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Trace Element Contamination as a Possible Cause of Infertility in Hungarian Resident Eastern Imperial Eagles (*Aquila heliaca*)

A nyomelem szennyezés mint az infertilitás lehetséges oka a Magyarországon élő parlagi sasok (*Aquila heliaca*) esetében

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2023

Abstract

Trace element analysis was done on five pairs of Eastern Imperial Eagles (*Aquila heliaca*), in order to try and find an explanation for their long-term infertility. The samples used were eggs and feathers from the vicinity of their nests. The elements examined were arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), mercury (Hg), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), and zinc (Zn). Anthropogenic activity is a major contributor to the release of these elements in the environment, which can be hazardous to the health of humans, wildlife, and ecosystems in inappropriate amounts. Raptors are often used as biological sentinels for an ecosystem, as their position on the food chain makes them susceptible to intoxication from trace elements via bioaccumulation and biomagnification. The analysis was done by inductively coupled plasma mass spectrometer (ICP-MS) and showed levels within the acceptable norms for all except nickel, manganese, and cobalt. A clear link for infertility due to intoxication could not be shown, and further investigation is needed to determine a more probable cause. Regardless, this was the first time that the elemental burden of *Aquila heliaca* was studied, and the values will be useful as a reference for further research.

Hosszú távú infertilitás okait kerestük fokozottan védett parlagi sas (Aquila heliaca) párok (n=5) esetében ahol felmerült a nyomelem szennyezés gyanúja. A felhasznált minták a fészkek környékéről származó tojások és tollak voltak. A vizsgált elemek az arzén (As), bárium (Ba), kadmium (Cd), kobalt (Co), króm (Cr), réz (Cu), higany (Hg), mangán (Mn), molibdén (Mo), nikkel (Ni), ólom (Pb) és cink (Zn) voltak. Az antropogén tevékenység nagymértékben hozzájárul ezen elemek környezetbe jutásához, amelyek nem megfelelő mennyiségben veszélyesek lehetnek az emberek, az élővilág és az ökoszisztémák egészségére. A ragadozó madarakat gyakran használják az ökoszisztémák bioindikátoraiként, mivel a táplálékláncban elfoglalt helyük miatt a bioakkumuláció és a biomagnifikáció révén érzékenyek a nyomelemek okozta mérgezésre. Az elemzést induktív csatolású plazma tömegspektrométerrel (ICP-MS) végezték, és a nikkel, a mangán és a kobalt kivételével minden elem esetében az elfogadható normákon belüli szintet mutatott ki. A mérgezés okozta infertilitás egyértelmű összefüggését nem lehetett kimutatni, és további vizsgálatokra van szükség a valószínűbb okok meghatározásához. Ettől függetlenül ez volt az első alkalom, hogy a parlagi sasok elemi terhelését vizsgálták, és az értékek hasznos referenciaként szolgálnak majd a további kutatásokhoz.

Figure 1 : Adult Eastern Imperial Eagle in Kiskunság National Park, Hungary (Márton Horváth, 2022)

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1. Introduction

As apex avian predators, birds of prey hold significant ecological roles. Their existence is currently threatened by a combination of human-induced factors. A significant amount of toxic elements are released into the environment through industrial activity. Monitoring toxic metals and trace elements in apex predators or animals elevated on a trophic level provides information on the extent of ecosystem contamination (Rodríguez-Álvarez *et al.*, 2022).

Birds of prey are regularly used as sentinel species for ecosystems, as their higher trophic level makes them susceptible to the effects of biomagnification and bioaccumulation of toxic compounds (Dmowski, 1999). Starting with Rachel Carson's "Silent Spring" in the 1960s, which rang alarm bells about the indiscriminate DDT (dichlorodiphenyltrichloroethane) pesticide use in the United States, awareness has grown on the chemical impact human activity has had on the environment and those that live in it.

In Hungary, careful monitoring of Eastern Imperial Eagles (*Aquila heliaca*) has been ongoing for more than 30 years (Horváth *et al.*, 2002) and those that study them are eager to know more about their health status. In general, reproductive success of Aquila heliaca is satisfactory, as it shows constant yearly increase (Horváth *et al.*, 2011). However, it has come to attention that certain pairs have had little success over the years. Each year they build a nest and get ready for the reproductive season spanning March to September, yet the eggs laid by these pairs do not hatch for a reason unknown to those that have been monitoring them through the years.

The adverse health effects of unusual concentrations of certain organic and inorganic molecules are varied yet present (Richard *et al.*, 2021). Abnormal levels of trace elements such as copper, cobalt, manganese, molybdenum, nickel, and zinc; and toxic elements like cadmium, mercury, lead, barium, arsenic, and chromium in the body are responsible for a plethora of health issues in terrestrial organisms (Rodríguez-Álvarez *et al.*, 2022). One of these is reproductive disorders, and in the hope of finding a reason for the unexplained infertility of these Eastern Imperial Eagles a toxicological investigation on their feathers and eggs was launched.

In May and June 2022, eggshell samples from those five nests were collected. Thanks to the careful recording of the previous years, feather samples from each territory were matched to the eggs. Our goal was to analyse the eggshells and feathers to determine the levels of heavy metals and trace elements present in them.

Environmental pollution has a big impact on raptors as they scavenge and hunt prey that is likely contaminated (Krone, 2018). In recent years, Eastern Imperial Eagles have changed habitats to populate agricultural flatlands where their prey is different to the small rodents they used to eat. A higher proportion of game species such as hare and pheasant make up their diet, thus competing with hunting activity (Horváth *et al.*, 2018). Hunting activity is widespread in Hungary, and it is likely to contribute to the release of toxic elements into nature. For example, lead shot pellets from hunting activity can be lost in water, which are then ingested by prey, or the game prey is directly shot and the contaminated carcass is scavenged (Krone, 2018).

1.1 Eggshells as a matrix

Dauwe *et al.*, (1999) compared eggshell heavy metal levels in passerine birds nesting in two different sites, one of which was heavily polluted. Cadmium, arsenic and lead were found at significantly higher concentrations in the polluted site than the reference one. In fact, lead levels were forty times higher than in the latter, while cadmium and arsenic were each almost four times higher. Other trace elements such as zinc and copper were not significantly elevated in the eggshell, although they were more elevated in the egg content similarly to the three previous elements.

Scheuhammer, (1987) theorised that trace elements such as cadmium and lead may interact with the metabolic pathway of calcium which would explain why they are more easily found in the eggshell than copper or zinc. Burger, (1994) studied the metal levels in the eggshells and contents of Herring Gulls (*Larus argentatus*) and Roseate Terns (*Sterna dougallii*). For egg contents, the levels found were as follows in order of lowest to highest concentrations; cadmium, mercury, lead, chromium, and manganese. For the eggshells in the same order; mercury, cadmium, lead, chromium and manganese.

It is advantageous for females to have the opportunity to excrete metals normally sequestered in bones such as mercury, lead and cadmium into their eggs. However, this is detrimental to their reproductive success as higher concentrations of these metals in eggs are embryotoxic (Burger, 1994).

1.2 Feathers as a matrix

Feathers are becoming more popular as a matrix to investigate levels of certain elements. The advantage is they can be collected from a nest's vicinity without disturbing the birds, and do not require more invasive techniques needed to obtain bone, kidney, blood, or liver samples. They are often collected opportunistically during ringing operations or other sampling activities, as was the case for us.

Elements such as heavy metals can bind to certain functional groups of proteins such as keratin, allowing them to be integrated into the feather's internal structure if they are present during growth. Birds can eliminate an important proportion of the body's burden by sequestering elements in their feathers. Considering the moulting period when analysing feather burden is important, as it may vary throughout seasons as elements gradually move from internal tissues to feathers (Dauwe *et al.*, 2003).

External contamination of feathers makes it difficult to determine the true level of contamination birds were exposed to. Even after cleaning, Pain *et al.*, (2005) found that feathers of Spanish Imperial Eagles kept in museums had levels of lead which correlated with the age of the specimen, proving how difficult it is to truly remove external sources of lead.

Feathers of birds of prey are good indicators for the extent of environmental heavy metal pollution. Nonetheless, simply relying on the values detected from feather samples is not enough to give a true representation of the actual load within an organism. A significant proportion of metals found in feathers are not active metabolically, as different organs will accumulate different concentrations. Furthermore, different metals will vary in concentration within parts of the body. There is a lack of research in using element concentrations of feathers from birds of prey as values reflecting the true toxicological status of the bird (Lodenius and Solonen, 2013).

There are many different factors which impact the concentration of certain elements in feathers, age of the feather and the type of metal are notable ones. The most important factor which may reduce the reliability of concentrations in feathers is external contamination. Cleaning before analysis is very important, but most routine laboratory methods are not sufficient to fully eliminate metals deposited externally via dust, gases, or liquids. Furthermore, metals may be deposited during preening via the uropygial gland, which coats the feather in a layer of oil which makes it even more difficult to decontaminate (Lodenius and Solonen, 2013). In fact, Dmowski, (1999) determined that in feather analysis, only mercury corresponded with internal levels which itself are distributed via the blood stream. The older the feather, the higher the concentration of elements deposited externally.

Further evidence on free-living Great Tits (*Parus major*) showed that zinc and mercury concentrations from feathers are mainly from endogenous sources than external contamination (Sidra *et al.*, 2022). Mercury, cadmium, copper, zinc, arsenic and chromium have a higher affinity for keratin than other metals (Dmowski, 1999).

A study on Swedish birds showed that zinc contamination is mainly internal, and that levels vary along the shaft most likely due to the variations in dietary zinc concentrations. Similar conclusions were drawn for copper, another essential element which varies over time depending on input. The concentration of essential elements is regulated by the organism, so any increase in the feathers is not necessarily proportional to environmental levels and may not indicate the true level of contamination. Cadmium on the other hand, is not an essential element and its levels do not vary according to the body's metabolism. This shows that a low-level stable cadmium contamination from the environment was deposited into the feathers mostly internally because it couldn't be washed away. This study also concluded that lead in feathers was found from both internal and external sources. (Ek *et al.*, 2004).

The detection of high levels of cadmium, cobalt, nickel and lead in feathers is less likely to be due to high amounts in blood during their growth. As such, elevated amounts detected in feathers is thought to be due to atmospheric pollution or from secretions of the uropygial gland during preening (Rodríguez-Álvarez *et al.*, 2022).

1.3 Feathers and moulting patterns in Aquila heliaca

The anatomy of feathers makes them well-suited to trap particles in a solid, liquid or gaseous form (Lodenius and Solonen, 2013). Our method did not specify the type of feather which was sampled, nor the part which was analysed yet it is important to consider how the stage of moult may affect the concentration of elements tested for. If the concentration in feathers is representative of the body's burden at a given time, different stages of feathers will have different levels. As feathers are fully formed and separated from the rest of the body, those which are moulted first are more likely to accumulate higher concentrations of metals while those that form later will have lower concentrations. Furthermore, feather segments most exposed to external conditions such as the tip and the vane contain higher levels of metals (Dauwe *et al.*, 2003).

In Central Europe, adults start to moult between March and April, which is finished by October to November. It takes two moults to replace the entire plumage, and 6-7 years for a bird to gradually replace the paler juvenile plumage to obtain the darker distinctive look of adult *Aquila heliaca* (Forsman, 2003). Our samples were taken during the moulting period, suggesting that any feathers collected around the nests would contain an elemental burden representative of the previous months.

1.4 Bioaccumulation/biomagnification in raptors

The risk of having toxic effects from certain elements such as mercury, cadmium and lead in birds increases with trophic levels and age of the animal due to bioaccumulation. Bioaccumulation refers to the net accumulation of metals over time within an organism from repeated contamination. Biomagnification refers to the build-up of some metals and other substances such as organic compounds throughout trophic levels. Meaning that the concentration ratio of a predator relative to its prey is much higher. This concept does not apply to all elements, for those such as copper and zinc are regulated by a healthy organism and excreted throughout its lifetime (Lodenius and Solonen, 2013).

