UNIVERSITY OF TURKU
Faculty of Science

Heavy metal exposure and estrous cyclicity in semi-captive Asian elephants (*Elephas maximus*)

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Biology
Master's thesis
Credits: 30 op

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September 2022
Turku

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

TURUN YLIOPISTO
Biologian laitos

ELINA TUOMIKOSKI: Heavy metal exposure and estrous cyclicity in semi-captive Asian elephants (*Elephas maximus*)

Pro gradu -tutkielma, 49 s, 1 liites.
Fysiologia ja genetiikka
Syyskuu 2022

Background: Pollutants cause significant harm to ecosystems and organisms and have become a major concern. While some toxic substances are natural, like heavy metals, and others are anthropogenic like PCBs and microplastics, human activities are the main contamination source of both. Even though pollutants affect all organisms, they often affect long-lived animals like eagles, whales and polar bears the most due to bioaccumulation, causing various negative effects. The effects on reproductivity are of special interest, as normal reproduction is vital for any species survival. The purpose of this thesis is to study the heavy metal concentrations in endangered Asian elephants (*Elephas maximus*) in Myanmar, using a semi-captive population where blood sampling is possible, and to perform a pilot study to measure estrous cyclicity in females. The aims are to map element concentrations in elephants, and to verify a method for estrous cyclicity measurements for future studies.

Methods and results: The heavy metal concentrations were measured from whole blood samples collected from 80 semi-captive Asian elephants and the effects of age, sex and geographical location on heavy metal concentrations were studied. Age had a significant negative effect on copper concentrations, and significant positive effect on the concentrations of chromium, nickel and lead. The concentrations of aluminium and nickel were significantly lower in females than in males, and the concentrations of vanadium and lead were significantly increased. The effect of camp was ambiguous. The estrous cyclicity was examined by measuring allopregnanolone levels from serum samples collected from 11 elephant females. The method allowed successfully characterising the estrous cycle profiles of 11 elephants that were classified into three categories based on their allopregnanolone levels: into cycling (n=5), non-cycling (=4) and indefinite (n=2). The effects of heavy metal concentrations on estrous cyclicity could not be confirmed due to small sample size (n=6), however, no abnormalities in estrous cycles in respect to heavy metal concentrations were observed.

Conclusion: In this thesis, the heavy metal concentrations and estrous cyclicity were measured for the first time in this study population. There were no signs of acute heavy metal poisoning, but there were some possible indicators of chronic exposure. Also, zinc levels were found to be lower than reference values, raising concerns of possible trace element deficiency. Estrous cycle profiles corresponded to the reproductive history or status of the elephants. Two elephants out of eleven had indefinite estrous cycles, however, their cycling profiles are likely to be affected by aging. This thesis facilitates future studies on element concentrations and allopregnanolone measurement in endangered Asian elephants, which can be used in e.g. conservational efforts.

Keywords: elephants, heavy metals, elements, allopregnanolone, estrous cycle

CONTENTS

1. INTRODUCTION

Environmental pollutants are substances or energy that damage organisms at relatively low concentrations. Most commonly the term is used to refer to heavy metals and man-made toxic organic chemicals like PCBs or dioxins. What is common to these substances is that they are stable and do not break down or do so very slowly. Organisms are exposed to such environmental pollutants via air, soil, water and food. Many environmental pollutants are fat-soluble and stored in fat tissue, and so may bioaccumulate because they do not exit the organism via an elimination system, like through urine or sweat. Bioaccumulation adds to the toxicity of substances: long-term storage of toxic substances enables long-term effects and leads to enrichening of the toxic load on an individual level as well as in the food chain leading to biomagnification (Popek 2018). Furthermore, biomagnification enables accumulation of diverse pollutants simultaneously, leading to multiple pollutants affecting the organism at the same time (Rhind 2009).

Due to human activity like industrialization, agriculture and mining, wildlife is exceedingly forced to live close by humans. This leads wildlife being increasingly exposed to pollutants (Rattner 2009). Environmental pollutants impact wildlife at different levels. They may e.g. impact health of individuals to the point that population ecology like population size and growth rates are affected, for example when adverse effects on fertility decelerate population recovery rate (Sonne et al. 2015, Desforges et al. 2018, Slabe et al. 2022). Such pollution creates additional stress on top of other challenges wildlife faces like habitat loss and climate change.

Environmental pollutants affect wildlife and human physiology on a wide scale, with effects on reproductivity being especially concerning (Rhind 2009). Impaired fertility is particularly dangerous to species which are long-lived and reproduce slowly, as their recovery after population decline may be challenging and, due to bioaccumulation of pollutants, the substances can have long-term effects. For example, killer whale (*Orcinus orca*) populations worldwide are predicted to collapse due to polychlorinated biphenyl (PCB) concentrations stored in their tissues (Desforges et al. 2018). Even though production of PCBs was banned 40-50 years ago in late 1970s and 1980s, they impact killer whale population dynamics half a century later. PCBs affect especially the reproductive system, immune function and calf survival in killer whales. In addition to killer whales, the impact of PCBs on reproduction has been studied in e.g. polar bears (*Ursus maritimus*). Sonne et al. 2015 found that bacula bone densities in polar bears in

Greenland and Canada had been negatively affected by PCBs, possibly leading to challenges in reproduction. Effects of PCBs on killer whale populations and on polar bear reproductivity are good examples of environmental pollutants adding critical negative impact on survival chances of an endangered, long-lived species facing multiple challenges at once.

One the most significant environmental pollutants are heavy metals. The term "heavy metal" refers to a group of chemical elements which are classified as metals or metalloids, have high density (over 5 g/cm^3) and mass, and many of which are toxic at low concentrations (Järup 2003, Balali-Mood et al. 2021, Rajkumar & Gupta 2022). Cadmium (Cd), lead (Pb) and mercury (Hg), for example, are among most common heavy metal pollutants. Heavy metals are natural substances and found in the environment like in the Earth's crust, soil and rock foundation. Some heavy metals, like zinc (Zn) and copper (Cu), are trace metals and essential to animal and plant metabolism as cofactors in enzymatic reactions in small concentrations. However, trace metals like all heavy metals become toxic when a certain threshold value is exceeded.

Heavy metals are released during natural phenomena like volcanic eruptions and soil erosion, or due to human activity like mining and burning fossil fuels (e.g. coal) (Rhind 2009). Heavy metals are stable elements, which contributes to their qualities as difficult hazards, and to their persistence in biological cycles. Most of the heavy metals accumulate in organisms, especially in long-lived mammals like eagles, whales and polar bears (Dietz et al. 1995, Krey et al. 2015, Balali-Mood et al. 2021, Slabe et al. 2022). Some but few, like aluminium (Al) can be secreted through elimination activities like feces (Greger & Baier 1984), urine (Jones et al. 2017), sweat (Genuis et al. 2010) and semen (Hovatta et al. 1998). Often organisms, including humans, are polluted simultaneously by several heavy metals at once, like the ringed seals (*Pusa hispida*) in the Canadian arctic being polluted simultaneously at least with Hg, Pb, Cd, Cu, Zn, selenium (Se) and arsenic (As) (Wagemann 1989). The effect of heavy metals is dependent on the dosage, route of exposure, duration of exposure and the combination of heavy metals affecting simultaneously (Rhind 2009, Jaishankar et al. 2014, Balali-Mood et al. 2021, Rajkumar & Gupta 2022).

Heavy metal exposure can be acute or chronic. Acute exposure means exposure of an organism to a large amount of heavy metals at once (Rajkumar & Gupta 2022). The acute exposure can be measured from blood or urine (Wiedner et al. 2011, Rajkumar & Gupta 2022). Symptoms of an acute exposure are, among others, nausea, skin lesions and

diarrhea (Rajkumar & Gupta 2022). Chronic exposure, on the other hand, means exposure to heavy metals for prolonged time in smaller amounts, for example if the water source of an animal or human is polluted (Rajkumar & Gupta 2022). Symptoms of chronic exposure are harder to detect than in acute exposure and may vary from anemia to cancer and impaired fertility (López-Botella et al. 2021, Rajkumar & Gupta 2022). Chronic exposure is measured from internal organs and tissues like liver and bone marrow (Wiedner et al. 2011, Rajkumar & Gupta 2022). For example, as a result of chronic exposure, Hg concentrations have been observed to exceed neurotoxicity thresholds in beluga whales (*Delpinapterus leucas*) (Krey et al. 2015), possibly leading to neurochemical and neurobehavioral changes.

On a cellular level heavy metals interfere cell mechanisms like growth, differentiation, proliferation and apoptosis. Mechanisms of action include oxidative stress, enzyme inactivation, disturbing the antioxidant defence, binding to macromolecules and hormone disruption (Balali-Mood et al. 2021). Body organs and tissues which have the most dynamic cellular activity, like ovary and testis, are most sensitive to heavy metals. In both sexes heavy metals affect especially the production of gametes (oogenesis and spermatogenesis) and endocrine function (Massányi et al. 2020). In addition to decreased fertility, heavy metal pollution may lead to, *inter alia*, genetic instability, cancer, nervous system disorders and birth defects (Balali-Mood et al. 2021).

The reproduction of both sexes can be affected by heavy metal exposure. Male reproductivity is harmed especially by heavy metals like Hg, Cd, As and Pb (most common heavy metals) via inhibition of spermatogenesis and steroidogenesis, as also due to induction of oxidative stress reactions in the testis, resulting in decreased sperm count and quality and changes in hormone production (Lopez-Botella et al. 2021). In females, Cd, Pb and Hg have been shown to induce alterations in estrous cycle length, changes in ovarian function, and increased fetal losses (Massányi et al. 2020). Heavy metals have also been shown to affect embryogenesis (Massányi et al. 2020). In addition to affecting reproductive systems and the production of gametes, successful conception and fetus development, even if offspring are born, heavy metals affect negatively on their survival chances (Hyvärinen & Sipilä 1984, Faires 2004, Rajkumar & Gupta 2022). Mammal offspring may be exposed to large amounts of pollutants via mother's milk, to where heavy metals and other fat-soluble pollutants transpose during production of milk. In birds, offspring are affected via the egg content and through eggshells (Burger 2007). The immune system of new-born and juveniles is undeveloped and sensitive, and therefore

most vulnerable to pollutants. Effects on reproductive systems and offspring survival may lead to decreased birth rates, increased juvenile mortality and, eventually, to population decrease. Therefore, heavy metals pose a risk especially to populations that are already under the threat of extinction. For example, the most abundant heavy metal, Pb, is known to affect eagle populations worldwide, (e.g. in the United States and Finland), just after their recovery from effects caused by pollutants DDT and PCBs in 1950-70s (Grier 1982, Slabe et al. 2022, WWF Suomi 2022). Eagles are exposed to Pb especially via ammunition bullets and cartridges used in hunting and sinkers used in fishing. Prolonged exposure to Pb is known to damage the nervous system, kidneys, bone marrow, and the reproductive system. In North America, Pb poisonings have been found to currently decrease the population growth of bald eagles (*Haliaeetus leucocephalus*) and golden eagles (*Aquila chrysaetos*) by 3,8 % and 0,8 % (Slabe et al. 2022).

Another example of a long-lived, endangered mammal that is increasingly forced to live close to humans, are elephants (Sukumar 2006, IUCN 2022, WWF 2022). Elephants live up to 60-80 years old (Lahdenperä et al. 2014). Close connection to humans potentially increases the risk of exposure of elephants to heavy metals. However, not much is known about elephants' heavy metal exposure, even though studying them is of interest e.g. for conservation purposes. One reason could be that heavy metal concentrations are challenging to measure from wild elephant populations, as sample collection like blood sampling, biopsy or organ collection, and especially long-term sampling, is not easily accessible.

Elephant populations are in decline worldwide, mostly due to habitat loss, poaching and human-elephant conflict (Sukumar 2006). Elephants suffer from poor reproductive rates, especially in captivity (Wiese 2000). Captive females have been observed to become acyclic, and even when calves are born, their mortality is higher in captivity than in the wild (Saragusty et al. 2009). Factors underlying the population decline both in the wild and in captivity are not entirely understood.

Elephants are found in sub-Saharan Africa and in South and Southeast Asia. There are three species of elephants: the African savanna (*Loxodonta africana*), the African forest (*Loxodanta cyclotis*) and Asian elephants (*Elephas maximus*). The elephants studied in this thesis are Asian elephants. There are about 38 500-52 500 wild Asian elephants fragmented around Southeast Asia, which form the total wild population. Over half of the wild elephants live in India, and the second largest population is in Myanmar. In addition to wild populations about 16 000 Asian elephants live in captivity (Sukumar 2006).

Captive populations can be totally captive, like those held in zoos or in otherwise restricted environments like tourist camps, or semi-captive, when elephants are used for human purposes like in logging industry but have regular unrestricted access to their surroundings. Semi-captive Asian elephant populations are found e.g. in Myanmar, India and Thailand. The largest with about 5000 individuals is found in Myanmar (Crawley et al. 2019).

As the captive population of Asian elephants is of significant size, it plays a major part in the survival of the species. However, captive populations are often not self-sustaining (Wiese 2000) and are maintained by capturing individuals from the wild. Capturing wild elephants possibly intensifies the population decline of Asian elephants in overall (Jackson et al. 2019). This, in addition to decreased birth rates, risks the self-sustainability of elephant populations and increases the risk of extinction. What is more, perinatal calf mortality has been detected to be much higher in captivity than in the wild (Saragusty et al. 2009). More studies are needed to comprehend the underlying reasons behind increasing infertility in elephant populations. One factor could be increased exposure to contaminants like heavy metals. In a study conducted in Sri Lanka, concentrations of Cd, Cu, Mg, Pb and Zn were measured and compared between wild and captive Asian elephant populations. It was observed that captive elephants had bigger concentrations than wild elephants. The reason for this is unknown but diet was proposed to be related (Jayasekera and Kuruwita 1996). Hence it is of special interest to study the link between heavy metals and reproduction.

