Age-related changes in Asian elephants' estrous cycle

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Reproductive senescence is recognized across species, but only a small number of them have a clear physiological reproductive cessation (i.e. menopause) and long post-reproductive lifespan. In female mammals, the reproductive ability is determined by the menstrual or estrous cycles and the associated hormones that prepare females for reproduction. Along with other factors, aging and reproductive history can cause changes in the qualities and prevalence of estrous cycle affecting fertility rates. Demographic data from long-lived Asian elephants has shown that female individuals of the species have relatively long post-reproductive lifespan although some of them are capable of reproducing beyond 60 years of age. However, the physiological processes and estrous cycle characteristics have not previously been investigated in Asian elephants. The aim of this thesis is to study how age, recent reproduction and some other key factors affect the estrous cycle characteristics in semi-captive Asian elephants to gain knowledge on the possible reasons for reproductive termination in the species.

Estrous cycle profiles were determined for 69 Asian elephants by measuring and analysing their serum concentrations of a progesterone metabolite, allopregnanolone. Based on cyclicity patterns, elephants were categorized to cycling and non-cycling individuals. Statistical models investigated how age, recent reproduction and additional factors affect the elephants' 1) mean allopregnanolone concentrations, 2) probability of having an estrus cycle and 3) cycle length. The results showed that age was not associated with mean allopregnanolone or cycle length. However, middle-aged elephants (~30-50 years old) had a higher probability of cyclicity than the younger (9-30 years old) and older (50-71 years old) elephants. Recent reproduction was not associated with mean allopregnanolone or cyclicity, but there was weak yet non-significant evidence on recent reproduction increasing cycle length.

In this thesis, Asian elephant's estrous cycle was investigated for the first time in the species' natural living environment and with a larger sample size. The observed age-related changes in estrous cyclicity were not as comprehensive as in truly menopausal species, but can be indicative of individual level menopause in some Asian elephants. As the demographic characteristics of the studied semi-captive elephants resemble more closely those of wild elephants than the elephants in zoos, the acquired information on how age and reproductive history affect the elephants' estrous cycle provides new insights that are relevant for the future research that investigates how evolution has shaped the species' physiology and reproductive lifespan. This thesis also gives new insights on the reproductive senescence patterns in long-lived animals in general, which is useful for the research of these species as individual-level physiological data is often very limited, particularly on animals living in their natural habitats.

Keywords: elephants, estrous cycle, allopregnanolone, age, reproductive senescence

CONTENTS

1. INTRODUCTION

Reproductive senescence is widely observed across species as a deterioration in reproductive physiology and decreasing likelihoods of reproduction with age (Nussey et al. 2008, Comizzoli et al. 2021). Classical evolutionary theory predicts that maintenance of an individual's vital functions is not selected for without maintenance of reproductive functions as it would serve no direct fitness benefit, which leads to most organisms retaining fertility close to death (Medawar et al. 1952, Hamilton 1966). Later research has shown that for various species ranging from mammals and birds to fish and insects, somatic and reproductive senescence follow separate trajectories suggesting independent selection on survival and fecundity. (Cohen et al. 2004, Holmes et al. 2003, Reznick et al. 2005, Kidd et al. 1985). However, for most of these species the post-reproductive lifespan remains short and a clear reproductive termination occurs only in some individuals.

Females of some long-lived species show significant post-reproductive lifespans and well-defined mechanisms of reproductive termination before the end of typical lifespan. Female humans and some toothed whale species live as much as 15-50 % of their lives post-reproductively (Chapman et al. 2019, Photopoulou et al. 2017, Levitis et al. 2013). For these species, ovarian activity decreases with age and eventually stops completely leading to females being unable to conceive, a point commonly described as menopause in humans (Laisk et al. 2018, Ellis et al. 2018). A recent study has also found evidence for menopause in chimpanzees as a wild population was found to live around 20% of their lives post-reproductively and experience similar hormonal transition as humans as they undergo menopause (Wood et al. 2023). It has been hypothesized that in species that require extended periods of infant care, such as humans and whales, the cessation of reproduction long before notable physiological senescence has evolved as an adaptation that allows females to assist the survival of existing offspring whilst avoiding further pregnancies that might pose a risk to their own survival (Williams 1957, Alexander 1974, Hawkes et al. 1998). Another evolutionary explanation states that a prolonged post-reproductive lifespan increases females' fitness by serving as an opportunity to assist their own offspring or other kin in reproduction (grandmother hypothesis) (Hawkes et al. 1998). Both of these hypotheses are supported by observed fitness benefits as in humans and whales females with a prolonged post-reproductive lifespan have more children or grandchildren. (Lahdenperä et al. 2004, Foster et al. 2012).

Evidence in support that menopause evolved as an adaptation in humans and whales has inspired research on other long-lived mammals, such as elephants. Similarly to humans and whales, elephants are long dependent on mother's care and live in socially complex groups where other female kin takes care of a calf in addition to biological mother (Fowler et al. 2006, Archie et al. 2011). As the presence of grandmothers has been shown to enhance their grandcalves' survival and decrease their daughters' inter-birth interval in Asian elephants (Lahdenperä et al. 2016), it is possible that some of them go through a menopause for similar adaptive reasons as humans and some whale species. Although the distribution of the age at last reproduction is wide in Asian elephants meaning that there is no clearly defined termination point for reproduction, they live on average 13-16 % of their lifespan post-reproductively (Chapman et al. 2019, Lahdenperä et al. 2014). Research on African elephants has indicated that aging females exhibit reduction in ovarian capacity, similarly to humans and whales, as the levels of progestagen metabolites decrease in aging African elephants (Freeman et al. 2013). The ovarian follicles, which secrete these hormones crucial to fertility, are reduced in number and become completely depleted through aging in some individuals of African elephants (Stansfield et al. 2012). As corresponding studies have not been conducted on Asian elephants, it has remained unclear whether they also experience age-related changes in reproductive physiology.

In general, female elephants show large variation in the timing of their first ovulation as it's known to occur between ages 3 and 23 years (Fowler et al. 2006, Hildebrandt, 2011). In free-ranging elephants it typically occurs around the early teenage years, but in captivity earliest pregnancies have been reported at ages 3,5 and 7 years for Asian and African elephants, respectively (Hildebrandt, 2011). Male elephants reach their physiological maturity at a similar age, but they are not likely to mate until their twenties as social maturity occurs later for them than for females (Fowler et al. 2006). Elephants breed around the year, although there is some evidence on estrous cycle seasonality (Thitaram et al. 2008, Yang et al. 2023).

A few distinctive features in elephants' estrous cycle differentiate them from other mammals. Elephants have the longest estrous cycle of any mammal studied to date, commonly 13-17 weeks in duration with observed extremes ranging from 11 to 22 weeks (Fowler et al. 2006, Hildebrandt 2011, Glaeser et al. 2012). While other mammals have only one surge of luteinizing hormone (LH), elephants have two of them, the first one being unovulatory and the second one being ovulatory (Brown et al. 2004). In addition, compared to most mammals, the circulating progesterone concentrations are relatively low in elephants. Instead, the major circulating progestins in elephants are progesterone metabolites 5-alpha-dihydroprogesterone (5α-DHP) and allopregnanolone (5α-pregnane-3α-ol-20-one) (Hodges et al. 1994, Heistermann 1997).

As in other mammals, elephants' estrous cycle consists of two phases, luteal and follicular phase (Figure 1). In the case of elephants, the luteal phase lasts commonly 6-10 weeks with observed extremes being 4 and 16 weeks while the follicular phase lasts commonly 4-6 weeks with extremes of 2 and 13 weeks (Fowler et al. 2006, Hildebrandt 2011, Glaeser et al. 2012). The luteal phase begins when a dominant follicle changes into corpus luteum (CL) after releasing an egg in ovulation. At the beginning of this phase the CL starts to excrete progesterone, which is metabolized into progestins 5-alpha-dihydroprogesterone and allopregnanolone. The elevated progestins inhibit follicular development and LH secretion. The degradation of the CL results in a fall in the levels of progesterone and its metabolites at the end of the luteal phase. At the beginning of the follicular phase the elevated follicle stimulating hormone (FSH) initiates two successive waves of follicular development, which both end with LH surges. The multiple follicles developed in the first follicular wave do not ovulate, but regress after the first LH surge. Occurring 3 weeks later, the second follicular wave results in one follicle that ovulates around 24 hours after the second LH surge. If fertilization happens the progesterone excretion that is started again by CL is soon replaced by forming placenta, which keeps the hormone's production up throughout the pregnancy (Fowler et al. 2006).

