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Seasonal variation in the physiological markers of stress in Asian elephants

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One of the fundamental concepts in evolutionary biology is to understand how the environment shapes individuals and, consequently, populations over time. However, although the basis for the relationships between environmental variation and vital rates (e.g. birth, death rates) is well-established, less is known of the physiological basis of these links in nature. This thesis aims to determine seasonal variation in different physiological markers of stress (cortisol, white blood cell ratio, oxidative stress levels and body weight) in a large semi-captive population of Asian elephants (*Elephas maximus*) employed daily in timber industry but foraging naturally in forests at night. Longitudinally measured cortisol, HL ratio and oxidative stress levels were extracted from 286-1712 fecal and blood samples of 88-209 elephants, depending on the marker. The elephants showed higher cortisol levels and HL ratios during cold season, indicating increased stress, and the lowest levels during monsoon season. There was an opposite pattern in oxidative stress (ROS) levels, where the highest ROS damage was observed during monsoon and the lowest during cold season. Based on these results, the damage from seasonal stress seems to accumulate slowly over time and shows only later in life. Further, the models showed a significant decline on body weight during hot season. This decline in body weight is likely to be caused by seasonal variation in food abundance, which also declines during hot season. Understanding how seasonal variation affects the Asian elephant population may help improving their working conditions, and more generally the conservation of these endangered animals.

Key words: seasonal variation; stress; cortisol; H/L ratio; oxidative stress; ROS; body weight

Contents

1 INTRODUCTION

1.1 Life history and environmental variation: what underlies the link?

One of the fundamental concepts in evolutionary biology is to understand how the environment shapes individuals and, consequently, populations over time. However, although the basis for the relationships between environmental variation and vital rates (e.g. birth, death rates) is well-established (McEwen and Wingfield 2003, Boonstra 2004, Schultner et al. 2013, Wingfield 2013), less is known of the physiological basis of these links in nature. A key concept for linking environmental variation to vital rates is understanding the underlying stress response, which can be induced by changing biotic and abiotic environmental conditions such as food availability (Kitaysky et al. 2007), social environment (Creel et al. 2013), predation (Hirschenhauser et al. 2000) or seasonal variation in climate (Romero et al. 2000). When a physiological stress response is triggered, the perturbation signal puts the animal physiologically into an emergency state so that survival is prioritised over other biological functions (Wingfield et al. 1998). While the term stress is generally used to describe situations where stress is caused by an unpredictable environmental change and the term stress response as a general response to this stressor (Selye 1956), in this thesis I use the term stress for both predictable and unpredictable changes and the term stress response to describe the physiological response triggered by them.

Stressful environments can have positive or negative fitness consequences: for example, harmful stress response is usually unpredictable, prolonged and eventually becomes long-term chronic stress (Monaghan 2014). In these situations the stress response is highly intense, and the individual is unable to adapt to it. However, stress response can be beneficial when it is a short-term solution to increase the likelihood of survival when homeostasis is disrupted. Moreover, periodical stress response caused by predictable factors such as seasonal variation can cause long-term physiological changes and in time shape life-history traits in many ways (Southwood 1988). A challenge for field studies in

nature is however to accurately quantify such stress responses using the correct molecular and physiological markers of stress.

1.2 Molecular and physiological markers of stress

In vertebrates, the stress response activates a cascade of molecular, physiological and behavioural responses that are highly conserved across the taxonomic group (Sapolsky et al. 2000, Boonstra 2004, Reeder and Kramer 2005), and that can be used in research to develop reliable markers of stress. One such marker is the glucocorticoid hormones. While the physiological response to changing conditions is complex overall and the mechanisms are pleiotropic, this cascade is mainly regulated by the hypothalamic–pituitary–adrenal (HPA) axis. It controls the release of stress hormones glucocorticoids that are usually emitted as a long-term coping mechanism to mediate homeostasis under changing conditions (i.e. allostasis) rather than as an immediate stress response. Although glucocorticoid secretion can highly increase when an acute response to environmental perturbations is needed, glucocorticoids are always secreted at a baseline level as a part of normal physiology (McEwen and Wingfield 2003, Wingfield and Romero 2011, Schultner et al. 2013). The baseline level does not always stay the same and can vary periodically in response to predictable environmental perturbations like seasonality, which affects the corticosterone response for example in birds (Lattin et al. 2016). Glucocorticoids are also known to mediate the physiology and behaviour of an individual during life-history transitions. For example, glucocorticoids have been used to determine stress response and health of individuals and populations during habitat disturbances (Walker et al. 2005).