Elephants' reproductive system has a few peculiarities. First, elephants reproduce around the year (Hildebrandt et al. 2011). Secondly, they have the longest known gestation period (18-22 months) in animals, and also the longest known estrous cycle among non-seasonal mammals (Hildebrandt et al. 2011), though the seasonality is debated (e.g. Wittemyer et al. 2007, more in discussion). The average length of estrous cycle in elephants varies from 13 to 18 weeks with follicular phase being, on average, 4-6 weeks long, and the luteal phase 6-12 weeks long (Brown 2000, Hildebrandt et al. 2011). What is more, during the follicular phase of the estrous cycle, elephants have two surges of luteinizing hormone (LH) instead of one, like in other mammals. The first surge is anovulatory (anLH), and only the second three weeks later is ovulatory (ovLH) (Brown et al. 2004). Two LH surges is not known to happen in any other species. The evolution and purpose of two LH surges are also unknown.

In addition, the predominant circulating progestins in elephants is not progesterone, like in most other mammals, but rather progesterone metabolites like 5-alpha-Dihydroprogesterone (5α-DHP) and allopregnanolone (5α-pregnane-3α-ol-20-one) (Heistermann et al. 1997, Hodges et al. 1997, Schwarzenberger et al. 1997). Because progesterone metabolites like 5α-DHP and allopregnanolone are dominant progestins in elephant females, their measurement gives more accurate assessment of the ovarian function in elephants than measuring progesterone (Heistermann et al. 1997, Hodges et al. 1997, Fieβ et al. 1999, Brown et al. 2004, Ghosal et al. 2010). Most of the research has been done on African elephants (Hodges 1998). However, based on current knowledge, the concentrations of progesterone metabolites between African and Asian elephants' plasma do not differ, at least not significantly (Schwarzenberger et al. 1997). More studies are needed on Asian elephants to confirm this.

In mammals, after ovulation, corpus luteum forms up and starts to excrete progesterone. The progesterone levels rise until the corpus luteum (CL) starts to degrade, after which the progesterone levels fall starting the cycle again (Cable & Grider 2022). The exception is when fertilization has happened, and the formed placenta starts to secrete progesterone after CL (Cable & Grider 2022). However, progesterone secretion forms a hormone peak. It indicates ovulation and cyclicity. Therefore, in mammalian studies, progesterone is usually measured to determine reproductive status and cyclicity of an animal (Cable & Grider 2022). In elephants, however, progesterone levels are lower, so they are harder to detect, and the measurements are not as reliable as measuring allopregnanolone and/or 5α-DHP, which is why allopregnanolone and/or 5α-DHP are preferably measured instead (Heistermann et al. 1997, Hodges et al. 1997, Fieß et al. 1999, Dehnhard et al. 2001, Ghosal et al. 2010). Fluctuations in allopregnanolone (as also 5α-DHP and progesterone) levels indicate ovarian function. When the allopregnanolone levels rise significantly for a specific period of time (about 10 weeks in elephants), it can be determined as the luteal phase, which is a result of ovulation (Ghosal et al. 2010).

My study objectives are to 1) measure the heavy metal concentrations in 80 semi-captive Asian elephants from whole blood samples in order to map current acute heavy meal load in the population 2) perform a pilot study to measure reproductive cycling in females by measuring the amount of serum allopregnanolone from 11 Asian elephants, 3) to compare the heavy metal concentrations to past reproductive success and current estrous cycle profiles (when available) of the elephants. The population used in this study is a semi-captive Asian elephant population in Myanmar working in timber industry. Semi-

captivity enables repeated sampling of the elephants, collection of invasive samples, like blood samples, and collection of long-term health data of the elephants. At the same time the elephants live in their natural climate, can roam in natural habitats, and socialize and reproduce even with the wild elephants in the region. Therefore, this semi-captive population resembles wild populations more closely than totally captive populations for example in zoos or in restricted habitats like sanctuaries or tourist camps. Also, the study elephants consume resources like water and food outside the camp in their free time, which could be a possible contamination source of heavy metals and other pollutants. One of the main sources of heavy metals in Myanmar are gold mines (Osawa & Hatsukawa 2015, Tun et al. 2020). Gold mining usually increases the concentrations of following heavy metals in the environment: Cu, Zn, As, Cd, Hg and Pb. High levels of these elements have been detected in Myanmar soil (Tun et al. 2020). In addition to gold mines, Cd, Hg and Pb concentrations build-up in soils also due to air pollution from industrial activity (WHO 2007). There are no previous heavy metal concentration or estrous cyclicity measurements done in the study population.

I hypothesize, that (1) heavy metal concentrations rise with age due to bioaccumulation (Dietz et al. 1995, Franson & Russell 2014, Slabe et al. 2022) and that (2) females will have smaller heavy metal concentrations than males due to the assumption that heavy metals stored in fat transit to bloodstream, fetus and milk during pregnancies and lactation, lessening the total heavy metal concentration in females in comparison to males (Ylitalo et al. 2001, Wade et al. 1997, Franson & Russell 2014, Desforges et al. 2018). I also hypothesize that (3) heavy metal concentrations will vary among elephants in geographically different locations (Dietz et al. 1995, Raubenheimer et al. 1998) depending on how close they are to mining sites and other potential heavy metal exposure sources, like polluted water source. I also predict that (4) some heavy metal concentrations will exceed reference values because local veterinarians have suspected heavy metal poisonings in local elephants and because there are mining sites, especially gold mines, near the study site (Osawa & Hatsukawa 2015, Tun et al. 2020). I also hypothesize that (5) the method for measuring reproductive cycling will prove to be valid for future studies and that there will be distinguishable results between cycling and non-cycling elephants. I also predict that (6) elephants with a normal reproductive history will show a normal cycling pattern and vice versa. Furthermore, I expect that (7) acyclic elephants have an increased level of heavy metals, when that acyclicity is not caused by

natural causes like lactating, given previous studies have showed heavy metals disrupting mammalian cycling (Rzymski et al. 2015, Massányi et al. 2020).

2. MATERIALS AND METHODS

2.1 Study population

Semi-captive elephants studied here work in Myanma Timber Enterprise (MTE) in Myanmar and are owned by the local government. The population is part of the largest captive Asian elephant population in the world. In MTE, elephants' work consists of riding, transporting logs and extracting timber. They work for 3-5 days a week, from 5 to 8 hours per day with a break at noon. Other time is free and unsupervised. Also, elephants have an annual 3-month long holiday during the hot season (March-May), and pregnant elephants are restrained from work halfway through pregnancy until one year from birth. During their free time, elephants can engage in normal species-specific behaviour, like roaming in the nearby forests and socializing. Mating happens through natural sexual selection during elephants' unsupervised time with other semi-captive or wild elephants, and breeding programs are not practiced. All elephants have a personal caretaker called a mahout that supervises his elephant's work and welfare (Crawley et al. 2019), and elephants are inspected by veterinarians regularly. The elephants live in logging camps distributed across the country. Camps are divided by age, health, or work status of the elephants. For example, the working, pregnant or lactating and tamed elephants are in different camps. Each elephant's health data is recorded into logbooks by local veterinarians, providing information like age, location, previous diseases and possible medication, maternal information (if captive-born), previous births etc. All elephants have a personal ID and a name. Here, the real IDs are not shown due to safety reasons, and elephants are coded from XXX1-XX85 instead (Table 1, Table 4). Elephants from age 18 until 53 are considered working adults. Over 53-year-old elephants are retired, and elephants under 18 are considered juveniles. Calves are tamed at the age of 4-5 years.

In Myanmar there are three seasons: 1) cool season from late October to March, 2) hot season from March until May, 3) rainy/monsoon season from June to late October. Even though elephants are considered non-seasonal breeders, some reproductive peaks have been reported to occur both in wild and captive populations (Hufenus et al. 2018). In the semi-captive population studied here, 41 % of births have been reported to happen between December and March, during the cool season meaning the conception had occurred between February and June, somewhat during the hot season and the holiday period of the elephants (Mumby et al. 2013). Duration of gestation in Asian elephants is 18-22 months, and females can, in theory, ovulate three times a year as the estrous cycle

is about 16 weeks long (Brown 2000). Bulls come into musth about once a year and it lasts for 2-3 months, but they can mate at any time of the year (Sukumar 2006, Keerthipriya et al. 2020). Physiology-wise both sexes become reproductively mature at about 10-14 years (Sukumar 2006), but start reproducing later, mean age at first birth in females being about 19 years old with a big range (Lahdenperä et al. 2014, Reichert et al. 2020, Toin et al. 2020). The mean age at first mating for males is a bit unclear, but it is assumed that males start reproducing only at the age of 30 and over (Sukumar 2006, Toin et al. 2020).

In this study population, about half of the elephants are captive-born, and half are originally wild born (Lahdenperä et al. 2018). The population is not self-sustaining, suffering from low birth rates and high calf mortality (Mumby et al. 2013, Jackson et al. 2019). The reasons behind poor reproductivity rates and calf survival are mostly unknown. However, insufficient milk production has been reported as one of the main reasons behind calf deaths in Myanmar (Saragusty et al. 2009) and taming has been known to be a stressful event for juveniles increasing their risk of death in the population in question (Crawley et al. 2019). Also, acyclicity is suspected to occur widely among the elephants in the study population, even though it has never been studied. The effect of pollutants on low reproductivity rates is also plausible, but not yet studied either.

During birth the birth-giving female is usually accompanied with another female from the herd, which helps with the delivery, acting like a "midwife". Calves are taken care by several females simultaneously, which is known as allomothering (Lahdenperä et al. 2016). Elephants can reproduce until late in life, some elephants giving birth in their 60s. Therefore, elephants are usually considered as non-menopausal mammals, though usually the reproductive rate declines with age gradually (Lahdenperä et al. 2014, Chapman et al. 2019). It is known that the presence of a grandmother enhances the survival chances of a calf (Lahdenperä et al. 2016). The reproductive status and history of the elephants studied here are shown in Table 1.

Table 1. Reproductive status and history of female Asian elephants included in analyses (n=49).

ID	Date of birth**	Reproductive status	Age at first birth	Births***	Age at last recorded birth	Year of last birth	Captive/ wild born
XXX1	22.12.1953	nonactive	16	9	53	2007	captive
XXX2	3.9.1959	nonactive	15	3	27	1987	captive
XXX3	6.12.1961	nonactive	15	6	29	1990	captive
XXX5	1.2.1954	nonactive	17	4	54	2008	wild

XXX6	30.11.1964	nonactive	14	3	29	1994	wild
XXX9	1.1.1956	nulliparous	-	-	-	-	wild
XX10*	1.1.1963	active	27	7	56	2019	wild
3456*	25.2.1967	paused	17	8	47	2014	captive
XX12*	30.11.1968	paused	16	6	47	2015	wild
XX13	30.11.1958	nonactive	20	4	53	2011	wild
XX15	1.1.1953	nonactive	35	2	52	2005	wild
XX16	30.11.1968	nonactive	18	4	39	2008	wild
XX82*	1.1.1966	active	25	9	54	2020	wild
XX18	30.1.1963	nonactive	19	3	33	1996	wild
XX19	11.6.1969	nonactive	22	3	25	2004	captive
XX23	1.1.1966	active	16	5	51	2017	wild
XX25	14.10.1973	nulliparous	-	-	-	-	captive
XX27	30.11.1967	paused	34	3	48	2016	wild
XX28	29.12.1976	nonactive	18	3	30	2007	captive
XX29	11.2.1976	active	17	3	44	2022	captive
XX30	12.5.1977	active	19	4	42	2020	captive
XX31*	7.6.1978	active	13	8	43	2021	captive
XX83*	5.5.1978	active	30	3	39	2017	captive
XX84*	4.4.1977	active	14	6	40	2017	captive
XX34	17.6.1996	active	18	2	23	2019	captive
XX38*	17.8.1987	active	21	4	33	2020	captive
XX39	5.3.1988	paused	18	4	29	2015	captive
XX40	3.10.1989	active	19	4	30	2019	captive
XX85*	24.8.1989	active	16	2	29	2019	captive
XX41	15.7.1989	nonactive	17	3	21	2010	captive
XX42	23.5.1989	nulliparous	-	-	-	-	captive
XX43	10.9.1990	active	19	4	28	2019	captive
XX44	9.3.1991	active	18	3	27	2018	captive
XX46	24.7.1992	nulliparous	-	-	-	-	captive
XX47	14.9.1992	paused	18	2	21	2014	captive
XX48	4.6.1993	active	23	2	27	2020	captive
XX49	8.9.1993	active	26	1	26	2020	captive
XX50	1.1.1995	nulliparous	-	-	-	-	captive
XX52*	13.3.1998	active	20	1	20	2018	captive
XX54*	10.8.1999	active	18	1	18	2018	captive
XX55	25.6.2000	nulliparous	-	-	-	-	captive
XX57	21.2.2003	active	15	1	15	2018	captive
XX59	6.5.2004	active	7	2	13	2018	captive
XX60	24.10.2004	premature	-	-	-	-	captive
XX61	31.12.2006	premature	-	-	-	-	captive

XX62	31.1.2007	premature	-	-	-	-	captive
XX63	10.7.2006	active	13	1	13	2020	captive
XX67	27.7.2010	premature	-	-	-	-	captive

Active = has given birth in less than 5 years
Paused = has given birth 5-10 years ago (potentially reproduces again)
Nonactive = has not given birth in over 10 years
Nulliparous = has not given birth even though in reproductive age
The classification is based on elephants' mean birth interval, which is reported to be around 5 years (Lahdenperä et al. 2014).

*These elephants are included in the estrous cyclicity study; but elephants XX81, XX82, XX83, XX84 and XX85 are not included in the heavy metal analyses due to lack of samples

**The date of birth, not only the year, was considered when calculating age at first birth

****Births do not equal pregnancies or survived calves

Among elephants studied here, the mean age at first birth is 19 years (median 18 years), min and max ages being 7 and 35. Mean age at last recorded birth for those who are classified as inactive is 37 years, median being 31,5 years (min=21 and max=54). Oldest reproductively active elephant gave birth at 56 years old. Six elephants are classified as nulliparous, which is 12,5% of the studied female elephants.