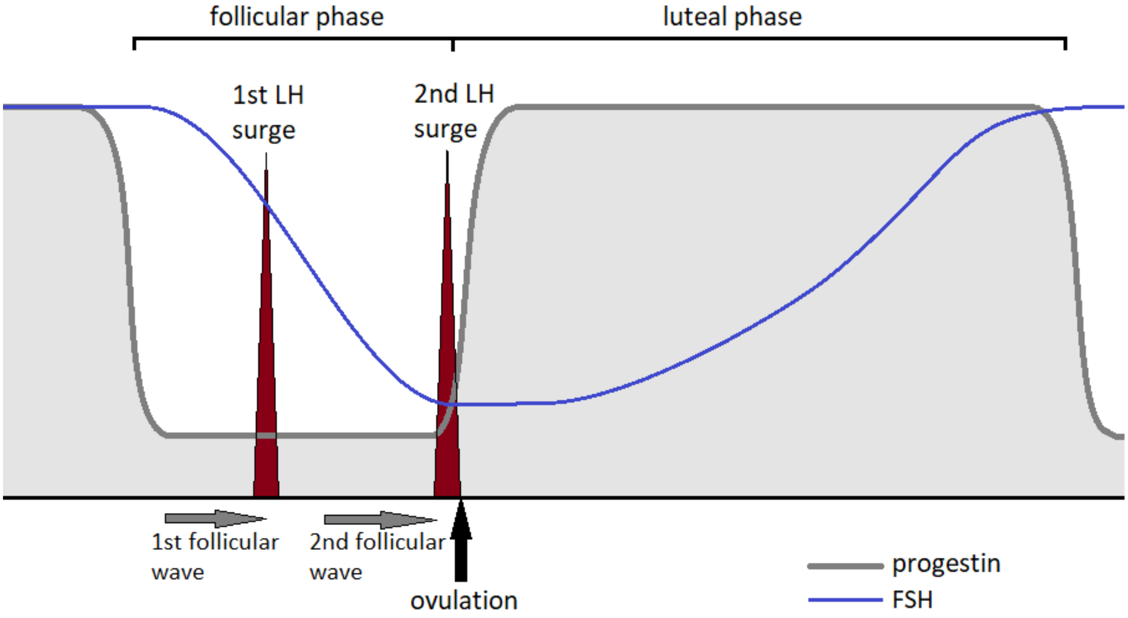

Figure 1. A model of elephant's estrous cycle. Proportional durations of phases are averages and can vary between individuals. Progestins include progesterone metabolites 5-alpha-dihydroprogesterone (5α-DHP) and allopregnanolone (5α-pregnane-3α-ol-20-one). LH = luteinizing hormone and FSH = follicle stimulating hormone.

As the secretion of progesterone forms a peak in mammals' estrous cycle, measurements of progesterone or its metabolites are often used as an indicator of their ovulatory status and cyclicity. According to previous studies, the level of serum progestins during the luteal phase of a reproductively normal elephant reaches a peak of above 0,5 ng/ml while during the follicular phase the level stays below 0,2 ng/ml (Brown 2004, Oliveira 2008). During pregnancy, the serum progestin levels have been measured to stay above 0,2 ng/ml through almost the whole pregnancy and to reach average and peak levels higher than those of a normal luteal phase (Oliveira 2008). The serum progestin tends to be high especially during the early stages of pregnancy and to stay at comparatively lower levels during the second half. Similar cyclic patterns and magnitudes have been observed in measurements of fecal allopregnanolone for both cycling and pregnant elephants (Hodges 1998, Ghosal 2012).

After giving birth elephants enter a non-cycling lactational anestrus state, lasting for around 46 weeks (Olsen et al. 1994, Brown 2000). Lactation inhibits reproduction and can be detected by low progestin concentrations (Olsen et al. 1994). As in other mammals, weaning reinitiates the estrous cyclicity, and a premature weaning or a death of a calf can reduce the length of the postpartum anestrus to only 8 weeks (Olsen et al. 1994, Brown and Lehnhardt 1995). Other causes for a shortened anestrus state can be a retained placenta (a condition where the placenta doesn't completely come out of the uterus during parturition) or conditions that reduce the milk production (Olsen et al. 1994, Brown and Lehnhardt 1995).

The main aim of this study is to investigate how age is associated with 1) mean allopregnanolone concentrations, 2) estrous cyclicity, 3) estrous cycle length in Asian elephants. To reach this aim, first, I determine estrous cycle profiles for 69 Asian elephant females by measuring and analysing their serum allopregnanolone concentrations by performing an enzyme linked immunosorbent assay (ELISA). Secondly, I categorize the elephants to cycling and non-cycling based on their cyclicity patterns. Thirdly, I investigate how age is associated with mean allopregnanolone, cyclicity and cycle length. In the statistical models, I also investigate how recent reproduction and elephant's origin (captive born vs wild born) are associated with the studied factors.

The studied elephants are part of a semi-captive population in Myanmar, which serves as a suitable study location for multiple reasons. Although the elephants are held in captivity for timber extraction during the day, they are free to roam in the forests in their social groups outside the working hours, which makes their living conditions and demographic characteristics resemble more closely those of wild elephants than the conditions and characteristics of the elephants in zoos. Both fertility and mortality rates in the studied population are comparable with free-ranging Asian and African elephants while in zoos the fertility rates are lower, and the infant mortality rate is double and lifespan only half of the ones in the studied population (Clubb et al. 2008, - 2009). As the purpose of the study is to gain knowledge on Asian elephant physiology and how evolution has shaped reproductive lifespan in the species' natural habitat, it is crucial to minimize human influence on the study outcomes. However, collecting blood samples regularly from such a large number of elephants would be much more challenging in a wild population than it is in the timber camps where the elephants are already given monthly health checks required by the local government and overseen daily by each elephant's personal caretaker. As Myanmar has a long history using elephants as a workforce in timber industry, each elephant's life events, including own and calf's birth dates have been recorded precisely for a century, which is crucial to this study as well.

Firstly, I hypothesize that average concentrations of allopregnanolone decline and the likelihood for a normal estrous cycle reduces after the age of 50 years, given the documented decline in the age-specific reproduction rate after 50 years of age in the semi-captive elephants of Myanmar (Lahdenperä et al. 2014). Secondly, I hypothesize that the captive-born elephants have higher allopregnanolone concentrations and higher likelihood for a normal estrous cycle than the wild born, as captive born elephants have previously been observed to have higher fertility than wild born (Lahdenperä et al. 2019). My third hypothesis is that the elephants with 3 years old or younger calves have lower levels of allopregnanolone and less cyclic change in the concentrations as some of them can be lactating, but also because of a relatively long inter-birth interval of around 6 years in the studied population (Lahdenperä et al. 2014) suggesting a possibly prolonged anestrus for the time when calves are still dependent on mother's care.

2. MATERIALS AND METHODS

2.1. Study population

The researched elephants belong to two semi-captive subpopulations owned by Myanmar Timber Enterprise, which manages the largest captive population of Asian elephants in the world (Sukumar 2006). The elephants are trained to work for timber industry for around 5-8 hours a day, but given a 4-month-long rest during the hot season (mid-February - mid-June). They are characterized as semicaptive as in the night time they are allowed to forage in the nearby forests unsupervised socializing and mating with both their tame and wild conspecifics. The elephants' breeding or calving is not controlled by humans and apart from occasional seasonal fruits and rice, they aren't supplied with food. The elephant's individual caretaker called mahout supervises his elephant's work and is responsible for its daily care including bathing, diet, defecation and health monitoring. Each elephant has their personal logbook that covers basic information such as birth date, sex, offspring and origin as well as detailed information on health checks carried out regularly by government veterinarians.

The elephants enter their training period around the age of 5 and are engaged with light work until being put in the mature workforce at the age of 18. All elephants are retired after reaching the age of 55, but their mahout keeps taking care of them until death. The work is done in groups of around six elephants managed by a head mahout and consists of dragging and pushing logs and extracting timber from felled trees. Pregnant females are restrained from work from halfway of the pregnancy and put back to light work one year after delivering. As the elephants aren't aided with their delivery, most of the calves are born outside human's watch, but as the mahouts are in frequent contact with their elephants, the birth dates are known precisely. The calves are measured and given a health check soon after birth, but they are cared for and nursed by their mother until around age 4 when lactation no longer supports their demands. In addition to a calf's biological mother, other related females within the social group take care of it as so-called allomothers (Fowler et al. 2006, Lahdenperä et al. 2016, Lynch et al. 2019). After spending their first 4 years with their mothers in relative freedom the elephants are tamed and paired with their mahout, whose relationship with the elephant can last their whole lifetime (Crawley et al. 2019).

As the net reproduction rate of the semicaptive elephants isn't high enough to maintain sufficient amount of workforce, more elephants need to be captured from the wild. Although all of the studied elephants have spent their recent years under similar conditions in semicaptivity, only the wild born are affected by the life-changing capture affecting both their age-specific reproduction rates and mortality. The wild-caught elephants have a reduced lifetime reproduction probability and increased mortality, but after the age of 45 they reproduce more likely than the captive borns making their age-specific reproduction rates resemble more closely those of wild populations (Lahdenperä et al. 2019, - 2018). While the ages of captive-born elephants are known from precise dates of birth, wild-caught elephants are aged by comparing their size and physical features at the time of capture to those of captive elephants of known age.

In the semi-captive Asian elephants the age at first reproduction is on average 20 and the last around 40 years with many continuing to bear calves beyond the age of 60 (Robinson et al. 2012, Lahdenperä et al. 2014, Sukumar 2003). The reproduction rate peaks at around 19-20 years of age and stays generally high at years 20-44, after which it shows a rapid decline (Lahdenperä et al. 2014). While the wild Asian elephants have an inter-birth interval of 2,5-4 years, the studied elephants in Myanmar give birth with an average interval of 6 years (Shoshani and Eisenberg 1982, Lahdenperä et al. 2014).

Myanmar has a tropical to sub-tropical climate with three seasons: 1) hot and dry inter-monsoonal season from mid-February to mid-May, 2) rainy southwest monsoon season from mid-May to October and 3) cool and dry northeast monsoon from October to mid-February. One of the camp sites of the studied elephants, Bago is closer to the coastline and has more annual rainfall and slightly warmer temperatures than the other site, Taungoo, which is located higher and more north. In the studied population, monthly birth rates are highest during the cool and dry season and in the beginning of the hot season as 41% of births occur between December and March. As this corresponds to conceptions occurring during the elephants' resting period between February and June, the maternal workload likely affects the probability of conception through effects on stress, estrous cycle or access to mates (Mumby et al. 2013).

