux length, tarsus width and tarsus depth (recorded in mm) were measured using a Vernier caliper or metal ruler. Body weight was recorded in grams using a spring scale. Weight of the crop content was estimated by holding the crop of the bird.

2.1.3 Estimating sex and age

Sex of birds was estimated directly in the field from the morphometric measurements, including beak height, tarsus width and tarsus depth (Løseth et al. 2019a).

Age was estimated from the tail feather length for both male and female nestlings according to Equation 1. The tail feather appears at day 30 and it grows with 4.95 ± 0.02 mm (mean ± SE) every day (Løseth et al. 2019a).

$$\text{Age} = 30 + (\text{tail length (mm)}/4.95) \qquad \text{(Equation 1)}$$

2.1.4 Body Condition Index (BCI)

The scaled mass index (Peig and Green, 2009) was estimated by standardized major axis (SMA) regression and used as an index of body condition of the white-tailed eagle nestlings. All linear body measurements (bill length, bill height, wing length, tail length, hallux length, tarsus width

and tarsus depth) were log transformed and compared to body mass transformed by the natural logarithm. Using Spearman rank correlation test, bill height and tail length showed the strongest correlation with body mass for males and females, respectively. However, since bill height was also strongly correlated with the body mass in females, only the bill height was used in further calculations for both males and females. This was used in BCI calculation by Equation 2:

$$BCI = M_i * (L_0/L_i)^{bSMA} \quad \text{(Equation 2)}$$

Where M_i is the body mass (g), L_0 is the mean bill height (mm), L_i is the bill height (mm) of the individual and b_{SMA} is the estimated slope of SMA regression on transformed variables (ln(body mass)~ln(bill height)). Results are presented in Appendix C.

2.1.5 Stable Isotopes

The stable carbon (^{12}C and ^{13}C) and nitrogen (^{14}N and ^{15}N) isotopes were analyzed in body feathers that were still growing at the time of sampling. The feather calamus was first detached from the feathers and the feathers were washed with Milli-Q water and cut into small pieces using stainless steel and glass tools. Homogenized feather material was then analyzed for its elemental and isotopic composition using a vario MICRO cube elemental analyzer coupled to an IsoPrime100 mass spectrometer. The reported stable carbon and nitrogen isotope values are expressed as δ (‰) (Løseth et al. 2019a).

2.2 Elemental analysis

2.2.1 Blood preparation

Eppendorf tubes containing 100-150 mg of whole blood were melted on ice and the blood was directly diluted with 0.5 mL concentrated nitric acid (HNO_3). The diluted blood was transferred into polyethylene ultraclave vials and decomposed at 50 bars nitrogen gas pressure for 2.5 hours using the UltraClave Microwave Digestion System produced by Milestone. The UltraClave report is presented in Appendix A. After the decomposition process, the samples were diluted with milliQ water to a final volume of 7-9 mL, the exact volume was noted, with a final HNO_3 concentration of 0.6 M. The samples were then transferred into conical-bottom tubes and used for ICP-MS analysis.

2.2.2 ICP-MS

Samples were analyzed with high resolution inductively coupled plasma mass spectrometer (HR-ICP-MS) using an argon gas. ICP-MS Instrument ELEMENT 2 produced by Thermo

Scientific was used. Each sample was analyzed for 36 elements using magnet settings of low, medium or high resolution (LR, MR, HR) to detect the specific element. Each isotope was scanned 3 times providing a mean and rsd value. The samples were run only once with a couple of repeating tests for a quality control.

2.3 Metallothionein analysis

A Metallothionein (MT) ELISA kit (MyBioSource) was used to analyze metallothionein concentration in plasma samples following the kit manual. The plasma samples were stored in -20 °C freezer until the day they were analyzed. One undiluted sample per nest was analyzed, with the sample from the oldest nestling preferentially used. The standards, blanks and the sample control (chicken serum) were added in triplicates while the plasma samples were added in duplicates with two samples added two times (2x duplicates) for quality control. The absorbance was read at 450 nm using the CYTATION 5 Imaging Reader produced by Biotek.

2.3.1 MT ELISA assay

The standard sample was prepared by adding 1.0 mL standard diluent into the Chicken MT lyophilized standard sample. The dilutions for the standard curve were then further prepared by adding 300 µL of standard diluent to 7 Eppendorf tubes and transferring 300 µL of standard sample from one tube to another providing a range of concentrations of 20, 10, 5, 2.5, 1.25, 0.625 and 0.312 ng/mL in tube 1, 2, 3, 4, 5, 6 and 7 respectively. Tube 8 contained only standard diluent, i.e. 0 ng/mL, and was used as a negative control.

One hundred µL of each standard sample and the negative control was then added into a 96-well microplate in triplicates. The wells for blank samples did not contain any solution at this stage. One hundred µL of pre-vortexed undiluted plasma samples from the nestlings and chicken serum used as a control were added into a 96-well microplate. The samples were incubated at 37 °C for 90 minutes. After incubation, the samples were washed twice with washing solution prepared by diluting washing buffer with double distilled H_2O (1:25, V:V). All wells, except for the blank sample wells, were coated with 100 µL biotinylated chicken MT antibody liquid. This liquid was prepared 30 minutes in advance by diluting concentrated biotinylated antibody with antibody diluent (1:100, V:V). The microplate was sealed with adhesive tape and incubated at 37 °C for 60 minutes. After incubation, the samples were washed three times with the washing solution. All wells, except for the blank sample wells, were coated with 100 µL enzyme-conjugate liquid that was prepared 30 minutes in advance by diluting concentrated enzyme-conjugate with enzyme-conjugate diluent (1:100, V:V). The microplate

was again sealed with adhesive tape and incubated at 37 °C for 30 minutes. The color reagent liquid was prepared 30 minutes in advance by mixing color reagent A with color reagent B (9:1).

After incubation, the samples were washed five times with washing solution and 100 µL of color reagent liquid was added to all wells, including the blank wells. The microplate was covered with aluminum foil and incubated for approximately 30 minutes at 37 °C until a blue color gradient of standard samples appeared. Further color development was stopped by adding 100 µL of color reagent C to each individual well (Figure 3). The absorbance value was read immediately at 450 nm.

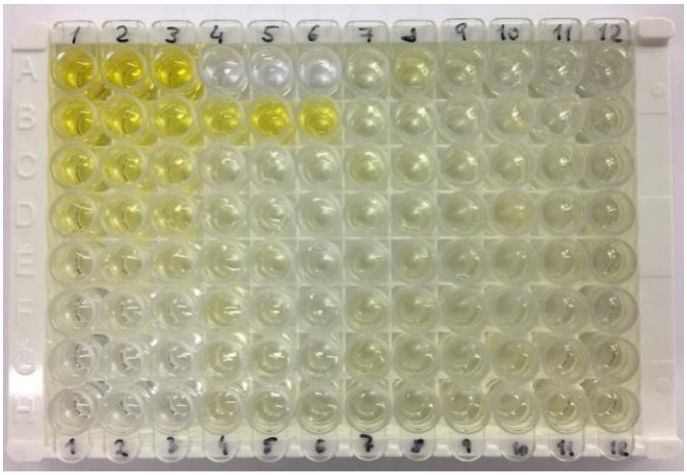

Figure 3: Metallothionein ELISA assay color development for standard samples (A1 – H3), blank sample (A4 – A6), chicken serum (B4 – B6) and plasma samples (C6 – H12).

2.4 Statistics

For statistical analysis and graphics, R version 3.4.3 (©R Development Core Team) and R Studio version 1.1.423 (©RStudio, Inc.) were used. Calculations were made in R Studio and Microsoft Excel version 16.19 (©2018 Microsoft Corporation). The significance level was set at 0.05 for all analysis. In case of two or three nestlings in one nest, only one nestling from that nest was randomly selected.

2.4.1 LOD and Data Treatment

The instrumental (IDL) and blank (LOD) reference limits of detection were calculated for each of the 36 metals. The ILDs were calculated by plotting the relative standard deviations (RSD) against concentration and the value at which the RSD gave approximately 25% increase was set as the instrumental detection limit. The LODs were calculated by multiplying the standard

deviations of the blanks by three. The blanks were also adjusted for the final dilution concentration of the samples and weight, giving the results in µg/L.

IDL and LOD values were compared and the higher of the two was used as a final threshold value for each metal. The detection frequency (DF) was calculated by dividing the number of detected samples (n> LOD) by the number of all samples (n). Metals with DF < 0.5, i.e. metals that were detected in less than 50% of the samples, were not used further in statistical analysis. The IDL, LOD and DF values are presented in Appendix B.

The values that were below the LOD were replaced by a substitution method which multiplies the LOD value by the DF value for each metal separately.

The median (min-max) values of the metal concentrations in the nestlings were chosen instead of the mean ± SE values due to the non-normal distribution of some of the metals.

2.4.2 Normality test

Metals were tested for normal distribution by Shapiro-Wilk test, quantile-quantile plots (QQ plot) and histograms. If the p-value was larger than 0.05 (p>0.05), the distribution of the data was not significantly different from normal distribution, i.e. normality could be assumed. Only Cu, K, Mn, Mo, Na, Zn satisfied the requirements for normal distribution when not transformed by natural logarithm (ln). After transformation by ln, As, Cd, Cu, Hg, K, Mn, Mo, Na and Se were seen to be normally distributed, while Ca, Fe, Mg, and Zn were not.

2.4.3 Univariate statistics

2.4.3.1 Correlations

To test a potential correlation between age, mass and BCI, the Spearman rank correlation test was used. The Spearman rank correlation test was also used to test for potential correlation between metals and age, mass and BCI. Results are presented in Appendix D.

2.4.3.2 Linear models

Linear models and generalized linear models were used to analyze which of the explanatory variables could best explain the variation between the metal concentrations. Linear models with normal distribution were used for metals that were normally distributed (As, Cd, Cu, Hg, K, Mn, Mo, Na, Se and Zn). Generalized linear models with gamma distribution were used for metals that were not normally distributed (Ca, Fe and Mg). All models were built with variable metal as a response variable and location, year, sex, stable isotopes and interaction between location and year as explanatory variables. If a possible interaction between location and the diet (stable isotopes) and/or an interaction between year and the diet (stable isotope) or an

interaction between the two stable isotopes could be assumed additional model was used to test for this.

Akaike's Information Criteria (AICc) was used on all models in order to select the best fitting model. AICc takes goodness of fit (R^2) and simplicity of the model into account to find the best fitting model (Burnham and Anderson 2004). The best fitting model was the one that had minimum AICc among all the other models and ΔAICc equal to zero. If several models, apart from the best fitting one, were discussed the ΔAICc value was specified. Linear models and the results from AICc test are presented in the appendix section (Appendix G).

2.4.4 Multivariate Analysis

Principal Component Analysis was used to visualize associations between metals and stable isotopes and to investigate the relationship between the two different locations and years and the metal concentrations in white-tailed eagle blood samples. All data were standardized by subtracting the mean value and dividing the data by the standard deviation. The data were not transformed by natural logarithm and the outliers were not removed. The first two principal components between variables and PCs, explained 29.7% (PC1) and 15.2 % (PC2) of the variation, respectively and the correlations were calculated.

2.4.5 Analysis of antioxidant defense

Five representative elements (As, Cd, Cu, Hg and Zn) were selected as explanatory variables for MT induction. These elements show the greatest affinity to metallothionein (Ruttkay-Nedecky et al. 2013) and were therefore expected to explain the variation in MT concentration in the nestlings' blood samples. All metals were normally distributed, and the interaction and additive effect between the metals was also tested.