These metals find themselves excreted in the faeces and deposited in feathers (Burger, 1994). Additionally, females will sequester these elements into their eggshells during breeding season, much like they do with calcium. As such, we can use the levels of elements found in eggshells as indicative of the levels present in the bird (Maegden *et. al*, 1982). This allows for a non-invasive method of evaluating trace element levels in birds.

A study of Korean raptors by Kim and Oh, (2016) showed that age and species of the raptor brings variability upon the levels of trace elements detected, for example in kestrels copper, lead, and cadmium can be significantly higher in adults compared to juveniles. In owls however, only lead levels were more elevated in adults, some of which showed potentially toxic levels.

1.5 Definition of essential elements and non-essential elements.

Certain elements which find themselves in living organisms are natural components of the environment, and are present in various concentrations in plants, soil, and animals. Others are present in significant amounts due to anthropogenic activity such as smelting, mining or metallurgic as well as agricultural. The combustion of fuels also releases a significant amount of metals in the air, present as fine particulates which penetrate into organisms and cause health problems (Manisalidis *et al.*, 2020). Most of these are metals (or metalloid in the case of arsenic) thus they will be referred to as metals or trace elements in this paper.

Elements classified as micronutrients are considered essential in certain amounts for the normal function of organisms. Of those which we sampled, they were copper (Cu), cobalt (Co), manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn). All of the above are defined as trace elements, because they are required in much smaller quantities than the other six elements (hydrogen, carbon, phosphorus, oxygen, nitrogen and sulphur) required to build most of the important molecules needed for a living system (Jomova *et al.*, 2022). However, there is still ongoing debate in the scientific community about the essentiality of some of these elements and for which species. The classification above follows the general most recent consensus that the author could find.

Non-essential elements tested were cadmium (Cd), mercury (Hg), lead (Pb), barium (Ba), arsenic (As), and chromium (Cr). Their prevalence in the environment has increased due to human industrial activity (Jomova *et al.*, 2022). These are subject to bioaccumulation and biomagnification in the food chain, which increases their risk to health. Just like in other organisms, toxic metals are associated with negative health effects in birds. These include behavioural alterations, impaired immune systems, decreased reproductive success and general disturbance of metabolism and physiological systems (Rodríguez-Álvarez *et al.*, 2022).

1.6 Effect of heavy metals on fertility and reproduction in birds of prey

One of the elements which has been heavily studied due to the numerous toxic effects it has on ecosystems is lead. Its widespread use in hunting ammunition for waterfowl and small game has drastically increased its concentrations in aquatic environments. Though birds of prey are rarely the direct target of such shots, they become contaminated by scavenging or preying on contaminated game (Krone, 2018).

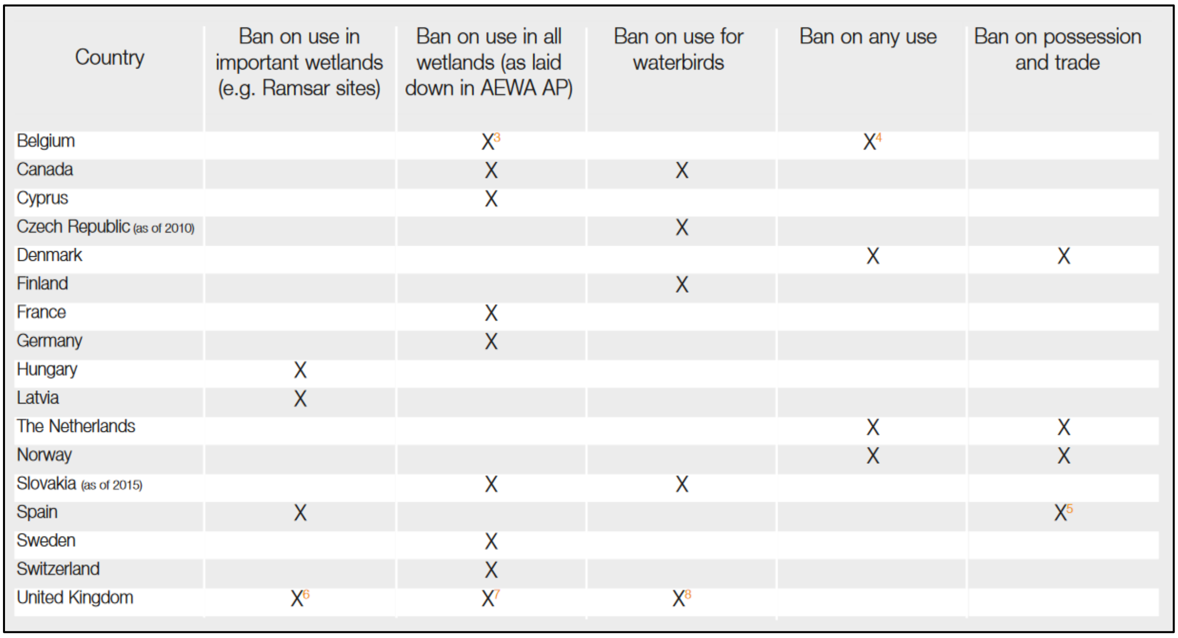
1.6.1 Lead

According to Krone, (2018) the main source of lead in birds of prey comes from oral ingestion of metallic lead particles, most often from hunting ammunition in the form of lead shot pellets or lead-based bullets. Lead from hunting can find its way into the prey of eagles via bullets or contaminate the water in which prey such as waterfowl live as spent shot. In fact, periodic blood lead level increases were found to correlate with hunting season in multiple studies (Pain, Bavoux and Burneleau, 1997); (Craighead and Bedrosian, 2008).

Lead pellets are often used to hunt flock birds such as waterfowl where multiple birds can be hit in one shot. Birds may have varying amounts of pellets in their bodies which allows many to survive long enough to be eaten by raptors as prey or carrion (Krone, 2018).

Waterfowl are particularly susceptible to lead poisoning due to their bottom-feeding habits and the intentional ingestion of grit which accumulates in the gizzard as part of normal digestion. This grit is often mixed with lead left over from hunting activity which finds its way at the bottom of the body of water and during digestion finds its way in the bloodstream (Sanderson and Bellrose, 1986). Though waterfowl are not a main source of prey for *Aquila heliaca* in Hungary, their scavenging behaviour exposes them to contamination via other game species. For example, Common Pheasants (*Phasianus colchicus)* are hunted using lead shot and they make up a significant proportion of Eastern Imperial Eagles' diet (Horváth *et al.,* 2018).

Thanks to the African-Eurasian Waterbird Agreement (AEWA) effective since 1999, the use of lead shot in wetland hunting is being phased out in Europe. However, a 2007 report showed that progress can still be made in legally prohibiting the use of lead for such hunting. Notably, Hungary has banned its use in important wetland sites (Figure 1), but nowhere else (AEWA, 2009). Unfortunately, *Aquila heliaca* and their prey are not geographically restricted to important wetland sites. Promisingly, Spanish Imperial Eagles in Doñana where an important wetland is located and a ban on the use of lead shot was implemented showed a decrease in its ingestion (Mateo *et al.*, 2007).



Country	Ban on use in important wetlands (e.g. Ramsar sites)	Ban on use in all wetlands (as laid down in AEWA AP)	Ban on use for waterbirds	Ban on any use	Ban on possession and trade
Belgium		X[3]		X[4]	
Canada		X	X		
Cyprus		X			
Czech Republic (as of 2010)			X		
Denmark				X	X
Finland			X		
France		X			
Germany		X			
Hungary	X				
Latvia	X				
The Netherlands				X	X
Norway				X	X
Slovakia (as of 2015)		X	X		
Spain	X				X[5]
Sweden		X			
Switzerland		X			
United Kingdom	X[6]	X[7]	X[8]		

Table 1: Type of ban in countries having phased out the use of lead shot (AEWA, 2009).

Scavenging is associated with higher-than-normal Pb levels, most likely due to the lead shot left behind by hunters. European Common Buzzards (*Buteo buteo*) and Cinereous Vultures (*Aegypius monachus*) were susceptible to this toxicity, most likely due to their scavenging feeding habits (Kim and Oh, 2016). The periodic increase of lead toxicity during hunting season is in accordance with those findings (Pain, Bavoux and Burneleau, 1997). Furthermore, lead isotopes from hunting ammunition were repeatedly matched with those found in raptors, which increases the probability that hunting lead is the source of contamination for birds of prey (Monclús, Shore and Krone, 2020).

1.6.2 Pathophysiology of lead ingestion

Aquila heliaca and other birds of prey consume lead particles present in the bodies of their previously shot prey, or while scavenging animals killed by hunters. Raptors have gizzards which can get filled by lead particles as they consume lead-contaminated prey, which gets further broken down mechanically and chemically, accumulating in the gizzard's mucosal folds. These small lead particles are digested and may remain in the gizzard or pass further into gastrointestinal tract. The combination of acid, enzymes and muscular action of the gizzard makes Pb^{2+} ions which are readily available for absorption in the small intestines (Martinez-Haro *et al.*, 2009). Low gastric pH and the fact that gizzards do not allow expulsion in the oral direction of large indigestible items increases retention time of those particles. This makes raptors susceptible to digestion and absorption of lead (Krone, 2018).

1.6.3 Effects of lead exposure in birds

Kendall *et al.*, (1996) showed that raptors which ingested lead-contaminated food demonstrated symptoms of acute and chronic lead intoxication. High levels of lead increased mortality, predation, and infection while subacute levels decreased reproduction and competitiveness for resources. An important mechanism of toxicity of lead is its ability to bind to proteins and especially enzymes, thus modifying their biological function. The consequences are a general derailment of many organ systems, explaining why the symptoms of lead intoxication are varied.

The central nervous system (CNS) is affected by severe degenerative changes, which often leads to altered behaviour. A lead burden of 4 mg/kg in gulls has been linked to delayed parenting, movement and feeding behaviour (Burger and Gochfeld, 1993). Numerous studies have also shown that low-level background lead is associated with increased activity of the antioxidant system (Monclús, Shore and Krone, 2020).

Furthermore, the immune system is impaired which leaves birds more susceptible to viral and bacterial infections. In addition to the loss of condition induced by the pathologies above, lead intoxication causes loss of body mass leading to emaciation. This is most likely due to feeding behavioural changes, partial paralyses and less specific metabolic alterations which prevent adequate nutrition (Kendall *et al.*, 1996). All the above reduce the chances of successful reproduction.

Haematopoietic disturbances are notable with lead intoxication. The activity of enzymes necessary for the synthesis of haeme such as delta-aminolevulinic acid dehydratase (ALAD) and haeme synthase are inhibited by lead. Haeme is important for the formation of haemoglobin as well as mitochondrial cytochromes such as P450 which are essential for numerous detoxifying processes. Anaemia will occur following chronic ALAD inhibition, even if it is on a low level (Sassa, Granick and Kappas, 1975).

Few studies have investigated on the effects of heavy metals specifically on raptor reproduction. He *et al*., (2020) showed that chronic lead exposure in juvenile female Japanese Quails (*Coturnix japonica*) caused it to accumulate in the ovaries, and ovarian development was delayed by high doses. These high doses (500-1000 ppm) caused histopathological changes to the ovarian tissue. Granulosa cells were disorganised, follicle atresia and interstitial cell degeneration was seen. Furthermore, they demonstrated that high lead concentrations significantly downregulated the expression of genes responsible for ovarian steroidogenesis, and oestradiol levels were also significantly lowered.

Lead exposure negatively affects reproduction parameters such as egg hatching rate, sperm motility and organ development in Domestic Pigeons *(Columba Livia domestica*) and Red-legged Partridges (*Alectoris rufa*) (Pain, Mateo and Green, 2019). Lead toxicity was shown to directly cause sterility, abortion, and higher neonate mortality. Buerger, Mirarchi and Lisano, (1986) showed that a single shot pellet resulted in early embryonic death and reduced hatchability (26%) in Mourning Dove (*Zenaida macroura*) eggs.

In male quails (*Coturnix coturnix*), chronic subtoxic lead exposure induced histological alterations in the testes which were significant enough to stop spermatogenesis (Almansour, 2009).