The studied population consists of 69 female Asian elephants. Most of the individuals were either between 9 and 20 (n=31) or between 50 and 71 years old (n=22). The elephants were grouped by their camp site, origin (captive/ wild born) and recent reproduction, which was defined by calving in past 3 years. There were 14 elephants that turned out to be pregnant during the study period based on parturitions reported after sample collection. The age distribution of the studied elephants is shown in Figure 2 by separating pregnant and non-pregnant individuals as most of the statistical analyses were done for non-pregnant individuals. The sample sizes of different groups are shown in Figure 3.

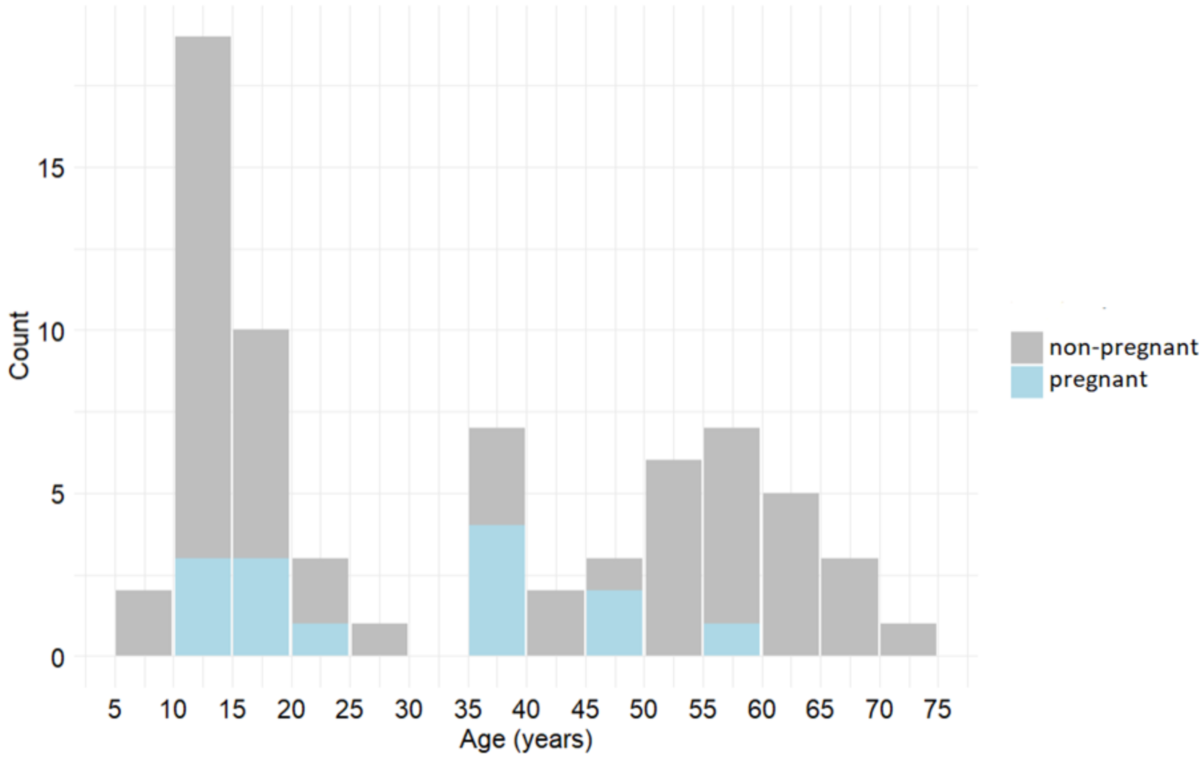

Figure 2. Age distribution of the study population

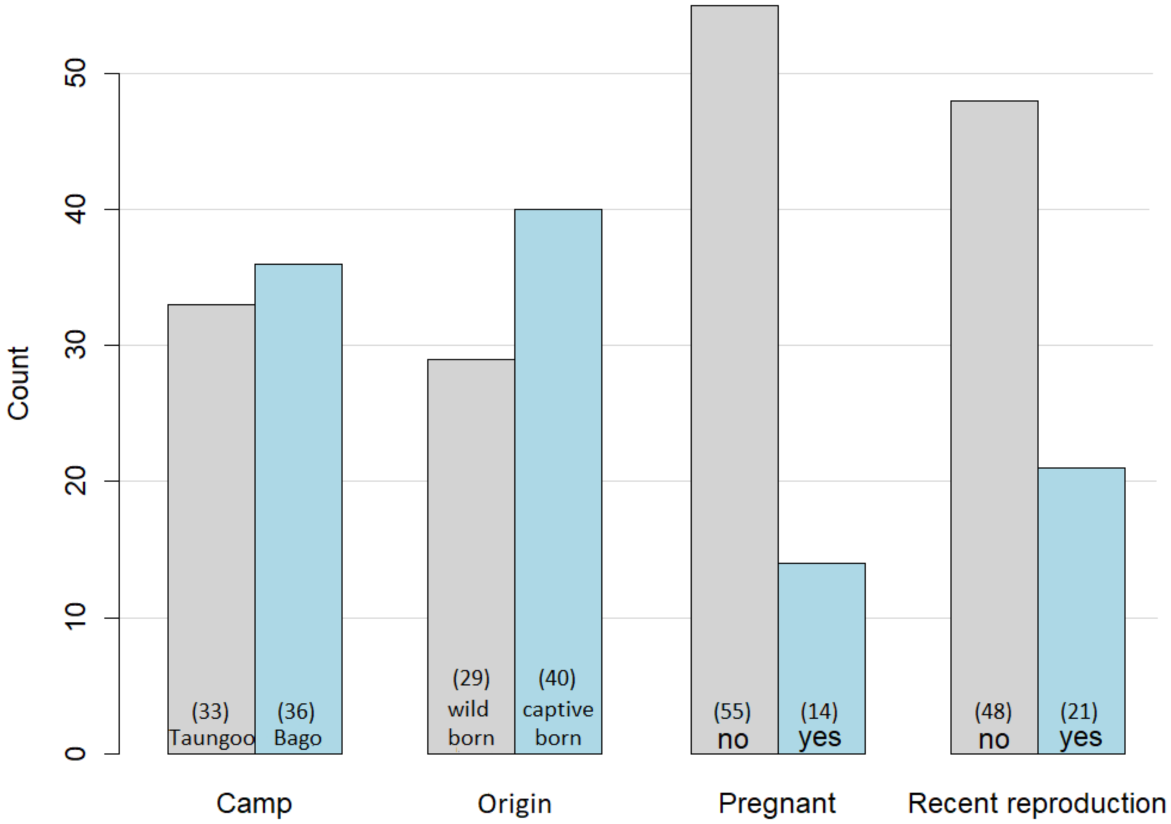

Figure 3. Sample size for different groups

The elephants' blood samples were collected twice a month between 14th of February and 28th of September 2021 by local veterinarians in Myanmar. The blood drawing was done from the elephants' ear vein and stored as serum samples in -20°C until transportation to University of Turku. In Turku, the serum samples were stored in -80°C.

2.2. Allopregnanolone ELISA

Serum 3β-allopregnanolone (5α-pregnan-3β-ol-20-one) levels were measured using an enzyme linked immunosorbent assay (ELISA) method validated for Asian elephant estrous cycle and pregnancy determination. ELISA kit was ordered from Leibniz Institute for Zoo and Wildlife Research in Berlin and tested for the first time in University of Turku (Tuomikoski 2022).

Prior to ELISA serum extractions were done as follows: 1,8ml of petroleum ether was added to 0,1ml of serum. Samples were shaken for 30 minutes in room temperature using an Eppendorf-tube shaker and then displaced in -80°C for 30 minutes. After this, for each sample the supernatant was transferred to a new Eppendorf tube. The samples containing supernatant were then evaporated by putting them on a heat block, which was warmed up to 55°C and placed inside a ventilated fume hood. After 1,5-2 hours when the samples were dry, 0,4ml of 100% methanol was added, followed by 0,6ml of mqH_2O, and vortexed.

ELISA was done by using a polyclonal antibody against 5α-pregnan-3β-ol-20-one-3-HS-BSA. The cross-reactivities for the antibody were: 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; < 0,1% 5β-pregnan-3α-20α-diol, 4-pregnen-20α-ol-3-one, 5β-pregnan-3α-ol-20-one, 5α-pregnan-20α-ol-3-one, 5α-pregnan-3β,20α-diol, 5α-pregnan-3α,20α-diol, testosterone, estradiol, cortisol, corticosterone. For each 96-well-plate 10 diluted standard samples of known hormone concentrations were analysed together with samples to determine a calibration curve. The first standard sample was prepared by diluting 20µl of standard stock (100ng/ml) into 180µl of 40% methanol to reach a proportion of 1:10 after which the sample was vortexed. The nine other standard samples were prepared by a serial dilution in 1:2 steps by diluting 100µl of a previous standard sample into 100µl of 40% methanol. As a result, the concentrations of the 10 standards were (pg/ml): 10 000; 5000; 2500; 1250; 625; 312,5; 156,25; 78,125; 39 and 19,5. A conjugate consisting of both the analyte of interest and peroxidase was used to perform a competitive enzyme immuno assay and to catalyze a color reaction. In the substrate solution chromogenic EIA 3,3',5,5'-

tetramethylbenzidine (TMB) was used as a substrate for peroxidase. The peroxidase of the conjugate converts the substrate into a blue product, which shifts to yellow by acidification with sulfuric acid in the termination of the enzyme reaction. Two control samples of a higher and a lower concentration were prepared beforehand from a pool of Asian elephant serum samples. For reagent quality control, non-specific binding (NSB) control and binding for the zero standard (B0) control were used.