The Shapiro-Wilk normality test and quantile-quantile plots (QQ plot) were performed. If the *p*-value was larger than 0.05 ($p>0.05$), the distribution of the data was not significantly different from normal distribution, i.e. normality could be assumed. The requirements for normal distribution were met and the data were not log transformed and the outliers not removed.
Linear models with normal distribution were performed to investigate the relationship between MT concentration (run as response variable) and the five representative elements (run as explanatory variables). PCA showed that the five elements are correlated. Therefore, separate models were run for each element independently. Sex and age were also included as explanatory variables.

3 Results

3.1 Metals

3.1.1 Metal concentrations in nestlings

The results show the nestlings' blood concentrations of 10 essential metals (Ca, Cu, Fe, K, Mg, Mn, Mo, Na, Se, Zn), 2 non-essential metals (Cd and Hg) and the metalloid As. All 13 elements were detected in > 50% of the samples, with As, Ca, Cd, Cu, Fe, K, Mg, Mn, Na, Se and Zn detected in 100% of the samples. Non-detected elements and elements detected in < 50% of the samples were not included in further analysis, but data are given in appendix. Median (min – max) blood metal concentrations in white-tailed eagle nestlings (µg/L) from Smøla 2015 (n=10), Steigen 2015 (n=8), Smøla 2016 (n=14) and Steigen 2016 (n=15) are presented in Table 1. The whole overview of metal concentrations, including arithmetic mean, standard deviation, standard error, median, minimum and maximum values, are presented in Table A4 and A5, Appendix E.

Table 1: Median (min – max) blood metal concentrations in white-tailed eagle nestlings (µg/L) from Smøla and Steigen in 2015 and 2016 (n=47).

	2015		2016	
Metal	**Smøla** (n=10)	**Steigen** (n=8)	**Smøla** (n=14)	**Steigen** (n=15)
As	$7.7x10^2$ ($2.1x10^2 – 3.2x10^3$)	$8.3x10^2$ ($3.4x10^2 – 1.5x10^3$)	$6.6x10^2$ ($3.5x10 – 3.3x10^3$)	$1.1x10^3$ ($3.4x10^2 – 2.8x10^3$)
Ca	$6.4x10^4$ ($4.9x10^4 – 8.2x10^4$)	$5.5x10^4$ ($1.8x10^4 – 7.9x10^4$)	$6.6x10^4$ ($3.8x10^4 – 8.2x10^4$)	$6.1x10^4$ ($2.4x10^4 – 8.2x10^4$)
Cd	0.85 (0.38 – 1.56)	0.70 (0.42 – 1.29)	0.64 (0.17 – 1.66)	0.77 (0.46 – 1.85)
Cu	$4.0x10^2$ ($3.4x10^2 – 5.0x10^2$)	$3.5x10^2$ ($2.6x10^2 – 4.0x10^2$)	$3.5x10^2$ ($2.8x10^2 – 4.2x10^2$)	$3.8x10^2$ ($2.7x10^2 – 4.1x10^2$)
Fe	$3.1 x10^5$ ($2.1x10^5 – 3.4x10^5$)	$2.7x10^5$ ($1.6x10^5 – 3.1x10^5$)	$2.6x10^5$ ($1.2x10^5 – 3.3x10^5$)	$2.6x10^5$ ($1.4x10^5 – 3.4x10^5$)
Hg	$1.0 x10^2$ ($5.7x10 – 1.4x10^2$)	6.2 x10 (3.5x10 – 9.1x10)	8.6x10 ($4.4x10 – 1.9x10^2$)	5.6x10 ($2.9x10 – 1.3x10^2$)
K	$1.3x10^6$ ($1.1x10^6 – 1.4x10^6$)	$1.1x10^6$ ($8.7x10^5 – 1.3x10^6$)	$1.1x10^6$ ($9.6x10^5 – 1.36x10^6$)	$1.2x10^6$ ($9.6x10^5 – 1.3x10^6$)
Mg	$6.2x10^4$ ($5.5x10^4 – 7.2x10^4$)	$5.6x10^4$ ($2.8x10^4 – 6.1x10^4$)	$5.8x10^4$ ($4.2x10^4 – 7.6x10^4$)	$5.9x10^4$ ($3.1x10^4 – 6.8x10^4$)
Mn	28.2 (23.5 – 40.0)	38.1 (11.7 – 58.8)	31.8 (19.4 – 54.0)	38.6 (11.3 – 79.2)
Mo	6.6 (3.8 – 8.2)	4.4 (2.1 – 7.4)	5.0 (2.9 – 8.9)	3.7 (2.3 – 7.4)
Na	$2.5x10^6$ ($2.1x10^6 – 2.6x10^6$)	$2.3x10^6$ ($1.9x10^6 – 2.6x10^6$)	$2.5x10^6$ ($2.2x10^6 – 2.7x10^6$)	$2.5x10^6$ ($2.0x10^6 – 2.9x10^6$)
Se	$7.2x10^2$ ($5.5x10^2 – 1.3x10^3$)	$6.0x10^2$ ($3.8x10^2 – 7.9x10^2$)	$5.6x10^2$ ($4.2x10^2 – 9.8x10^2$)	$5.7x10^2$ ($3.5x10^2 – 9.3x10^2$)
Zn	$5.2x10^3$ ($4.3x10^3 – 5.7x10^3$)	$4.6x10^3$ ($3.6x10^3 – 5.1x10^3$)	$4.6x10^3$ ($3.9x10^3 – 5.6x10^3$)	$4.8x10^3$ ($3.1x10^3 – 5.6x10^3$)

3.1.1.1 Hg:Se ratio

The Hg:Se molar ratio ranged from 0.022 to 0.131 and Se and Hg were shown to correlate (p=0.007).

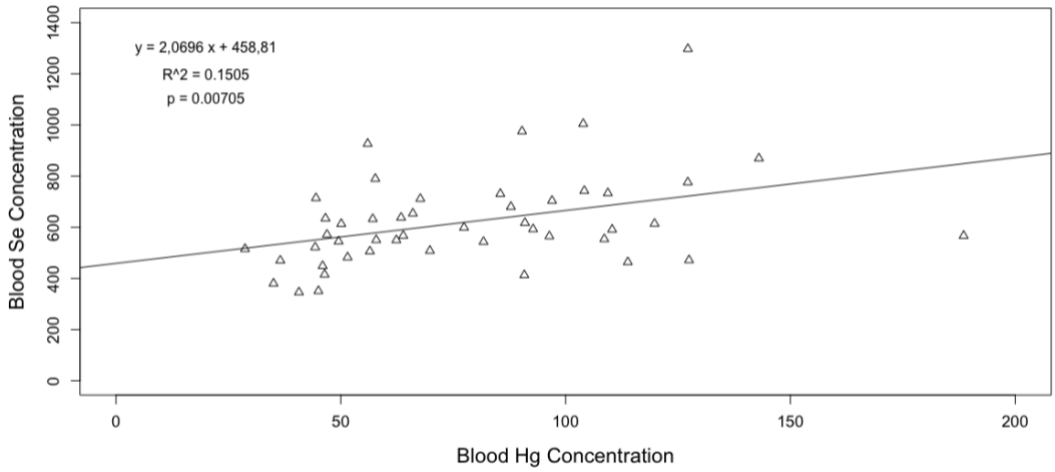

Figure 4: Correlation between Hg and Se in the blood of white-tailed eagle nestlings. The concentrations are in µg/L.

3.1.2 Variation in blood metal concentration by location and year

3.1.2.1 Variation by Location

Only three metals were significantly different between Smøla and Steigen when samples from the two years were combined in the analyses (Figure 5 A, B and C). Hg (R^2 = 0.11, t = -3.40 and p = < 0.01), Mg (R^2 = 0.20, t = 2.42 and p = 0.02) and Mo (R^2 = 0.19, t = -3.21 and p = < 0.01) concentrations were significantly different between Smøla and Steigen. Since the metal concentrations distributions were non-normal for several metals, separate models were performed as described in the methods. The model selection suggested that location (ΔAICc = 0), as a sole explanatory variable, was the most significant variable that produced the best models explaining the variation in Hg, Mg and Mo concentrations. Age (R^2 = 0.10, t = 2.21, p = 0.03) was a significant variable explaining variation in Hg concentration, indicating increased Hg with increasing age at sampling. The models and AICc results are presented in Table A10.

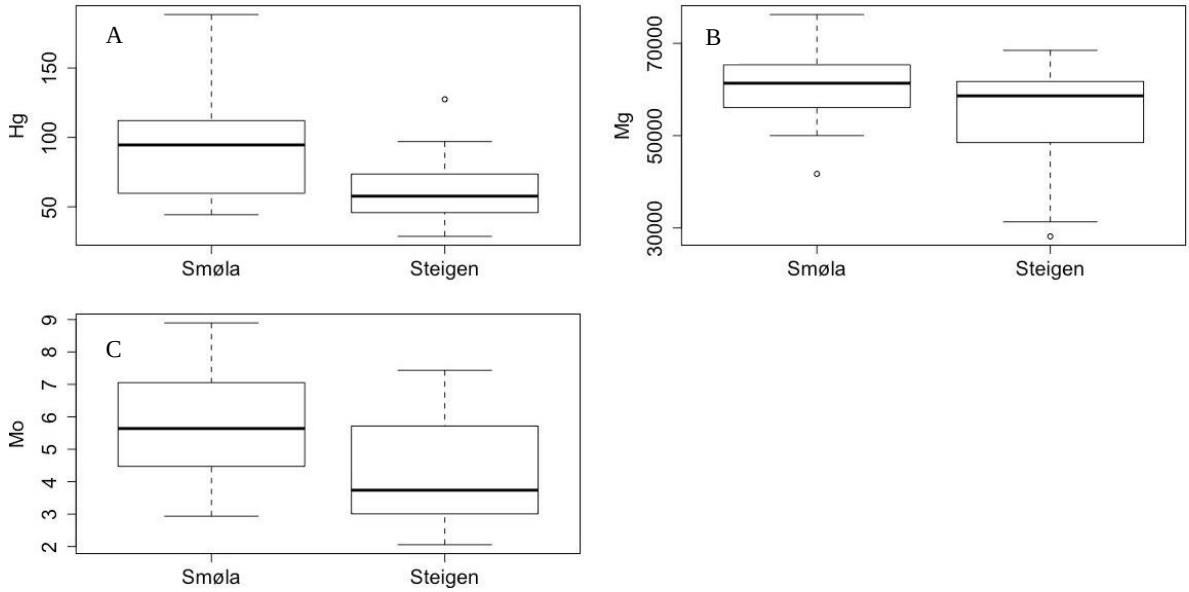

Figure 5: Blood concentrations of Hg (A), Mg (B) and Mo (C) from Smøla (n=24) and Steigen (n=23). All concentrations are in µg/L. The box shows the interquartile range (25-75th percentile), median (bold line) and the whiskers that represent highest and lowest values within 1.5 times the interquartile range plus upper quartile and lower quartile, respectively. Potential outliers are denoted by a circle.

3.1.2.2 Variation by Year

For all metals, except for Na, there were no significant differences in blood concentrations between nestlings from 2015 and 2016 when the concentrations from the two locations were combined. Na showed significant difference ($R^2 = 0.08$, $t = 2.03$, $p = 0.05$) between 2015 and 2016 when tested by a linear model (Figure 6). The best model explaining the variation in Na metal concentrations was the model with year ($\Delta AICc = 0$) as an only explanatory variable (Table A10).