1.6.4 Lead distribution in the bodies of birds

After oral ingestion, lead distribution in the body varies according to time and absorption before elimination from the gastrointestinal tract. Soon after ingestion, it is rapidly deposited in soft tissues primarily the kidneys and liver, as well as growing feathers and bone. Bone is where the highest concentrations are normally found and there it is particularly immobile. This is of particular importance for laying females where medullary absorption is highest, especially during chronic exposure (Franson and Pain, 2011). The increased concentration of lead in the bones of females before and during the breeding season may account for the sex differences in the immune responses of male and female birds. Rocke and Samuel, (1991) found that male mallards were more sensitive to the immunosuppressive effects of lead than females, likely because the females were able to sequester lead in their bones more easily.

Levels may depend on age of the birds as well as sex. Feathers may contain higher lead concentrations if the birds were exposed during the growing period. In juvenile birds, lead feather concentrations were well-correlated with levels in other tissues such as bone and kidney (Golden *et al.*, 2000). Lead does accumulate in calcium-containing tissues such as bones, feather and hair, and females are able to release lead in a similar manner to calcium during laying season (Grúz, 2017).

1.7 Brief ecology of Eastern Imperial Eagles

1.7.1 Range and distribution

Eastern Imperial Eagles (*Aquila heliaca*) (Savigny, 1809) of the Accipitridae (eagle) family share the particularity with other *Aquilinae* of having feathers on their tarsi unlike other bare-legged members of the family. They are sometimes referred to as “booted eagles”.

The Carpathian basin has the largest and most Western population of Eastern Imperial Eagles outside of Russia and Kazakhstan (Horváth *et al.*, 2011). Their upper elevation limit in central and Eastern Europe is 2000 metres (BirdLife International, 2016). These birds can nest in low mountainous areas such as the Mátra, Bükk and Zemplén mountains although they prefer low-land territories in the vicinity of mountains. There they can be found in clusters of trees or electrical pylons (Meyburg and Kirwan 2013) surrounded by wide open (often agricultural) land which serves as their hunting ground. Human activity will force them to retreat to middle-range mountains, which was observed in the 1990s (Horváth *et al.*, 2011).

The IUCN Red List (International Union for Conservation of Nature) regional assessment in Europe classified Eastern Imperial Eagles as ‘Least Concern’ in 2020 with resident populations in the Carpathian Basin. This is a more promising status than *Aquila heliaca* as a species which holds a ‘Vulnerable’ status. In Europe, their current population trend is increasing, with 3,900-6000 mature individuals (1,900-3000 pairs) recorded as of 2020 worldwide. 270-390 breeding pairs are estimated in the EU28, with 150-230 recorded in Hungary as of 2017 (BirdLife International, 2016). Half of the world’s breeding population was found in Europe in 2002 (Horváth *et al.*, 2002).

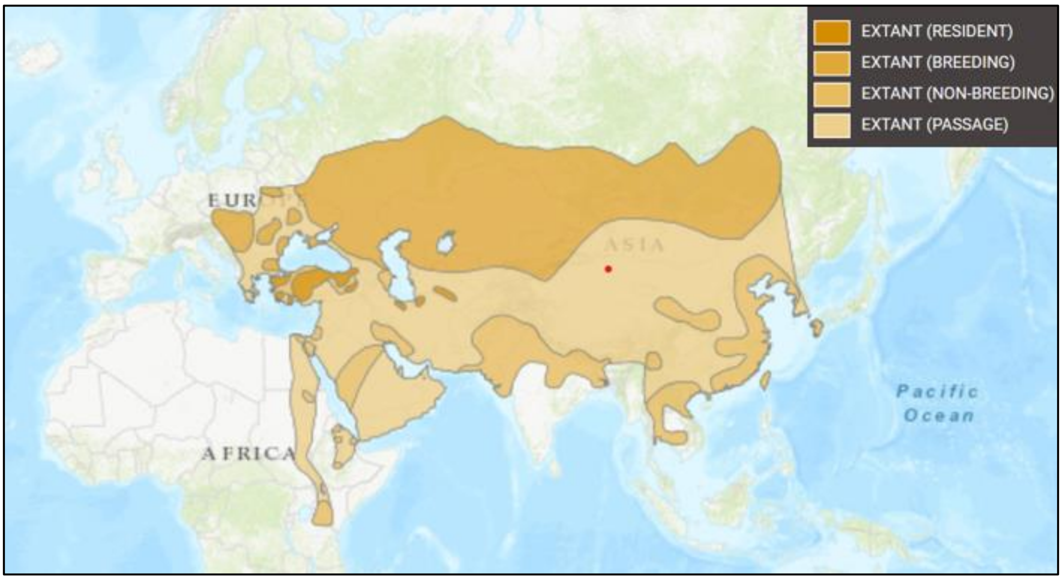


Figure 2: Geographic distribution of different types of populations of Aquila heliaca (BirdLife International, 2016).

Resident population refers to a group of individuals of a particular bird species that remain within a specific geographic area year-round without long-distance migrations. They are also known as sedentary because they stay in the same breeding and non-breeding habitats throughout the year (Clements *et al.*, 2022). Population of resident Eastern Imperial Eagles are present over most of Eurasia from Austria to Russia and as far south as Ethiopia (BirdLife International, 2016).

Breeding population refers to birds within a species that engage in reproductive behaviour such as building nests, laying eggs and raising offspring in a particular area during a season (Clements *et al.*, 2022). Breeding populations of Eastern Imperial Eagles are recorded from Eastern Europe (Bosnia and Herzegovina) to Central Asia (BirdLife International, 2016).

Non-breeding populations are usually present in a more favourable area (habitat, temperature, food availability) where the birds have migrated to feed and survive. No reproductive behaviour is seen in these places, which range from the Middle East to South East Asia (BirdLife International, 2016).

Other than the locations mentioned above, vagrant (birds outside of their expected range) Eastern Imperial Eagles have been recorded all over Eurasia and parts of North and West Africa such as Morocco, Lybia and Togo. Eastern Imperial Eagles are considered full migrants as a species, despite some individuals remaining in Europe during the winter months.

1.7.2 Feeding ecology

In Hungary, Eastern Imperial Eagles were documented to have a wide prey diversity with 129 different species recorded. Their diet evolves with the availability of prey that varies over the years. Previously, the Common Hamster (*Cricetus Cricetus*) and European Ground Squirrels (*Spermatophilus citellus*) were significant, but these species have become too scarce to provide a reliable source of prey, even if the hamster is still important in their diet. More recently, small game species such as the European Hare (*Lepus europaeus*) and the Common Pheasant (*Phasianus colchicus*), both plentiful in Hungarian lowlands made up the majority of their diet. In addition, Eastern Imperial Eagles preyed on corvids, pigeons, waterbirds, other rodents, the young of roe deer as well as other raptors and owls to a lesser extent (Horváth *et al.*, 2018). As such, Eastern Imperial Eagles take advantage of their opportunistic feeding habits, scavenging, and even engaging in kleptoparasitism from other species in order to adapt to their changing environment (Danko, 2012).

1.7.3 Breeding

The Carpathian Basin is the most western point of the Eastern Imperial Eagle's breeding range, breeding pairs have been observed there since proven recording has begun (Horváth *et al.*, 2011). As such, conservation efforts in this important location are numerous and effective as demonstrated by the increasing population numbers and the regional 'Least Concern' IUCN status. In contrast, Eastern Imperial Eagles are decreasing globally and are considered to be 'Vulnerable' (BirdLife International, 2016).

In Hungary, breeding pairs are found in the central Great Hungarian Plains as well as the Northern Hungarian Mountains (Mátra, Bükk and Zemplén). Since the 1990s as monitoring increased, the expansion of the breeding range from mountain forests to lowland plains was recorded, with up to 50% of the Hungarian population. Pairs that nest in the mountains can often forage 10-15 km away from their nests as opposed to lowland-breeding pairs who's foraging habitats are usually within a 3-8 km radius from the nests (Horváth *et al.*, 2011). This could be explained by the fact that lowlands are often composed of extensive agricultural fields and grasslands which house a higher concentration of prey, and as such there is no need to travel more.

This 8-year study between 2001 and 2009 determined that the population trend for Eastern Imperial Eagles increased yearly on average by 10% which was the highest recorded since 1980, doubling the population. This varied depending on habitat, with mountainous breeding pairs decreasing yearly by 5 % on average while lowland breeding pairs were closer to +15% on average. By 2009, of 105 breeding pairs recorded as many as 85% bred in lowland agricultural habitats (Horváth *et al.*, 2011). A recent technical report of nesting pairs between 2017 and 2019 showed that numbers in Hungary increased from 230-250 to 285-300 nesting pairs during those three years (Horváth *et al.*, 2020).

Breeding pairs will often have multiple nests in one territory, though one nest may be suitable for several seasons and can thus be used for consecutive years. These nests often grow to 100-150 cm as the pair will add materials to reinforce it before the chicks hatch in late spring and fledge by the end of summer (Meyburg and Kirwan, 2020). Their incubation period lasts 43 days with chicks fledging between 65-80 days after (Sós-Koroknai, 2020).

1.7.4 Reproductive success

In the same 8-year period where the population dynamics of *Aquila heliaca* were studied, so was breeding success. 142 breeding failures were recorded, defined as a complete lack of offspring born and not including chick mortality.

Typically, annual breeding success was from 59-75% with a fledgling success in accordance with the increasing population. 0.91-1.30 fledged successfully on average per pair (Horváth *et al.*, 2011). A fledgling chick does not guarantee survival to reproductive age, but since mortality is highest in the beginning of life it highly increases the chances of a successful population renewal.

Table 2: Causes of breeding failures, mortality of eggs and chicks of Imperial Eagles in Hungary between 2003 and 2009 (Horváth et al., 2011)

	Breeding failure			**Mortality**		
Cause	Incubation	Chick-rearing	Total	Egg	Chick	Total
Storm	18 (16 %)	15 (54 %)	33 (23 %)	37 (15 %)	34 (42 %)	71 (22 %)
Disturbance	15 (13 %)	2 (7 %)	17 (12 %)	27 (11 %)	3 (4 %)	30 (9 %)
Unfertilized eggs	7 (6 %)		7 (5 %)	23 (10 %)		23 (7 %)
Poisoning	2 (2 %)	1 (4 %)	3 (2 %)	4 (2 %)	3 (4 %)	7 (2 %)
Cainism					3 (4 %)	3 (1 %)
Shooting to the nest	2 (2 %)		2 (1 %)	3 (1 %)		3 (1 %)
Mortality of parents	1 (1 %)		1 (1 %)	2 (1 %)		2 (1 %)
Illegal logging	1 (1 %)		1 (1 %)	2 (1 %)		2 (1 %)
Abnormal embryo				1 (0 %)		1 (0 %)
Haywire coiled on chick					1 (1 %)	1 (0 %)
Nest-robbing					1 (1 %)	1 (0 %)
Unknown	68 (60 %)	10 (36 %)	78 (55 %)	139 (58 %)	36 (44 %)	175 (55 %)
Total	114 (100 %)	28 (100 %)	142 (100 %)	239 (100 %)	81 (100 %)	320 (100 %)

In the 2017-2019 study on nesting pairs, it was determined that 78-79% of the breeding pairs managed to lay eggs. In those that laid eggs, breeding success was 1.8-1.9 chicks per pair. Accounting for those that did not lay eggs successfully, breeding success for the species overall in that period was of 1.4-1.5 chicks per pair (Horváth *et al.*, 2020). As the above table shows, up to 60% of breeding failure occurred at the incubation stage for reasons other than those listed (Horváth *et al.*, 2011).