2.2. Blood sampling

Two kinds of blood samples were taken for this study: whole blood samples for heavy metal analyses and serum samples for allopregnanolone analyses. All blood samples were taken via ear vein by trained veterinarians according to the local and University of Turku ethical protocol, after which they were stored in -20 ºC until cold-chain transportation to Turku. In Turku, the samples were stored in -80 ºC.

Whole blood samples for heavy metal analyses were collected from 80 elephants aged 4-67 years between November 2019 – June 2020 into 2 ml Vacuette lithium-heparin tubes. About 1-2 ml of blood was collected. Of these 80 individuals 43 were females and 37 males. Mean age was 32 years and median 38 for those whose age at the time of sampling was known, n=68. Exact age was not known for 12 young male elephants which were under 8 years old during the analyses, even though their birthdates were obtained later and are shown in Table 3. Camp, sex, age at sampling and date of birth of elephants sampled for heavy metal analyses are seen in Table 3. Reproductive history of sampled females is shown in Table 1.

Serum collection for estrous cycle analyses was conducted about every 2 weeks for 38-42 weeks during November 2018 – August 2019 from 11 elephants (n=11). About 1-2 ml

of serum was collected per sample. The age of elephants varied from 19 to 56 with average age being 39 and median 40. In total 192 serum samples were analysed. For six of these elephants, there were also had blood samples for heavy metal analyses.

2.3. ICP-MS

To obtain heavy metal concentrations, inductively coupled plasma mass spectrometry (ICP-MS) was performed by the Ab Spectrochem Oy, according to ICP-MS standards. In summary, the whole blood samples were digested in nitric acid (HNO_3) using microwave digestion technique before elemental analysis. Elemental analysis was done with ICP-MS. The concentrations of following 20 heavy metals were measured (full element names listen in Table 3): Al, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Sr, Ag, Pb, Be, Ga, Cd, Cs, Ba, Tl and U. Elemental analysis quality control was conducted by analysing two Certified Reference Materials (CRM): Bovine Liver (SRM 1577c, NIST, USA) and Dogfish Liver (DOLT-4, NRC - CNRC, Canada) (Table 2). The method was first validated on bovine whole blood, and the contamination risk caused by Vacuette lithium-heparin tubes was inspected.

Table 2. Measured concentrations, standard deviations and recovery rates for ICP-MS with the CRMs Bovine Liver 1577c and Dogfish Liver DOLT-4.

	Bovine Liver 1577c					Dogfish Liver DOLT-4				
Element	Measured value (µg/kg)	SD (µg/kg)	RSD %	Certified value (µg/kg)	Recovery %	Measured value (µg/kg)	SD (µg/kg)	RSD %	Certified value (µg/kg)	Recovery %
Al	1297	57,3	4,5	800	162	138147	2111,6	1,5	200000	69
V	0	6,6	10,4	8,17	0	665	92,3	13,9	600	111
Cr	37	38,6	71,8	53	69	2041	244,5	12	1400	146
Mn	10283	297,3	2,9	10460	98	9324	220,5	2,4	-	-
Co	308	8,5	2,7	300	103	226	8,6	3,8	250	90
Ni	125	7,6	6,3	44,5	281	1384	110,6	8	970	143
Cu	275416	522,0	0,9	275200	100	32879	586,4	1,8	31200	105
Zn	186415	309,3	0,2	181100	103	135506	3648,3	2,7	116000	117
As	11,8	4,4	44,1	19,6	60	9124	282,2	3,1	9660	94
Se	1961	102,0	5,2	2031	97	10585	466,2	4,4	8300	128
Sr	96,3	3,0	3,1	95,3	101	5391,3	107	2	5500	98
Ag	2,6	0,8	33,6	5,9	45	899,7	32,8	3,7	930	97
Pb	63,7	2,6	4,1	62,8	101	209,1	8,3	4	160	131
Be	0,7	2,9	1153,9	-	-	0,0	16,3	2521	-	-
Ga	8,1	1,7	23,2	-	-	47,0	8,3	17,4	-	-
Cd	93,4	6,0	6,4	97	96	24069,4	581,2	2,4	24300	99
Cs	21,4	1,1	5,4	21,7	99	101,0	6,5	6,4	-	-
Ba	319,4	33,0	11,9	-	-	1169,9	69,9	6	-	-
Tl	0,7	0,2	28,5	-	-	11,7	1,2	9,2	-	-
U	0,7	0,2	23,6	-	-	61,1	4,5	7,2	-	-

2.4 Statistics

Limits of detection (LODs) were calculated for each element using formula k x 3 x SD of an acidified blank, k=1. In cases where at least 15 % of samples were below the LOD, the element was excluded from statistical analyses. Here, elements that were 15%<LOD were Mn, Co, As, Ag, Be, Ga, Cd, Cs, Tl and U (Table 3). The remaining 11 elements out of 20 were modelled, those being Al, V, Cu, Cr, Ni, Zn, Se, Sr, Pb, Cs and Ba (Table 3). Age, sex and geographical location of elephants at the time of sampling were chosen as predictor variables in statistical analyses. Age was coded as a linear variable, and sex as a 2-level factor variable. Geographical location was grouped into seven categories based on elephant working camps, which are categorized based on elephants' life stage and working ability, e.g. is it working or currently being tamed (Table 7). Statistical analyses were performed using RStudio version 1.4.1066 using following packages: DHARMa for model quality assessment, mgcv and nlme for linear models and ggplot2 for plotting. Linear models (LM) were used for comparison of means and significance level of $p<0,05$ was used. The tested terms predicting the concentration of each heavy metal were sex, age, camp, sex+age, sex+camp, age+camp, sex*age, sex*age+camp, sex+age+camp. The interaction between sex and age was tested to investigate whether the age-effects were different for females and males due to their behaviour, life-history and possible exposure differences. The model selection was done according to Akaike information criterion (AIC) values, where the model with the lowest AIC value is selected as the best fit to the data. If the difference in AIC values between two models was less than 4, I chose the simpler model. When element data was not normally distributed, it was $\log_{10}$-trasformed for analyses and back-transformed for tables. Residuals of chosen models were tested to verify that the conditions of models were met.

The heavy metal and cyclicity data were comparable only for a such limited sample number, that the statistical analyses would have lacked power. Therefore, the analyses were simplified and results interpreted visually/approximately. The elephants were grouped by their cyclicity status obtained here, and their element concentrations were compared per element. Also the total mean concentrations of the groups were compared, using a t-test to obtain the p-value. Also, the mean total concentrations of all the elephants were compared visually to study the chance of outliers.

2.5 Allopregnanolone ELISA

Serum samples were pre-diluted into 40 % methanol (MeOH) (Appendix 1) before allopregnanolone levels were measured via enzyme immunoassay (EIA, ELISA) using the double-antibody technique (Appedix 2). ELISA kit validated for Asian elephant estrous cycle and pregnancy determination was ordered from Leibniz Institute for Zoo and Wildlife Research in Berlin (IZW Wildlife Endocrinology Lab). Serum extractions and ELISA were done according to the protocols (Appendix 1, Appendix 2). In summary, the extraction of serum samples was done as follows: 1,8 ml of PetrolEther (PE) was added to 0,1 ml serum. Samples were shaken for 30 minutes in room temperature (RT) using an Eppendorf-tube shaker, after which samples were displaced in -80 ºC for 30 minutes. Next, the supernatant of a sample was transferred to a new Eppendorf tube, and this was repeated for all the samples. Samples containing supernatant were then put on a heat block warmed up to 55 ºC and samples were evaporated until dry for about 1-2 hours. After evaporation, 0,4 ml of 100 % MeOH was added, following with 0,6 ml of mqH_20, and vortexed.

ELISA assay was done using an antibody against 5alpha-pregnan-3beta-ol-20-one 3HS:BSA. The antibody cross-reacted followingly: 650% 5α-pregnan-3α-ol-20-one (Allopregnanolone); 100% 5α-pregnan-3β-ol-20-one (3β-Allopregnanolone); 72% 4-pregnen-3,20-dione (P4); 22% 5α-pregnan-3,20-dione (5α-DHP); < 0,1% for 5β-pregnan-3α-20α-diol, 4-pregnen-20α-ol-3-one, 5α-pregnan-20α-ol-3-one, 5α-pregnan-3β,20α-diol, 5α-pregnan-3α,20α-diol, testosterone, estradiol, cortisol, corticosterone. The standard curve consisted of 10 diluted standard samples prepared so, that first 10µl of standard sample (100 ng/ml) was diluted in 1:10 proportion into 180µl of 40 % MeOH and vortexed, after which nine other standard samples were prepared via serial dilution in 1:2 steps by diluting 100µl of previous standard into 100µl of 40 % MeOH. As a result, the standards were of following concentrations (pg/ml): 10 000, 5000, 2500, 1250, 625, 312,5, 156,25, 78,125, 39 and 19,5. For the assay, 96-well-plate-frames were used by filling them with anti-rabbit-IgG-coated 8 well strips. Peroxidase (POD) was used to label the analyte and catalyze the color reaction. Chromogenic EIA 3,3',5,5'-tetramethylbenzidine (TMB) containing substrate solution was used for peroxidase. To control reagent quality, non-specific binding (NSB) control and binding for the zero standard (B0) control were used. For assay control, two control samples of low and higher concentrations were used. Control samples were pooled Asian elephant serum samples and prepared beforehand.

Briefly, ELISA was conducted followingly: first, the plate was washed once with 300 µl/well with washing solution containing Tween 80 pure concentrate 1:2000 in mqH20 (0,5 ml/L). Then 20 µl of extracted serum samples, control samples or standards were pipetted per well, following with 100 µl of diluted 5α conjugate dilution (1:10 pre-diluted conjugate stock diluted 1:400 into assay buffer; assay buffer consisting of IZW assay buffer 5x concentrate diluted 1:5 into mq$H_2$0), following with 100 µl of prepared 5α antibody dilution (1 part of 5α antibody to 2000 parts of assay buffer) except into NSB wells. Into NSB wells 100 µl of assay buffer was pipetted instead. Then, the plate was covered and protected from light, and incubated over night at 4 ºC with slight shaking. On the second day, the plate was first washed 4 times with 300 µl/well with cold (+4 ºC) washing solution. Then, 150 µl of cold substrate solution mix (1 part of substrate solution A + 1 part of substrate solution B) was added per well. The plate was then covered and light protected again, and shaken slightly in + 4 ºC for 40 min. After this, 50 µl of stop solution 2M H2SO4 was added per well. Pipetting was done using a repeater pipet when convenient. The order of samples on the plate was randomized beforehand.

Absorbance was measured at 450 nm. Samples and standards were measured in duplicate assuming a CV < 5%. Standard curve was plotted using 4PLC fitting. Samples were back calculated for tables and figures.

3. RESULTS

3.1. Heavy metals

The heavy metal concentrations (range, quartiles and median) of the sampled 80 semi-captive Asian elephants are summarized in Table 3. Zn was found at the highest concentrations, median concentration being 3276,5 µg/kg. U, Tl, Ga, Be, Ag and Cd were detected the least in the analysed samples, with their concentrations staying lower than 5 µg/kg. The largest range between samples was in Ba where the difference between minimum and maximum concentrations was 48-fold. The second largest range with a 37-fold difference between smallest and biggest concentrations, was in V.

Table 3. Range and quartiles (Q25, median, Q75) for element concentrations (µg/kg) in whole blood of Asian elephants (*Elephas maximus*). LOD=Lower limit of detection (LOD) and proportion of values below LOD (%<LOD). N = 80.

Element	Range (min-max) µg/kg	Q25	Median	Q75	LOD	%<LOD
Aluminium (Al)	40,3 - 904	150	234	361	5,04	0
Vanadium (V)	<LOD - 109	15,9	29,7	46,9	2,88	0
Chromium (Cr)	38,2 - 429	94,8	151	213	11,9	0
Manganese (Mn)	<LOD - 24,7	2,39	5,14	7,60	2,61	25
Cobalt (Co)	<LOD - 8,47	0,82	1,18	2,51	2,25	73
Nickel (Ni)	9,52 - 132	16,0	21,5	26,8	2,34	0
Copper (Cu)	477 - 981	679	741	813	3,24	0
Zinc (Zn)	2170 - 4330	2910	3280	3530	8,82	0
Arsenic (As)	<LOD - 29,9	4,19	6,75	11,1	5,31	34
Selenium (Se)	103 - 817	237	300	419	15,0	0
Strontium (Sr)	31,6 - 203	66,0	80,9	90,7	1,44	0
Silver (Ag)	<LOD - 3,60	0,17	0,26	0,35	1,89	99
Lead (Pb)	8,42 - 52,8	17,2	20,9	26,8	1,98	0
Beryllium (Be)	<LOD	0	0,04	0,19	13,1	100
Gallium (Ga)	<LOD - 4,59	0,12	0,64	1,36	1,71	81
Cadmium (Cd)	<LOD	0,31	0,36	0,45	2,97	100
Caesium (Cs)	<LOD - 14,7	3,05	4,73	6,56	2,07	6,3
Barium (Ba)	4,30 - 194	15,4	39,7	64,7	2,97	13,8
Thallium (Tl)	<LOD	0	0,02	0,05	2,07	100
Uranium (U)	<LOD	0,03	0,03	0,04	0,88	100

The total element concentrations of elephants are depicted in Table 4. Male XXX7, aged 60, had the biggest total element concentration having 5993 µg/kg. Female XX15 aged

67 had the smallest total element concentration: 3579 µg/kg. Mean total element concentration was 4994 µg/kg and median 5020 µg/kg. Total detectable element concentrations ranged from 3579 µg/kg to 5993 µg/kg.