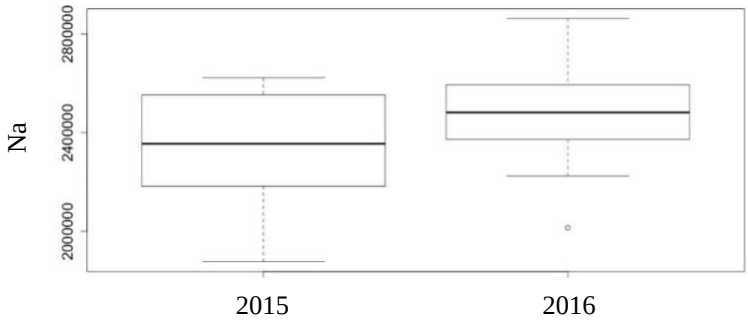

Figure 6: Blood concentrations of Na from 2015 (n=18) and 2016 (n=29). The concentration is in µg/L. The box shows the interquartile range (25-75th percentile), median (bold line), and the whiskers that represent highest and lowest values within 1.5 times the interquartile range plus upper quartile and lower quartile, respectively. Potential outliers are denoted by a circle.

3.1.2.3 Variation by Location and Year

Only Cu (R^2 = 0.15, F = 5.55, p = 0.03), K (R^2 = 0.23, F = 9.64, p = < 0.01) and Zn (R^2 = 0.14, F = 5.33, p = 0.03) were significantly different between Smøla and Steigen when the samples from the two years were combined, and between 2015 and 2016 when samples from the two locations were combined. Samples from Smøla 2015 were significantly different from other samples in each case. After a log transformation, all three metals were shown to be normally distributed and a normal linear model was used. Results are presented in Figure 7 (A, B and C). The AICc results suggest that the interaction between location and year (ΔAICc = 0) produced the best models explaining the variation in Cu, K and Zn concentrations. In addition, the age of the nestlings (R^2 = 0.12, t = 2.52, p = 0.02) was a significant variable explaining variation in K concentration, indicating increased K concentrations with increased age at sampling. The models and AICc results are presented in Table A10.

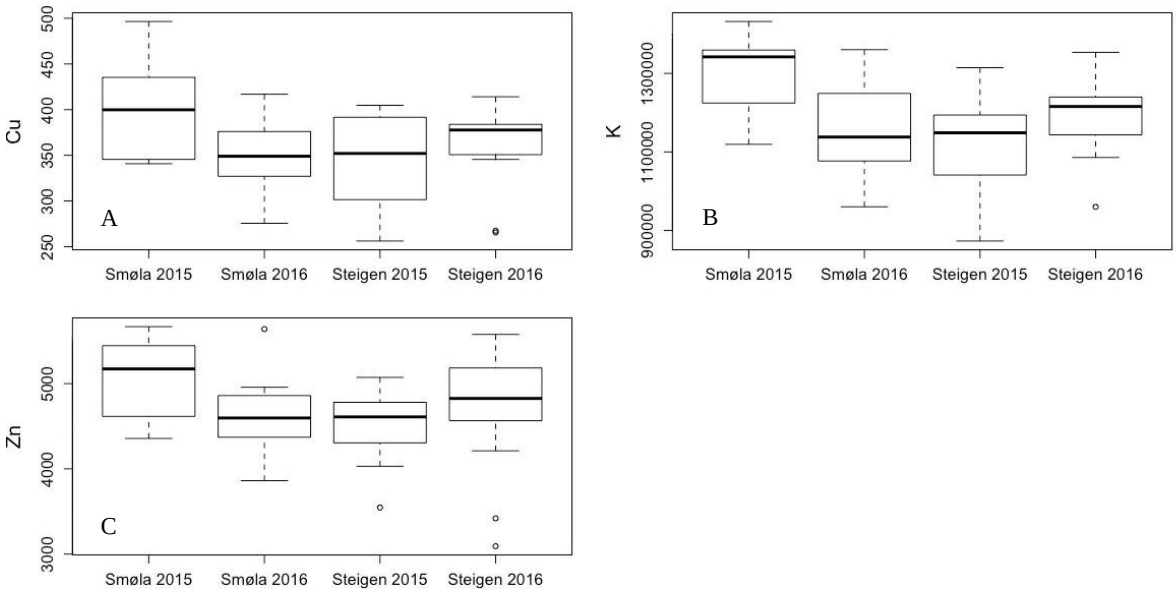

Figure 7: Blood concentrations of Cu (A), K (B) and Zn (C) from Smøla 2015 (n=10), Smøla 2016 (n=14), Steigen 2015 (n=8) and Steigen 2016 (n=15). All concentrations are in µg/L. The box shows the interquartile range (25-75th percentile), median (bold line), and the whiskers that represent highest and lowest values within 1.5 times the interquartile range plus upper quartile and lower quartile, respectively. Potential outliers are denoted by a circle.

3.1.3 Variation in blood metal concentration by sex

There were no significant differences between the two sexes (M = 24, F = 23) regarding the 13 metals detected in the blood of the nestlings. The metal closest to showing a slight difference, but not significant, between the blood metal concentrations in males and females was Hg (R^2 = 0.06, t = 1.72, p = 0.09). This can be seen in Figure 8. Sex as a sole explanatory variable was rated as the fourth best model with ΔAICc = 7.79 (Table A10).

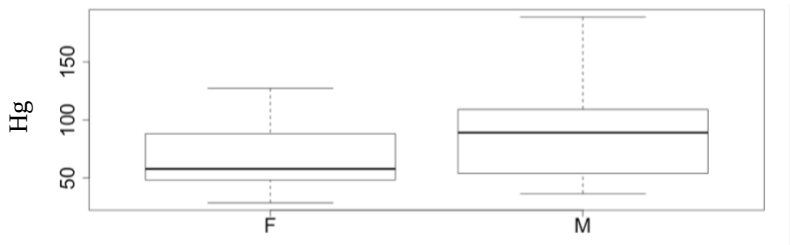

Figure 8: Blood concentrations of Hg from females (n=23) and males (n=24). The concentration is in µg/L. The box shows the interquartile range (25-75th percentile), median (bold line), and the whiskers that represent highest and lowest values within 1.5 times the interquartile range plus upper quartile and lower quartile, respectively.

3.1.4 Diet and Trophic level

Diet and the trophic level have significantly influenced concentrations of three elements (As, Fe and Se). The interaction between $\delta^{13}C$ and $\delta^{15}N$ (R^2 = 0.44, t = 11.03, p = < 0.001) best explained the variation in As concentrations in the whole blood of the nestlings. Stable nitrogen (R^2 = 0.19, t = 8.70, p = 0.002) was rated as the second best explanatory variable for variations in As concentrations and stable carbon (R^2 = 0.15, t = 8.90, p = 0.007) was rated as the third best explanatory variable for variations in As concentrations. Stable nitrogen and stable carbon were also significant in explaining variations in Fe concentrations. Stable nitrogen (R^2 = 0.11, p = 0.02) was rated as the best sole explanatory variable in Fe concentrations while stable carbon (R^2 = 0.10, p = 0.03) was rated as the second best explanatory variable. Only stable carbon (R^2 = 0.14, t = 24.24, p = 0.009) significantly explained variation in Se concentrations and this model was rated as the best model. The models and AICc results are presented in Table A10.

3.1.5 Spatial and Temporal Analysis

The results of the principal component analyses of blood metal concentrations, stable isotopes ($\delta^{13}C$, $\delta^{15}N$) and Hg:Se ratio is visualized in Figure 9A. Zn, Mg, Cu, K, Mo, Se, Ca, Hg, Fe and Na were significantly explained by the first principal component, PC1 (29.7%, all $p < 0.01$), while $\delta^{13}C$, $\delta^{15}N$, Mn, Na, Ca, Cd, Mo, Hg, Fe and Hg:Se were significantly explained by the second principal component, PC2 (15.2%, all $p < 0.01$). The correlation values for each variable and the two principal components are presented in Table A9, Appendix F.

The PCA plot for blood metal concentrations for nestlings at Smøla and Steigen displayed large variations between the two locations (Figure 9B). The same holds for the variation in blood metal concentrations in 2015 and 2016 (Figure 9C). PC1 explained 29.7% of the variation while PCA2 explained only 15.2%. The PC components and loadings for the first five PC components

are presented in Table A6 and Table A7, respectively. The total number of PC components was sixteen. The eigenvalues and variance (%) for each of the 16 components are presented in Table A8, Appendix F.

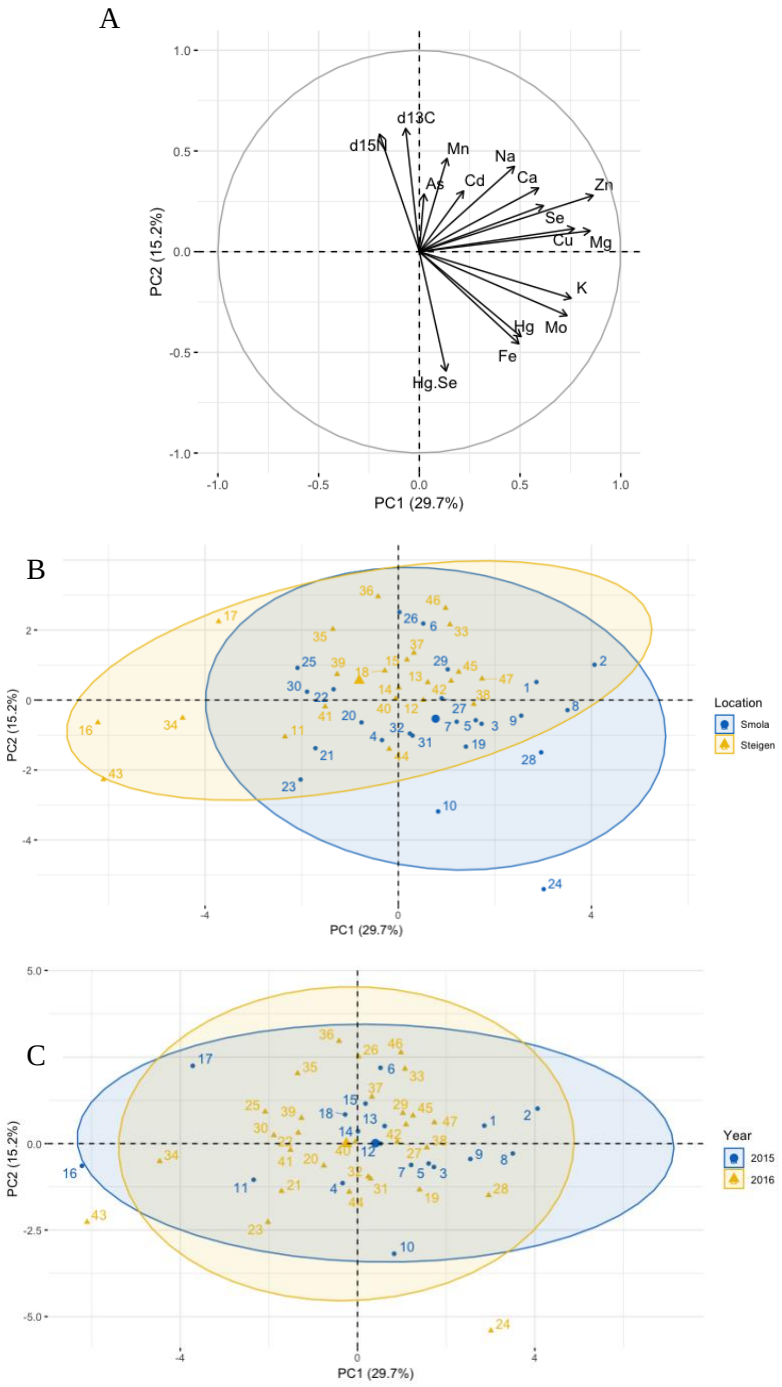

Figure 9: A: PCA loading plot showing correlations between metal concentrations (As, Ca, Cd, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Se and Zn), stable isotopes ($\delta^{15}N$, $\delta^{13}C$) and Hg:Se ratio. B: PCA score plot of explanatory variable location (Smøla, Steigen) for blood metal concentrations. C: PCA score plot of explanatory variable year (2015, 2016) for blood metal concentrations. PCA component 1 explains 29.7% of the variation while component 2 explains 15.2% of the variation.