Figure 3 : *Aquila heliaca* juveniles (Aurore Quéromain, 2022)

2. Materials and Methods

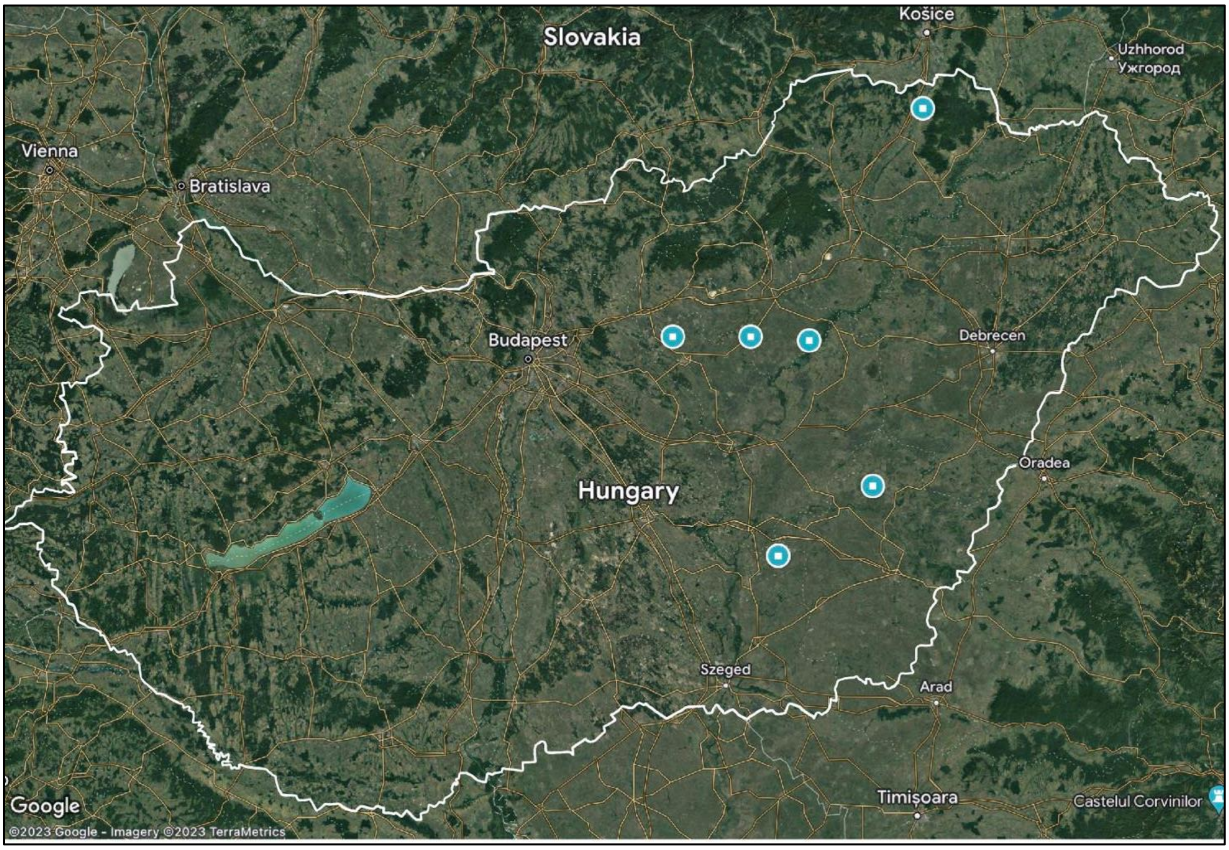


Figure 4: Approximate nest locations of the infertile pairs (Aurore Quéromain, 2023)

The focus was on Eastern Imperial Eagles in Hungary, which are specially protected under Hungarian law. They are under careful safeguarding by Magyar Madártani és Természetvédelmi Egyesület (MME) also known as the Hungarian Ornithological and Nature Conservation Society or simply BirdLife Hungary.

The monitoring and field sample collection for the study was undertaken in the frame of the Eastern Imperial Eagle conservation program of MME BirdLife Hungary, supported by the EU LIFE Nature Fund (PannonEagle LIFE, LIFE15NAT/HU/000902). The laboratory analyses were undertaken and financed with the cooperation of MME and the University of Veterinary Medicine, Budapest.

MME has been monitoring nests all over Hungary, working with rangers from local national parks to record precisely where they are. *Aquila heliaca* pairs tend to stay within the same territory even if they may change nests throughout the years. In lowland habitats, their foraging range is around 3-8 km from their breeding territory, there is little impingement on territory between pairs (Horváth *et al.*, 2011). As such, anything gathered from the nest's vicinity can confidently be attributed to the closest nest. Every year, teams go to each nest to record data. Pellets are picked apart to determine the prey composition of the pair in that area. Eagle feathers from the vicinity of the nest are also taken for genetic analysis, as well as anything of importance such as regurgitated pellets. The chicks in each nest are counted, weighed, measured and their age is estimated (Horváth *et al.*, 2020).

MME's team along with rangers from various national parks in Hungary worked together in the May and June 2022 to ring chicks and record data about that season's Eastern Imperial Eagles. Annually, around 100-150 breeding birds were surveyed.

Figure 5: Ringing of Aquila heliaca juveniles (Aurore Quéromain, 2022)

At each nest, a specialised tree climber climbed the tree to put the eagle chicks in a bag and lowered them down so that data could be collected. The number of chicks were recorded along with their weight, and age was estimated according to leg bone measurements and state of juvenile plumage.

On top of this, pellets and prey bird feathers found in the nest and its vicinity were collected and, from that the species of prey was determined. Eggshell fragments, whole eggs if present as well as feathers were also gathered as part of ongoing research on *Aquila heliaca* genetics in the Carpathian Basin (Zsinka, Pásztory-Kovács and Kövér, 2023).

Five Eastern Imperial Eagle nests with recurrent infertility since 2011 were targeted for this study. We explored the possibility that essential and non-essential elements present in abnormal levels in the bodies of those eagles could be responsible for this long-term breeding failure. Eggs from each nest and the matching feathers from 2022 (or from previous years if none could be collected in 2022) were sent to the Ecology department of the University of Veterinary Medicine in Budapest (UVMB) for analysis.

Most studies investigating the toxicology of environmental contamination in birds use samples from livers. This method was too invasive and terminal for the birds, as such we could not use it due to the conservation status of Eastern Imperial Eagles which did not justify the death of birds for research purposes. The use of feathers and eggshells as samples to identify trace element contamination is not widespread enough in literature. This makes it difficult to obtain reliable reference values allowing to determine if levels are elevated enough to cause toxicity and subsequent reproductive disorders. We acknowledge that this is a limitation of our study.

2.1 Reagents and analytical standards

Trace chemical analysis needs special care due to the unusually low concentrations of the elements normally present in the samples. Contamination or leakage at the time of measurement can quickly undermine the reliability of the results obtained, as such the instrument must be well-calibrated using trace analysis quality reagents. The samples were analysed using inductively coupled plasma mass spectrometry (ICP-MS).

For sample preparation, trace analysis quality reagents are used. Hydrogen peroxide (30 m/m%, AnalaR NORMAPUR), nitric acid (69 m/m%, Aristar), hydrochloric acid (37 m/m%, Aristar) and ethanol (AnalaR NORMAPUR) are purchased from VWR International Ltd. (Leicestershire, UK). Deionized water (18.2 MΩ/cm) is produced by a Purite Select Fusion 160 BP water purification system (SUEZ Water Technologies & Solutions, Trevose, PA USA). For the calibration of the inductively coupled plasma mass spectrometer (ICP-MS) a multi-element standard solution (Instrument Calibration Standard 2, TruQms) is used; the internal standard elements is added by using Internal Standard Mix (TruQms), both purchased from Perkin Elmer Inc. (Waltham, MA USA). Quality control standards is prepared from bovine liver (Standard Reference Material 1577c) obtained from Merck KGaA, (Darmstadt, Germany). The argon gas used is of 4.8 purity and purchased from Messer Hungarogaz Ltd. (Budapest, Hungary).

2.2 Sample preparation

The preparation of feather samples was preceded by a multi-stage washing and drying process, during which: (1) the samples were washed with hot tap water; (2) soaked in 50 v/v% ethanol for 15 minutes; (3) dried in a drying oven at 105 °C for 60 minutes, (4) soaked in 50 g/L EDTA solution for 1 hour; (5) rinsed twice with high purity water; (6) dried in a drying oven (105 °C; 2 hours). As such, the values for the feathers are given as mg/kg dry weight.

The egg samples were prepared as such: the egg yolk and white were mixed in the beginning of sample preparation, and the previous steps for the feathers were repeated. The egg samples were not dried, and the results are in mg/kg wet weight.

From each sample 0.5 g is weighed into a CEM MARS XPreSS Teflon vessel, mixed with 5 mL of hydrogen peroxide and 5 mL of nitric acid, and digested in a CEM MARS6 microwave digestion system (CEM Corporation, Matthews, North Carolina, USA) with the following parameters: ramp in 35 min; temperature at 200 °C; hold for 50 min; MW power at 1700 W; 40 places. After digestion, the vessels are emptied and washed into 50 mL polypropylene (PP) tubes (Deltalab, Rubí Barcelona, Spain). Then they are made up to 25 mL by deionised water, and before analysis diluted 5-times in 12 mL PP tubes (Deltalab, Rubí Barcelona, Spain) by deionised water after adding 0.4 mL ethanol and 100 µL of internal standard solution to them, containing 1 µg/mL bismuth (Bi), germanium (Ge), indium (In), scandium (Sc) and yttrium (Y). The quality control and blank samples are prepared the same way. 0.15 M hydrochloric acid solution is used for the cleaning of the Teflon vessels between the digestion rounds.

2.3 Instrumentation

Determination of the elements is carried out by a Perkin Elmer NEXION 2000 (Perkin Elmer, Waltham, MA, USA) ICP-MS instrument in helium KED mode (low and high cell gas flow 5.6 and 6.7 mL/min, respectively) by applying the following instrument parameters: solid-state RF generator, 34.5 MHz LumiCoil, free running; RF power: 1600 W; Nebuliser type: Meinhard plus quartz, low internal volume, type C; Plasma gas flow rate: 15 L/min; Auxiliary gas flow rate: 1.2 L/min; Nebuliser gas flow rate: 1.06 L/min.

2.4 Validation of the analytical method

In order to assess the reliability of the analytical method including sample preparation, several validation parameters are established according to the relevant guidelines (Commission Decision 2002). Limits of detection (LOD) and limits of quantitation (LOQ) are calculated as three and ten times the standard deviation of concentration values of the blank samples, respectively. Trueness is determined by analysing SRM 1577c certified reference material (CRM). Spiked samples of the CRM are used for those elements which does not have certified values or the measured value are below the LOQ. The precision is determined as the relative standard deviation of the concentration values of ten replicates of the same CRM or spiked CRM samples. Percentages are used to express both precision and trueness. Linearity is evaluated by using the equations of the calibration curves. The *r* values of the calibration curves of each analytical isotope are ≥ 0.999. Matrix effect is not studied separately as the internal standard solution used in each sample is compensated for this effect. The detected isotopes, the LODs and LOQs of each element as well as the precision and trueness are shown in Table X and Y. Precision is accepted if the deviation of the measured parameter does not exceed 10%, trueness values are accepted below ±20%. The recoveries for the quality control standards are between 90 and 115% for each measured element (Table 2).

Table 3: Measured isotopes, limit of detections of the elements and quality control (QC) of the ICP-MS measurements.