Table 4. Total concentration of detectable %<LOD<15% elements (listed in Table 3) per elephant. Highest and lowest total element concentrations are highlighted. Camp, sex, date of birth and age at sampling for heavy metal analyses in November 2019 – June 2020 also stated. The table is sorted by ID in a growing order.

ID	Sex	Date of birth	Age at sampling ± 0,5 years	Camp	Total (µg/kg)
XXX1	F	22.12.1953	67	OLD	4220
XXX2	F	3.9.1959	61	OLD	4587
XXX3	F	6.12.1961	59	OLD	4957
XXX4	M	1.1.1963	57	OLD	4431
XXX5	F	1.2.1954	66	OLD/SICK	4760
XXX6	F	30.11.1964	56	OLD	4974
XXX7	M	30.11.1960	60	MATERNITY/REST	**5993**
XXX8	M	30.11.1964	56	OLD	5535
XXX9	F	1.1.1956	64	OLD/SICK	4029
XX10*	F	1.1.1963	57	MATERNITY	5582
XX11	M	30.11.1956	64	OLD/SICK	4892
XX12*	F	30.11.1968	52	OLD	5741
XX13	F	30.11.1958	62	OLD/SICK	3960
XX14	M	30.11.1961	59	OLD/SICK	4618
XX15	F	1.1.1953	67	OLD/SICK	**3579**
XX16	F	30.11.1968	52	MATERNITY	4985
XX17	M	30.11.1965	55	OLD	5186
XX18	F	30.1.1963	57	OLD	5184
XX19	F	11.6.1969	51	OLD/SICK	5172
XX20	M	30.11.1967	53	OLD/SICK	4843
XX21	M	30.11.1968	52	OLD/SICK	5354
XX22	M	30.11.1971	49	OLD	4995
XX23	F	1.1.1966	54	MATERNITY	5541
XX24	M	30.11.1970	50	OLD	5444
XX25	F	14.10.1973	47	OLD	5698
XX26	M	22.4.1974	46	NA	4702
XX27	F	30.11.1967	53	MATERNITY/REST	4986
XX28	F	29.12.1976	44	WORKING	4339
XX29	F	11.2.1976	44	NA	4796
XX30	F	12.5.1977	43	WORKING	4621
XX31*	F	7.6.1978	42	MATERNITY	5137

XX32	M	7.4.1979	41	KONGI	5833
XX33	M	1.1.1973	47	WORKING	5435
XX34	F	17.6.1996	24	OLD	4556
XX35	M	22.10.1981	39	WORKING	5297
XX36	M	16.2.1984	36	WORKING	4831
XX37	M	22.1.1985	35	WORKING	5386
XX38*	F	17.8.1987	33	MATERNITY	4780
XX39	F	5.3.1988	32	OLD	5213
XX40	F	3.10.1989	31	MATERNITY	5115
XX41	F	15.7.1989	31	WORKING	4550
XX42	F	23.5.1989	31	MATERNITY	5410
XX43	F	10.9.1990	30	TC	5303
XX44	F	9.3.1991	29	MATERNITY	3582
XX45	M	25.5.1992	28	WORKING	4725
XX46	F	24.7.1992	28	MATERNITY	4305
XX47	F	14.9.1992	28	WORKING	4777
XX48	F	4.6.1993	27	WORKING	5460
XX49	F	8.9.1993	27	MATERNITY	5295
XX50	F	1.1.1995	25	NA	4737
XX51	M	3.3.1997	23	KONGI	5444
XX52*	F	13.3.1998	22	MATERNITY	5045
XX53	M	24.6.1999	21	KONGI	5648
XX54*	F	10.8.1999	21	MATERNITY	4292
XX55	F	25.6.2000	20	WORKING	4535
XX56	M	21.10.2001	19	WORKING	4964
XX57	F	21.2.2003	17	TC	5291
XX58	M	17.2.2004	16	MATERNITY/REST	4807
XX59	F	6.5.2004	16	TC	4727
XX60	F	24.10.2004	16	MATERNITY/REST	5556
XX61	F	31.12.2006	14	TC	5524
XX62	F	31.1.2007	13	TC	5146
XX63	F	10.7.2006	14	TC	5246
XX64	M	12.10.2007	13	TC	5168
XX65	M	10.7.2008	12	TC	5053
XX66	M	21.4.2009	11	MATERNITY/REST	5089
XX67	F	27.7.2010	10	TC	5225
XX68	M	28.2.2012	8	TC	5045
XX69	M	01.07.2014	NA	TC	5568
XX70	M	22.09.2014	NA	TC	5482
XX71	M	23.10.2014	NA	TC	4978
XX72	M	24.07.2014	NA	WORKING	4672

XX73	M	30.06.2015	NA	TAMING	4615
XX74	M	02.02.2015	NA	TAMING	5484
XX75	M	28.06.2015	NA	TAMING	5195
XX76	M	05.02.2015	NA	TAMING	4691
XX77	M	26.02.2015	NA	TAMING	4996
XX78	M	16.06.2015	NA	TAMING	4493
XX79	M	22.04.2016	NA	TAMING	4516
XX80	M	02.03.2016	NA	TAMING	5602

*These elephants are included in the allopregnanolone measurements

In general, sex did not have a significant effect on heavy metal concentrations (Table 5, Figure 1). However, the concentration difference between females and males was statistically significant in Al (B= 0,03 ± 0,07), V (B=0,04 ± 0,07), Ni (B=0,003±0,05) and Pb (B=0,02±0,03) (Table 5), where females had bigger concentrations of Pb (25%) and V (14%), and males of Al (24%) and Ni (26%). Females had also bigger Cr (18%) (B=0,1±0,05), Se (12%) (B=0,19±0,04), Cs (6%) (B=0,67±0,06) and Ba (24%) (B=0,32±0,12) concentrations compared to males, though the difference was not statistically significant. Males had slightly bigger Cu (9%) (B=0,13±22,0), Zn (5%) (B=0,06±103,1) and Sr (4%) (B=0,54±0,03) concentrations but these differences did not reach statistical significance. Overall, in 6/11 elements females had bigger concentrations than males (Table 5), even though when comparing total element concentrations between females and males, females seem to have a slightly smaller element burden (Figure 1).

Table 5. Mean and the range of element concentrations (µg/kg) in whole blood of Asian elephants per sex. Linear model (LM) for comparison of means.

Element	**Mean (µg/kg)**					
	Females	N**	Males	N**	*p*	t
Al *	207 (165 - 260)	41	271 (223 - 330)	34	**0,03**	2,21
V*	25,7 (24,5 - 40)	40	22,2 (18,1 - 27,3)	34	**0,04**	-2,14
Cu	723 (694 - 751)	41	796 (759 - 833)	34	0,13	1,54
Cr*	151 (127 - 181)	41	124 (105 - 146)	34	0,10	-1,66
Ni*	20,3 (17,7 - 23,3)	41	27,4 (22,0 - 34)	34	**0,00**	3,14
Zn	3170 (3020 - 3320)	41	3350 (3220 - 3490)	34	0,06	1,94
Se*	314 (270 - 366)	41	276 (246 - 310)	34	0,19	-1,33
Sr*	77,9 (69,7 - 87)	41	81,5 (74,1 - 89,5)	34	0,54	0,61
Pb*	23,9 (21,7 - 26,4)	41	17,9 (15,9 - 20,1)	34	**0,02**	-2,31
Cs*	4,44 (3,7 - 5,32)	41	4,19 (3,47 - 5,06)	34	0,67	-0,43
Ba*	29,5 (19,4 - 44,8)	38	22,4 (15,7 - 31,9)	31	0,32	-0,99

*Geometric means, values $\log_{10}$-transformed for the analyses and back-transformed for the table.
**NA:s were excluded from the analyses, which modifies the N.

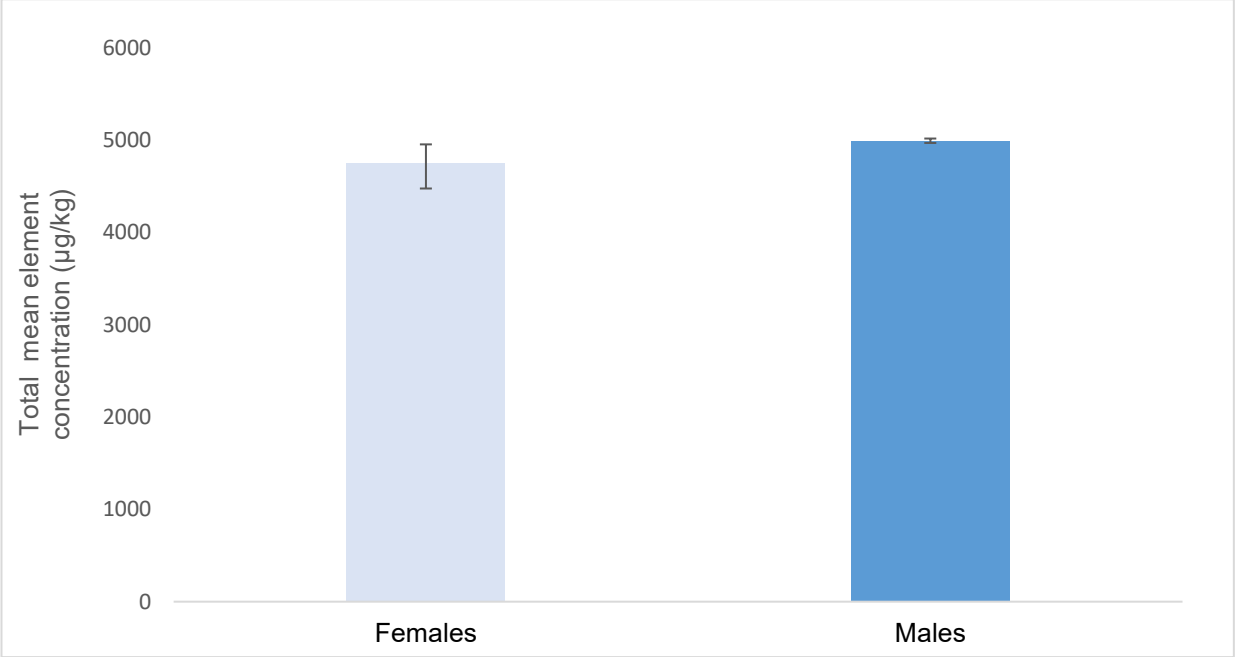

Figure 1. Total mean element concentrations and standard errors in semi-captive female and male Asian elephants (p=0,96; t=0,86; females n=43, males n=37; df=78).

The effect of age is shown in Table 6. Age did not have a significant effect on concentrations of the following heavy metals: Al (B = 0,063 ± 0,002), V (B=0,107±0,005), Zn (B=0,095±2,52), Se (B=0,876±0,001), Sr (B=0,393±0,001) and Cs (B=0,767±0,001), which is over half of the analysed elements. In Cu (B=0,000037 ± 0,53), Cr (B= 0,047±0,003), Ni (B=0,01±0,003) and Pb (B=0,022±0,001), age did affect the concentrations statistically significantly (p<0,05). In Cu the effect was negative, and the concentrations decreased with age, and in Cr, Ni and Pb the effect was positive, meaning that concentrations rose with age. Age had a slightly positive effect also on the concentrations of Al, V and Ba, though the effect was not statistically significant. In addition to Cu, age had a negative but statistically non-significant effect on the concentrations of Zn. In Se, Sr and Cs the effect of age was slightly negative, though almost non-existent. In Ba, the effect of age was statistically different for females and males, where the concentrations decreased with age for females, and increased with age for males (Figure 2). The effect of age was then tested separately on females and males, and the result was that the negative effect of age on Ba concentrations in females was not statistically significant (p=0,61, n=38), and only the positive effect of age on male Ba concentrations was statistically significant (p=0,0001, n=31).

Table 6. The effect of age on the element concentrations (Linear model, n=75).

Element	**Slope**	***p***	**t**	**df**
Al*	0,003	0,063	1,89	74

V*	0,003	0,107	1,63	74
Cu	-2,349	**0,000037**	-4,39	74
Cr*	0,003	**0,047**	2,02	74
Ni*	0,004	**0,01**	2,64	74
Zn	-4,26	0,095	-1,69	74
Se*	0	0,876	-0,16	74
Sr*	-0,001	0,393	-0,86	74
Pb*	0,003	**0,022**	2,35	74
Cs*	0	0,767	-0,30	74

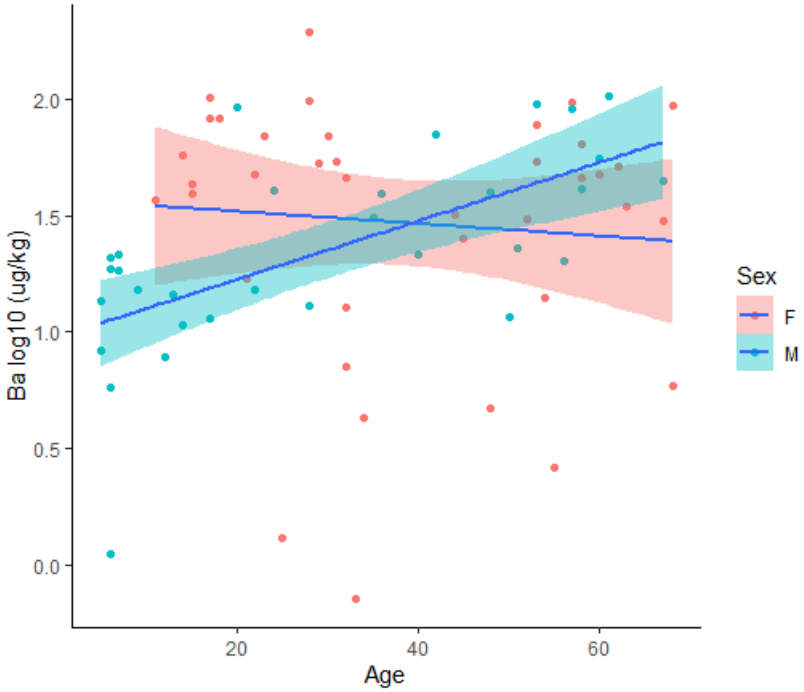

Figure 2. The effect of age*sex on the Ba concentrations in Asian elephant whole blood samples (Linear model, p=0,012; n=69).