3.2 Metallothionein

The MT concentrations ranged from 4.98 to 10.78 ng/ml plasma with a mean (±SE) concentration of 7.73 ± 0.27 ng/ml. No statistically significant relationships were found between MT concentrations in the nestling blood and metal concentrations of As, Cd, Cu, Hg and Zn. The best model selected by the AICc model selections in explaining the variation in MT concentrations only included Zn (ΔAICc = 0) as an explanatory variable (Table A11, Appendix G). However, according to the results from the linear model, the variation in MT concentrations was not significantly explained by Zn (R^2 = 0.05, t = 1.28, p = 0.21).

No significant differences were found in MT concentrations between nestlings of different sex (R^2=0.004, t=-0.36, p=0.74) or varying ages (R^2=0.005, t=-0.37, p=0.71).

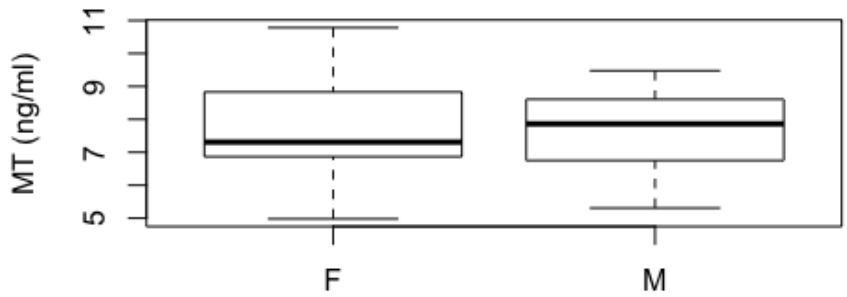

Figure 10: Concentration of metallothionein in plasma of males (n = 16) and females (n = 15) from both locations (Smøla, Steigen) and years (2015, 2016). The box shows the interquartile range (25-75th percentile), median (bold line) and the whiskers that represent highest and lowest values within 1.5 times the interquartile range plus upper quartile and lower quartile, respectively.

4 Discussion

4.1 Metal concentrations in nestlings

The metal concentrations found in the nestlings were compared to concentrations found previously in birds of prey and to toxic reference values reported in the literature. Some of the metals exceeded these reference values as will be further discussed below separately for the non-essential and essential elements.

4.1.1 Non-essential metals

Median concentrations of As varied from the lowest value $6.6x10^2$ µg/L in Smøla 2016 to the highest value $1.1x10^3$ µg/L in Steigen 2016. These concentrations were one order of magnitude higher compared to the reference threshold value of 2.0x10 µg/L in the whole blood of aquatic bird species (Burger and Gochfeld 1997). A study on Bonelli's eagle (*Aquila fasciata*) nestlings suggested that elevated levels of As were most common for industrialized areas and coal combustion sites (Ortiz-Santaliestra et al. 2015). While Smøla is located in a close proximity to Trondheim, an industrialized city, Steigen is located in a rather pristine environment in the north of Norway. Interestingly, the highest concentrations were found in Steigen, indicating an increase in As pollution northwards. Therefore, besides industrialization, other causes should be considered. Coal combustion is an improbable cause of elevated As concentrations since the only coal burning power plant in Norway is situated on Svalbard. However, As can be carried by the air currents from Europe and be deposited in Norway with higher deposition in the north (Steinnes et al. 1994; Bustnes et al. 2013). As concentrations above the threshold values have been linked to decreased body mass and reduced hatching and breeding success in great tits (Janssens et al. 2003; Eeva et al. 2009).

The concentrations of Hg ranged from the lowest in Steigen 2016 (5.6x10 µg/L) to the highest in Smøla 2015 ($1.0x10^2$ µg/L). These values slightly exceeded the threshold value of 3.0x10µg/L that was shown to induce oxidative stress in Griffon vultures (Espín et al. 2014). The Hg comes primarily from the diet and the concentrations of Hg in the whole blood directly reflect Hg concentrations in the diet (Scheuhammer et al. 1998). Hg has been shown to negatively affect reproduction, especially in higher trophic level species (Alvárez et al. 2013). However, it is the organic form of Hg, MeHg, that is of a special concern due to its known adverse effects on egg hatchability, neurobehavioral underdevelopment and the fact that it biomagnifies through the food web (Burger 2002; Alvárez et al. 2013). Demethylation is a key process in neutralizing potential adverse health effects of MeHg by turning MeHg into

inorganic Hg (Eagles-Smith et al. 2009). Se is believed to play an important role in demethylation of MeHg acting as its initiator (Eagles-Smith et al. 2009). The significant correlation between Hg and Se concentrations and their ratio <1 in the whole blood of nestlings therefore indicated that these nestlings had levels of Se high enough to suppress possible toxic effects of Hg. Hg was the only metal that showed some concentration differences between male and female nestlings, although not significant (p=0.09). Males had higher Hg concentrations compared to females. In breeding adult birds, the levels of Hg are often lower in females due to Hg depuration into their eggs (Robinson et al. 2012). However, this is not the case here since only nestlings were sampled. In this study, female nestlings might have lower Hg concentration due to their larger size. It has been documented that some aquatic species with higher growth rate have lower Hg concentrations than slow-growing species due to somatic growth dilution (Karimi et al. 2007; Ward et al. 2010; R. Wang and Wang 2012). This is a result of an unequal increase in the net rate of biomass gain compared to the Hg gain (Karimi et al. 2007). Somatic growth dilution might thus be an explanation for the results obtained in this study.

Median concentrations of Cd ranged from 0.64 µg/L in Smøla 2016 to 0.85 µg/L in Smøla 2015. In the study on Griffon vultures, Cd blood concentrations above 0.5 µg/L caused an induction of GPx and CAT activity by 33 and 44%, respectively (Espin et al. 2014a). However, a study on eagle owls (*Bubo* bubo) showed that Cd concentrations above 3 µg/L can cause GPx and CAT activity inhibition by 26 and 32%, respectively (Espin et al. 2014b). The Cd concentrations in white-tailed eagle nestlings in the present study was close to the threshold values found for Griffon vultures (Espín et al. 2014). Cd toxicity has been linked to egg shell thinning, kidney and testicular damage in several bird species (Garcia-Fernandez et al. 1995; Leach, et al. 2018).

4.1.2 Essential metals

The concentrations of essential metals were generally consistent with reference values found in previous studies (Stout et al. 2010; Vainio 2018; Zdziarski et al. 1994; Mochizuki et al. 2002) and well below the NOAEL (Santolo et al. 2007). The median concentrations did not vary greatly between the two locations (Smøla and Steigen) and the two years (2015 and 2016). For almost all metals (Cu, Fe, K, Mg, Mo, Na, Se, Zn), the median concentrations were highest in Smøla 2015. This might be due to the age of the birds captured. On average, the birds captured in 2015 at Smøla were 10 days older than birds captured in Steigen (both years) and Smøla 2016. It therefore seems that the older the birds, the higher the blood element content. A similar

pattern has been found in 11 bird species from Doñana National Park, where concentrations of adult individuals were higher compared to the nestlings (Alvárez et al. 2013). The internal as well as external concentrations of metals tend to increase with age as a result of prolonged metal accumulation over time (Berglund et al. 2011).

From the essential metals, only Fe was found above the reference value (Stout et al. 2010). The median concentrations ranged from $2.6x10^5$ in Smøla 2016 and Steigen 2016 to $3.1\ x10^5$ in Smøla 2015, which was 10-fold higher than the avian reference level ($1.7x10^4$ µg/L) for adult northern goshawks (Stout et al. 2010). In Hispaniolan Amazon parrots (*Amazona ventralis*) the mean Fe concentration in plasma was $4.25x10^3$ (Osofsky et al. 2001), almost 100 times lower than concentrations found in this study. However, the concentration of Fe in plasma is expected to be lower than the concentration in the whole blood as Fe is the main component of hemoglobin in red blood cells.

The median concentrations of the rest of the essential metals (Ca, Cu, K, Mg, Mn, Mo, Na, Se and Zn) were found below or comparable to reference values from previous studies. In comparison to a study on northern goshawks from Norway both Se and Zn concentrations found in this study were similar to those found in northern goshawks (Dolan et al. 2017). The concentrations of Se were approximately 60 times lower than the highest reference concentrations with NOAEL (Santolo et al. 2007). The Zn concentrations were also below the NOAEL reference levels ($12.6x10^3$ - $16.6x10^3$ µg/L) for avian species (Zdziarski et al. 1994). The median concentrations of Ca, Cu, K, Mg and Na were comparable to or below the metal concentrations found in adult northern goshawks from Pennsylvania (Stout et al. 2010). The study on northern goshawks did not find any adverse health effects at these metal concentrations (Stout et al. 2010).

4.2 Spatial and Temporal Differences

There were no significant differences between the two years when samples from the two locations were combined, except for Na. Na is an important electrolyte necessary for regulating body fluids and proper function of nerve and muscle cells (Strazzullo and Leclercq 2014). A possible explanation for why there could be significant differences in Na concentrations between years is the average temperature and the rate of respiration and sweating. In higher temperatures, sweating increases and the loss of Na is more significant (Bates and Miller 2008). However, the average temperatures in summer 2015 and 2016 were almost identical. This is

therefore an improbable cause of this finding. The findings of no temporal differences (except for Na) were also supported by PCA analysis. The PCA1 and PCA2 (Figure 9C) explained only 29.7% and 15.2% of the variation in metal concentration, respectively. There were no specific patterns observed for the two years and the data were very spread.

Regarding spatial differences, only Hg, Mg and Mo were significantly different between Smøla and Steigen when samples from the two years were combined in the analyses. All three elements were significantly higher in Smøla. Potential factors that could affect varying metal concentrations between Smøla and Stiegen include diet, industry, long-range transport and the age of the birds. Diet is a main source of internal metal contamination, especially in nestlings that do not yet come to contact with environment outside their nest (Eulaers et al. 2011a). From the PCA graph, we see a relationship between Hg and $\delta^{15}N$, suggesting that Hg contamination increased with trophic level. Hg was also negatively correlated with $\delta^{13}C$, indicating that the source of the diet also played a role in Hg contamination. It has been earlier shown that PFAS concentrations in white-tailed eagle nestlings increase with trophic level (Løseth et al. 2019a) and Hg followed the same trend. The diet and trophic level of the nestlings were not significantly different between Smøla and Steigen but the PCA results imply that Hg variation can be explained by carbon source and trophic position. The two essential metals, Mg and Mo, that were also significantly different between Smøla and Steigen do not follow any trend with neither $\delta^{13}C$ nor $\delta^{15}N$ on the PCA graph (Figure 9A). There is therefore no clear indication of the diet being an influencing factor in the Mg and Mo concentration differences between Smøla and Steigen.