						QC (ERM-CE278k)			
Element	Isotope (m/z)	LOD* (mg/kg)	LOQ* (mg/kg)	Precision %	Trueness %	Certified value (mg/kg)	Measured value (mg/kg)	Measured with spike** (mg/kg)	Recovery (%)
Arsenic (As)	75	0.0041	0.014	4.4	1.5 – 14.7	0.0196 ± 0.0014	<LOQ	1.110 ± 0.049	109
Barium (Ba)	135	0.13	0.42	2.5	-4.0 – 3.5	N.A.	<LOQ	1.005 ± 0.025	100
Cadmium (Cd)	111	0.0088	0.029	7.3	-1.6 – 19.8	0.0970 ± 0.0014	0.1060 ± 0.0077	N.A.	109
Chromium (Cr)	52	0.17	0.58	4.4	-8.0 – -4.0	0.053 ± 0.014	<LOQ	0.995 ± 0.044	97
Cobalt (Co)	59	0.0016	0.0052	5.1	-0.1 – 14.5	0.300 ± 0.018	0.322 ± 0.018	N.A.	107
Copper (Cu)	65	0.17	0.55	4.0	-1.4 – -10.8	257.2 ± 4.6	273.0 ± 10.8	N.A.	106
Lead (Pb)	207	0.11	0.36	2.7	-12.5 – (-4.9)	0.0628 ± 0.0010	<LOQ	0.955 ± 0.026	97
Manganese (Mn)	55	0.066	0.020	2.4	-12.0 – (-5.2)	10.46 ± 0.47	9.48 ± 0.22	N.A.	91
Mercury (Hg)	201	0.20	0.66	2.8	8.2 – 17.6	N.A.	<LOQ	1.113 ± 0.031	112
Molybdenum (Mo)	95	0.020	0.068	2.2	-3.4 – 3.8	N.A.	3.30 ± 0.13	3.32 ± 0.07	101
Nickel (Ni)	60	0.060	0.20	2.8	-5.5 – 3.2	0.0445 ± 0.0092	<LOQ	1.059 ± 0.029	98
Zinc (Zn)	68	1.0	3.3	3.3	-1.3 – 8.1	181.1 ± 1.0	189.3 ± 6.3	N.A.	105

3.Results

Table 4: Measured elements in egg and feather samples of Aquila heliaca. The column “measurement” refers to the sample weight.

				Measuremen	As	Ba	Cd	Co	Cr	Cu	Hg	Mn	Mo	Ni	Pb	Zn
Sample	ID	Date	Location	g	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Feather_1	AQU HEL 1			0.1926	0.0076	<0,13	<0,0088	0.0037	<0,17	9.26	<0,20	0.14	<0,020	0.35	2.21	121
Feather_2	AQU HEL 2			0.0536	<0,0041	<0,13	<0,015	0.0073	<1,0	10.8	<0,40	0.74	<0,070	0.41	<0,11	92.3
Feather_3	AQU HEL 3			0.0687	0.062	<0,13	<0,0088	0.015	<0,30	11.7	<0,20	1.05	<0,18	0.12	1.02	87.7
Feather_4	AQU HEL 4			0.1998	0.025	<0,13	<0,0088	0.0030	<0,30	10.4	<0,20	0.25	<0,020	0.10	6.30	98.9
Feather_5	AQU HEL 5			0.2036	0.084	<0,13	<0,0088	0.011	<0,30	10.2	<0,20	0.37	<0.035	0.11	3.91	100
Egg_1		17/06/2011	Sarud	0.5005	0.018	18.6	<0,0088	0.012	<0,17	0.87	<0,20	2.83	<0,020	0.26	0.337	4.1
Egg_2		20/06/2022	Udvarnok	0.5019	<0,0041	17.7	<0,0088	0.0078	<0,17	0.70	<0,20	1.57	0.021	0.18	<0,11	6.0
Egg_3		22/06/2022	Göncruszka	0.5069	0.0070	12.6	<0,0088	0.010	<0,17	1.05	<0,20	5.03	0.027	0.12	0.20	17.5
Egg_4		24/06/2022	Székesmajor	0.5006	0.015	25.6	<0,0088	0.020	<0,17	0.88	<0,20	1.59	<0,020	0.16	0.26	10.1
Egg_5		08/06/2022	Horvát dűlő	0.5143	0.011	32.3	<0,0088	0.013	<0,17	0.82	<0,20	1.98	<0,020	0.14	0.66	2.2
Egg_6		17/07/2022	Sand	0.5147	<0,0041	26.5	<0,0088	0.0076	<0,17	1.15	<0,20	1.07	<0,020	0.12	0.70	9.9
Detection limit (for 0.5 g sample*)					0.0041	0.13	0.0088	0.0016	0.17	0.17	0.20	0.066	0.020	0.060	0.11	1.0

There were four elements which could not be measured by the laboratory method. These were cadmium, chromium, mercury, and molybdenum (except for two egg samples, but the amount was very close to the undetectable limit) due to undetectable levels in the eggs and feathers. In the case of barium, levels could be measured in the egg but not the feathers.

The level of arsenic which could be measured in the feathers were 0.0076, 0.025, 0.062, and 0.084 mg/kg while the eggs were 0.0070, 0.011, 0.015, 0.018 mg/kg. In both samples of the second nest, the levels were below the detectable level. Barium was not present in high enough amounts in feathers, but in eggs it was detected at 12.6, 17.7, 18.6, 25.6, 26.5, and 32.3 mg/kg.

In the feathers, cobalt was detected at 0.0030, 0.0037, 0.0073, 0.011, and 0.015 mg/kg while the eggs were 0.0076, 0.0078, 0.010, 0.012, 0.013, and 0.020 mg/kg. For copper the feathers were at 9.26, 10.2, 10.4, 10.8 and 11.7 mg/kg while the eggs were 0.70, 0.82, 0.87, 0.88, 1.05, and 1.15 mg/kg.

Manganese was detected at 0.14, 0.25, 0.37, 0.74, and 1.05 mg/kg in the feathers while the eggs had 1.07, 1.57, 1.59, 1.98, 2.83, and 5.03 mg/kg. Molybdenum had only 2 detectable levels in the eggs, which were 0.021 and 0.027 mg/kg. In the feathers, nickel was present at 0.10, 0.11, 0.12, 0.35 and 0.41 mg/kg while the eggs contained 0.12, 0.12, 0.14, 0.16, 0.18, and 0.26 mg/kg. Lead levels in the feathers were 1.02, 2.21, 3.91, and 6.30 mg/kg while the eggs contained 0.20, 0.26, 0.337, 0.66, and 0.70 mg/kg. Finally, zinc was found at 87.7, 92.3, 98.9, 100 and 121 mg/kg in the feathers while in the eggs it was at 2.2, 4.1, 6.0, 9.9, 10.1 and 17.5 mg/kg.

Table 5: Comparison of arsenic levels in eggs and feathers in mg/kg.

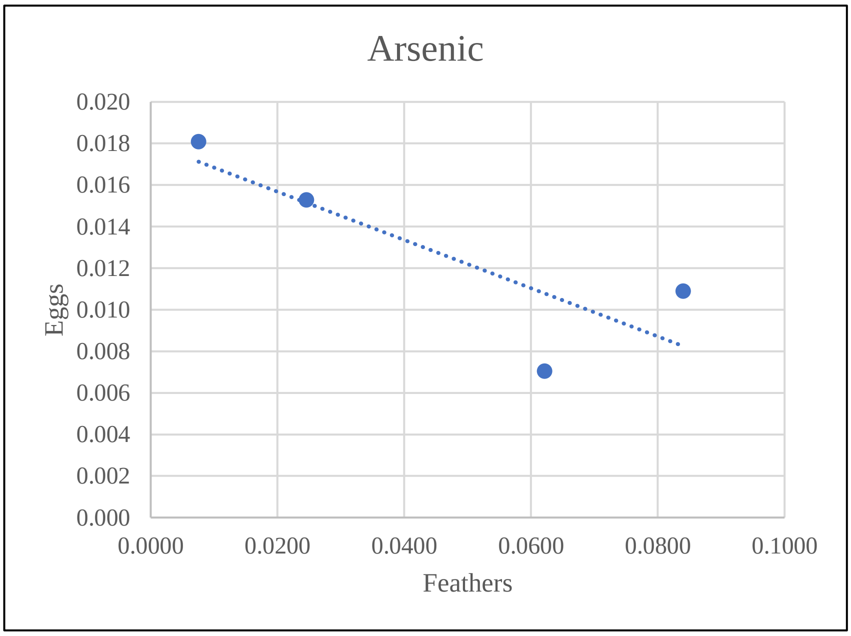


Table 6: Comparison of cobalt levels in eggs and feathers in mg/kg.

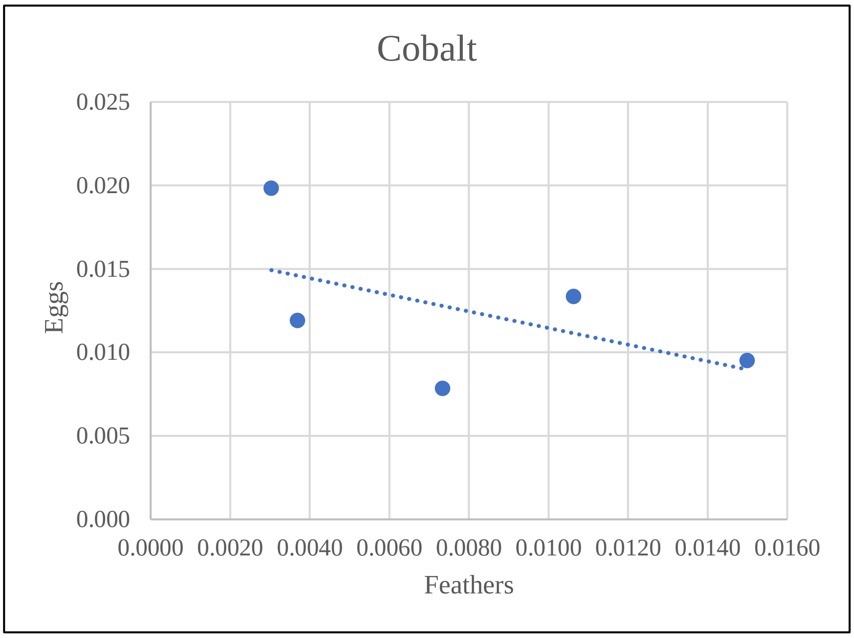


Table 7: Comparison of copper levels in eggs and feathers in mg/kg

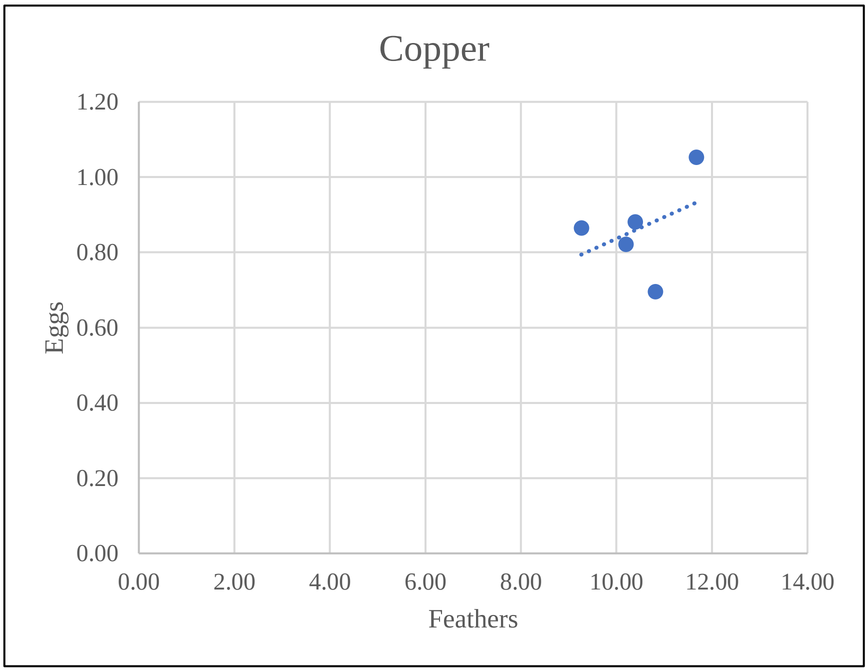


Table 8: Comparison of manganese levels in eggs and feathers in mg/kg.