The effect of camp is shown in Table 7. In overall, camp had a statistically non-significant effect on heavy metal concentrations in semi-captive Asian elephants studied here. However, in one element, in Zn, concentrations seemed to differ significantly among camps, being largest in the camp called Kongi, and the smallest in the camp for old and sick elephants. There were significant differences also in the following camps and element concentrations: Cu levels were statistically significantly higher in Taming camp than in others, Sr levels were significantly lowest in Old/Sick camp in comparison to others, and Pb levels differed significantly in other camps than in for old and for old and sick elephants.

Table 7. Geometric mean element concentrations (µg/kg) in whole blood of Asian elephants per camp. Means with the same letter are not statistically different, b = p<0,05.

Element	Camp**						
	Kongi	**Maternity/Rest**	**Old**	**Old/Sick**	**Taming**	**TC**	**Working**
*Al **	154,149 [a]	250,497 [a]	299,580 [a]	198,734 [a]	236,595 [a]	181,323 [a]	249,540 [a]
*V**	16,136 [a]	33,088 [a]	28,390 [a]	31,514 [a]	21,951 [a]	19,781 [a]	28,153 [a]
Cu	765,667 [a]	711,706 [a]	741,571 [a]	675,000 [a]	917,375 [b]	805,091 [a]	741,154 [a]
*Cr**	88,928 [a]	138,512 [a]	154,530 [a]	153,935 [a]	121,856 [a]	118,075 [a]	153,807 [a]
*Ni**	20,754 [a]	20,737 [a]	26,237 [a]	24,534 [a]	21,613 [a]	16,353 [a]	32,709 [a]
Zn	4175,000 [b]	3280,765 [b]	3261,143 [b]	2921,667 [b]	3147,375 [b]	3129,846 [b]	3129,846 [b]
*Se**	248,991 [a]	296,322 [a]	245,751 [a]	364,973 [a]	318,926 [a]	352,306 [a]	269,492 [a]
*Sr**	89,453 [a]	82,260 [a]	76,780 [a]	56,901 [b]	79,167 [a]	81,705 [a]	94,644 [a]
*Pb**	12,312 [b]	23,167 [b]	20,849 [a]	19,200 [a]	16,875 [b]	26,902 [b]	20,544 [b]
*Cs**	4,610 [a]	5,162 [a]	5,541 [a]	2,501 [a]	5,761 [a]	3,050 [a]	4,250 [a]
Ba	35,238 [a]	30,078 [a]	21,652 [a]	33,187 [a]	8,115 [a]	34,557 [a]	29,574 [a]

**Kongi n=3; Maternity/rest n=17 except V and Ba n=16; Old n=14; Old/Sick n=9 except Ba n=7; Taming n=8 except Ba n=6; TC n=11; Working n=13 except Ba n=12

The median values of heavy metals obtained in this thesis were compared to heavy metal concentrations measured in other studies on elephant element concentrations (Table 8). All values measured here are lower than values measured in Jayasekera & Kuruwita 1996 from whole blood samples from captive and wild Asian elephants in Sri Lanka, and lower than values measured in Sach et al. 2020 from whole blood samples from captive Asian elephants in UK zoos (Table 8). Values are also lower than concentrations measured in Raubenheimer et al. 1998, which is most probably due to Raubenheimer et al. having measured concentrations from African elephant ivory in Southern Africa. The most comparable results are from Wiedner et al. 2011, where they studied element concentrations from Asian elephants' whole blood samples in American zoos and circuses. It should be noted that means and medians are not entirely comparable, and the results presented in median values are usually lower than the mean values when data is log distributed.

Table 8. Reference values of measured elements and values measured here.

Article:	Jayasekera & Kuruwita 1996		Wiedner et al. 2011	Sach et al. 2020	Raubenheimer et al. 1998	Values measured here
Sample type:	whole blood, wild Asian elephants in Sri Lanka	whole blood, captive Asian elephants in Sri Lanka	whole blood, captive and private circus Asian elephants in America	plasma, captive zoo Asian elephants in UK	ivory, wild African elephants in Southern Africa	whole blood, captive Asian elephants in Myanmar

Sample amount:	n=7	n=8	n=33	n=11	n=25	n=80
Method:	atomic absorption spectrophotomtre		inductively coupled plasma mass spectroscopy	ICP-MS	ARL 34000 inductively coupled plasma optical emission spectroscope	ICP-MS
Element	Mean (µg/kg)*	Mean (µg/kg)*	Mean (µg/kg)*	Mean (µg/kg)*	Mean (µg/kg)*	Median (µg/kg)
Cd	383,84	389,33	NA**	0,063	400	0,37
Cu	1541,22	3422,19	766	893,659	2200	741
Pb	1455,45	4877,79	17	0,185	8700	20,9
Zn	4624,44	5562,30	6136	659,024	20000	3280
Cr	-	-	44	-	3700	151
As	-	-	19	0,39	8000	6,75
Se	-	-	382	142,927	-	300
Mn	-	-	NA	1,854	600	5,14
Co	-	-	NA	-	720	1,18
Ni	-	-	NA	-	890	21,5
Al	-	-	NA	-	6200	234
*	*Originally informed as µmol/l. Converted to µg/kg using 1,025 as blood plasma density (Benson 1999)	*Originally informed as µmol/l. Converted to µg/kg using 1,025 as blood plasma density (Benson 1999)	*Converted from µg/g to µg/kg	*Mean obtained from two median values. Values converted from mg/l to µg/kg using 1,025 as blood plasma density (Benson 1999)	*Converted from µg/g to µg/kg	
**			**NA = over 70 % of samples were below LOD or nondetectable			

3.2. Estrous cycle profiles

Generally, the concentrations of allopregnanolone remained below 1 ng/ml during the study period, making rise in allopregnanolone levels easily distinguishable as then the concentrations reached over 1 ng/ml for several weeks. Minimum and maximum values of allopregnanolone measured were 0,1 and 2,9 ng/ml. Mean allopregnanolone values and cycling status of the elephants included in this study are summarized in Table 9. Their reproductive history and date of birth are found in Table 1.

Table 9. Mean allopregnanolone levels and estrous cycling status of elephants which hormone profiles were measured in this study. Allopregnanolone values as mean ± SEM. Sampling period: 2018/11-2019/8.

ID	Mean allopregnanolone (ng/ml) ± SEM	SD	Estrous cycling status	N
XX10	0,603±0,032	(0,192-1,014)	cycling	13
XX82	0,853±0,044	(0,100-1,606)	cycling	17
XX81	0,795±0,027	(0,335-1,254)	indefinite, probably cycling	17
XX12	0,455±0,023	(0,113-0,796)	cycling	15
XX31	0,371±0,01	(0,184-0,558)	non-cycling	19
XX83	0,435±0,018	(0,092-0,777)	indefinite	19
XX84	0,281±0,011	(0,115-0,447)	non-cycling	15
XX38	1,226±0,056	(0,273-2,179)	cycling	17
XX85	0,274±0,017	(-0,056-0,604)	indefinite, probably non-cycling	20
XX52	0,271±0,13	(0,050-0,492)	non-cycling	17
XX54	0,248±0,008	(0,098-0,399)	non-cycling	20

Allopregnanolone profiles of (presumably) cycling and non-cycling females are presented in Figures 4 and 3. In non-cycling elephants, the allopregnanolone concentrations stayed under 1 ng/ml, the biggest value reaching only 0,76 ng/ml (Figure 3). The cycle profiles were flat, with no signs of rising allopregnanolone levels indicating ovarian activity. In cycling elephants, on the other hand, the allopregnanolone levels fluctuated periodically over 1 ng/ml, with the maximum value reaching almost 3 ng/ml, indicating ovarian activity (Figure 4). The mean variation of allopregnanolone concentrations during one cycle length is presented in Figure 5. The average length of a cycle was about 16 weeks.

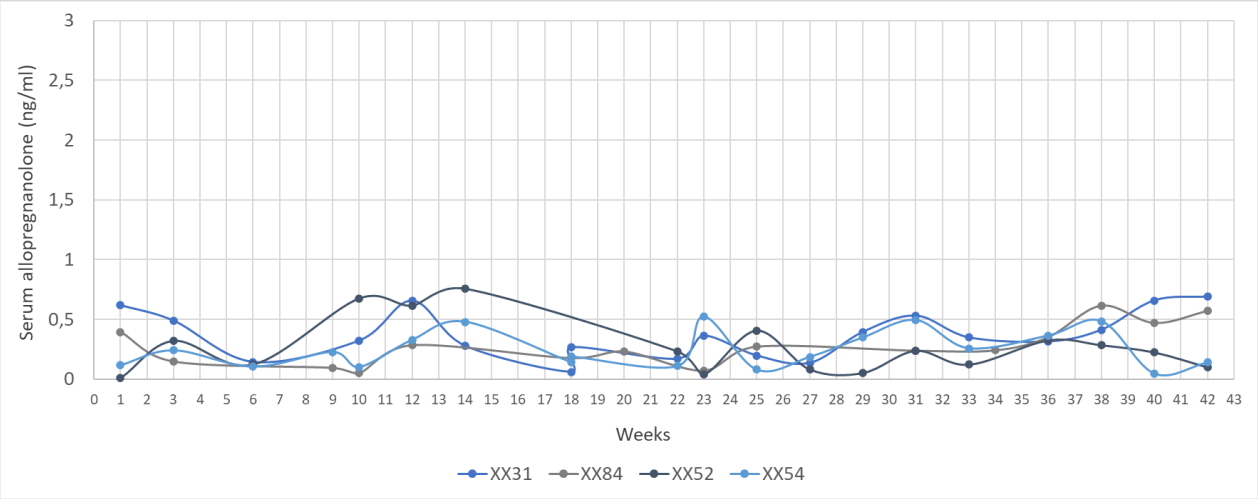

Figure 3. Non-cycling elephants' cycle profiles during the sampling period (42 weeks, n=71).

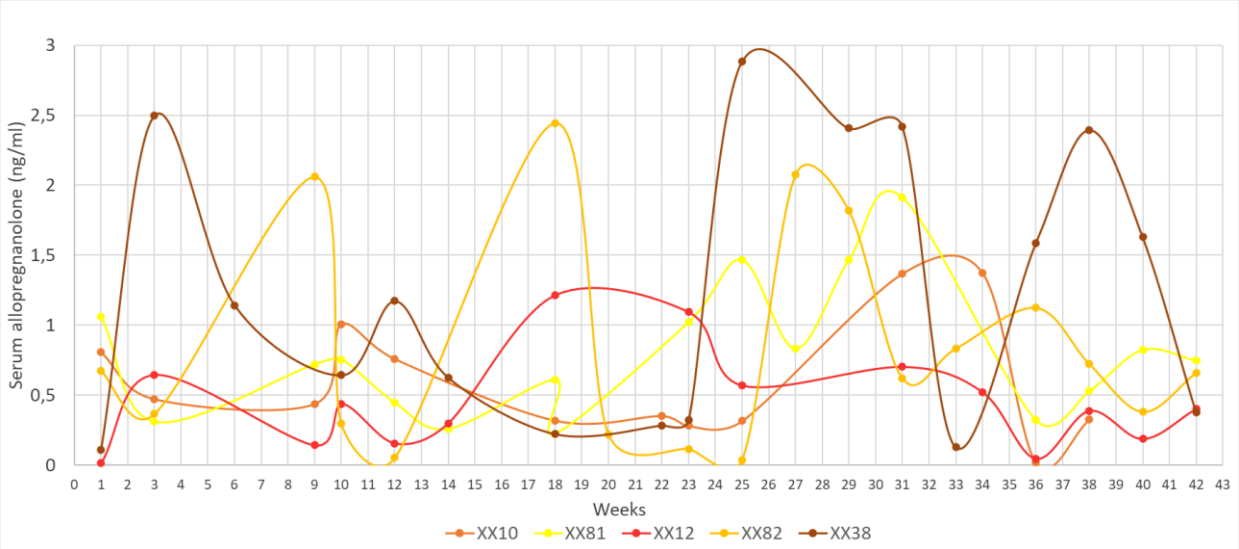

Figure 4. Cycling elephants' estrous cycle profiles during the sampling period (42 weeks, n=79).

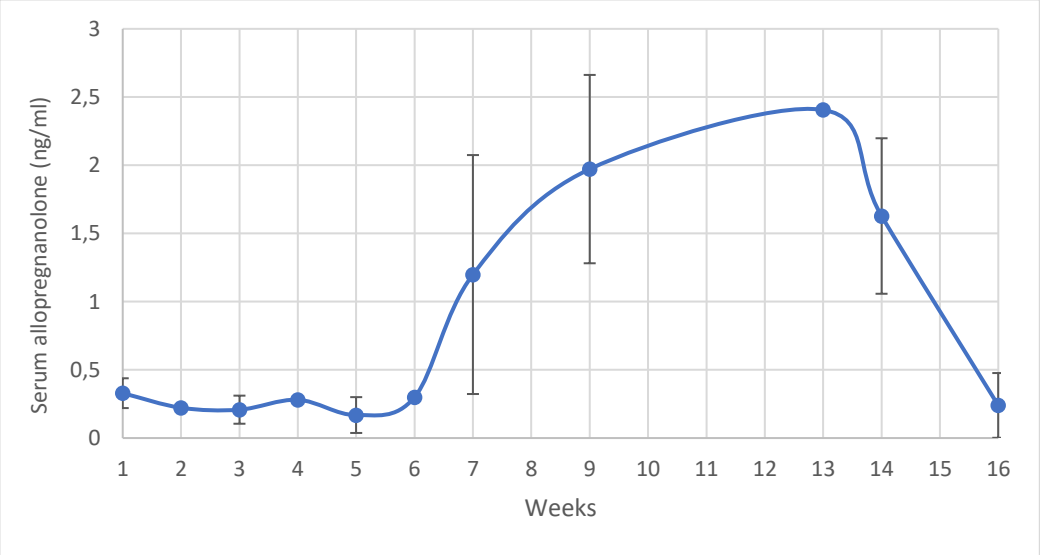

Figure 5. Mean profile of serum allopregnanolone concentrations with standard errors throughout the estrous cycle in ovulating semi-captive Asian elephants (4 cycles, n=23). Follicular phase weeks 1-6, luteal phase weeks 6-16.