The assay was performed in following steps: First, IZW assay buffer 5x concentrate was diluted 1:5 in mqH_2O for the preparation of conjugate- and antibody solutions. Conjugate solution was prepared by diluting a stock of 1:10 pre-diluted 5α-conjugate 1:400 in assay buffer and antibody solution was done by diluting 5α-antibody stock 1:1200 in assay buffer. Next, the anti-rabbit-Ig-G-coated plate was washed once with wash solution containing Tween 80 pure concentrate 1:2000 in mqH_2O (0,5ml/L) by using 300µl of wash solution per well. 20µl of extracted serum samples, control samples and standard samples were then pipetted on the plate. Each sample type was pipetted in duplicate and the serum samples' order on the plate was randomized beforehand. 100µl of conjugate solution was added to all wells after which 100µl of antibody solution was added to all wells except for the NSB-wells where 100µl of assay buffer was added instead. The plate was then covered with a plate seal and protected from light and let incubate overnight at 4°C with light shaking. On the second day, the plate was first washed 4 times by using 300µl of cold wash solution (4°C) per well. Cold substrate solution mix consisting of substrate solution A and substrate solution B in a ratio of 1:1 was then added 150µl/well. The plate was covered and protected from light again and put to 4°C with slight shaking for 40 minutes. After this, 50µl of stop solution 2M H_2SO_4 was added to all wells. After letting the plate reach room temperature in around 10 minutes optical density (OD) was measured at 450nm. Pipetting of reagents was done using a repeater pipet.

A CV <5% was assumed between ODs of duplicates on a plate and <15% between all control samples of one type across all plates. A standard curve was then plotted by first substracting the average OD of the NSB duplicates from the average OD of samples' and standards' duplicates and dividing these values (OD-NSB) by the average OD of B0 duplicates. These values were then used to plot a standard curve by using four parameter logistic curve (4PLC) regression model from which the concentrations of serum samples were calculated.

The calculated concentrations of allopregnanolone were then plotted as cyclicity profiles for each elephant. A baseline allopregnanolone value was calculated individually for each profile using R’s package “hormLong”, an iterative process developed for Asian elephants (Fanson et al. 2015). In this process for each elephant, data points with values above the mean plus 1.25 times the SD were removed and the process repeated until no values exceeding the mean plus 1.25 times the SD remained. The remaining data points defined the baseline for that individual. Luteal phases were defined as concentrations greater than baseline for at least two consecutive weeks, with a total duration of at least 4 weeks. Follicular phases were defined as concentrations below the baseline for at least two consecutive weeks. Single point fluctuations above or below baseline were assigned to the same phase as the surrounding points. Elephants were categorized as cycling if they had at least one complete estrous cycle that consisted of a lutheal and follicular phase that met the criteria. The described criteria for a normal estrous cycle was adapted from previously used criteria for an estrous cycle in Asian elephants (Glaeser et al. 2012).

For each individual, a mean value was calculated for the length of both lutheal and follicular phase and the cycle length was calculated as the sum of these mean values. The mean and peak concentrations of an individual were taken from the whole study period regardless of cyclicity.

2.3. Statistics

Statistical analyses were performed using RStudio version 4.3.0 (RStudio Team 2023). The effect of pregnancy on mean allopregnanolone concentration was investigated using a t-test (n=69). After excluding the pregnant individuals (n=14), three separate models were conducted for non-pregnant individuals to investigate how elephant’s age is associated with 1) mean allopregnanolone, 2) cyclicity, 3) cycle length. First, linear regression model was applied to analyse differences in mean allopregnanolone using age (linear association with age), age^2 (quadratic association with age), recent reproduction status (reproduced in the past 3 years vs not), camp (Taungoo vs Bago) and origin (wild-captured vs captive-born) as explanatory terms predicting the mean allopregnanolone. Secondly, logistic regression was used to model the probability of cyclicity (an individual cycled vs not) with binomial distribution and logit link function. The same explanatory variables were included in this model. These two models had a sample size of 55. Thirdly, another linear model was applied only for the cycling elephants (n=30) to analyse differences in mean cycle length using the same explanatory terms. The models’ predicted values were generated and plotted using R’s commands “lm” and “ggplot”.

All terms that had a P-value $\lesssim 0.1$ as well as age, as the term of main interest, were kept in the final model as the dataset was relatively small. The remaining non-significant terms were added to this final model one by one separately from the other nonsignificant terms and their statistics were reported from these models. Shapiro-Wilk test was performed to the linear models to confirm a normal distribution of residuals.

3. RESULTS

3.1. Mean allopregnanolone

The mean and peak allopregnanolone of an individual were on average 0.58 ng/ml and 1.58 ng/ml, respectively. The highest measured concentration was 5.06 ng/ml and the lowest 0.00 ng/ml. Mean values for mean and peak allopregnanolone in different groups, attained from the raw data, are shown in Table 1.

Table 1. Mean and peak allopregnanolone for elephants in different groups, attained from the raw data (n=69). Pregnant individuals are shown as their own group and excluded from all the other groups.

Variable	**Group**	**Mean allopregnanolone (ng/ml)**	**Peak allopregnanolone (ng/ml)**
Reproductive status	Pregnant (n=14)	0.7535	2.222
	Non-pregnant (n=55)	0.5326	1.4164
Recent reproduction	Has 1-3-years old calves (n=11)	0.5113	1.4739
	No 1-3-years old calves (n=44)	0.5379	1.4020
Camp	Bago (n=29)	0.5587	1.4617
	Taungoo (n=26)	0.5034	1.3659
Origin	Captive born (n=33)	0.5510	1.4289
	Wild born (n=22)	0.5049	1.3977

The pregnant elephants had a mean level of 0.75 ng/ml, which was significantly higher than the mean of the non-pregnant elephants, 0.53 ng/ml ($t = -4.19$; $P = 0.00037$). The individual peak values were also higher in the pregnant elephants as the peak values were 2.22 ng/ml and 1.42 ng/ml for the pregnant and non-pregnant elephants, respectively ($t = -2.93$; $P = 0.0087$). Because of the elevated allopregnanolone levels and absence of cycles (section 3.2.), the pregnant elephants were excluded from all further analyses.

The results of the analysis show that age did not have a significant effect on the mean allopregnanolone among non-pregnant females (F1,53 = 1.92, $P = 0.17$, Table 2). A mild tendency for decreasing levels with increasing age was observed and the linear model estimated that each added year decreased the mean allopregnanolone of an individual by 0.29% (0.0017 ng/ml) (Figure 4, Table 2). The squared age term was not associated with mean allopregnanolone levels either (F1,52 = 0.39, $P = 0.54$, Table 2), and neither were the other tested terms camp, origin or recent reproduction. The elephants from camp Bago were estimated to have 9.7% (0.049 ng/ml) higher mean level than those from Taungoo (F1,52 = 0.87, P = 0.36, Table 2). The predicted mean level for the elephants that have 1-3 years old calves was 4.9% (0.025 ng/ml) lower than for those that have not given birth in the past 3 years (F1,52 = 0.14, P = 0.71, Table 2). The wild born individuals were estimated to have 2.1% (0.011 ng/ml) higher mean allopregnanolone than those that are born in captivity (F1,52 = 0.020, P = 0.89, Table 2). However, all these estimated differences between different groups were statistically non-significant (Table 2).

Table 2. Effects of recent reproduction, age, age^2, origin and camp on mean allopregnanolone by a linear regression model (n=55). Reference levels for Camp, Recent reproduction and Origin are "Bago", "no" and "wild born", respectively. Recent reproduction was defined by calving in past 3 years. Terms retained in the final model are shown above the solid line.

Effect	**Estimate**	**Standard error**	***F* value**	***P***
Intercept	0.5906	0.04933		
Age	-0.001698	0.001224	1.9229	0.1713
Camp (Taungoo)	-0.04891	0.05256	0.8657	0.3565
Age^2	-6.779×10^{-5}	1.090×10^{-4}	0.3866	0.5368
Recent reproduction (yes)	-0.02486	0.06577	0.1428	0.7070
Origin (captive born)	-0.01066	0.07506	0.0202	0.8876

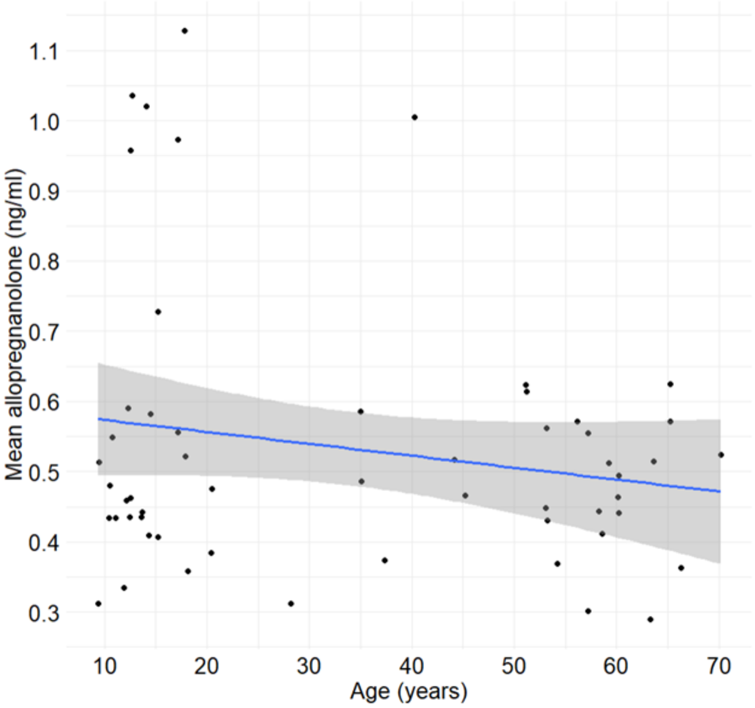

Figure 4. The association between age and mean allopregnanolone (n=55). Raw data is shown as dots and the model's predicted values as a blue line with 95% confidence intervals in grey.