Local pollution sources such as traffic and industry could also lead to differences in metal concentrations (Bustnes et al. 2013). Smøla is an island situated in a close proximity to an industrialized Norwegian city, Trondheim, and other urbanized areas. Steigen on the other hand, is situated in a more pristine environment in the north of Norway. Therefore, it was expected that higher level of metals will be found in Smøla. Many metals have, however, trans-boundary origin and come often to Norway from different parts of world via the long-range atmospheric transport (Bustnes et al. 2013). Environmental Hg concentrations are often higher in urban areas due to a greater incidence of industries and traffic (Han et al. 2014). It has also been documented that recent Hg trends increase eastward in Arctic biota (Rigét et al. 2011) and decrease northwards in the North Sea area (Wängberg et al. 2007). This was found in the present study too. Hg concentrations are, however, also influenced by atmospheric transport and can

originate from distinct sources (AMAP 2005). Elevated concentrations of Mg are often found on and around agricultural lands and arise from overuse of commercial fertilizers and agrochemicals (Senesi et al. 1999). Mg that is introduced into the soils can potentially leak out with the precipitation into the surrounding water bodies, resulting in more Mg available for the aquatic species (Senesi et al. 1999). As there are more agricultural lands around Smøla than there are around Steigen, the use of agrochemicals might have led to Mg concentration differences between the two locations. Similar to Hg, concentrations of Mo in the environment increase with increasing urbanization. A potential anthropogenic source of Mo is fossil fuel combustion (Harkness et al. 2017; Chappaz et al. 2012). The significant difference in Mo concentration between the two locations could be therefore assigned to more evident urbanization close to Smøla than to Steigen.

In addition, the significant difference between Smøla and Steigen could be possibly assigned to the age of the nestlings. On average, the nestlings from Smøla were 10 days older that the nestlings from Steigen. It has been documented that levels of polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs) are higher in older nestlings (Løseth et al. 2019a). In this study, Hg ($p = 0.03$) showed significant difference with age. In snow petrels (*Pagodroma nivea*), Hg has been shown to increase with increasing age due to bioaccumulation of MeHg (Tartu et al. 2014). The same could happen in white-tailed eagle nestlings from Smøla and Steigen in this study. The concentrations of the most metals do not increase with increasing age as the bioaccumulation of inorganic compounds is generally lower (or none) compared to the bioaccumulation of organic compounds. An exception to the rule is the organic form of Hg, which is lipophilic and thus accumulates with similar pattern as other organic pollutants (Mason, Reinfelder, and Morel 1995).

When the samples were divided into four groups according to the location and the year (Smøla 2015, Smøla 2016, Steigen 2015 and Steigen 2016), there were three metals (Cu, K and Zn) that were significantly different in Smøla 2015 compared to Smøla 2016 and Steigen both years. As mentioned before, the nestlings from Smøla 2015 were in average 10 days older than all the other nestlings. However, results suggest that only K was significantly influenced by age ($p = 0.02$). There were no previous studies that investigated accumulation of K with age neither in raptors nor other birds and mammals. Therefore, the reason for a significant relationship between K and age is unknown but could be related to developmental changes.

4.2.1 Effect of diet on the metal concentration

The diet and the trophic level significantly influenced the concentrations of three elements, As, Fe and Se in this study. The data on stable isotopes showed that concentration of As and Fe were influenced by both trophic level ($\delta^{15}N$) and carbon source ($\delta^{13}C$), while Se was influenced only by carbon source ($\delta^{13}C$). A study on white-tailed eagle nestlings found that more contaminated nestlings were feeding at lower trophic level and that concentrations of PCBs, OCPs and PBDEs decreased with increasing $\delta^{13}C$, that is with more a marine based diet (Løseth et al. 2019a). Fe, As and Se each followed different trends as seen on the PCA graph (Figure 9A).

As aligned in the same direction as $\delta^{15}N$ and $\delta^{13}C$, meaning that As relates positively with trophic level and marine based diet. It has been documented that As bioaccumulates in both freshwater and marine food chain but biomagnification through the food chain is not frequent (Rahman et al. 2012). A Spanish study on Bonelli's eagle nestlings reports that concentrations of As also increased with closer proximity to industrialized zones (Ortiz-Santaliestra et al. 2015). In our study the sampling location did not play a significant role in the As concentrations.

Fe, on the other hand, was related negatively to $\delta^{15}N$ and $\delta^{13}C$ on the PCA graph (Figure 9A). This means that the concentration of Fe increased with decreasing trophic level and more terrestrial based diet. It has been suggested that levels of Fe can be affected by local sources and windblown mineral dust (Bustnes et al. 2013). Possibly because Fe in mineral dust can easier attach to or be inhaled by terrestrial species rather than aquatic ones. If such species serve as a source of diet for the eagles, they might have an influence on the concentrations of Fe in the nestlings.

From the model analysis the concentration of Se was significantly influenced by the dietary source ($\delta^{13}C$). However, this is difficult to confirm with the PCA graph (Figure 9A) as the arrow lies rather horizontally and therefore shows no specific connection to dietary source or trophic level. Although it is hard to explain how the diet affects the level of Se in white-tailed eagle nestlings in this study, several studies describe the effect of elevated Se levels in birds. Se accumulation was linked to reduced fertility in American kestrels (*Falco sparverius*) (Santolo et al. 2007) and oxidative stress in eastern screech owls (*Megascops asio*) (Wiemeyer and Hoffman 2007). In this study, the levels of Se were below NOAEL and no such effects were therefore presupposed.

4.3 Effect on metallothionein induction

Metallothionein was detected in all plasma samples of white-tailed eagle nestlings. The MT concentrations ranged from 4.98 to 10.78 ng/ml plasma with a mean concentration of 7.73 ± 0.27 ng/ml. There is very limited data on MTs in raptors, especially for MT measures in plasma, also from other species. Previous studies investigated MTs in kidneys and liver of diverse avian species. MTs are primarily synthetized in the liver and kidneys and these organs expectedly contain higher MT levels than blood (Wang et al. 2014).

Metallothionein concentrations have been studied in kidney of seabirds from Atlantic coast of Canada. The mean MT concentrations in kidneys of juvenile double crested cormorant (*Phalacrocorax auritus*) ranged from 14.4±4.23 µg/g wet weight (w.w.) in Ile aux pommes to 24.4±8.65 µg/g w.w. in Manawagonish Island (Elliott et al. 1992). A 5 year study on adult scoter (*Melanitta* spp.) from Pacific northwest of Canada found kidney MT values ranging from 2.7 to 416.8 µg/g w.w., and liver MT ranging from below detection to 499.0 µg/g w.w. (Barjaktarovic et al. 2002). Metallothionein concentrations were also measured in liver and kidneys of fledging Cory's shearwater (*Calonectris diomedea*) where the mean concentrations were found to be 24.76±16.58 µg/g w.w. in kidneys and 106.05±40.59 w.w. in liver (Stewart et al. 1996).

Metallothioneins are affected by several metals, including non-essential metals (As, Cd, Hg and Pb) and some essential metals (Cu, Zn) when the levels are above the threshold values (Elliott and Scheuhammer 1997; Barjaktarovic et al. 2002; Elliott et al. 1992). In previous studies, MTs were found to positively correlate with Cd, Cu and Zn (Stewart et al. 1996). However, most studies find positive correlations only between Cd and MTs (Barjaktarovic et al. 2002; Elliott et al. 1992; Elliott and Scheuhammer 1997). In the present study, the effect of five metals (As, Cd, Cu, Hg and Zn) on MT concentrations was tested. There were no significant results, suggesting that the detected levels of the five metals were too low to potentially induce MT production. There were neither positive nor negative correlations found between MT and the five metals (As, Cd, Cu, Hg and Zn) and MT and the biological factors (age, sex). The results of the present study suggest that levels of MTs in the blood of white-tailed eagle nestlings are low and it is difficult to see any relationship between the metal concentrations and MT induction, probably related to the low elemental contamination in the studied nestlings.

4.4 Considerations

In this study, only whole blood and plasma were used as matrices for metals and MT analysis, respectively. Previous studies used mostly kidney and liver samples to analyze metal concentrations in various avian species and therefore the results were sometimes difficult to compare. As previously mentioned, there are both advantages and disadvantages when using either blood or tissue samples. While blood can be sampled without sacrificing the animal, neither kidney nor liver samples can be so. Therefore, the greatest advantage in using whole blood instead of tissue samples is the non-destructive way of obtaining the sample. However, blood metal concentrations only reflect the recent contamination loads, mostly from the diet (Martínez-López et al. 2005), and thus cannot be used to study long-term exposure to metals. Likewise, to study MT induction, only plasma samples were used, while the highest concentrations of MTs are found in liver and kidneys as these are the organs of MTs synthesis (Wang et al. 2014). It could be expected that if liver and/or kidney samples had been analyzed, the concentrations would be higher. However, using plasma was the best option as the least destructive sampling was required.

White-tailed eagle nestlings were used in this study to monitor metal pollution in Norwegian coastal areas. As mentioned before, the use of nestlings has several advantages including the easier capture process and sampling. However, as these nestlings were on average eight weeks old, only a short-term exposure to the metals could be investigated. The metal contamination in the nestlings is very recent and comes primarily from the diet and thus might not reflect the overall contamination in the area. Using nestlings as biomonitors might be therefore less effective than using adults when long-term contamination trends are monitored.

To study MT induction, ELISA kit designed for analyzing levels of MT in chickens was used. The concentrations of MTs in eagle nestlings were on the lower end of the standard curve and were much lower than the chicken serum control sample. Although using an ELISA kit designed for chickens was the most suitable kit on the market to study MTs in the eagle nestlings it did not find any significant associations with the metals. The non-significant results might be due to a non-optimal kit or simply because the metal concentrations were mainly background levels and thus maybe not high enough to induce MTs in the nestlings. MTs were earlier studied using differential pulse polarographic assay (Thompson and Cosson 1984; Rotchell et al. 2001), Hg saturation assay (Lucia et al. 2009) and Ag saturation assay (Stewart

et al. 1996; Elliott and Scheuhammer 1997) and the suitability of these assays should be compared to ELISA.

Gnerally, it seems that the metal contamination in white-tailed eagle nestlings from Smøla and Steigen is low. The metal concentrations found in this study were consistent with or lower than the reference values from previous studies. The overall health status of the nestlings was not studied here but it is known that these nestlings are generally in good health (Løseth et al. 2019b), have low metal concentrations and therefore also no evident induction of MT.

5 Conclusion

In this work, whole blood concentrations of 13 metals (As, Ca, Cd, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Se and Zn) were studied in white-tailed eagle nestlings. Of those metals, Ca, Cd, Cu, K, Mg, Mn, Mo, Na, Se and Zn were consistent around or below the reference values found in previous literature. Only As, Hg and Fe were in some cases above the reference values. Nestlings from Smøla 2015 showed highest median concentrations for almost all metals and this could possibly be explained by the age of the nestling, which were in average 10 days older than the nestlings from other locations and years.

Temporal differences were not observed between the two years (except for Na), suggesting that the levels of metals in the environment and the diet were steady and did not significantly increase or decrease over this time. Spatial differences were observed only for Hg, Mg and Mo. These were most likely due to urbanizations differences and diet.

The diet and the trophic level significantly influenced As, Fe and Se levels in the nestlings. As increased with increasing trophic level and marine based diet, while the trend was completely opposite for Fe. From the results, Se was significantly influenced only by the diet.

Regarding MT induction, MTs were detected in low levels and neither As, Cd, Cu, Hg and Zn nor any biological factors (sex and age) significantly explained variation in MT concentration. The low levels of MTs found in the plasma of white-tailed eagle nestlings might be related to the low levels of metals in these birds.

To conclude, the results of this study indicate that metal contamination in white-tailed eagle nestlings is low and the blood concentrations of metals do not seem to have an effect on MT induction.