From the five cycling elephants, two had repetitive cycles during the sampling period (XX82, XX38) and three had only one visible cycle (XX10, XX81, XX12) (Figure 3). Elephants' XX10 and XX12 hormone profiles show one cycle 16 weeks in length: follicular phase lasting about 6 weeks in both and luteal phases 10-11 weeks. Elephant XX81 cycling profile shows a long period of heightened allopregnanolone levels, with a drop in the middle. Altogether the phase is about 18 weeks long, from which the allopregnanolone levels are over 1 ng/ml for 9 weeks. Elephants XX82 and XX38 seem to have more several luteal peaks during the sampling time. Elephant XX82 seems to have 3 cycles 10-12 weeks in length (follicular phase lasting from 3 to 5 weeks and the luteal phase from 6 to 8 weeks). Elephant XX38 has the highest allopregnanolone levels reaching up to 2,9 ng/ml. It also has one clear cycle visible where the follicular phase

lasts 6 weeks (or more) (weeks 17-23) and the luteal phase 10 weeks (weeks 23-33). One luteal phase is also visible at the beginning of the hormone profile, lasting from 10 to 14 weeks (weeks 1-10/14). At the end there is visible a third luteal peak (6 weeks long, weeks 35-41), with a possibility of a fast follicular phase (about 2 weeks) before it, but which is not possible to confirm due to distant datapoints. Three of the four ovulating elephants were about 51-56 years old, with the youngest being 32 years old, at the time of sampling. All ovulating elephants had calved several times in the past, and their reproductive status is classified as active or paused (XX12) (Table 1).

The timing of the luteal phases/ovulation does not seem to overlap between cycling elephants (Figure 3), giving no signs of synchronization across different females. A small exception are peaks of XX38 and XX82 during weeks 26-30, but it is most likely due to coincidence rather than any other reason.

Estrous cycle profiles of two elephants were classified as indefinite: allopregnanolone values get over 1 ng/ml, but do not reach higher concentrations leaving peaks relatively low and quick (Figure 6). Peaks do not follow each other in a typical follicular-luteal phase timing. This indicates some ovarian activity, but not enough to maintain normal cycling.

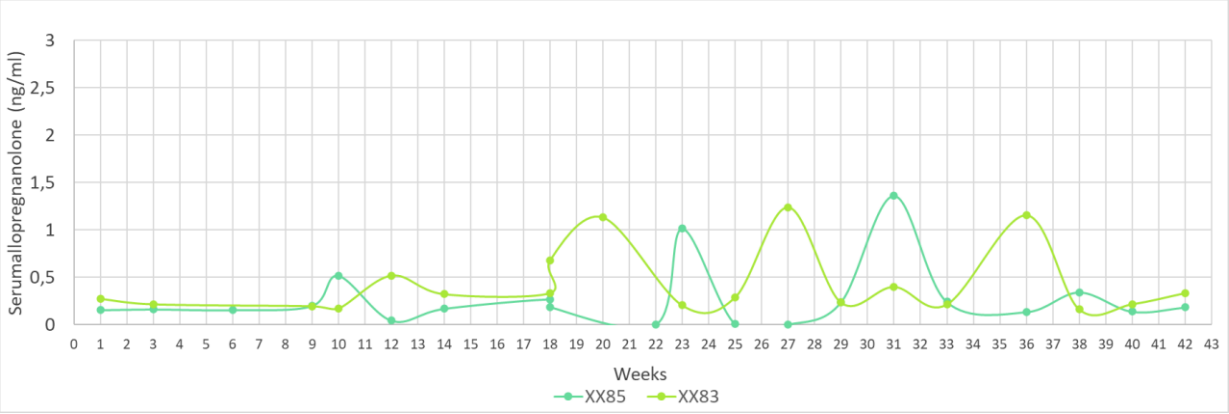

Figure 6. Allopregnanolone profiles that are not clearly anestrous nor normal: allopregnanolone levels increase at times, but the peaks do not last for over 5 weeks and repeat too fast after each other (n=39).

3.3. Heavy metals and estrous cyclicity

Only six elephants had both the heavy metal and cyclicity data obtained in this study (Table 4). Half of the elephants were classified here as cycling, and half of them as non-cycling (Table 9). Non-cycling elephants (n=3) had slightly bigger concentrations than cycling elephants (n=3) in 7/11 of the studied elements, and lower levels of element concentrations in 4/11 of the elements, those being Ba, Cu, Zn and Se (Figure 7, Figure

8). Even though cycling elephants had bigger concentrations in less than a half of measured elements, the total mean concentration in cycling elephants seems to be higher than the total mean of the non-cycling elephants, even though the difference is not statistically significant (t-test, p=0,9, df=4). This might be biased due to the high (over 100 µg/kg) concentrations of Cu, Se and especially Zn (Figure 8). Only the concentrations of the elements which had LOD<15% (Table 2) were included into the statistical analyses and compared.

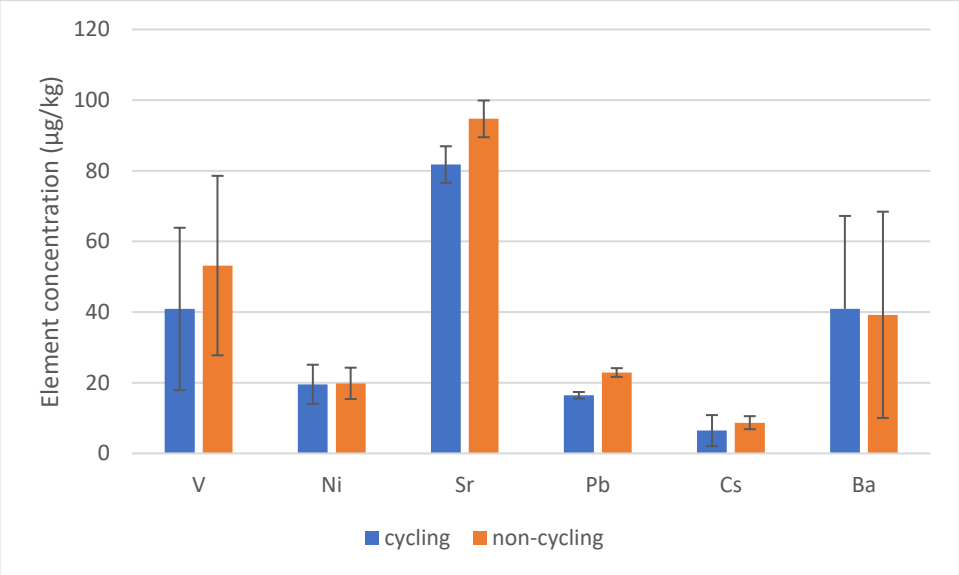

Figure 7. Mean heavy metal concentration and standard errors of elements which concentration in cycling (n=3) and non-cycling (n=3) elephants was under 100 µg/kg in total.

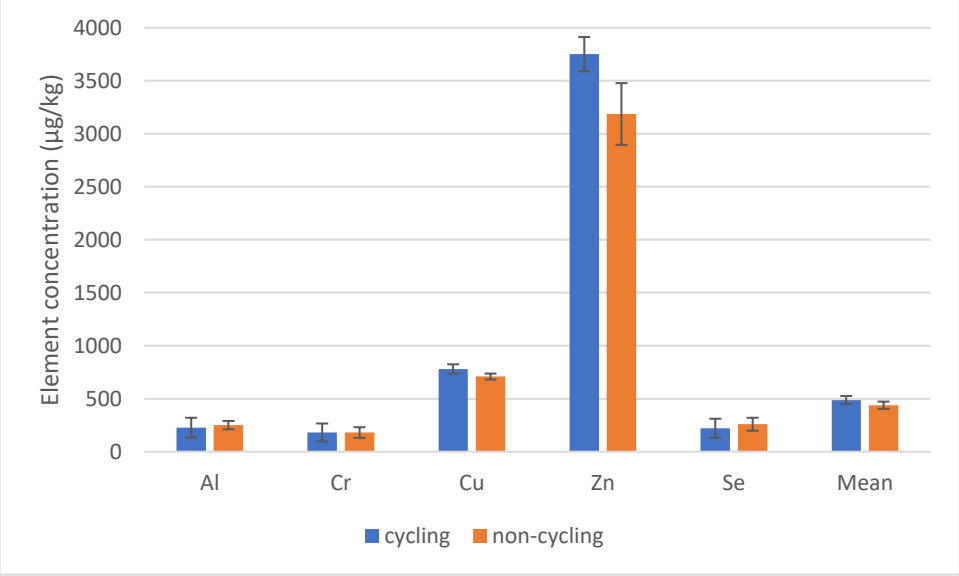

Figure 8. Mean heavy metal concentration and standard errors of elements which concentration in cycling (n=3) and non-cycling (n=3) elephants was over 100 µg/kg in total. Also the total mean element concentration between cycling and non-cycling elephants.

All the elephants studied for heavy metals and cyclicity had relatively similar element concentrations and there were no outliers in their group (Figure 9).

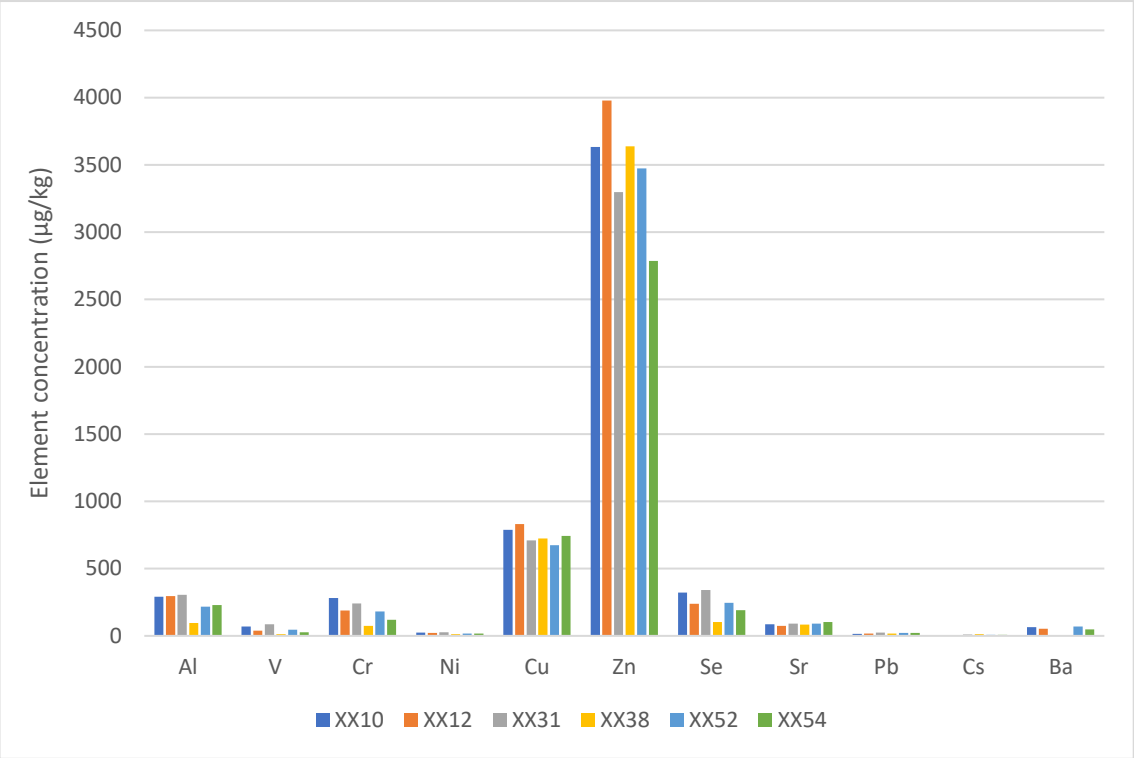

Figure 9. Element concentrations of each elephant which had both the heavy metal data and cyclicity data obtained in this study (n=6). There are no outliers detectable from the figure.

4. DISCUSSION

The aims of the thesis were to study heavy metal concentrations in 80 semi-captive Asian elephants in MTE and to form an overview of their current heavy metal load, and to measure allopregnanolone levels in 11 female elephants in order to study their reproductive cycles, test a method for future use, and to examine whether heavy metal load has any link to cyclicity status. I succeeded to map the acute heavy metal load of elephants and categorized elephants by their estrous cycle profiles into cycling or non-cycling. The used methods were found out to be convenient and reusable in future studies. The results deepen our knowledge on elephant health measurements and can have applicability in elephant conservation efforts.

4.1. Heavy metals and the effect of age

My first hypothesis was that age will have a positive effect on heavy metal concentrations, metals bioaccumulating with time (Dietz et al. 1995, Franson & Russell 2014 and Slabe et al. 2022). However, I found that age did not have a clear effect and the element concentrations rose with age only in about a half of analysed elements: in Ni, Pb, Cr, Al, and V, of which the rise was statistically significant ($p<0,05$) in Ni, Pb and Cr. In Se, Sr and Cs, age did not seem to have almost any effect, concentrations of the elements not changing with age. Opposing the original prediction, age had a negative effect on Cu and Zn concentrations, where age had a statistically significant negative effect in Cu. Ba differed from other elements so that age affected Ba concentrations in interaction with sex: in females, the Ba concentrations decreased with age, though without a statistical significance, and in males, the concentrations increased significantly with age. Curiously, Ba was the only element where age and sex had a statistically significant interaction. The different effects of age on different element concentrations reflect the variety in how elements function, e.g. how they accumulate. For example, Pb seems to easily accumulate with age. In Slabe et al. 2022 and Franson & Russell 2014, Pb concentrations increased with age in bald and golden eagles when chronic exposure was measured from femur and carcasses. Here, according to the prediction, Pb concentrations also increased significantly with age. It is likely that the studied elephants suffer from chronic Pb exposure, due to its bioaccumulation. This assumption is strengthened by Pb values exceeding reference values from Wiedner et al. 2011 (Table 7). However, it is not possible to verify here whether elephants in this study population suffer from chronic exposure or not.