3.2. Cyclicity

Based on estrous cycle characterization that was done for each elephant with the hormLong-package, 30 individuals had a normal estrous cycle and 39 were either pregnant (n=14) or on an anestrus state (n=25). Examples of each of these hormone profile types are shown in Figures 5, 6 and 7. Pregnant individuals were excluded from the cyclicity analysis as they are proving a recent capability for having a normal estrous cycle for being pregnant yet don't show it in their hormone profile, where allopregnanolone levels stay higher and a normal cyclicity pattern is absent. The age distribution of the elephants included in the cyclicity analysis is shown separately for cycling and non-cycling individuals in Figure 8. The proportions of cycling individuals in each group, attained from the raw data, are shown in Table 3.

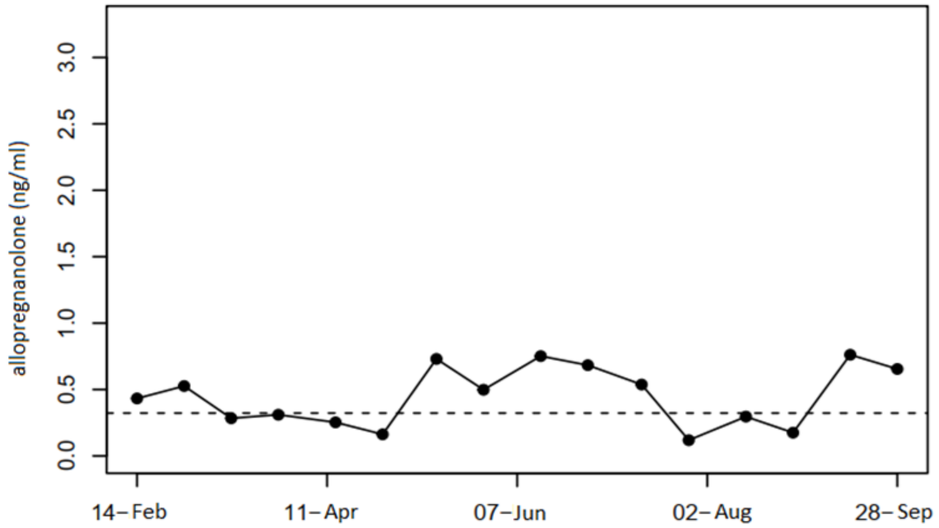

Figure 5. Allopregnanolone profile of a cycling individual. The dashed line shows the baseline for the individual calculated by hormLong.

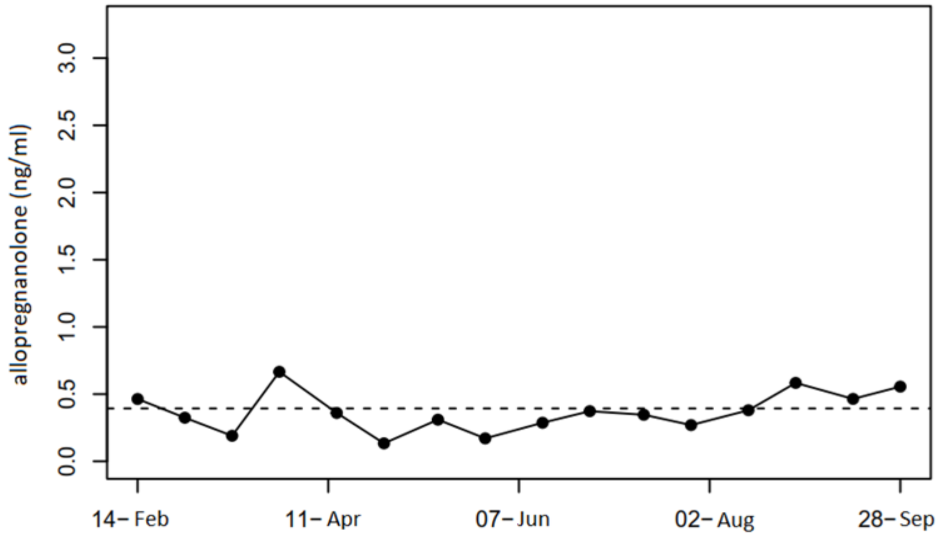

Figure 6. Allopregnanolone profile of a non-cycling individual. The dashed line shows the baseline for the individual calculated by hormLong.

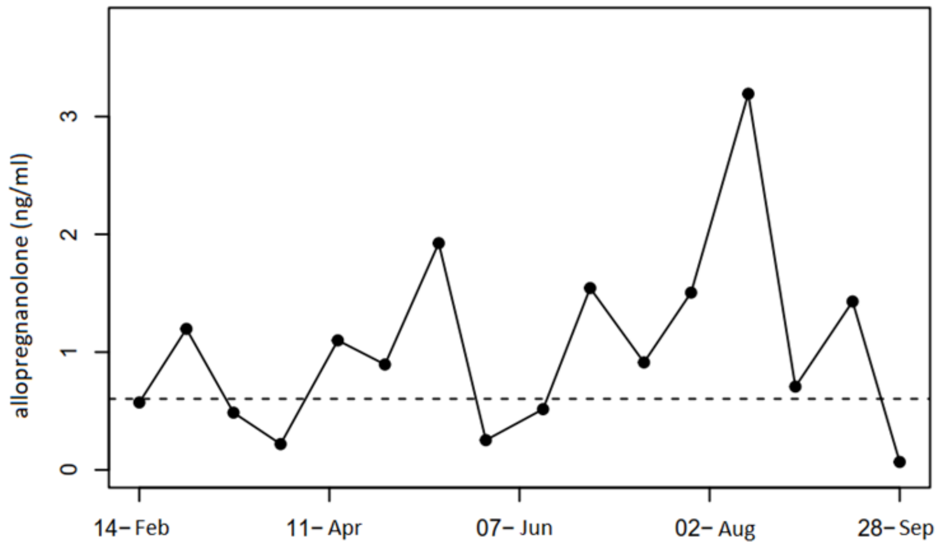

Figure 7. Allopregnanolone profile of a pregnant individual. The dashed line shows the baseline for the individual calculated by hormLong.

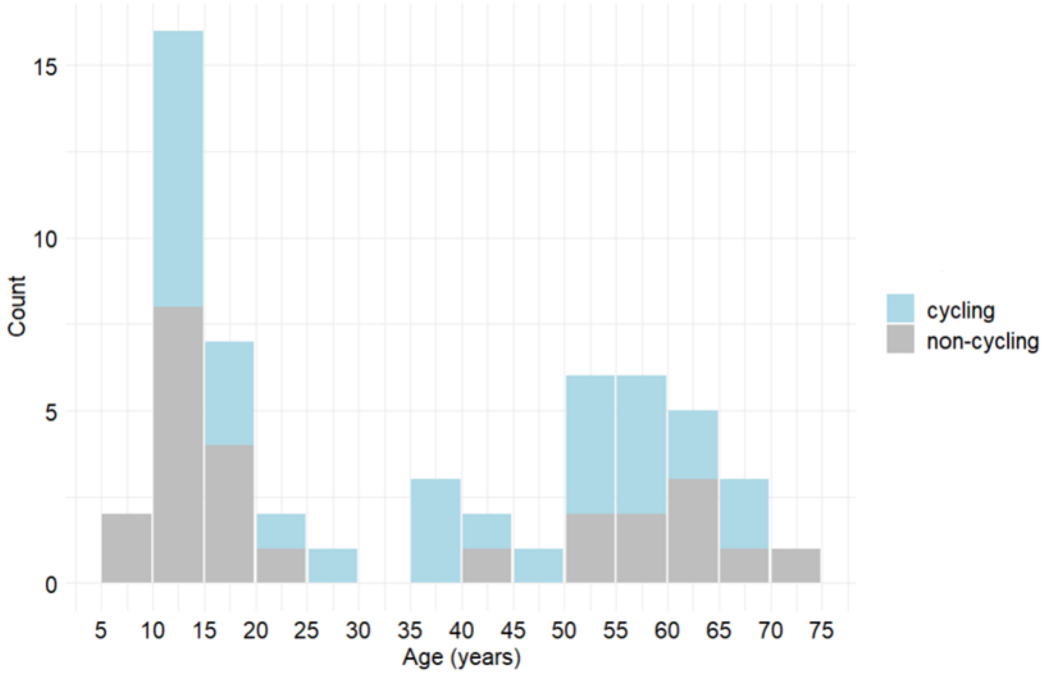

Figure 8. Age distribution of the cycling and non-cycling elephants (n=55). Pregnant individuals are excluded from the figure.

Table3. The proportion of cycling individuals out of all non-pregnant individuals in different groups, attained from the raw data (n=55).

Variable	**Group**	**Proportion of cycling individuals**
Recent reproduction	Has 1-3-years old calves (n=11)	0.7273
	No 1-3-years old calves (n=44)	0.5000
Camp	Bago (n=29)	0.4138
	Taungoo (n=26)	0.6923
Origin	Captive born (n=33)	0.4848
	Wild born (n=22)	0.6364

The analysis showed that the probability of cyclicity correlated negatively with squared age meaning that the young and old elephants were less likely to have a normal estrous cycle than the middle-aged elephants ($\chi^2 1 = 4.95$, P = 0.026, Table 4, Figure 9). There was no significant association with the probability of cyclicity and linear age ($\chi^2 1 = 0.73$, P = 0.39, Table 4). According to the model, the probability of having a cycle was 0.49 at the age of 15, 0.82 at the age of 40 and 0.57 at the age of 60. These ages were suitable reference points for this data because of sufficient age specific sample sizes. The elephants from Taungoo were estimated to have 170.16% higher odds of having a cycle than those from Bago (Odds ratio (OR) (95% CLs): 2.70), and this effect showed weak significance ($\chi^2 1 = 2.88$, P = 0.089, Table 4). The model predicted that wild born elephants would have had 71.10% higher odds of having a cycle than those that are born in captivity (OR (95% CLs): 1.71) and that recent reproduction would have increased the odds by 29.43% (OR (95% CLs): 1.29), yet these effects were not statistically significant (origin: $\chi^2 1 = 0.38$, P = 0.54; recent reproduction: $\chi^2 1 = 0.095$, P = 0.76, Table 4).