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Appendix A – UltraClave Report

MLS Microwave Report Systemtest: MWT AG
Application: pressPREP
Report: 05.02.2018 15:16:38

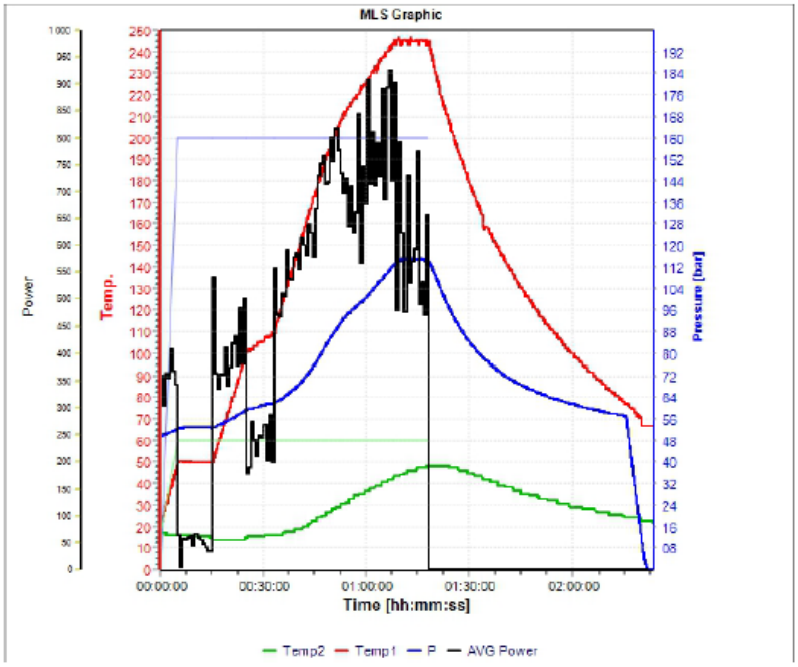

Figure A1: UltraClave temperature profile showing the temperature of UltraClave Microwave Digestion System (green), temperature of the samples (red) and the pressure of nitrogen gas (blue).

Appendix B – Limits of Detection

Instrumental Detection Limits (IDLs):
The IDLs were calculated by plotting the relative standard deviations against concentration and the value at which the RSD gave approx. 25% increase was set as the instrumental detection limit (Figure A2).

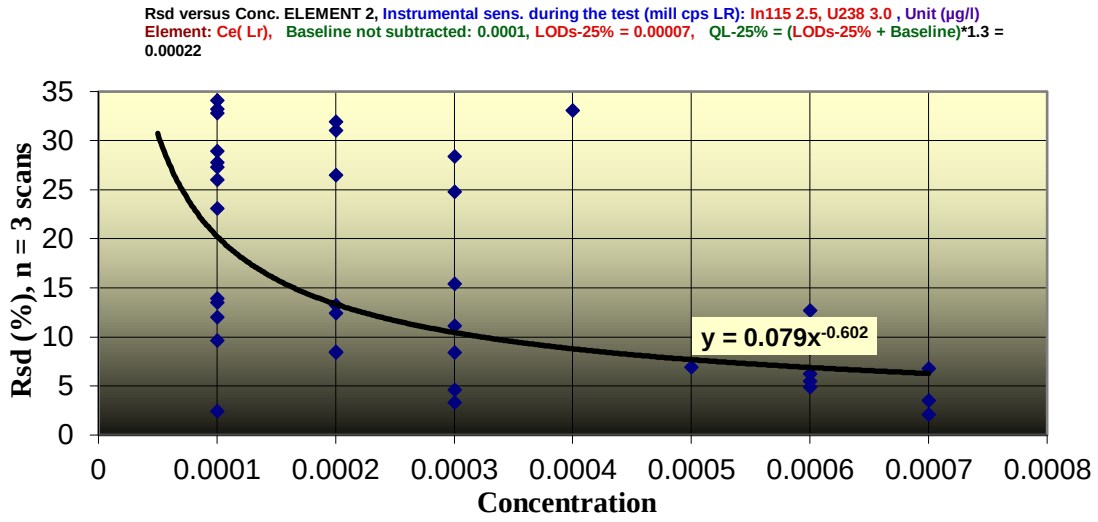

Figure A2: Relative standard deviations of sample detection as it goes towards low concentrations.

Blanks Limit of Detection (LODs):
The blank LODs were calculated by multiplying the standard deviations of the blanks by three. The blanks were also adjusted for the final dilution concentration of the samples and weight, giving the results in µg/L.
The detection frequency (DF) was calculated by dividing the number of detected samples (n> LOD) by number of all samples (n). Metals with DF < 0.5, i.e. metals that were detected in less than 50% of the samples were not used further in statistical analysis (Table A1).

Table A1: Limits of detection and detection frequency for blood samples. Units are given in µg/L. Metals with DF < 0.5 were not used for further statistical analysis.

Element	IDL	LOD	MAX	DF
Al	13.33	81.54	81.54	0.17
As	1.67	0	1.67	1
Ca	133.33	2215.04	2215.04	1
Cd	0.13	0	0.13	1
Cu		0	0.00	1
Fe	1.33	1966.51	1966.51	1
Hg	0.07	24.55	24.55	0.99
K	333.33	3826.51	3826.51	1
Mg	6.67	0	6.67	1
Mn	0.40	0	0.40	1
Mo	1.33	0	1.33	0.99
Na	666.67	2858.80	2858.80	1
Ni	1.00	50.81	50.81	0.02
Pb	0.01	5.81	5.81	0.32
Se	10.00	0	10.00	1
Zn	1.67	169.86	169.86	1

Appendix C – Body Condition Index

Using Spearman rank correlation test, bill height showed the strongest correlation with body mass for males and females. Bill height was therefore used in Equation 2 to calculate BCI.

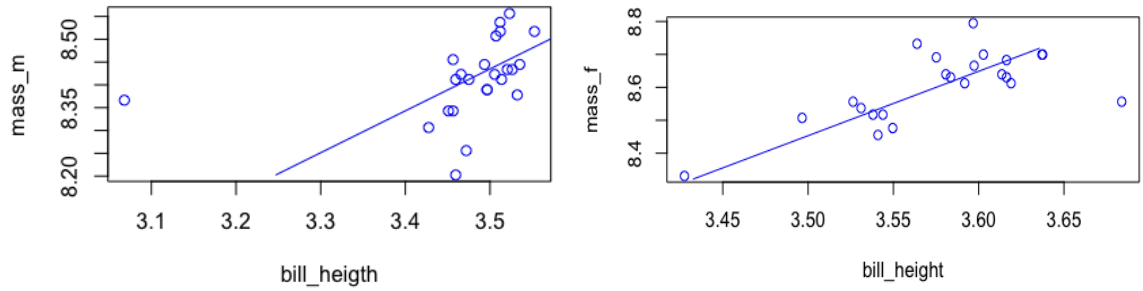

Figure A3: Scatter plot showing the correlation between male's body mass and bill height (left) and female's body mass and bill height (right).

Appendix D – Correlation tests

To test a potential correlation between age, mass and BCI, Spearman rank correlation test was used. The Spearman rank correlation test was also used to test for potential correlation between metals and age, mass and BCI.

Table A2: Spearman rank correlation test showing correlation coefficient rho (r_s) and a significance level (p-value) for correlations between age, mass and BCI for males and females. Values in bold show significant correlation between variables.

	Age		Mass	
Males	p-value	r_s	p-value	r_s
Mass	0.092	0.351		
BCI	0.451	0.161	**<0.01**	0.787
Females	p-value	r_s	p-value	r_s
Mass	**<0.01**	0.755		
BCI	0.242	-0.254	0.103	0.349

Table A3: Spearman rank correlation test showing correlation coefficient rho (r_s) and a significance level (p-value) for correlations between elements and age, mass and BCI. Values in bold show significant correlation between variables.

	Age		Mass		BCI	
Metal	p-value	r_s	p-value	r_s	p-value	r_s
As	0.703	0.057	0.604	0.078	0.880	-0.023
Ca	0.237	-0.176	0.726	0.053	0.284	0.160
Cd	0.316	0.150	0.985	-0.003	0.357	-0.137
Cu	0.138	0.220	0.600	0.078	0.204	-0.189
Fe	0.138	0.220	0.782	0.041	0.511	-0.098
Hg	**0.009**	0.378	0.451	-0.113	**0.021**	-0.336
K	**0.009**	0.375	0.811	-0.036	**0.039**	-0.302
Mg	0.319	0.148	0.445	-0.114	0.184	-0.197
Mn	**0.002**	-0.434	**0.004**	-0.413	0.108	-0.238
Mo	0.098	0.244	0.182	0.198	0.734	0.051
Na	0.269	-0.164	0.877	0.023	0.841	0.030
Se	**0.036**	0.307	0.262	0.167	0.576	-0.084
Zn	0.653	0.067	0.451	0.113	0.438	-0.116

Appendix E – Metal concentrations in nestlings

Table A4: Whole blood essential metal concentrations in nestlings (µg/L) grouped according to location and year (Smøla 2015, Smøla 2016, Steigen 2015 and Steigen 2016).

Location/Year	Metal	Count	Ar.Mean	SD	SE	Median	Min	Max
Smøla 2015	Ca	10	64817	8134	2572	64431	49686	82124
Smøla 2016	Ca	14	65628	10176	2719	66512	38573	82246
Steigen 2015	Ca	8	54573	20355	7196	55517	18682	79390
Steigen 2016	Ca	15	59604	18428	4758	61690	2476	82209
Smøla 2015	Cu	10	399.48	52.45	16.59	399.81	340.79	496.47
Smøla 2016	Cu	14	351.51	38.88	10.39	348.98	275.54	416.84
Steigen 2015	Cu	8	343.90	54.95	19.43	352.02	256.18	404.74
Steigen 2016	Cu	15	361.24	42.70	11.03	377.70	265.54	414.07
Smøla 2015	Fe	10	305794	40428	12784	314028	214346	344723
Smøla 2016	Fe	14	257489	60370	16134	265937	127560	335002
Steigen 2015	Fe	8	259879	50283	17777	276333	162817	317833
Steigen 2016	Fe	15	255524	52799	13632	265667	146520	341760
Smøla 2015	K	10	1302376	103196	32633	1342134	1119442	1432203
Smøla 2016	K	14	1160513	120946	32324	1138021	960389	1360465
Steigen 2015	K	8	1119491	140400	49639	1148816	873236	1314528
Steigen 2016	K	15	1189099	94663	24441	1215793	960187	1353663
Smøla 2015	Mg	10	63212	4940	1562	62439	55579	72116
Smøla 2016	Mg	14	59609	9186	2455	58561	41709	76234
Steigen 2015	Mg	8	53115	11428	4040	56224	28175	61761
Steigen 2016	Mg	15	54910	10904	2815	59037	31326	68471
Smøla 2015	Mn	10	30.01	5.79	1.83	28.20	23.47	39.96
Smoøla 2016	Mn	14	33.15	10.69	2.86	31.77	19.40	53.95
Steigen 2015	Mn	8	38.96	15.06	5.32	38.09	11.73	58.76
Steigen 2016	Mn	15	41.64	19.47	5.03	38.55	11.27	79.19
Smøla 2015	Mo	10	6.30	1.55	0.49	6.63	3.81	8.21
Smøla 2016	Mo	14	5.43	1.71	0.46	4.97	2.94	8.90
Steigen 2015	Mo	8	4.34	1.96	0.69	4.40	2.06	7.44
Steigen 2016	Mo	15	4.31	1.50	0.39	3.74	2.27	7.43
Smøla 2015	Na	10	2420921	186345	58927	2506855	2144180	2622172
Smøla 2016	Na	14	2487037	137895	36854	2482101	2228008	2689519
Steigen 2015	Na	8	2276677	223884	79155	2305420	1875866	2569286
Steigen 2016	Na	15	2460732	217479	56153	2481573	2014306	2862459
Smøla 2015	Se	10	775.52	228.11	72.13	722.38	553.97	1297.19
Smøla 2016	Se	14	581.90	141.70	37.87	557.39	415.18	975.31
Steigen 2015	Se	8	585.03	151.30	53.49	602.17	380.04	789.67
Steigen 2016	Se	15	571.00	147.85	38.17	567.27	346.07	926.54
Smøla 2015	Zn	10	5104	477	151	5172	4356	5667
Smøla 2016	Zn	14	4578	465	124	4596	3861	5639
Steigen 2015	Zn	8	4501	485	171	4609	3546	5072
Steigen 2016	Zn	15	4765	725	187	4825	3092	5576

Table A5: Whole blood non-essential metal concentrations in nestlings (µg/L) grouped according to location and year (Smøla 2015, Smøla 2016, Steigen 2015 and Steigen 2016).