Cd, Zn and Se have also been found to increase with age, for example in Dietz el al. 1995 where the concentrations were measured from livers and kidneys of polar bears, and in Vizuete et al. 2022 where the concentrations were measured from livers, kidneys and feathers of seagulls. Here, Cd levels were so low it wasn't even included in the statistical analyses, Zn decreased with age, and Se did not really show any variation with age. However, this doesn't exclude the possibility of chronic Cd, Zn and Se poisoning in the studied elephants, even though there are no signs of an acute poisoning measurable in blood samples. The results here could actually imply Zn deficiency in the studied elephants, especially in older ones and in females (Table 4, Table 5).

Blood is not the optimal tissue to study long term effects or accumulation of heavy metals, as blood reflects mainly short-term exposure to elements. Long-term exposure can be studied from tissues where regeneration is slow or non-existent, like from teeth, bones or internal organs such as the liver. However, blood is easier to collect than, for example, internal organs so it allows bigger sampling sizes and monitoring of the concentration levels in individuals and in populations. Also, blood is considered a good medium to measure trace elements (Wiedner et al. 2011). Nevertheless, in the future, it would be of interest to study the chronic heavy metal exposure of the elephants, and as collection of samples like internal organs can be challenging, toenails could provide a compromise: in Sach et al. 2020 toenails were observed to be the best bioindicator of element intake in comparison to other relatively easily collectable samples like feces, plasma and urine. What is more, MTE could consider collecting and studying organs like kidneys and liver post-mortem.

4.2. Heavy metals and the effect of sex

The second hypothesis was that heavy metal concentrations would differ between males and females so that females would have smaller heavy metal burden due to pollutant excretion during pregnancies and lactation (Ylitalo et al. 2001, Wade et al. 1997, Franson & Russell 2014, Desforges et al. 2018). Opposing the hypothesis, in more than half of the analysed elements, those being V, Cr, Se, Pb, Cs and Ba, females had bigger concentrations than males. The difference was statistically significant in V and Pb ($p<0,05$). On the other hand, when comparing the total heavy metal concentrations between females and males, females had a smaller heavy metal load, which is in line with the original hypothesis (Figure 1), but the difference was not statistically significant ($p=0,96$). Besides, males had bigger concentrations in elements considered more toxic:

Al ($p<0,05$), Cu, Ni ($p<0,05$), Zn and Sr. However, the difference between total element concentrations between sexes is small, and statistically nonsignificant ($p=0,96$). The sex difference could be explained also by size difference in sexes: males are bigger than females and consume more food and water, which are the most likely contamination sources. In addition, total element concentrations in males may be bigger due to higher Zn concentrations as they are higher in general than other elements (Zn concentrations being over 3000 µg/kg vs. other elements having concentrations between 4-800 µg/kg) and its concentration may easily distort total element concentrations and their comparison (Table 3).

Sex differences in element concentrations have not been unambiguous in other studies either (Burger 2007). For example, in Franson & Russell 2014, Pb concentrations were detected to be higher in female eagles than in male eagles, in line with the results here. However, there are studies where Pb concentrations do not really differ significantly between sexes, like in Helander et al. 2009 and Vizuete et al. 2022. Unclear sex differences raise questions of heavy metal metabolism and how it differs from metabolism of other pollutants and possibly also between sexes. For example, it has been suggested that Mn is metabolised differently in females and males (Berglund et al. 2011). Also, it is a possibility that excretion of heavy metals through reproductive organs and functions in females is not as significant as previously thought, because in contrast to heavy metals, other environmental pollutants like PCBs and organochlorines have been consistently reported to be found at lower levels in females than in males (Ylitalo et al. 2001, Wade et al. 1997, Desforges et al. 2018). Sex differences in heavy metal concentrations might also be affected by different diets between sexes, as in many species females and males have been observed to have dissimilar foraging and/or hunting habits (Lei et al. 2020, Schuppli et al. 2021), and diet has been proposed to have a significant influence on element concentrations (Osawa & Hatsukawa 2015, Sach et al. 2020).

It could have been assumed that more of the elements would have had a similar sex dependent age effect as there was in Ba (Figure 2), where the concentrations decreased with age in females, but not statistically significantly, and increased with age in males ($p<0,05$). This kind of effect would suit the assumption that females lessen their heavy metal burdens through pregnancies and lactation or through eggs and eggshells, whereas in males, heavy metals bioaccumulate with age. However, this hypothesis was not supported by the overall results in this thesis. Also, when assuming there to be sex differences (female<male), due to reproductivity of females, it would make sense to

assume that heavy metal concentrations increase in males with age but decrease in females in general. In the future, it could be interesting to study how specific heavy metals function and what affects their accumulation. Overall, more studies could concentrate on the effects of few heavy metals or trace elements at once, so the mechanisms of action, accumulation and excretion of each element could be more closely studied.

4.3. Heavy metals and the effect of camp

The third hypothesis was that heavy metal concentrations will vary among elephants living in different logging camps, as the sites where camps are located might be differently polluted by, *inter alia*, gold mines situated in Myanmar. Here, camp had an effect on the concentrations of Zn, Pb, Cu and Sr (Table 7). Zn concentrations differed in all camps on a range from about 2920 µg/kg in camp Old/Sick to 4175 µg/kg in camp Kongi with a statistical difference. Pb concentrations were lowest in the camp Kongi (about 12,3 µg/kg), and significantly higher in camps Maternity, Taming, Tc and Working, the highest concentration being in camp Tc (26,9 µg/kg). The concentrations of Cu in Taming camp were significantly higher than in other camps, and the concentrations of Sr in camp Old/Sick were significantly lower than in other camps. Thus, the element concentrations of Zn, Pb, Cu and Sr differed significantly between camps like predicted but in general, however, the element concentrations did not vary among camps remarkably.

Gold mines usually increase the concentrations of Cu, Zn, Pb, As, Cd and Hg in the environment. These elements have been detected to be present in high concentrations in Myanmar soil (Osawa & Hatsukawa 2015, Tun et al. 2020). Polluted soil might contaminate the vegetation and water in the area, and thus accumulate in animals living in the site, like in elephants. Here, the concentration of Hg was not measured, but Cu, Zn, Pb and As were, and the three first mentioned elements showed variation in concentrations between camps studied. It could indicate that gold mines in Myanmar might possibly be one source of heavy metal exposure in elephants in different camps, but future studies should focus more on the effect of geographic proximity to such mines to confirm this. However, from Cu, Zn, Pb, As and Cd, only Pb levels were slightly elevated in comparison to concentrations measured in Wiedner et al. 2011 (Table 8). It seems, therefore, that heavy metal leaks from gold mines do not cause acute poisonings in elephants. However, they might be a source of chronic exposure not studied here, and they also might affect elephants differently depending on where elephants live and roam.

What is more, gold mines are only one suspected source of exposure, but their effect has not been directly studied, and other exposure sources should also be considered in future studies.

Sampling sites have been demonstrated to have a significant effect on the element levels in African elephants (Raubenheimer et al. 1998) and in other animals (Dietz et al. 1995, Slabe et al. 2022, Vizuete et al. 2022). However, in most of the studies, the concentrations have been measured from several tissues at once, and it has been detected that heavy metal concentrations vary in different organs or mediums. Also, the effect of a factor, like age or camp, also depends on the tissue sample. For example, Cd seems to accumulate the most in kidneys, as Cd levels have been higher in, *inter alia*, polar bears' and sea gulls' kidneys than in livers (Dietz et al. 1995, Vizuete et al. 2022). In Vizuete et al. 2022, the sampling site had an effect on Cd concentrations only when examining the kidney concentrations, but not when examining liver concentrations. This highlights the differences between element function, and that when examining element concentrations and what affects them, individual characteristics should be taken into account in analyses.

The aim here was to compare different locations, and working camps were used as a correlate of this. However, camps are not necessarily geographically very distant from one another, and my study would have benefitted from detailed information on the geographic coordinates of these logging camps. Also, the sample size varied from 3 individuals to 17 animals per individual camp, which is a relatively small number for reliable statistical analyses. Taking this into consideration, the information on spatial variation is limited, since the camp classification may differ more sociologically (e.g. whether elephant is pregnant or working or old) than geographically. Therefore, these results should be interpreted with caution. In future studies, geographic distance to e.g. mining sites should be preferred in order to address geographic variation and its causes more precisely.

4.4. Heavy metals and reference values

The fourth hypothesis was that some of the heavy metal concentrations would exceed reference values, because there have been suspicions of heavy metal poisonings among the studied elephant population in Myanmar. Most comparable results are from Wiedner et al. 2011 where they measured the concentrations of Cu, Pb, Zn, Cr, As, Se, Mn, Co, Ni and Al from 33 captive and private circus Asian elephants in America (Table 8). Wiedner

at al. 2011 note that the concentrations measured in their study provide somewhat like baseline values for measured element concentrations. Compared to their values the concentrations of Zn, As, and Se are lower in the population studied here, Cu concentrations are similar, and the concentrations of Pb, Cr, Al and Ni are elevated (Table 8). The levels of Pb do not differ remarkably: 17 µg/kg (Wiedner et al. 2011) vs. 20,86 µg/kg (here); but the levels of Cr differ notably: 44 µg/kg vs 150,76 µg/kg, as do Al levels: <LOD vs. 234,30 µg/kg. Ni levels measured here were 21,47 µg/kg, in comparison to being undetectably low in Wiedner et al. 2011. Ni reference values from elephant whole blood were not found (Table 8), but in cow whole blood Ni has been reported to be measured at 11 µg/kg (0,011 mg/kg), and normal levels for "humans, cattle, dogs, and rats" has been reported as 2-7 µg/kg (0,0020-0,0027 mg/kg) in serum (Eisler 1998) and 0,2 µmol/l in urine (TTL 2022). In comparison to those concentrations, Ni levels measured here seem slightly elevated. Increased levels could indicate elephants being exposed to Pb, Cr and especially Al and Ni more than what could be suspected from normal diet and habitat. On the other hand, decreased levels of Zn, which are almost half smaller than in Wiedner et al. 2011, and also smaller than values in Jayasekera & Kuruwita 1996, could indicate Zn deficiency, Zn being an important trace element.

The concentrations of Cd, Mn, Co, Ni and Al were so low in Wiedner et al. 2011 the values were informed as NA (Table 8). Here, the measured levels of Cd, Mn and Co were also undetectably low, as were the concentrations of As, Ag, Be, Ga, Cd, Tl and U (Table 3). Undetectable levels do not necessarily mean lack of elements or lack of occasional exposure. For example, As is almost impossible to detect from blood, as it integrates into nonvascular tissues in a few days after exposure (Mayo Clinic Laboratories 2022), leaving possible poisoning most often undetected.

In this thesis, the levels of Al, Ni and Cr were found to be notably bigger than their reference values. Pb values were also higher than in Wiedner et al. 2011, but much lower than in Jayasekera & Kuruwita 1996, where the values are very high, over 1000 µg/kg (Table 8). The possibility of elephants being exposed to risky levels of these elements cannot be excluded. However, in general, the concentrations measured here do not raise concerns of acute poisoning in the studied Asian elephant population. On the other hand, elephants might suffer from chronic exposure and possibly accumulation of metals in internal organs, which is not detectable here. Also, even if the whole population is not at considerable risk, single individuals could be, as heavy metal concentrations may vary between individuals a lot. Individual risk assessment was not done here but would be an

interesting possibility to examine in further studies. Also, in future studies, Hg concentrations could also be examined, this metal being a quite common and toxic pollutant, associated especially with gold mining activities (Osawa & Hatsukawa 2015, Tun et al. 2020).

4.5. Estrous cyclicity profiles

The fifth and sixth hypotheses were that 5) the method for measuring reproductive cycling in Asian elephants would give distinguishable results between cycling and non-cycling elephants, as analyses of progesterone metabolites have been shown to be reliable in monitoring cyclicity (Fieß et al. 1999, Brown 2000, Ghosal et al. 2010) and 6) that elephants with a normal reproductive history will mostly show a normal cycling pattern and vice versa (Thitaram et al. 2008). Here, due to successful allopregnanolone measurements, the cycle profiles of elephants were classified in three categories: cycling (Figure 3), non-cycling (Figure 4) and indefinite (Figure 6), fulfilling the fifth prediction. In non-cycling elephants, no clear allopregnanolone surges were detected (Figure 3), which indicates no ovarian activity. In cycling elephants, the allopregnanolone levels rose remarkably for over 5 weeks in a row, indicating the luteal phase of the cycle and ovarian activity (Figure 4). In indefinite elephants the allopregnanolone levels were mainly low, but rose a few times over 1 ng/ml, differentiating the profile from non-cycling elephants, but not reaching high enough allopregnanolone levels and for enough time to be classified as cycling elephants (Figure 6). In line with the sixth hypothesis, the cycling patterns of the elephants' studied were observed to mostly correspond to the reproductive histories of the elephants.