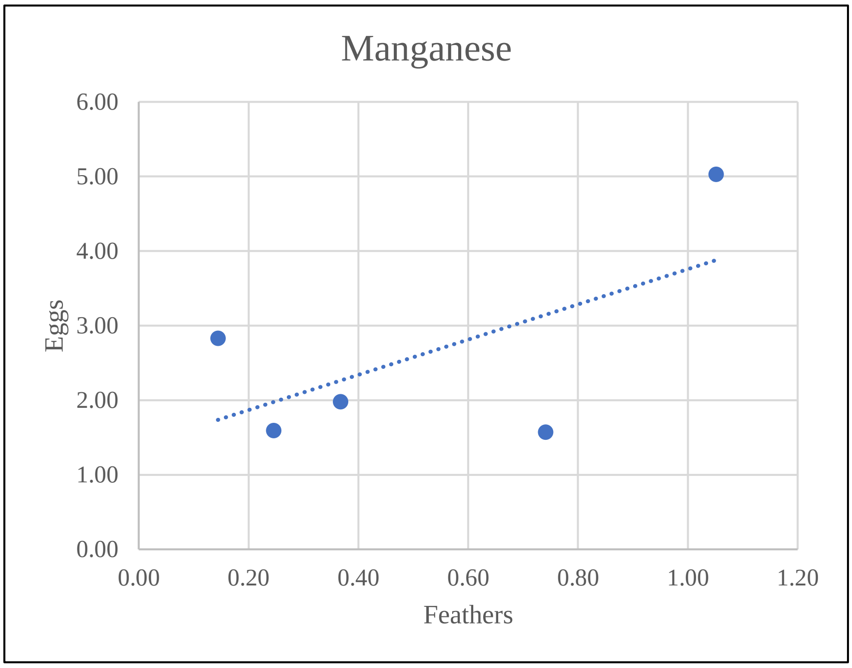


Table 9: Comparison of nickel levels in eggs and feathers in mg/kg.

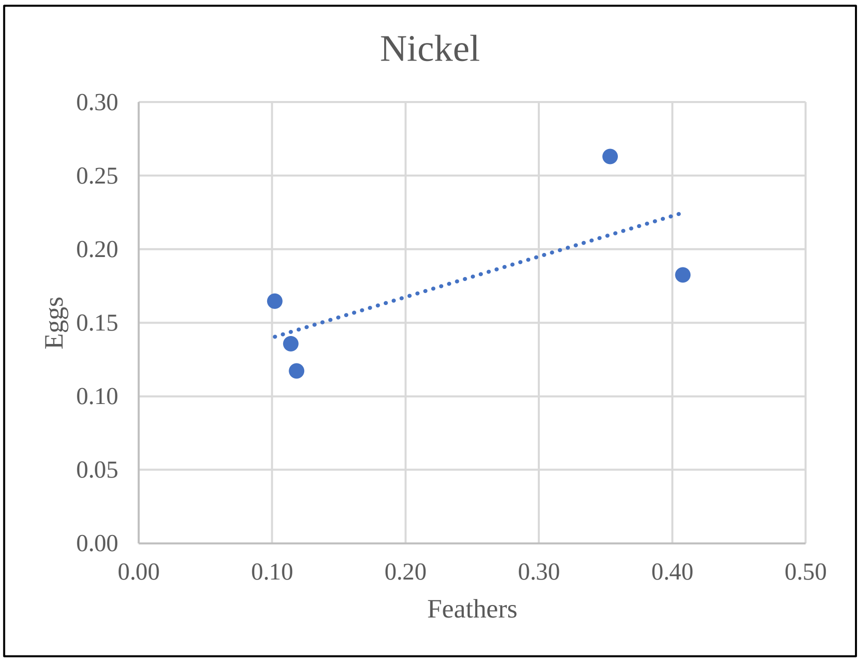


Table 10: Comparison of lead levels in eggs and feathers in mg/kg.

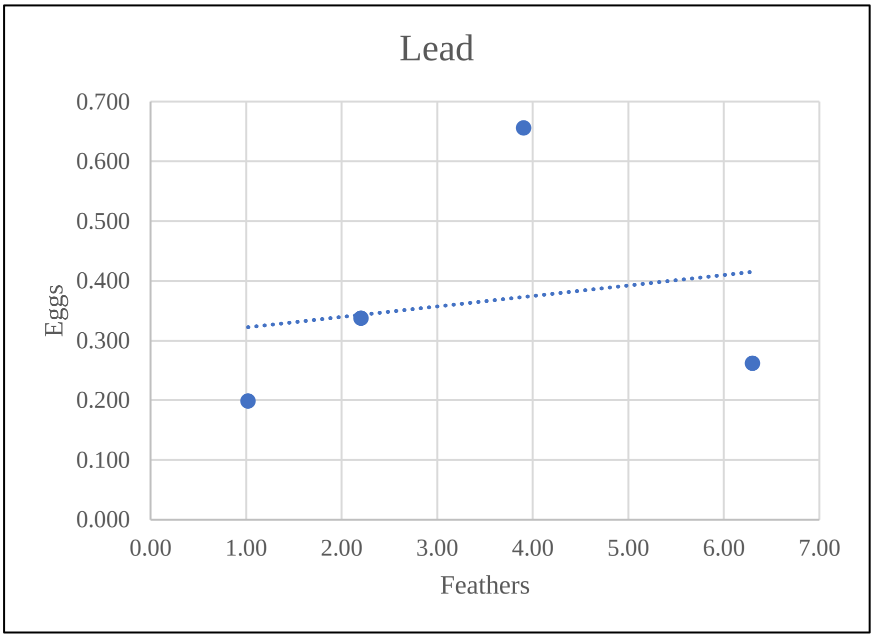


Table 11: Comparison of zinc levels in eggs and feathers in mg/kg.

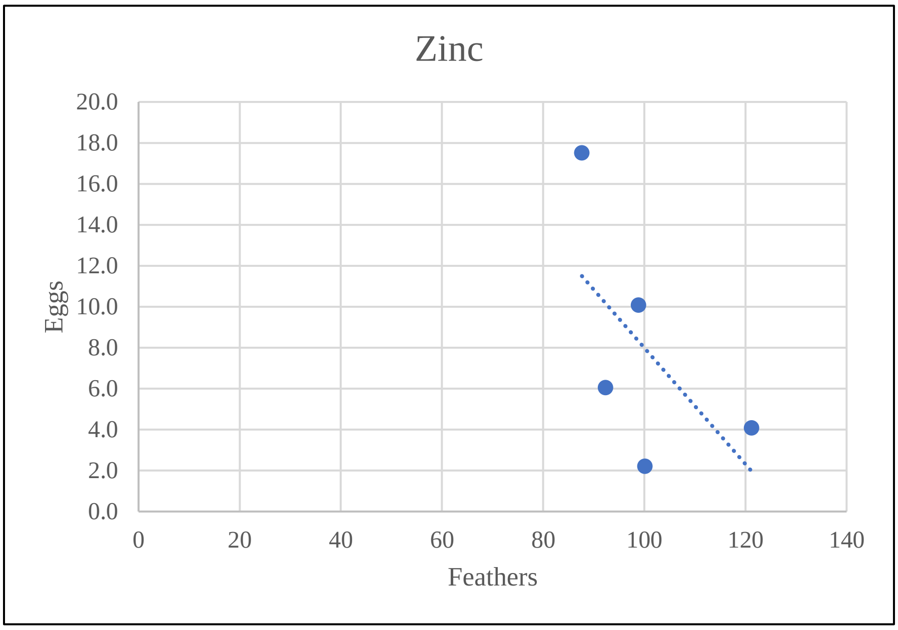


Table 12: Mean, median, standard deviation (SD) and interquartile range (IQR) of all the elements detected in feathers.

Feathers				
Elements	Mean value (mg/kg dry weight)	Median value (mg/kg dry weight)	SD	IQR
As	0.0446	0.0434	0.0348	0.0473
Ba	No values above the detection limit			
Cd	No values above the detection limit			
Co	0.0079	0.0073	0.0050	0.0069
Cr	No values above the detection limit			
Cu	10.470	10.390	0.8794	0.6179
Hg	No values above the detection limit			
Mn	0.51	0.37	0.3775	0.4949
Mo	No values above the detection limit			
Ni	0.22	0.12	0.1488	0.2394
Pb	3.36	3.06	2.2922	2.5969
Zn	100	99	12.8749	7.791

Table 13: Mean, median, standard deviation (SD) and interquartile range (IQR) of all the elements detected in eggs.

Eggs				
Elements	Mean value (mg/kg wet weight)	Median value (mg/kg wet weight)	SD	IQR
As	0.013	0.013	0.0049	0.006
Ba	22.22	22.1	7.1986	8.401
Cd	No values above the detection limit			
Co	0.012	0.011	0.0045	0.0047
Cr	No values above the detection limit			
Cu	0.91	0.87	0.1652	0.1777
Hg	No values above the detection limit			
Mn	2.34	1.79	1.44	1.04
Mo	No values above the detection limit			
Ni	0.16	0.15	0.06	0.06
Pb	0.43	0.34	0.23	0.39
Zn	8.3	8.0	5.48	5.4733

4. Discussion

All of the values detected were beneath the Meant Tolerated Values (MTL) established for avian species (*Front Matter | Mineral Tolerance of Animals.*,2005). Grúz, (2017) who used a similar method to sample different bird species in Eastern Hungary concluded that most of the elements they sampled were within the normal range. They noted that due to biomagnification, species on a higher trophic level (such as *Aquila heliaca*) could be more susceptible to show toxic symptoms.

A similar study in Spain showed that levels in birds of prey were greater than background yet none reached the levels necessary to cause toxicity (Pérez-López *et al.*, 2008).

4.1 Arsenic

The mean detected value for arsenic was 0.013 mg/kg for eggs and 0.0446 mg/kg for feathers which was below what a similar Hungarian study found. In the feathers of apparently healthy Hungarian wild birds, values ranged from 0.35 ± 0.21 mg/kg for the Barn Swallow (*Hirundo rustica*) to 0.65 ± 0.56 mg/kg for Little Owls (*Athene noctua*) (Grúz, 2017). Our values for arsenic were well below any threshold we could find, suggesting it is not a cause for concern in Hungarian Eastern Imperial Eagles.

Arsenic is a toxic element classed as a persistent environmental pollutant, coming from natural and anthropogenic sources, present in the air, soil and water. In waters it is dissolved or in fine particulate form from natural deposits, industrial and agricultural effluents. In soil, it can come from pesticides, the product of burning fossil fuels, industrial effluents and lastly it is naturally present in the earth's crust (Patel *et al.*, 2023).

The effects of arsenic on organisms are varied, from neurologic, cardiovascular, dermatologic and carcinogenic effects with variations according to its chemical form. It generates reactive oxygen species (ROS) which are themselves the source of many negative health effects. The accepted permissible limit for daily intake of arsenic in air, water and food are 10 µg/l, 0.5 mg/kg, and 1.5 mg/kg respectively. These values not only apply to humans but also wildlife (Patel *et al.*, 2023).

In birds, impairment of normal liver function as well as the teratogenic effects of arsenate on offspring have been noted. Specifically in male birds, a notable increase in kidney tumours has been observed. Furthermore, arsenic can induce chromosomal damage in bone marrow cells, and though the exact mechanism has not been demonstrated it is suggested that the formation of ROS and the inhibition of DNA repair is important (Francis, 2017). Arsenic also affects the avian central nervous system as well as the development of chicks and their survival (Wilson, Petersen and Troy, 2004). Feathers are an appropriate tool for measuring arsenic load, as the element binds well to sulphur groups in keratin (Grúz, 2017).

4.2 Barium

The effects of barium are varied on organisms especially at chronic low doses, it is not considered as an essential element with a biological role. This element is present abundantly in the earth’s crust, water, and plant sources. As such, contamination is inevitable in predators such as raptors which feed on a wide variety of herbivorous prey. Anthropogenic sources such as industrial manufacture of paints, ceramics and adhesives (Dauwe *et al.*, 1999).

Barium carbonate is the most common source in plant materials, the break down by stomach acid allows it to be assimilated by animals. In the past, barium carbonate was used as a rodenticide. The toxicity of soluble barium salts is dose-dependent, chronic exposure can lead to cardiovascular and renal damage as well as neuromuscular disorders. Furthermore, a strong correlation between prenatal exposure and congenital defects in newborn children has been recorded (Peana *et al.*, 2021).