Location/Year	Metal	Count	Ar.Mean	SD	SE	Median	Min	Max
Smøla 2015	As	10	997.13	904.58	286.05	769.65	208.87	3161.35
Smøla 2016	As	14	911.65	954.99	255.23	656.52	35.30	3272.36
Steigen 2015	As	8	894.50	469.44	165.97	834.42	339.08	1502.58
Steigen 2016	As	15	1275.51	767.56	198.18	1078.55	340.49	2779.43
Smøla 2015	Cd	10	0.86	0.40	0.13	0.85	0.38	1.56
Smøla 2016	Cd	14	0.68	0.36	0.09	0.64	0.17	1.66
Steigen 2015	Cd	8	0.78	0.33	0.12	0.70	0.42	1.29
Steigen 2016	Cd	15	0.97	0.40	0.10	0.77	0.46	1.85
Smøla 2015	Hg	10	100.48	25.39	8.03	104.04	57.10	143.00
Smøla 2016	Hg	14	87.39	42.21	11.28	86.02	44.29	188.52
Steigen 2015	Hg	8	66.53	19.91	7.04	61.94	35.03	90.82
Steigen 2016	Hg	15	60.23	25.38	6.55	55.97	28.69	127.46

Appendix F – PCA loadings and scores

Table A6: PC scores for white-tailed eagle nestlings (n = 47) on the first five principal components.

ID	PC1	PC2	PC3	PC4	PC5
WTE.15.SM-1.2	2.83	0.51	2.25	1.58	0.17
WTE.15.SM-2.2	4.02	1.00	3.44	-1.16	0.39
WTE.15.SM-3.1	1.71	-0.67	-1.11	-1.84	0.86
WTE.15.SM-4.1	-0.33	-1.13	1.93	0.32	-0.79
WTE.15.SM-5.1	1.59	-0.57	0.55	-0.54	0.03
WTE.15.SM-6.1	0.51	2.16	-0.28	0.75	0.60
WTE.15.SM-7.1	1.20	-0.61	0.02	1.81	0.61
WTE.15.SM-8.1	3.47	-0.28	0.86	-0.54	-0.31
WTE.15.SM-9.1	2.52	-0.44	1.88	-1.27	0.76
WTE.15.SM-10.1	0.82	-3.15	-0.54	-0.58	0.96
WTE.15.ST-2.1	-2.32	-1.04	1.00	0.94	-1.53
WTE.15.ST-3.1	0.51	0.00	0.94	-2.49	-0.11
WTE.15.ST-4.2	0.61	0.50	0.20	0.38	1.02
WTE.15.ST-5.1	0.01	0.35	0.77	-1.31	-2.17
WTE.15.ST-6.1	0.18	1.14	-0.08	0.66	1.08
WTE.15.ST-7.1	-6.15	-0.64	2.80	-0.57	0.98
WTE.15.ST-8.2	-3.68	2.22	-1.41	1.68	0.56
WTE.15.ST-9.1	-0.28	0.83	0.46	0.01	0.54
WTE.16.SM-1.2	1.39	-1.32	-0.24	1.00	-0.66
WTE.16.SM-2.1	-0.75	-0.63	-2.34	-1.12	0.70
WTE.16.SM-3.2	-1.70	-1.36	-2.88	-0.46	0.38
WTE.16.SM-4.1	-1.32	0.31	-1.57	0.91	0.94
WTE.16.SM-5.1	-2.00	-2.25	1.01	-0.40	-2.28
WTE.16.SM-6.1	2.98	-5.35	-0.92	2.24	-1.13
WTE.16.SM-7.2	-2.06	0.91	1.29	2.20	0.28
WTE.16.SM-8.3	0.02	2.49	2.39	0.66	1.27
WTE.16.SM-9.1	0.89	0.05	1.71	1.73	-0.08
WTE.16.SM-10.1	2.93	-1.48	-3.18	-0.87	1.65
WTE.16.SM-11.1	1.01	0.87	-1.60	-0.11	-0.57
WTE.16.SM-12.1	-1.87	0.24	-1.31	0.21	2.00
WTE.16.SM-13.1	0.29	-1.00	-0.45	0.28	1.01
WTE.16.SM-14.1	0.24	-0.95	-0.53	1.10	-0.45
WTE.16.ST-1.1	1.06	2.13	-1.08	-1.95	-1.46
WTE.16.ST-2.1	-4.42	-0.51	0.45	0.54	-0.31
WTE.16.ST-3.1	-1.34	2.01	-1.02	-2.44	-2.62
WTE.16.ST-4.1	-0.41	2.93	-1.56	1.48	-1.81
WTE.16.ST-5.2	0.32	1.33	-0.79	1.02	-0.53
WTE.16.ST-6.1	1.55	-0.12	-0.41	2.47	-2.57
WTE.16.ST-7.1	-1.25	0.73	0.47	0.21	0.63
WTE.16.ST-8.1	-0.05	0.04	0.23	-1.63	1.04
WTE.16.ST-9.1	-1.50	-0.18	-0.93	-1.92	-1.20
WTE.16.ST-10.2	1.08	0.54	-1.69	0.17	0.08
WTE.16.ST-11.1	-6.04	-2.24	0.34	-1.18	0.48
WTE.16.ST-12.1	-0.18	-1.38	1.65	-0.60	0.35
WTE.16.ST-13.1	1.24	0.80	-0.24	1.31	1.17
WTE.16.ST-14.2	0.97	2.60	-1.26	0.06	-0.01
WTE.16.ST-15.2	1.72	0.60	0.79	-2.72	0.06

Table A7: PC loadings of the 13 metals, $\delta^{13}C$, $\delta^{15}N$ and Hg:Se ration on the first five principal components.

	PC1	PC2	PC3	PC4	PC5
As	0.01	0.18	0.01	0.38	-0.08
Ca	0.27	0.20	-0.34	0.28	0.25
Cd	0.10	0.19	0.07	-0.43	-0.44
Cu	0.35	0.07	0.11	-0.22	-0.16
Fe	0.23	-0.29	-0.04	-0.20	0.01
Hg	0.23	-0.27	0.32	0.36	-0.23
K	0.35	-0.15	0.24	-0.10	-0.13
Mg	0.39	0.07	-0.15	0.21	0.15
Mn	0.06	0.30	-0.30	0.04	-0.50
Mo	0.34	-0.20	-0.04	-0.04	0.38
Na	0.22	0.27	-0.30	0.13	-0.08
Se	0.28	0.15	0.40	-0.16	0.19
Zn	0.40	0.18	-0.03	-0.06	-0.09
$\delta^{13}C$	-0.03	0.39	0.41	0.21	0.14
$\delta^{15}N$	-0.09	0.37	0.41	0.14	0.01
Hg:Se	0.06	-0.38	0.10	0.46	-0.40

Table A8: Principal components with eigenvalues, variance (%) and cumulative variance (%).

	Eigenvalue	Variance (%)	Cumulative Variance (%)
PC1	4.75	29.66	29.66
PC2	2.43	15.19	44.84
PC3	2.12	13.24	58.09
PC4	1.78	11.12	69.20
PC5	1.24	7.75	76.95
PC6	0.88	5.48	82.43
PC7	0.67	4.22	86.65
PC8	0.57	3.53	90.18
PC9	0.48	3.00	93.19
PC10	0.33	2.07	95.25
PC11	0.23	1.44	96.69
PC12	0.20	1.27	97.96
PC13	0.16	1.00	98.97
PC14	0.12	0.77	99.73
PC15	0.03	0.20	99.94
PC16	0.01	0.06	100

Table A9: Correlation coefficients (R^2) to PC1 and PC2 and p-values for white-tailed eagle blood samples.

PC 1			PC 2		
	Correlation (R^2)	p-value		Correlation (R^2)	p-value
Zn	0.86	5.98E-15	$\delta^{13}C$	0.61	4.82E-06
Mg	0.85	5.19E-14	$\delta^{15}N$	0.58	1.64E-05
Cu	0.77	2.56E-10	Mn	0.46	1.04E-03
K	0.75	9.60E-10	Na	0.42	3.00E-03
Mo	0.73	4.58E-09	Ca	0.32	3.05E-02
Se	0.62	3.95E-06	Cd	0.30	3.93E-02
Ca	0.59	1.16E-05	Mo	-0.32	2.98E-02
Hg	0.51	2.93E-04	Hg	-0.42	3.14E-03
Fe	0.49	4.17E-04	Fe	-0.46	1.22E-03
Na	0.47	8.03E-04	Hg:Se	-0.59	1.12E-05

Appendix G – Models

Table A10: Regression models explaining concentrations of 13 metals (As, Ca, Cd, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Se and Zn) in the whole blood of white-tailed eagle nestlings (n = 47) including potential effect of location, year, diet ($\delta^{13}C$, $\delta^{15}N$), sex and age. The models were selected based on Akaike's Information Criteria (AICc) adjusted to a small sample size. Only the models with ΔAICc < 2.00 were considered as promising models that could possibly explain the variation in metal concentrations.

As	(Intercept)	Location	Year	Sex	$\delta^{13}C$	d15N	Loc:Year	$\delta^{13}C$: $\delta^{15}N$	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~d13C*d15N	138.67				7.16	-9.39		-0.51	5	-51.38	114.22	0.00	0.997	0.44	<0.001
~d15N	-0.21					0.49			3	-60.18	126.91	12.70	0.002	0.19	0.002
~d13C	14.89				0.44				3	-61.18	128.91	14.70	0.0006	0.15	0.007
~Location	6.32	+							3	-63.13	132.82	18.61	9.08E-05	0.08	0.06
~Location+Year+Sex+d13C+d15N	7.91	+	+	+	0.28	0.27			7	-58.31	133.49	19.28	6.50E-05	0.25	0.03
~Location*Year	6.55	+	+				+		5	-62.32	136.10	21.88	1.77E-05	0.11	0.23
~sex	6.67			+					3	-64.86	136.29	22.07	1.61E-05	0.007	0.57
~Year	6.59		+						3	-65.04	136.63	22.41	1.35E-05	5.47E-05	0.96

Fe	(Intercept)	Location	Year	Sex	$\delta^{13}C$	d15N	Loc:Year	$\delta^{13}C$: $\delta^{15}N$	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~d15N	0.072					0.0006			3	4.36	-2.17	0.00	0.33	0.11	0.02
~d13C	0.091				0.0005				3	3.98	-1.40	0.77	0.23	0.10	0.03
~Location+Year+Sex+d13C+d15N	0.077	+	+	+	0.0003	0.0006			7	9.01	-1.15	1.02	0.20	0.27	0.01
~Year	0.080		+						3	2.98	0.59	2.76	0.08	0.06	0.10
~d13C*d15N	0.020				-0.0029	0.0048		0.0002	5	5.18	1.11	3.27	0.07	0.14	0.32
~Location	0.080	+							3	2.12	2.31	4.48	0.04	0.02	0.30
~Location*Year	0.079	+	+				+		5	4.30	2.87	5.04	0.03	0.11	0.22
~Sex	0.080			+					3	1.75	3.05	5.22	0.02	0.008	0.54