The allopregnanolone profiles of cycling (Figure 4) and non-cycling elephants (Figure 3) measured here are similar to progestin profiles of cycling and non-cycling elephants in previous studies, but with generally higher progestin concentrations (Brown et al. 2004, Thitaram et al. 2008). Higher hormone levels could perhaps partly be explained by using an antigen against a specific progesterone metabolite, not a progesterone antigen. Schwarzenberger et al. 1997 seemed to use the same antibody as used in this study for measuring 20-oxo-P values in Asian elephants, and their concentrations of plasma 20-oxo-P concentrations during the luteal phase were 2.19 ± 0.16 ng/ml, which is similar to progestin concentrations during luteal phases measured in this study (Figure 4, Figure 5). However, curiously, they reported to not have detected allopregnanolone, which could be

due to cross-reactivity of the antigen and/or not being capable of distinguishing between different hormone levels.

The sampling period lasted for 42 weeks (2018/11-2019/8), which means that in theory the sampling period could have fitted from 2 to 3,5 estrous cycles depending on cycle length. The cycle length in Asian elephants has been detected to vary from 12 to 16 weeks, e.g. the average cycle length of Asian elephants in Thailand was 14,6 weeks where follicular phase lasted on average 6 weeks and the luteal phase 8,5 weeks (Thitaram et al. 2008). This is in line with the mean cycle profile in this thesis, where the cycle was on average 16 weeks long, follicular phase lasting for 5 weeks and the luteal phase for 10 weeks (Figure 5). The length of the estrous cycle is determined by the timing of the anovulatory LH surge during the follicular phase (Thitaram et al. 2008).

In cycling elephants observed here, there were no clear repetitive cycles detected during the sampling period. This might be explained by short sampling period and distant sampling time points. However, in the cycling elephants, the allopregnanolone levels rose significantly, confirming ovarian activity, and at least one cycle was detectable in four out of five elephants, number XX81 being the exception. In XX81 the allopregnanolone levels indicate clear ovarian activity, but the timing of ovulation and luteal phase are challenging to interpret from the profile (Figure 4). No synchronization of the estrous cycles was detected in this study (Figure 4). Synchronization of estrous cycles and the timing of ovulation is a debated topic in elephants and in mammals in general (Weissenböck et al. 2009, Clarke et al. 2012). There is no strong evidence for synchronization of the estrous cycles in elephants, nor for the lack of it (Thitaram et al. 2008, Hildebrandt et al. 2011).

Three out of five elephants that were classified as cycling in this thesis turned out to be pregnant at the time of sampling: XX10, XX82 and XX38, as they gave birth to calves in 2019 and 2020 (Table 1). In Thitaram et al. 2008 “normal cycling and pregnant” is used as one of the categories for normally cycling elephants, in addition to classes “normal cycling” and “pregnant, postpartum period and normal estrous cycle”. The other cycling elephants, XX81 and XX12, were most probably not pregnant at the sampling time. Their reproductive status has been classified as paused and last time they calved in 2014 and in 2015 (Table 1). If the elephants were pregnant after that, their pregnancies stopped. However, it is possible that both elephants will calve again, as they have been reproductively active in the past and show signs of normal cycling. On the other hand,

both of them are over 50 years old, and female elephants' reproductivity has been shown to decrease after age of 50 (Lahdenperä et al. 2014).

Four elephants were classified as non-cycling (Figure 3). Their acyclicity is most likely due to lactational anestrous, as these elephants had given birth in 2015-2018 (Table 1), and Asian elephants are known to lactate from 2 to 8 years (Ochs et al. 2001). The time of sampling was from November 2018 until August 2019. All these elephants have been reproductively active, and XX31 gave birth to a new calf in 2021, meaning it very likely got pregnant straight after ovulating again after the lactational anestrous.

Two elephants studied here showed cycling profiles that were classified as "indefinite" (Figure 6). Their allopregnanolone levels were lower than allopregnanolone levels in ovulating elephants, but higher than allopregnanolone levels in non-cyclic elephants, raising occasionally even over 1 ng/ml. These "peaks" lasted only for a maximum of 2 weeks, not being enough to interpret it as the luteal phase which follows ovulation. However, there was some activity in the ovaries, but not enough to lead to ovulation. This might be due to multiple small corpora lutea or CL-like structures (luteinized unovulated follicles) that develop between the LH surges in both ovaries (Hermes et al. 2000). Their development is linked to serum progesterone rise a few days before the ovulatory LH surge (Hermes et al. 2000). Perhaps they secrete levels of progestins that are observed in the indefinite cycling profiles of elephants XX85 and XX83, explaining some levels of detected allopregnanolone. It is unclear in what reproductive stage these elephants were during the sampling period. XX85 gave birth in April 2019, which is in the middle of the sampling time. XX83 gave birth in 2017, and in theory could be in lactational anestrous. However, it is possible that XX83 had stopped lactating during the sampling period and that its estrous cycle was (slowly) activating. Elevated reproductive hormone levels have been observed in non-cycling females also before (Brown et al. 2004, Thitaram et al. 2008).

The current reproductive status of most of the elephants that were sampled for the cyclicity analyses was "active", except for two, which were classified as "paused" (Table 1). However, both seem to be cycling, and have an active reproductive past, so they are probably experiencing normal reproductive decline associated with age (>50) (Lahdenperä et al. 2014). Therefore, elephants classified here as cycling and non-cycling all show normal allopregnanolone profiles according to their reproductive status. Indefinite elephants' allopregnanolone profiles are not as clear, but closer examination of their behaviour, reproductivity and health status at the time would likely explain the

cycling profiles. In future, it would be of interest to include elephants classified as "nulliparous" into cyclicity studies, and elephants which are suspected to have become acyclic after reproductive activity. Also, weekly sampling would be ideal for studying cyclicity, and the longer the sampling period, the better.

Even though there were no acyclic elephants recognized in this study and all the elephants have a reproductive history, in general, elephants' reproductive rates have decreased for years both captivity and in the wild, and calving problems have increased, especially in captive elephants, stressing the need to unravel the causes (Wiese 2000, Saragusty et al. 2009). In addition to decreased reproduction in comparison to wild elephants, captive elephant females attain puberty at younger age than wild ones (Brown 2000) and give birth more often to male calves than female calves, skewing the birth ratio (Saragusty et al. 2009) for unknown reasons. Free-ranging elephants, on the other hand, reproduce longer than captive ones, with less calving problems or stillbirths and maintain a normal sex ratio (Brown 2000, Saragusty et al. 2009). Usually, the health statuses between cycling and non-cycling elephants do not "markedly differ" (Thitaram et al. 2008) and there are no clear consistent endocrine anomalies found which would explain non-cyclicity in elephants (Brown 2000). However, hyperprolactinemia, high body fat and elevated insulin levels have been suggested as possible contributors to non-cyclicity in elephants (Brown et al. 2004, Morfeld & Brown 2016). More studies on elephant reproductivity are needed in order to clarify the possible risk factors of elephant reproductivity.

4.6. Cyclicity and heavy metals

The last hypothesis was that acyclic elephants will have increased heavy metal levels in comparison to cycling elephants, if the acyclicity is not due to lactational anestrous (Allen 2006, Lueders et al. 2012). Here, there were 4 elephants observed not to be cycling, but all of them were lactating at the sampling time, so there were no elephants observed on which to examine this hypothesis.

Also, as all the studied elephants were reproductively active, it is of no surprise that heavy metal concentrations in cycling and non-cycling elephants did not remarkably differ from each other (Figure 7, figure 8). There were also no individual outliers (Figure 9). The sample size (n=6) was so small no trustworthy conclusions can be made about the state of the population.

Six elephants included in this study in the heavy metal analyses are classified as nulliparous, which is 12,5% of the studied female elephants. Nulliparous elephants are common in other study populations too (Thitaram et al. 2008). Nulliparous elephants have not given birth, but it is unclear whether they have mated or been pregnant. These elephants' cyclicity status would be of special interest to study: are they not ovulating and cycling or does something else lead to them not reproducing, and could it be linked to e.g. heavy metal exposure. In some cases, nulliparous doesn't mean incompatibility to calve, but just that the elephant hasn't calved yet, given the elephants' age at first birth has a big range (Table 1) (Reichert et al. 2020).

It is also worth remembering that reproductivity is dependent on the males as well. Their reproductive systems are vulnerable to pollutants and other disturbances (Hovatta et al. 1998, Lopez-Botella et al. 2021), and so the fertility decrease in elephant populations might also be caused by abnormalities in male reproductivity, like in sperm quality. Therefore, males should be included in fertility studies.

4.7. Conclusions

My results have several relevance. First, based on results in this thesis, measuring acute heavy metal exposure from whole blood can be used as an applicable method for estimating population and individual level heavy metal exposure in elephants. Based on the results, chronic exposure could be a more potential health risk than acute heavy metal poisoning. However, chronic exposure is challenging to examine due to difficulties in obtaining suitable samples like organs or ivory. Whole blood measurements, on the other hand, provide a relatively easy way to monitor element concentrations in Asian elephants, meaning that not only heavy metal levels but also trace element levels can be monitored, helping in identifying possible health risks, like heavy metal poisoning or trace element deficiency. However, in addition to monitoring element levels, it is of importance to monitor the general health status of the elephants, because element concentrations do not necessarily correlate with symptoms, and symptoms can appear only some time after the exposure (Rajkumar & Gupta 2022).

Second, as elephants suffer from decreased fertility, heavy metal measurements enable to study whether element levels in Asian elephants may affect reproductivity. Even though it was not possible to reliable study this issue here, this thesis provides relevant methods for future studies: ICP-MS measurement and whole blood, or e.g. toenail, sampling

enable element monitoring, and allopregnanolone ELISA from serum samples enables to monitor estrous cycles and to detect abnormal cycling profiles. Many commercial kits for measuring progesterone are not suitable and/or sensitive enough to study elephants, due to different hormonal excretion in comparison to other more-studied mammals. Therefore, there is a lack of a common, accessible and reliable laboratory method to study ovarian activity in elephants. Method used in this study, however, could be suggested as an alternative for such. Allopregnanolone levels can also be measured from fecal or urine samples, if it facilitates weekly sampling in comparison to serum sample collection (Ghosal et al. 2012, Brown 2000).

Also, the effect of season could be more closely studied, as the lack of it might contribute to acyclicity of some captive elephants. The effect of season on estrous cyclicity has been debated. In some studies, no effect of season on estrous cyclicity have been detected on captive Asian elephants (Brown et al. 2004), like the length of the cycle (Thitaram et al. 2008). In others, season and other ecological conditions like vegetation or seasonal workload might have had an effect on some aspects of ovarian function and reproductive endocrinology (Wittemyer et al. 2007, Thitaram et al. 2008, Hildebrandt et al. 2011, Mumby et al. 2013, Hufenus et al. 2018). Therefore, it might be assumed that season does affect reproductivity in the wild, but not in captivity perhaps due to different climate, habitat and diet especially in western zoos and other facilities.

As African and Asian elephants are different species, results obtained from studying one species are not necessarily applicable to the other species, even though their physiologies seem to function similarly. Nevertheless, it has been emphasised that there is a need to study both species separately, and to study different elephant populations in different parts of the world (Thitaraman et al. 2008).

This thesis gives a snapshot of the element load and cyclicity status in semi-captive Asian elephants working in the logging industry in Myanmar. The methods described and the information obtained here can be used in future studies for example to optimize sample collection to fit different study questions. Studying element concentrations in Asian elephants may help to prevent poisonings, maybe even deaths, and broaden our knowledge on the health of the elephants in general. Estrous cyclicity studies, in turn, contribute to research on elephant reproduction and will help to understand the reasons underlying poor reproductivity rates. Results can then be used in conservational efforts to revive and preserve the population of endangered Asian elephants.

5. ACKNOWLEDGEMENTS

Firstly, I want to thank my supervisors Virpi Lummaa, Tapio Eeva and Diogo dos Santos for their patient help and guidance throughout the whole thesis. Secondly, I want to thank Katja Anttila for valuable help and support with the laboratory work. Also, I want to greatly thank everyone at Ab Spectrochem Oy and Jella Wauters and others from IZW involved in the process for smooth collaboration. Last but not least, I want to thank MTE for the provided samples. And Jenni Kauppi, and others not mentioned here but who contributed to the process.

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7. APPENDICES

Appendix 1: Serum extractions

1. Add 1,8 ml PetrolEther (PE) to 0,1 ml serum
2. Shake for 30 min
3. Freeze samples at – 80 ºC for 30 min.
4. Transfer the supernatant into a new tube.
5. Evaporate until dryness at + 55 ºC.
6. Add first 0,4 ml 100 % methanol and vortex (immediately after evaporation when tube is still warm).
7. Add then 0,6 ml mqH_2O and vortex again.

Appendix 2: Assay protocol for 5alpha-pregnan-3beta-ol-20-one EIA provided by Leibniz Institute for Zoo and Wildlife Research (user manual, version 1-2021)

Assay day 1:

1. Wash plate once with 300 µl/well wash solution.
2. Pipet 20 µl 40 % methanol into NSB and B0 wells.
3. Pipet 20 µl standard of the standard curve into standard curve wells.
4. Pipet 20 µl (diluted) sample into sample wells.
5. Add 100 µl conjugate dilution o all wells using a repeater pipette.
6. Add 100 µl assay buffer to the NSB wells.
7. Add 100 µl antibody dilution to all wells except the NSB with a repeater pipette.
8. Cover the plate with a lid and incubate over night at + 4 ºC, light protected, slight shaking.
9. Store the wash solution at + 4 ºC overnight for the next day.

Continuation next day – Assay day 2:

10. Mix cold substrate solution fresh (50/50 solution A/B=8 ml A + 8 ml B)
11. Wash plate 4 times with 300 µl cold wash solution with a repeater pipette.
12. Add 150 µl/well cold substrate solution mix to all wells with a repeater pipette.
13. Cover the plate with a lid and incubate 40 min at + 4 ºC, light protected, slight shaking.
14. Add 50 µl/well stop solution (2 M H_2SO_4) with a repeater pipette,
15. Let the plate allow reaching approximately room temperature (ca. for 10 min) before reading it with a plate reader,
16. Wipe off the plate bottom with a clean tissue, make sure the wells are free from air bubbles and read optical density at 450 nm with a plate reader.