Table 4. Effects of recent reproduction, age, origin and camp on cyclicity by a logistic regression (n=55). Reference levels for Camp, Origin and Recent reproduction are "Bago", "wild born" and "no", respectively. Recent reproduction was defined by calving in past 3 years. Terms retained in the final model are shown above the solid line.

Effect	**Estimate**	**Standard error**	χ^2	***P* (>Chi)**
Intercept	-2.7464	1.2969		
Age^2	-0.002459	0.001342	4.9507	0.02608
Age	0.1900	0.09960	0.7270	0.3939
Camp (Taungoo)	0.9938	0.5922	2.8842	0.08945
Origin (captive born)	-0.5371	0.8772	0.3832	0.5359
Recent reproduction (yes)	0.2580	0.8396	0.0953	0.7576

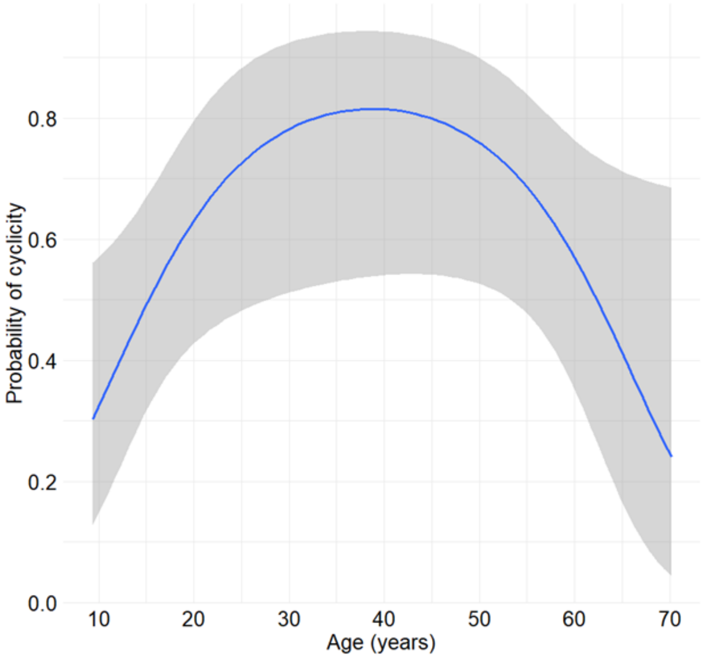

Figure 9. The association between age and predicted probability of cyclicity (n=55). The model's predicted values are shown as a blue line with 95% confidence intervals in grey.

3.3. Cycle length

The elephants that were categorized to have a normal estrous cycle had a mean cycle length of 99.19 days, the longest being 156 and the shortest 62 days. Mean cycle lengths in different groups, attained from the raw data, are shown in Table 5.

Table 5. Mean cycle length for elephants with a normal estrous cycle in different groups, attained from the raw data (n=30).

Variable	Group	Mean cycle length (days)
Recent reproduction	Has 1-3-years old calves (n=8)	112.25
	No 1-3-years old calves (n=22)	94.44
Camp	Bago (n=12)	104.83
	Taungoo (n=18)	95.43
Origin	Captive born (n=16)	100.25
	Wild born (n=14)	97.98

There was a mild tendency for decreasing cycle length with increasing age (Figure 10). The model predicted that for each added year in age the cycle length would have shortened by 0.28% (0.29 days), yet the effect of age showed no statistical significance (F1,27 = 1.56, P = 0.22, Table 6). The results of the analysis showed that recent reproduction increased the cycle length by 20% (19.06 days), but this trend was not significant (F1,27 = 2.88, P = 0.10, Table 6). The cycle length was estimated to be 14% (13.38 days) longer in the elephants from Bago than in those from Taungoo (F1,26 = 2.00, P = 0.17, Table 6) and 13% (12.54 days) longer in the wild born elephants than in those that are born in captivity (F1,26 = 0.82, P = 0.37, Table 6), yet these differences were not significant.

Table 6. Effects of recent reproduction, age, origin and camp on cycle length by a generalized linear model (n=30). Reference levels for Recent reproduction, Camp and Origin are "no", "Bago" and "wild born", respectively. Recent reproduction was defined by calving in past 3 years. Terms retained in the final model are shown above the solid line.

Effect	**Estimate**	**Standard error**	***F* value**	***P***
Intercept	104.7622	9.8793		
Recent reproduction (yes)	19.0635	10.5360	2.8837	0.1010
Age	-0.2926	0.2342	1.5609	0.2223
Camp (Taungoo)	-13.3782	9.4696	1.9959	0.1696
Origin (captive born)	-12.5381	13.8688	0.8173	0.3743
Age^2	-0.002033	0.02329	0.0076	0.9311

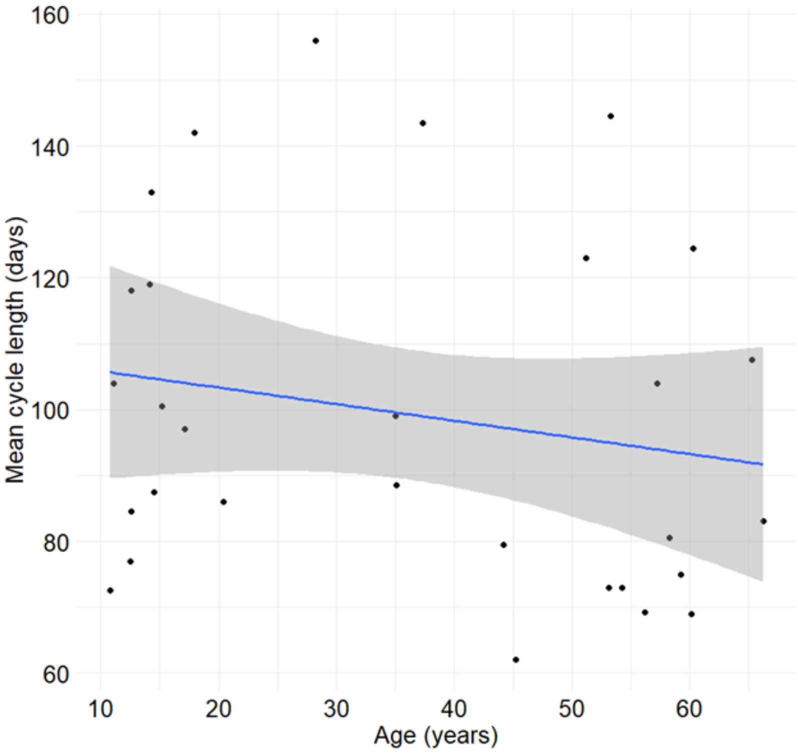

Figure 10. The association between age and mean cycle length (n=30). Raw data is shown as dots and the model's predicted values as a blue line with 95% confidence intervals in grey.

4. DISCUSSION

This study aimed to provide insight into the reproductive senescence of female Asian elephants by investigating how aging along with some other key factors affect their reproductive physiology in a population of 69 semi-captive Asian elephants. After determining estrous cycle profiles for the elephants and categorizing them to cycling and non-cycling individuals, hypotheses concerning mean allopregnanolone, cyclicity and cycle length were tested with the statistical analyses of the data. This is the first time Asian elephant’s estrous cycle has been analysed in natural living environment and with a larger sample size, as previous studies have been conducted on captive elephants that regularly suffer from reproductive abnormalities and/or there has been a smaller sample size (Glaeser et al. 2012, Thitaram et al. 2008, Olsen et al. 1994).

4.1. Effects of age on mean allopregnanolone

The first hypothesis expected allopregnanolone mean levels to decline after 50 years of age as the age-specific reproduction rate has been observed to decline steadily after 50 years of age in a previous study on semi-captive Asian elephants in Myanmar (Lahdenperä et al. 2014). There was no clear support for this by the results as the observed negative correlation between mean allopregnanolone and age remained non-significant. One explanation for why the decline after age 50 was not as noticeable as expected can be the unexpected discovery of pregnancies in the studied elephants making more middle-aged than old elephants being excluded from most of the analyses. As the allopregnanolone concentrations are considerably higher during pregnancy, demonstrated by the results of this study and previous ones, it made no sense comparing these individuals with non-pregnant ones and hence the pregnant elephants needed to be excluded from the statistical models (Oliveira et al. 2008, Hodges 1998). These excluded elephants, for being proven to be fertile and therefore having a recent capacity for normal cycling, are more likely to have higher allopregnanolone levels when non-pregnant than the included elephants, which weren’t proven to be fertile (as they were non-pregnant). This can be assumed as previous studies have reported non-cycling elephants to have lower levels of mean progestins than cycling elephants (Brown et al. 2004, Dissanayake et al. 2018). As these excluded elephants made a larger share of 30-50 years old than over 50 years old elephants, it should be noted that in reality, there could be a more considerable decline in allopregnanolone levels after the age of 50 than what was observed in this study. The observed non-significant trend for decreasing concentrations with increasing age, being relatively close to significance, indicates that there is a possibility for a real negative association that could be proven by future studies with larger sample sizes.