In birds, few studies have investigated the toxic effects of soluble barium salts. Furthermore, acceptable thresholds in diet and organisms of wild birds have not been determined. Chronic exposure is associated with cardiovascular disorders and muscular hypertension in experimental animals. In King (*Somateria spectabilis*) and Spectacled Eiders (*Somateria fischeri*) living in Alaskan oil development areas, blood barium levels were higher in females, and its presence in ducklings proves a maternal source (Wilson, Petersen and Troy, 2004).

Barium could not be detected in feathers in our analysis, only in the eggs. Our mean value was 22.22 mg/kg, the author could not find reference values in literature for eggs. However, Wilson, Petersen and Troy, (2004) found mean levels at 0.2 µ/g in the blood of Alaskan eiders. It is unknown how representative egg levels are of the body’s total burden, we acknowledge this limitation.

4.3 Cobalt

There are few studies investigating the specific effects of cobalt in wild birds, and even less its presence in feathers. In most studies, cobalt levels are generally low and thought to pose no risk to bird organisms (Burger *et al.*, 2018). Though naturally occurring, it is released in large quantities from petroleum, metal and mining industries. It is essential in trace amounts to synthesise vitamin B12 as methylcobalamin, as well as a co-factor for enzymes such as mutases and synthetases. In toxic amounts, some of the most notable effects of cobalt are gastrointestinal disturbances, cardiomyopathies, motor disorders, polycythaemia, and hyperlipemia due to pancreatic cell damage. Furthermore, it negatively affects embryonic development by inhibiting the growth of the amnion as well as inducing brain lesions in ovo (Domingo, 1989).

We found mean cobalt levels of 0.0079 and 0.012 mg/kg for feathers and eggs respectively. Norouzi *et al.*, (2012) detected a mean feather value of 0.0012 mg/kg in

Iranian Chukar Partridges (*Alectoris chukar*) sampled in a protected area without observable health effects. Our values were slightly higher but cobalt overload is infrequent and its toxicity is relatively mild compared to other metals (Jomova *et al.*, 2022) thus it is unlikely to cause adverse health effects at those levels.

4.4 Copper

Another essential micronutrient, copper only becomes toxic when the liver is unable to regulate its levels or dietary intake is too high. It is needed for a myriad of enzymatic processes, notably haemoglobin synthesis, antioxidant mechanisms, growth and energy metabolism. The toxic dose of copper for birds is high, (250-1000 mg Cu/kg feed) which is unlikely to occur in the wild (Grúz, 2017).

In healthy South African Barn Owls (*Tyto alba*) a non-pathogenic level is considered to be 2.29 ± 0.579 µg/g in primary feathers (Ansara-Ross, Ross and Wepener, 2013). In Hungarian birds, the lowest levels were seen in the feathers of Barn Swallows at 13.34 mg/kg, while in seemingly healthy Hungarian Sparrowhawks (*Accipiter nisus*), the highest levels of copper detected were at 65.45 ± 17.66 mg/kg in feathers. Higher levels were attributed to the proximity of industrial factories (Grúz, 2017). Our mean feather and egg values were at 10.470 and 0.91 mg/kg respectively, which is below even the lowest values of the cited study. Therefore, it is unlikely to be a causative factor of infertility in these eagles.

4.5 Manganese

Burning diesel fuel is one of the main sources of environmental manganese contamination. Though essential for the organism, in vertebrate sub-lethal exposure is associated with teratogenic effects, altered growth, haemoglobin formation disorders and behavioural disturbances (Rodríguez-Álvarez *et al.*, 2022).

In this study, mean manganese concentrations were at 0.51 mg/kg for feathers and 2.34 mg/kg for eggs. This is far higher than the average levels found in the feathers of Alaskan Bald Eagles (*Haliaeetus leucocephalus*), (0.0343 mg/kg) where birds did not show signs of toxic contamination (Burger and Gochfeld, 2009). It would be interesting to further explore the effects of manganese in birds, and whether the levels we detected could have negative consequences on their health and reproduction. Few studies have looked into the subject, and previous research is not enough to draw a conclusion on these concentrations.

4.6 Nickel

Extensively used in the metal and chemical industries, nickel is also present naturally in the environment though most of it today is from anthropogenic sources. Mining, the burning of fossil fuels, car traffic and fertiliser use all spread nickel into the ecosystem. On top of that, plants accumulate nickel, which makes it bioavailable to the food chain, becoming more toxic for animals on a higher trophic level (McIlveen and Negusanti, 1994) such as *Aquila heliaca*.

Nickel in the atmosphere is present as particulate form, where it is readily inhaled. Run-off from industrial sites will contaminate water sources as well, rendering it available to wildlife. Nonetheless, the proportion of nickel in soil and vegetations is threefold that of atmospheric and water concentrations, suggesting toxicity is a greater threat when ingested (Outridge and Scheuhammer, 1993).

As the use of lead shot is becoming scarcer, hunters will switch to different ammunition. However, the environmental safety of these alternative bullets is not necessarily higher. Steel may contain nickel in significant quantities, which may be enough to induce toxicity in birds if they ingest contaminated prey, though this has yet to be proven (Brewer *et al.*, 2003).

Though its biological role has not clearly been defined, the evidence suggests that it can be considered an essential micronutrient, it is subject to some homeostatic control. Nickel in higher amounts can cause reduced growth, disturb reproduction and the metabolism of other trace elements (notably iron). This can occur during chronic exposure, starting with dietary levels as low as 5µg/g. It is probable that moulting removes much of the body's burden, as nickel levels can be unusually high in the feathers compared to the other tissues (Outridge and Scheuhammer, 1993).

Furthermore, Ansara-Ross, Ross and Wepener, (2013) showed a significant positive relationship between the burden in feathers and the liver. Feathers will readily accumulate nickel, suggesting they are an appropriate matrix for evaluating nickel levels.

Wiemeyer, Jurek and Moore, (1986) showed there may be a link between breeding status and nickel burden. In Turkey Vultures (*Cathartes aura*), breeding pairs had twice the amount of nickel in feathers and kidneys than non-breeding ones. This suggests that reproduction leads to a higher accumulation of nickel, though this is perhaps due to increased bodily requirements.

In South African Barn Owls (*Tyto alba*), feather levels were found at 0.00407 mg/kg (Ansara-Ross, Ross and Wepener, 2013). Our results showed much higher values of 0.22 mg/kg for feathers and 0.16 mg/kg for the eggs. The tissues of birds from uncontaminated environments should not have more than 0.0001-0.005 mg/kg. In a contaminated environment, tissue levels can increase from 0.005-0.08 mg/kg (Outridge and Scheuhammer, 1993), which *Aquila heliaca* exceeded. These concentrations are theoretically enough to cause significant toxic effects, though proving their negative role in reproduction is beyond the scope of this study.

Nickel toxicity is rarely described in wild animals. A study by Eastin and O'Shea, (1981) showed that even levels up to 0.8 mg/kg in diet were not enough to cause adverse health effects in adult mallards, although ducklings were more sensitive. However, there is currently little evidence which would allow for the definition of a real tissue threshold that could induce negative health consequences.

These toxic health effects include reduced immune function and a disturbance of normal thermoregulation. The impact of nickel on reproduction includes a reduced number of offspring, reduced weight, and increased mortality of neonates. Nickel can induce teratogenic effects, though this is poorly studied in birds. Malformations in chicken embryos following nickel administration have been described (Outridge and Scheuhammer, 1993).

The feather levels may have risen due to their exposition to the external environment and subsequent contamination. However, none of the other metals susceptible to similarly contaminate feathers such as cadmium, lead, or mercury were increased. This suggests that the elevated concentrations could be significant, yet with the data available, no conclusions can be drawn on their possible reproductive impact.

4.7 Lead

Lead use is widespread in hunting activities due to its presence in bullets and shot, which contributes to environmental contamination. As one of the most notorious trace elements susceptible to cause adverse health effects it was justified to test for lead in infertile eagles. High levels of lead in the body of raptors increases mortality, predation and infection while subacute levels are enough to decrease reproduction and competitiveness for resources (Kendall *et al.*, 1996).

Multiple investigations on the effects of high lead concentrations on breeding parameters yielded contradictory results. In Spanish Imperial Eagles there was no link between lead and the viability of eggs. Other species of raptors showed no association between high lead levels and fecundity, nestling mortality as well as eggshell thickness (Monclús, Shore and Krone, 2020). A link between lead and decreased breeding parameters was shown on Spanish Bonelli's eagles (Pain, Mateo and Green, 2019). Our results did not show a positive association either, suggesting the subject of infertility in certain breeding pairs is more complex than heavy metal intoxication.

The following values were gathered by Franson and Pain, (2011) to determine thresholds for lead in various tissues. For subclinical poisoning in Falconiformes, blood levels were 20 < 50 µg/dl, liver was 2 < 6 mg/kg (wet weight) and kidneys were 2 < 4[d] mg/kg (wet weight). For clinical poisoning the values were 50-100 µg/dl, 6-10 mg/kg ww and 4-6[d] mg/kg ww respectively. The background concentration of lead was determined at <20 µg/dl (or <0.2 mg/kg) for blood, <2 mg/kg in the liver and kidneys, and <10 mg/kg dry weight in bone.

There does not seem to be a level at which lead has no effect on birds, because certain enzymes such as haem-synthase were inhibited at a blood lead concentration of <5 µg/dl (<0.05 mg/kg), which is astonishingly low. Therefore, any lead present in the body has a negative effect on the organism.

It is difficult to find reliable thresholds for feathers, most likely due to the perceived unreliability of feather values because of external contamination, though Burger and Gochfeld, (2009) suggested that feather levels around 4 mg/kg would be enough to cause harmful health effects due to bioaccumulation. In Hungarian wild birds, levels ranged from 5.36 ± 1.46 mg/kg for Barn Swallows to 1.34 ± 0.67 mg/kg in Common Buzzards (*Buteo buteo*) (Grúz, 2017). In general, the lowest lead concentrations are found in feathers and eggs relative to other tissues, and feathers are not the best suited to evaluate dietary lead exposure (Ek *et al.*, 2004). We detected far lower levels than the threshold determined by Burger and Gochfeld, (2009), suggesting that lead levels in Hungarian *Aquila heliaca* are not threatening to their health.

Liver analyses of Hungarian raptors showed mean values of 0.58 mg/kg (Sós-Koroknai, 2020) which remains below the threshold for subclinical poisoning of 2 < 6 mg/kg established by Franson and Pain, (2011). This supports the assumption that lead poisoning is not a major threat to wild Hungarian raptors.

4.8 Zinc

Zinc is widely used in industries such as mining, milling and smelting. Galvanisation, where a zinc coating is present on the surface of metals to prevent corrosion makes this element susceptible to contaminate environments (Beyer *et al.*, 2004).
It is a micronutrient, necessary as a co-factor for enzymes responsible involved in the metabolism of carbohydrates and proteins. Furthermore, this element is essential for the proliferation and differentiation of keratinocytes, which then form skin and feather. This may be why high levels accumulate in the feathers sampled.

Animals will tolerate an excess of zinc in their diets as they are able to regulate levels through excretion, providing the levels are not high enough to prevent effective homeostasis. As such, high concentrations of zinc are not dangerous from a toxicological perspective. In fact, there is a positive relationship between cadmium and zinc concentrations in many species of birds. High levels of zinc provide a protective effect which reduces the absorption and accumulation of cadmium (Rodríguez-Álvarez *et al.*, 2022). In excess, it is associated with degenerative changes of the exocrine pancreas, decreased motor function and typhlitis, though these levels are hard to reach (>2200 mg/kg feed) (Beyer *et al.*, 2004).

Ek *et al.*, (2004) also determined that zinc content in feathers is internally sourced, yet higher levels are no cause for concern. The levels in seemingly healthy Hungarian wild birds ranged from 110.64 ± 14.62 mg/kg in little owls to 157.21 ± 57.30 mg/kg for owls (Grúz, 2017). In South African owls, levels were detected at 75.1 ± 13.5 μg/g though these values varied greatly according to species (Ansara-Ross, Ross and Wepener, 2013). Our mean feather results were 100 mg/kg and 8.3 mg/kg for eggs. Considering the high levels necessary to cause toxicity and the similar values in other Hungarian birds, it is unlikely that our results are alarming.