Another way to assess stress levels is from the ratio of white blood cells in the circulating system. Most vertebrates have five types of white blood cells: lymphocytes, neutrophils, eosinophils, basophils and monocytes. These cells are morphologically conserved across taxa except for neutrophils, which are in some species replaced by heterophils (Jain 1993). Despite the morphological differences between neutrophils and heterophils, both have the same immunological

functions as primary phagocytic cells that are produced in response to inflammation, infection and stress (Jain 1993, Harmon 1998, Thrall 2004). Lymphocytes on the other hand are a diverse group with multiple different immunological functions such as regulating immunologic memory and antibody production (Campbell 1996). Neutrophils/heterophils and lymphocytes often make up nearly 80% of all white blood cells and are especially affected by stress and directly correlate with stress hormone levels (Dhabhar et al. 1995). In detail, stress or glucocorticoid treatment increases the number of neutrophil/heterophil and lowers the number of lymphocytes, and this opposite reaction has been used as a comparative ratio (HL ratio) to measure stress response in many animals such as mice (Van Dijk et al. 1979), sows (Kranendonk et al. 2005), cows (Anderson et al. 1999) and humans (Dale et al. 1975). Unlike hormonal response to stress, white blood cell response is slow, ranging from several hours to days. However, HL results should be comparable with glucocorticoid results in animals as the HL response is partly mediated by HPA axis (Saad and El Ridi 1988, Brenner et al. 1998). Many earlier studies indicate that HL ratio mediated by glucocorticoids varies seasonally in vertebrates (Saad and El Ridi 1988) and for example, high HL ratios have been linked to seasonal stress during migration in birds (Owen and Moore 2006).

Stress often leads to cellular damage, such as oxidative stress, which is a third commonly used correlate of stress response. Oxidative stress refers to the imbalance between the production of reactive oxygen species (ROS) and antioxidant defences, and is a common phenomenon at the cellular level as almost all intracellular activities generate reactive oxygen species (ROS) (Sies 1986, Mittler 2002, Kohen and Nyska 2002). They are created mainly in the mitochondria when oxygen is used in oxidative phosphorylation to produce ATP for the cell's biological processes. Oxygen can be used to release a lot of energy, so it is a major source of fuel for cells. Oxygen level in cells increases oxidation rate, which is harmful for the cell structures and processes (Yakes and Van Houten 1997, Halliwell 2003, Kujoth 2005, Valko et al. 2006). Oxidative stress happens

when an organism is not capable of stopping the damaging effects of oxidation when ROS is produced, in other words the balance between the damaging effects and damage control is off. Oxidative stress can create damage in all bio-molecules, causing cell senescence and aging (Berlett and Stadtman 1997, Epel et al. 2004, Richter and Zglinicki 2007). Oxidative stress is a result of basic cellular metabolism, but it can also indicate the level of physiological damage caused by stress from for example seasonal changes in the environment (Bhat et al. 2008). It is possible that oxidative stress has an impact on life histories over longer time scales, because the accumulating damage from ROS may affect the physiology of the species and ultimately lead to changes for example in reproduction (Christe et al. 2012). However, the underlying physiological pathways that are affected by the environmental variation and stress are still poorly known.