In comparison to menopausal species, such as humans and chimpanzees, the observed reduction of 0.29% in mean allopregnanolone per year in Asian elephants is marginal. In humans the level of urinary progestin has been observed to drop into half between ages 35 and 50 years while in chimpanzees the level of progestins decreases by one third in the same time period as both of these species have been observed to experience menopause around the age of 50 years (Ferrell et al. 2005, Wood et al. 2023). In many non-menopausal species, such as horses and rats, the mean levels of progesterone or its metabolites have been observed to be unaffected by age (Ginther et al. 2008, Steger et al. 1982). However, in a study on cattle, plasma progesterone was observed to be lower in old cattle than in young ones, suggesting that some level of decrease in the level of progesterone and its metabolites can be a quality of reproductive senescence also in species that don't experience a menopause (Hori et al. 2018).

4.2. Effects of age on cyclicity

The second part of the first hypothesis expected the likelihood for a normal estrous cycle to decline after the age of 50 years consistent with previous observations of declining age-specific reproduction rates in the semi-captive Asian elephants (Lahdenperä et al. 2014). This was supported by the analysis of cyclicity as both young and old elephants were shown to have lower probability of cyclicity than the middle-aged ones. These results are in line with previous observations of a rapid decline in reproduction rates after the age of 50 years in semi-captive Asian elephants and could be suggestive of menopausal deterioration in reproductive physiology (Lahdenperä et al. 2014). One explanation for why the likelihood of a normal cycle was also lower in young elephants in comparison to the middle-aged ones is that some of the youngest elephants might not have reached their physiological maturity fully, 21 of them being only 9-15 years old. This can be assumed as the timing of the first ovulation in elephants varies between 3 and 23 years of age, although in the vast majority it occurs around the early teenage years according to previous research (Fowler et al. 2006, Hildebrandt 2011).

The observed probability of cyclicity being as high as 0.57 at the age of 60 years in the studied Asian elephants suggests that the physiological cessation in reproduction long before death is not as widespread as in species where all individuals undergo menopause. As a comparison, in humans, 95% of women go through menopause before the age of 55 (Cramer et al. 1994). As most comprehensive studies on the long-lived menopausal species aside from humans define the age at menopause based on last reproduction, it remains unclear, whether cessation of reproduction always comes together with total termination of cyclicity in the individuals defined as menopausal. In non-menopausal species the age at onset of acyclicity is known to vary greatly between individuals of a species as for example in mice, the average age at cessation of estrous cyclicity has been observed to range from 11 to 16 months between different groups of mice (Felicio et al. 1984).

4.3. Effects of age on cycle length

The analysis of cycle length provided more information on possible age-related changes in the characteristics of estrous cycle. As previous studies have observed the length of an estrous cycle to be most commonly 13-17 weeks in reproductively normal Asian elephants, it can be assumed that progressive changes in the cycle length could affect the elephant's fertility (Fowler et al. 2006, Hildebrandt 2011). According to the analysis, age did not have a significant effect on cycle length, although a mild tendency for a decreasing cycle length with increasing age was observed. As the frequency of the sample takes, two samples per month, was not regarded to be suitable for accurate measurements of cycle phase lengths, especially follicular phase length, it remains unclear, whether lengths of specific phases are affected by age. A progressive shortening of the luteal phase could be indicative of decreasing fertility, if this happens as a consequence of corpus luteum's decreasing progesterone production ability, because a sufficient progesterone production by CL is crucial for the early part of pregnancy (Fowler et al. 2006). On the other hand, changes in the properties of a follicular phase could have effects on follicular development (Fowler et al. 2006). For these reasons, it remains an interesting topic for future studies to investigate, whether the length of the whole estrous cycle or the luteal or follicular phase are affected by aging in Asian elephants.

In humans the length of menstrual cycle has been observed to be around 2 days shorter in 45-49 years old women than in women below 20 years of age, although after 50 years of age the length of the cycle increases, possibly due to menopause occurring around this age and affecting the sample observed (Li et al. 2023). As the observed non-significant trend for decreasing cycle length with increasing age was relatively close to significance in the studied Asian elephants and proportionally similar to the observed decrease in human's cycle length, it remains interesting whether future studies with larger sample sizes could find a real association between cycle length and age. However, in chimpanzees the cycle length has been observed to increase with age unlike in humans, although there has been evidence of chimpanzees exhibiting menopause, which indicates that the connection between cycle length and fertility is complex (Videan et al. 2006). The cycle length has been shown to increase with age also in non-menopausal species, such as horses (Ginther et al. 2008).

4.4. Effects of origin

Secondly, I hypothesized the captive-born elephants to have higher allopregnanolone concentrations and higher likelihood for a normal estrous cycle than the wild born as captive born elephants have been observed to have higher fertility than wild born in previous studies (Lahdenperä et al. 2019). This was not supported by the results as the two groups showed no significant difference in mean concentrations or probabilities of cyclicity. Although these results can't suggest a physiological mechanism behind previous observations of reduced lifetime reproduction probability in wild caught elephants, it should be noted that the studied elephants' age at capture might have affected the results. The effect of capture has previously been observed to be stronger the older the elephant is at the moment of capture, meaning that the earlier the elephant has been caught the more closely its age-specific reproduction rate and lifetime reproduction probability resembles those of the captive born elephants (Lahdenperä et al. 2019). As in this study, a large majority of the wild-caught elephants were captured before the age of 12, it is hence possible that significant reductions in allopregnanolone concentrations and probability of cyclicity can only be noticed in elephants with a higher age at capture in comparison to captive born elephants. Furthermore, the studied elephants had been in captivity for long periods of time at the time of the study, and the negative impact of wild-capture on fertility rates has been shown to wane off with time over the years (Lahdenperä et al. 2019).

4.5. Effects of recent reproduction

The third hypothesis was that elephants with 3 years old or younger calves have lower levels of allopregnanolone and less cyclicity pattern in their hormone profiles because the studied population has previously been observed to have a relatively long interbirth interval of around 6 years (Lahdenperä et al. 2014), which suggests a possibly prolonged anestrus for the time when calves are still dependent on mother's care. This was not supported by the results concerning mean allopregnanolone and probability of cyclicity as recent reproduction did not have a significant effect on these variables. However, there was weak yet non-significant evidence that recent reproduction would increase the cycle length, which can be seen as a change in cyclicity pattern. Although the hypothesis was motivated by an aim to explain why Asian elephants have a relatively high inter-birth interval in comparison to other animals, the observed increase of 19.06 days in cycle length would not be sufficient to explain the multiple year difference to other animals, such as humans, which have an inter-birth interval of around 2.7 years (Rutstein 2011). As the probability of cyclicity was not found to change as a result of recent reproduction, the high inter-birth interval in Asian elephants is likely not explained by a prolonged anestrus either. As the elephants with 1-3 years old calves did not differ in concentrations or probability of cyclicity in comparison to other elephants, this study does not suggest lactational anestrus to be prolonged beyond 46 weeks, which is the duration that has been reported by previous studies (Olsen et al. 1994, Brown 2000).

In humans, it has been found that the timing when menstrual cycles return after parturition can vary greatly from 6 weeks to several months and depends largely on the duration of breast-feeding (Chauhan et al. 2022, Aryal 2007). Research on cattle has found that longer estrous cycle before breeding might be associated with lower conception rates in the species, which could be explained by longer estrous cycle resulting in a persistent follicle with higher potential for lower quality oocyte (Cushman et al. 2007). However, recent reproduction was not associated with longer cycles in this study on cattle. As the observed non-significant evidence on recent reproduction increasing cycle length in the studied Asian elephants was close to significance, it remains an interesting question, whether recent reproduction increases cycle length and whether this affects the fertility rates and the length of inter-birth interval in Asian elephants.

4.6. Effects of camp

The elephants from Taungoo were estimated to have a higher chance for cyclicity than the ones from Bago, yet this difference was not statistically significant with a $P < 0.1$. The observed trend is nevertheless interesting and can be explained by differences in environmental conditions between these two camp sites. Taungoo experiences less annual rainfall and slightly colder temperatures for being inland, higher and more north than Bago, which is located closer to the coastline. As the length of the estrous cycle as well as the length of the luteal phase have been observed to be longer in the rainy season in semi-captive Asian elephants, it can be assumed that the amount of rainfall can affect the properties of the cycle (Yang et al. 2023). However, as the effects of environmental conditions, such as rainfall and temperature, on probability of estrous cyclicity has not been studied before, it remains unclear whether the obtained weak evidence of camp site's effects on cyclicity is due to differences in environmental factors or other causes or whether the observed weak difference was due to chance.

4.7. Strengths and limitations

The natural living conditions of the studied semi-captive Asian elephants makes their demographic characteristics, such as fertility rate and lifespan, resemble more closely those of wild elephants than the elephants in zoos, which makes this study population well suited for the purpose of studying how evolution has shaped their reproductive physiology. The studied population of 69 elephants can be considered as a relatively large sample size for a longitudinal study, as regular blood sample collection would be challenging for such a large number of individuals especially in wild populations. The regular health checks and laboratory facilities already being established in the semi-captive population enabled reliable and continuous collection of the blood samples. Also, the ages of the captive born elephants of this study, as well as those wild born that were captured at a young age, are known well, unlike in studies of wild elephants, which makes this study population well suited for research of age-related changes. As some of the studied elephants are born in the wild but caught later and some are born in captivity, the chosen semi-captive population also provides an opportunity to study the effects of the capture from the wild.

The low number of middle-aged elephants in this study can limit the accuracy of the results as the assessment of the effects of continuous age requires sufficient representation of all age groups. As the deterioration in reproductive physiology is expected to occur at some point after middle age, including more individuals also in the group of middle-aged elephants could help more accurately describe the changes that are related to the reproductive senescence of Asian elephants. Another limitation of this study is the inability to regard changes in concentration at the level of an individual as only mean concentrations were analysed. The assessment of variation in concentrations within individuals as well as concentrations of luteal and follicular phases could be done in future studies with a higher sample take frequency to provide more accurate information on changes in the estrous cycles. In this study, the use of mean values for a set time period that was the same for all individuals also disregards the possible differences in the starting points of individual cycles leading to some elephants having proportionally more luteal phases than other elephants affecting the obtained mean values. However, the effect of different starting points of the cycles was regarded to be small as most elephants experienced more than one full cycle in the study period and the differences in starting points were presumably random with respect to the elephants' age or group within a tested variable. A longer study period would enable a more accurate measurement of estrous cycle lengths as an average cycle length could be calculated for each elephant based on a higher number of full cycles.