4.9 Elements which could not be detected in the samples

The following elements were not present in high enough amounts to be detected by ICP-MS in feathers or eggs. Particularly in the case of cadmium and mercury, their potential negative effects on reproduction are non-negligeable. As such, we cannot disregard their possible impact on the infertility of those eagles, we can only state that they could not be detected in the matrices analysed.

4.9.1 Cadmium

Cadmium is a non-essential element whose atmospheric levels rose steeply during the Industrial Revolution, peaking between 1970 and 1980. Thankfully, levels decreased to half of their peak values a decade later in the 1990s and this trend continues. Despite this, human activities continue to increase cadmium pollution in the environment. Bird habitats located near polluted areas such as mines or other industrial sites tend to show higher levels of cadmium (Dauwe *et al.*, 1999). Birds living in coastal areas are at higher risk than inland terrestrial birds such as *Aquila*, because pelagic environmental contamination far exceeds that of the atmosphere.

Cadmium is readily accumulated in organisms, thus birds at a higher trophic level such as Eastern Imperial Eagles are at risk of intoxication. In Hungary, cadmium levels in the feathers of wild birds were highest in Barn Swallows at 0.13 ± 0.06 mg/kg. Higher concentrations in agricultural areas could be linked to fertiliser use (Grúz, 2017).

Respiratory uptake of cadmium is more efficient than intestinal absorption, however this does not diminish the role that dietary cadmium plays in intoxication. Cadmium is accumulated in avian tissues over time and poses a real toxicological risk, though levels are lowest in terrestrial birds like *Aquila heliaca*. (Wayland and Scheuhammer, 2011). Feathers are a notable source of excretion for cadmium, between 1-28% of the body's burden can be removed in this manner. As such they are a reliable tool to measure levels in birds, while eggs are not such an important source of cadmium (Burger, 2008).

However, when using feathers as a matrix for estimating toxicological burden, one must account for the external contamination from airborne cadmium which may skew the true levels. The kidneys and liver remain the organs where the most cadmium is found, with approximately 67-97% percent of the body's burden. Some studies found that the feather cadmium burden was correlated with the kidneys and liver's while others did not. There is also the issue of external contamination from the uropygial gland during preening, as this gland is also a place of cadmium accumulation.

Chronic cadmium exposure is the source of toxicity in wild birds, acute poisoning has not been reported (Wayland and Scheuhammer, 2011). Low-level chronic intoxication is enough to cause reproductive and behavioural alterations which may negatively affect breeding success. Laying hens (*Gallus domesticus*) experimentally injected with cadmium intraperitoneally showed up to a 31% reduction in egg laying compared to a saline-injected control group (Timothy D. Nolan, Dan Brown, 2000) . Scheuhammer, (1996) showed that egg hatchability of Zebra Finches was not affected by dietary cadmium supplementation.

In male Japanese Quail (*Coturnix coturnix japonica*), dietary cadmium supplementation reduced fertility. Histological examination of the testes showed decreased germinal cell maturation as well as other changes which led to the severe decrease or total absence of sperm (Richardson, Fox and Fry, 1974). With regards to behaviour, relatively low concentrations of cadmium ingested experimentally were enough to induce significant changes in the fear response of young ducklings which the authors theorised could be relevant to wild birds (Heinz, Haseltine and Sileo, 1983). Any behavioural alterations could also have an impact on parental success.

The question of using feathers as a dependable matrix for determining toxic cadmium threshold is still without answer. Wayland and Scheuhammer, (2011) determined that studies were still inconsistent in proving that internal sequestration is the source of the levels in feathers and have been unable to reliably correlate hepatic and renal burden with that of feathers. However, cadmium has a particular affinity for keratin proteins, thus high blood cadmium levels mean that the metal will be deposited in the feathers during their growth which guarantees an internal source of cadmium in feathers (Rodríguez-Álvarez *et al.*, 2022).

4.9.2 Chromium

Sometimes classified as an essential element, chromium is mainly ingested through diet. As little as 2.80 mg/kg is enough (Burger and Gochfeld, 2009) to cause adverse effects on embryonic development and hatching. In Hungary, levels were measured at 1.69 ± 0.44 mg/kg in Barn Swallows (Grúz, 2017). In birds of prey, levels of 2.02 ± 0.603 µg/g were reported (Ansara-Ross, Ross and Wepener, 2013).

4.9.3 Mercury

Mercury contamination of humans and their environment has been omnipresent in toxicological studies for the last hundred years. Its presence in nature due to soil accumulation and via industrial processes such as coal-burning, its toxicological impacts are numerous. Unlike other elements, mercury is non-essential for organisms and as such the toxic threshold is much lower than copper for example. However, it is difficult to properly evaluate the impact of mercury and its different forms (inorganic or organic) and how they interact with different tissues, as metabolism changes its final form in organisms. This is an issue which can be generalised to other environmental contaminants (Shore *et al.*, 2011).

The source of mercury in non-maritime areas generally comes from agricultural seeds coated with mercury-containing fungicides. Small granivores (birds and mammals) consume these, and are eaten in turn by predators, which are themselves poisoned as a result. Thankfully, these fungicides are no longer used today but the environmental contamination persists. The majority of the health effects from mercury exposure are subtle yet important, and usually affect behaviour, neurochemistry and endocrine systems (Rattner, Scheuhammer and Elliott, 2011). These effects combined with teratogenicity is why we chose to test for mercury in the infertile *Aquila heliaca*. Reproductive failure can be attributed to malfunction of the reproductive organs as well as changes in behaviour which can impair mating.

A well-known property of mercury is the ease at which it is biomagnified over trophic levels, though the exact mechanism is not well understood. Methylmercury is the main form in which it is absorbed through diet. Even at sublethal levels, mercury intoxication can indirectly affect reproduction by modifying nesting behaviour, as well as directly through toxicity to the embryo. Offspring exposed to mercury through maternal transfer or direct environmental contamination can in turn suffer from increased mortality, neurological impairment and behavioural changes which prevent survival to adulthood.

Thresholds for mercury poisoning vary widely according to species and tissues examined. Most commonly, kidney and liver samples were used to determine toxic doses. In experimentally infected raptors, lethal median liver levels were set at 63 mg/kg (range 18.4-127) (Shore *et al.*, 2011). Brain concentrations higher than 15 mg/kg in adults and >3 mg/kg in embryos were associated with mortality. Studies on free-living Common Loons (*Gavia immer*) showed levels >0.3 mg/kg were enough to reduce reproductive success severely, mostly due to behavioural alterations. Egg-laying was decreased, and adults were less likely to stay in their nesting area. The concentration of mercury in eggs in numerous species showed that concentrations >1 mg/kg were associated with decreased hatchability (Scheuhammer *et al.*, 2007). Two raptors species, White-tailed Eagles (*Haliaeetus albicilla*) and American Kestrels (*Falco sparverius*) showed egg concentrations associated with adverse reproductive effects at 1.15 and 2 mg/kg respectively (Shore *et al.*, 2011).

During a period of feather growth, levels in feathers reflect the mercury concentration in the blood. In fact, about 70-93% of the total mercury burden of the body can be stored in feathers. Mercury from the blood will bind to the internal feather matrix, meaning it will not be removed by cleaning and as such is considered a good sample type for measuring heavy metal contamination. In Hungary, levels of mercury in Sparrowhawks (2.72 ± 1.08 mg/kg) exceeded the limit observed by Shore *et al.*, (2011) for Common Loons which caused a decrease in breeding success (Grúz, 2017).

4.9.4 Molybdenum

This element is rarely included in toxicological panels because its accumulation to levels that are problematic for health is rare. It naturally occurs in the environment in association with other elements. Levels will rise due to mining, coal-burning, industrial activity such as metallurgy, and agricultural fertiliser use. It can also be found in surface and groundwater in minuscule amounts (a few µg/l) but concentrations increase with industrial activity.

Essential in trace amounts for plants and microorganisms, legumes will accumulate it. In animals it is also essential for various enzymatic processes, such as its presence in xanthine oxidase. The range is narrow between toxicity and deficiency, molybdenum toxicity is primarily in its antagonist interaction with copper, where it can induce deficiency (Jarrell, Page and Elseewi, 1980). In a rural environment, molybdenum from raven feathers were detected at 0.80 ± 0.45 µg/g, rising to 1.23 ± 0.73 µg/g in an industrial setting (Adout *et al.*, 2007).This suggests that a certain amount of molybdenum in wild healthy birds is not necessarily pathogenic.

5. Summary

There were no significantly high elemental burdens which could be determined as the cause of infertility. Certain elements such as manganese, cobalt and nickel were increased, but their role in infertility could not be directly proven. Most of them were within acceptable range based on similar studies (Grúz, 2017); (Ansara-Ross, Ross and Wepener, 2013); (Burger and Gochfeld, 2009). The elements which were most likely to cause infertility at low doses (cadmium, mercury) (Richardson, Fox and Fry, 1974); (Scheuhammer, 1987); (Scheuhammer *et al.*, 2007) were those that had such low levels that they could not be detected by our methods. This further suggests that environmental intoxication is not the sole cause of infertility for these pairs of Eastern Imperial Eagles.

The concentrations in eggs and feathers were higher than those of other similar studies for manganese, cobalt, and nickel. All the other elements were within normal levels or in accordance with those found in healthy bird populations. Although manganese, cobalt and nickel can all have negative effects on breeding success, we cannot prove that they were the cause in these pairs. Due to a lack of overwhelming evidence, a clear correlation could not be shown. Nonetheless, further investigation into the concentrations of these metals and their direct physiological impact on wild birds of prey would aid our understanding of elemental burdens and their health effects.

It was interesting to evaluate the trace element burden of Eastern Imperial Eagles in Hungary, as it had never been done before in this species. Previous research on reproductive and other health issues caused by trace element contamination, particularly the use of lead in hunting ammunition justified this investigation (Buerger, Mirarchi and Lisano, 1986); (Rodríguez-Álvarez *et al.*, 2022); (Monclús, Shore and Krone, 2020).

There are many factors which can impact reproductive outcome, such as habitat disturbance and prey scarcity (Horváth *et al.*, 2011). It was unlikely that we would find a direct causation between infertility and abnormally elevated trace elements in this study design as the limitations were numerous. For example, we could not rule out by physical exam any anatomical or physiological abnormalities which would also prevent reproductive success. A lack of control around the sampling also makes it difficult to draw conclusions. Feathers and eggs show the burden of previous months, yet we cannot be certain that the concentrations detected in feathers and eggs correspond to those seen in tissues such as blood, liver, and kidneys. Furthermore, the threshold at which concentrations become toxic or sub-toxic is species-specific, and no previous studies have been done to determine that threshold on this population of *Aquila heliaca*.

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Zsinka, B., Pásztory-Kovács, S. and Kövér, S. (2023) 'University of Veterinary Medicine Budapest, Department of Ecology, Molecular Ecology Research Group Budapest, January 2023'.

Acknowledgements

The following people have been invaluable for their help and support in writing this paper.

First, Dr Péter Fehérvári, my thesis supervisor who helped me structure and guide me in my research, as well as translations.

Many thanks to Dr László Könyves, Piroska Szabó, and Dr Balázs Berlinger from the Animal Hygiene Department of UVMB for doing the trace element analysis, helping with the analytical method, and explaining it all to me.

I would like to thank Dr Viktoria Sós-Koroknai from the Budapest Zoo who connected me with MME and shared knowledge about the eagles. Thank you also to Márton Horváth and Márton Árvay from MME. I also thank Atilla Bereczky, the national park directorates, and all the national park rangers who took us to the different nesting locations for allowing me to accompany them on their yearly sampling Together, they gave me the birding bug and welcomed me in the field with them. All the data about eagle infertility came from MME's work throughout the years, for which I am very grateful for.

Lastly I want to thank my family, my partner, and my dear friend Cecily Boarder for being there for me during that time, and of course my ever-present shadow and desk buddy, Buck.

Figure 6: Tree climber going to collect chicks from a nest in Jászság National Park, Hungary (Márton Horváth, 2022)