Hg	(Intercept)	Location	Year	Sex	$\delta^{13}C$	d15N	Loc:Year	Age	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Location	4.45	+							3	-21.00	48.55	0	0.53	0.2	0.001

~Location+Year+Sex+d13C+d15N	1.46	+	+	+	-0.042	0.162			7	-16.54	49.95	1.40	0.26	0.34	0.003
~Location*Year	4.58	+	+				+		5	-19.75	50.96	2.41	0.16	0.25	0.72
~Age	3.50							0.011	3	-23.95	54.47	5.92	0.03	0.10	0.03
~Sex	4.16			+					3	-24.89	56.34	7.79	0.01	0.06	0.1
~Year	4.39		+						3	-25.11	56.77	8.22	0.01	0.05	0.12
~d13C	5.77				0.079				3	-25.75	58.05	9.50	0.00	0.03	0.28
~d15N	3.83					0.031			3	-26.27	59.10	10.55	0.00	0.004	0.68

K	(Intercept)	Location	Year	Sex	$\delta^{13}C$	d15N	Loc:Year	Age	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Location*Year	14.08	+	+				+		5	44.28	-77.09	0	0.60	0.23	0.004
~Age	13.77							0.003	3	40.98	-75.40	1.69	0.26	0.12	0.02
~Sex	13.96			+					3	39.21	-71.86	5.23	0.04	0.05	0.15
~Location	14.01	+							3	39.14	-71.72	5.37	0.04	0.04	0.16
~Year	14.01		+						3	38.67	-70.78	6.31	0.03	0.03	0.29
~d13C	13.74				-0.013				3	38.35	-70.13	6.96	0.02	0.01	0.48
~d15N	14.09					-0.008			3	38.18	-69.79	7.30	0.02	0.004	0.68
~Location+Year+Sex+d13C+d15N	13.13	+	+	+	-0.032	0.020			7	41.56	-66.24	10.85	0.00	0.14	0.28

Mo	(Intercept)	Location	Year	Sex	$\delta^{13}C$	d15N	Loc:Year	Loc: $\delta^{15}N$	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Location	1.72	+							3	-15.58	37.71	0.00	0.58	0.19	0.003
~Location*d15N	3.21	+				-0.11		+	5	-14.20	39.86	2.15	0.20	0.23	0.26
~Location*Year	1.81	+	+				+		5	-14.86	41.19	3.48	0.10	0.21	0.35
~d15N	3.56					-0.14			3	-17.74	42.04	4.33	0.07	0.11	0.02
~Location+Year+Sex+d13C+d15N	0.36	+	+	+	-0.09	-0.03			7	-13.53	43.92	6.21	0.03	0.25	0.03
~d13C	-0.19				-0.09				3	-19.34	45.24	7.53	0.01	0.05	0.15
~Year	1.61		+						3	-20.08	46.71	9.00	0.01	0.02	0.42
~Sex	1.54			+					3	-20.39	47.34	9.63	0.00	0.001	0.80

Se	(Intercept)	Location	Year	Sex	$\delta^{13}C$	$\delta^{15}N$	Loc:Year	Year: $\delta^{13}C$	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~d13C	8.62				0.12				3	-0.99	8.54	0.00	0.35	0.14	0.009
~Year:d13C	6.75		+		0.01			+	5	1.24	8.98	0.44	0.28	0.22	0.16
~Location*Year	6.62	+	+				+		5	0.54	10.37	1.84	0.14	0.20	0.10
~Year	6.50		+						3	-2.35	11.26	2.72	0.09	0.09	0.04
~Location+Year+Sex+d13C+d15N	8.56	+	+	+	0.11	-0.004			7	2.51	11.85	3.31	0.07	0.26	0.03
~Location	6.46	+							3	-3.10	12.75	4.21	0.04	0.06	0.09
~d15N	5.46					0.07			3	-3.52	13.60	5.06	0.03	0.05	0.15
~Sex	6.36			+					3	-4.35	15.26	6.72	0.01	0.01	0.48

Zn	(Intercept)	Location	Year	Sex	$\delta^{13}C$	$\delta^{15}N$	Loc:Year	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Location*Year	5104.96	+	+				+	5	-362.70	736.87	0.00	0.30	0.14	0.03
~Year	4836.58		+					3	-365.68	737.91	1.04	0.18	0.02	0.37
~Location	4797.66	+						3	-365.84	738.23	1.37	0.15	0.01	0.48
~d13C	5266.26				27.88			3	-366.07	738.69	1.82	0.12	0.001	0.78
~d15N	5091.70					-25.72		3	-366.07	738.70	1.84	0.12	0.001	0.80
~Sex	4715.88			+				3	-366.08	738.71	1.84	0.12	0.001	0.82
~Location+Year+Sex+d13C+d15N	8700.36	+	+	+	101.20	-142.71		7	-365.17	747.21	10.34	0.002	0.040	0.89

Ca	(Intercept)	Location	Year	Sex	$\delta^{13}C$	$\delta^{15}N$	Loc:Year	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Location	0.090	+						3	-9.50	25.55	0.00	0.54	2.10E-01	0.05
~d15N	0.084					0.0005		3	-10.77	28.09	2.54	0.15	7.00E-02	0.25
~Year	0.091		+					3	-11.34	29.23	3.68	0.09	0.01	0.63
~Sex	0.091			+				3	-11.45	29.46	3.91	0.08	0.0004	0.94
~d13C	0.091				-1.53E-06			3	-11.45	29.47	3.92	0.08	6.87E-07	0.99
~Location*Year	0.090	+	+				+	5	-9.09	29.64	4.09	0.07	0.25	0.54
~Location+Year+Sex+d13C+d15N	0.078	+	+	+	-0.0004	0.0004		7	-9.15	35.18	9.63	0.004	0.24	0.46

Cd	(Intercept)	Location	Year	Sex	$\delta^{13}C$	$\delta^{15}N$	Loc:Year	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Location	-0.40	+						3	-29.46	65.48	0.00	0.39	0.05	0.12
~Location*Year	-0.26	+	+				+	5	-28.00	67.46	1.98	0.14	0.11	0.11
~d13C	0.58				0.05			3	-30.60	67.76	2.28	0.12	0.007	0.57
~Sex	-0.25			+				3	-30.62	67.80	2.32	0.12	0.007	0.59
~d15N	-0.81					0.04		3	-30.66	67.89	2.41	0.12	0.005	0.65
~Year	-0.28		+					3	-30.77	68.09	2.62	0.10	0.0002	0.91
~Location+Year+Sex+d13C+d15N	5.84	+	+	+	0.18	-0.21		7	-28.38	73.64	8.16	0.007	0.10	0.51

Cu	(Intercept)	Location	Year	Sex	$\delta^{13}C$	$\delta^{15}N$	Loc:Year	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Location*Year	5.98	+	+				+	5	30.53	-49.60	0.00	0.35	0.15	0.03
~Location	5.91	+						3	27.38	-48.19	1.40	0.17	0.03	0.27
~Year	5.91		+					3	27.31	-48.05	1.54	0.16	0.03	0.29
~Sex	5.87			+				3	26.98	-47.41	2.18	0.12	0.01	0.48
~d15N	6.03					-0.01		3	26.81	-47.07	2.53	0.10	0.004	0.67
~d13C	5.72				-0.009			3	26.79	-47.02	2.58	0.10	0.003	0.72
~Location+Year+Sex+d13C+d15N	5.59	+	+	+	-0.02	0.001		7	28.43	-39.99	9.61	0.003	0.07	0.69

Mg	(Intercept)	Location	Year	Sex	$\delta^{13}C$	$\delta^{15}N$	Loc:Year	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Location	0.091	+						3	12.12	-17.67	0.00	0.66	0.11	0.02
~Location*Year	0.090	+	+				+	5	12.60	-13.74	3.93	0.09	0.13	0.37
~d15N	0.087					0.0003		3	10.00	-13.44	4.23	0.08	0.03	0.24
~Sex	0.092			+				3	9.98	-13.41	4.26	0.08	0.03	0.25
~Year	0.091		+					3	9.39	-12.22	5.45	0.04	0.004	0.66
~d13C	0.093				6.25E-05			3	9.31	-12.06	5.61	0.04	0.00	0.83
~Location+Year+Sex+d13C+d15N	0.083	+	+	+	-0.0002	0.0004		7	13.04	-9.22	8.46	0.01	0.15	0.19

Mn	(Intercept)	Location	Year	Sex	$\delta^{13}C$	$\delta^{15}N$	Loc:Year	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*

~Location	3.42		+						3	-23.63	53.82	0.00	0.34	0.05	0.15
~Sex	3.44			+					3	-24.04	54.64	0.82	0.23	0.03	0.26
~Year	3.47		+						3	-24.56	55.68	1.86	0.14	0.006	0.61
~d13C	2.89				-0.03				3	-24.59	55.74	1.92	0.13	0.005	0.65
~d15N	3.39					0.008			3	-24.69	55.95	2.13	0.12	0.0003	0.91
~Location*Year	3.39	+		+				+	5	-23.53	58.52	4.70	0.03	0.05	0.91
~Location+Year+Sex+d13C+d15N	5.85	+		+	+	0.05	-0.11		7	-22.40	61.68	7.86	0.007	0.09	0.53

Na	(Intercept)	Location	Year	Sex	$\delta^{13}C$	$\delta^{15}N$	Loc:Year	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Year	14.67		+					3	52.18	-97.81	0.00	0.47	0.08	0.05
~Location*Year	14.70	+	+				+	5	53.72	-95.98	1.83	0.19	0.14	0.31
~Location	14.71	+						3	50.82	-95.08	2.73	0.12	0.08	0.25
~d15N	14.81					-0.008		3	50.29	-94.01	3.79	0.07	0.007	0.58
~d13C	14.82				0.006			3	50.23	-93.90	3.91	0.07	0.004	0.66
~Sex	14.70			+				3	50.17	-93.78	4.03	0.06	0.002	0.78
~Location+Year+Sex+d13C+d15N	14.86	+	+	+	0.01	0.005		7	54.06	-91.25	6.55	0.02	0.150	0.21

Table A11: Regression models explaining MT concentrations in the whole blood of white-tailed eagle nestlings (n = 31) including potential effect of As, Cd, Cu, Hg, Zn, sex and age. The models were selected based on Akaike's Information Criteria (AICc) adjusted to a small sample size. Only the models with ΔAICc < 2.00 were considered as promising models that could possibly explain the variation in MT concentrations.

	(Intercept)	As	Cd	Hg	Cu	Zn	Sex	Age	df	logLik	AICc	ΔAICc	weight	R^2	*p-value*
~Zn	5.10					0.0006			3	-54.68	116.25	0	0.25	0.05	0.21
~Hg	7.20			0.0066					3	-55.11	117.10	0.86	0.16	0.03	0.37
~Cu	6.05				0.0046				3	-55.23	117.34	1.09	0.14	0.02	0.45
~Age	8.31							-0.0086	3	-55.46	117.81	1.57	0.11	0.005	0.71
~Sex	7.82						+		3	-55.47	117.83	1.58	0.11	0.004	0.74
~Cd	7.61		0.13						3	-55.51	117.91	1.67	0.11	0.002	0.83
~As	7.75	-2.5E-05							3	-55.53	117.96	1.71	0.11	0.0002	0.94