4.8. Future research

4.8.1. Seasonal effects and estrous synchrony

Seasonal variation in the properties of Asian elephant's estrous cycle provides another interesting topic for future research. Although elephants breed around the year, there is some evidence on seasonality and estrous synchrony. Most calves are born at the timing of most abundant feed resources as is observed in both Asian and African elephants, and in the former such timing of birth has been shown to enhance calf survival as well (Mumby et al. 2013, Moss 2006). In Myanmar's climate, this time of increasing feed resources is during the rainy season from mid-May to October. Ensuring parturition at a favourable time can be aided by an increased frequency of estrous cycles at a certain period as this is observed in African elephants (Wittemyer et al. 2007). A study on captive Asian elephants in Thailand found that the length of the follicular phase was shorter in the winter and summer time, while a more recent study on semi-captive Asian elephants found the whole cycle length and luteal phase to be longer in the rainy season (Thitaram et al. 2008, Yang et al. 2023).

In captive African elephants, estrous synchrony has been observed among zoo elephants kept in same facilities (Weissenböck et al. 2009, Bechert et al. 1999). Elephants in a group appear to synchronize their cycle with that of the dominant female as a consistent synchrony was shown to appear only after the re-occurance of the dominant female's progesterone cycle after its lactational anestrus.

In this study, the average concentrations of a given date of sample collection across all individuals shows cyclic change in time, which could indicate estrous cycle synchronization. The concentrations appeared to be higher in the rainy season from mid-May to October in comparison to the hot season from mid-February to mid-May, but the significance of this difference was not tested as the data didn't include samples from the cool season (Figure 11).

Another reason for leaving out the analysis of seasonal differences is that the sample date was not randomized in the immunoassay protocol because of an aim to minimize sample collection time from freezer and consequent differences in samples' time spent in room temperature. This means that the same assay plate contained mostly samples from the same date making possible plate specific variations affect selectively some dates more than others. Possible reasons for the seasonal differences in allopregnanolone concentrations include seasonal variation in environmental conditions as well as seasonal variation in workload, which leads to variation in stress. The elephants are given a 4-month-long rest starting from mid-February, which could explain why allopregnanolone concentrations seem to be lowest at that time and rise up until the end of the rest period, mid-June, when the elephants can be expected to be physically most recovered. However, the elephants included in this study were not subject to heavy work load during the time of the study. Adaptive mechanisms that lead to conception and birth occurring at a timing that is optimal for calf survival could also explain the observed seasonal differences in the hormone concentrations. Nevertheless, further studies with year-round data are needed to investigate seasonal differences in the properties of elephant's estrous cycle and their possible causes.

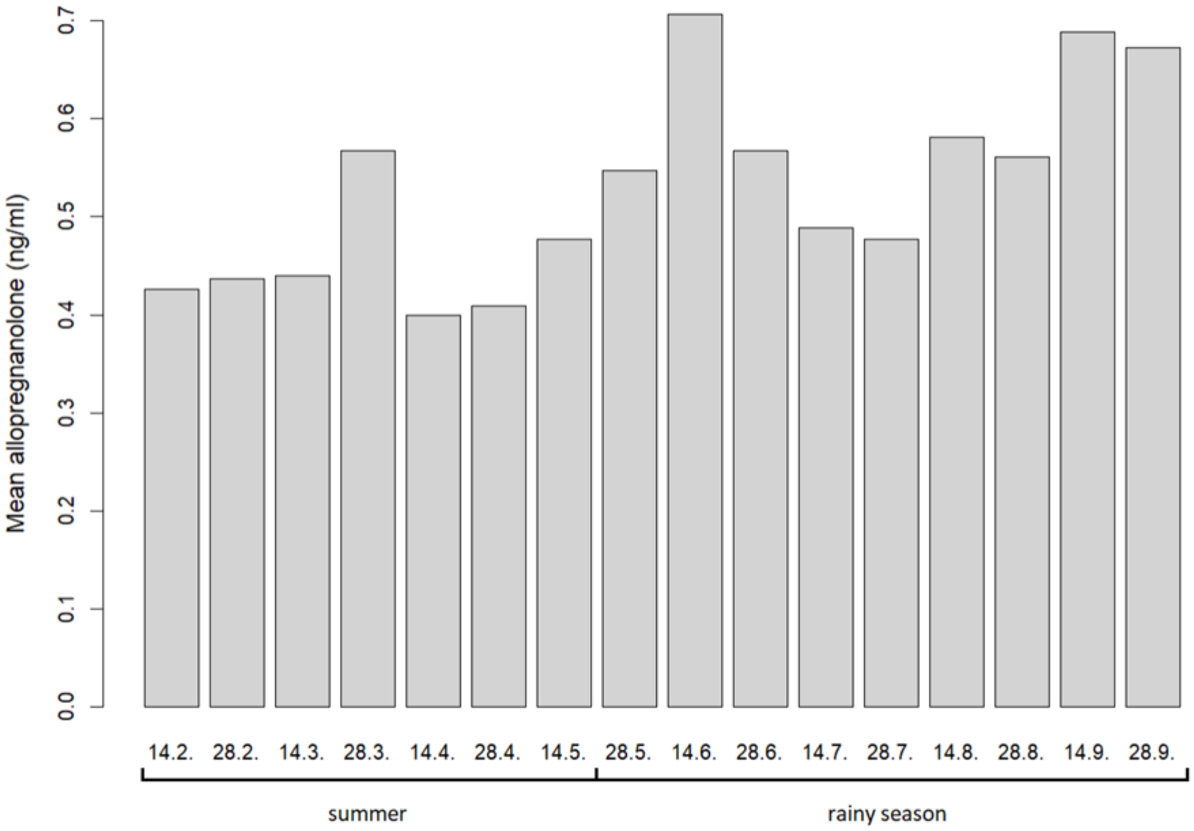

Figure 11. Mean allopregnanolone for sampling date (n=55). Pregnant individuals were excluded.

4.8.2. Peak allopregnanolone

As part of the statistical analyses of this study, a linear model was applied to analyse differences in the peak values of allopregnanolone in the cycling elephants using age, age^2, recent reproduction status, camp and origin as explanatory terms. The results indicated that increasing age could be associated with a slight decrease in peak concentrations, but this effect or those of other tested terms did not show significance. This analysis was not reported along with the main results as it was noted that the sampling frequency of two samples per month was not suitable for detecting true peak values of a cycle as concentrations can potentially rise and fall considerably between two sample takes. However, an individual typically experiencing a luteal maximum multiple times in this length of study period decreases this error in peak measurements and the error is random with respect to individuals. As aging has been shown to be associated with declining mean progestagens in previous studies with African elephants, it can be assumed that there would also be a reduction in peak levels (Freeman et al. 2013). Both mean and peak levels of progesterone metabolites are important objectives of research in the topics concerning fertility as a decline in either can indicate a reduction in ovarian function and reproductive performance. The current study’s results on peak values can be regarded as directional information on allopregnanolone levels in Asian elephants, but studies with a higher sampling frequency should be conducted to investigate whether aging or other factors are associated with a reduction in peak levels of the hormone.

4.9. Conclusions

This study provides new information on the reproductive physiology of Asian elephants and how some observed changes can be related to aging. The probability for a normal estrous cycle was observed to decline after middle age, which is the time when reproduction rates have previously been observed to decline rapidly in Asian elephants (Lahdenperä et al. 2014). These findings suggest that a deterioration in reproductive physiology would occur in some individuals of Asian elephants already relatively early considering their life span, making it possible that they experience a menopause similarly to humans and some whale species. As some of the Asian elephant individuals are still capable of reproducing at well over 60 years of age close to their death, menopause can’t be suggested to be a species-specific characteristic in Asian elephants as in truly menopausal species, but possibly a characteristic of some individuals, which would vary greatly in their timing of the reproductive termination.

However, the mechanisms for the loss of cyclicity with age in some Asian elephant females remains unclear. As the mean concentrations of a major determinant of ovarian function, allopregnanolone, as well as the length of the estrous cycle, were not found to be affected by age, further studies are needed to investigate the underlying physiological changes behind the loss of cyclicity in some individuals. Furthermore, although this study did not find evidence for anestrus to be prolonged beyond one year after calving, the observed weak evidence of recent reproduction affecting the length of the cycle makes it an interesting topic for future research to investigate whether reproduction affects the properties of an estrous cycle after one year of lactational anestrus. Further studies on the effects of seasonal changes and environmental factors are also needed to more accurately explain the observed variation in estrous cyclicity and the properties of an estrous cycle in Asian elephants.

More generally, my thesis brings novel insights on physiology of reproductive senescence in animals living in natural habitats, where physiological data is scarce and studies usually focus on population-level measures of reproduction with age, such as age at last birth. Reproductive senescence is a natural phenomenon in most mammals, characterized by gradually declining reproduction rates with age as a result of normal aging process (Nussey et al. 2013, Lemaître et al. 2020). So far, only humans and some toothed whale species have been shown to have a true menopause with a total loss of reproductive capacity in all females of the species after a certain age limit, occurring decades before the end of their typical lifespan (Laisk et al. 2018, Ellis et al. 2018). My study suggests that long-lived Asian elephants cannot be regarded as a species with a similar phenomenon in all older females.

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