Poor environmental conditions are already known to be associated with reduced survival or reproduction and they have more recently been evidenced as an important source of stress and reduced physiological performances (Hirschenhauser et al. 2000). However, quantifying the environmental stress in natural populations has been challenging because of the absence of reliable method allowing measuring the stress in an environment, where many factors within the environment vary simultaneously. For example, baseline HPA function has not been often assessed in free-ranging mammals because taking plasma samples from individuals in under 3 minutes from capture is difficult but necessary to prevent stress during capture from interfering with the glucocorticoid levels. In order to prevent stress from capture interfering with the results, glucocorticoids can be also measured from fecal samples but knowledge of the individual and the time the sample was produced are needed, which is difficult to obtain from free-ranging populations. One possibility is to measure the levels of both glucocorticoids and HL ratio and using these results to demonstrate how the signals of stress (glucocorticoids, immune response) translate into damage at the cellular level (oxidative stress) and furthermore how stress affects body weight, a proxy of body condition. Studying the effects of stress on multiple

levels gives a good overview on its long-term consequences. Learning about the interactions between ecological variations, physiological markers of stress and their effects on population dynamics helps to determine the relationship between environmental effects and life-histories.

1.3 Seasonal variation and stress

As seasonal changes in stress parameters drive the investment in survival versus reproduction (Cornelius et al. 2012), studying seasonality gives insight on the long-term effects of stress in individuals and populations. Even though seasonal variation affects the physiology of most of living beings (Nelson and Demas 1996, Romero 2002a, Touma and Palme 2005, Mormède et al. 2007, Bale and Hayward 2010), there are some exceptions. For example, bank voles do not show any major seasonal changes in their physiology (Peacock et al. 2004). Such species-specific patterns are explained by different environments and life histories.

Since season affects the physiology of most animal species, understanding physiological impacts of stressful conditions and the functions of HPA axis is important in explaining how animals respond to these changing environments. In fact, there is evidence that such ecologically changing environment can influence physiological parameters and fitness in birds (Kitaysky et al. 1999, Casto et al. 2001). Moreover, while breeding season often increases stress in vertebrates (Lattin et al. 2016), less is known about the effects of seasonal variation on stress in species that reproduce throughout the year. Interestingly, studies investigating the long-term effects of seasonal variation on stress at the physiological level are rare and these effects on long-lived species are mostly unknown.

Stress physiology especially in free-ranging populations is difficult to study because of the issues with sampling (discussed earlier). Moreover, studies that focus more on the short-term effects of stressful environments may not show clearly how the effects are manifested later in an individual's life (Wingfield 2013).

There has been a lack of longitudinal studies of stress and environmental variation overall, which is understandable since assessing the significance of temporal variation requires longitudinal studies over long timescales. This approach requires more work than focusing on spatial variation and simply collecting samples only once without repeated measurements.

1.4 The Asian elephants

In order to determine how the effects of stress build up and become noticeable over time, I used longitudinal data on Asian elephants (*Elephas maximus*), long-lived and aseasonally breeding mammals that regularly experience seasonal variation in their habitats. The Asian elephant population in this study is part of the largest semi-captive Asian elephant population in the world, owned by and working for the Myanmar Government. The climate in Myanmar is subtropical and tropical monsoon climate with three distinct seasons (cool, hot and monsoon), where the elephants are working during monsoon and cool season from 5 to maximum 8 hours per day (Mumby et al. 2015b). Although elephants do not in general have a specific breeding season within a year but instead, are able to reproduce throughout the year, a previous study on this Myanmar population found that their birth rate varied considerably between the different months of the year, being highest in cold season and lowest in monsoon. Similarly, mortality varies seasonally with the lowest mortality occurring during monsoon compared to relatively higher rates in cold and hot seasons (Mumby et al. 2015). The population has become unsustainable because of low birth rate and high infant mortality, but the causes are still largely unknown. There is also a population living in the wild, which also may no longer be sustainable (Mumby et al. 2013). Therefore, understanding in detail how the seasonal environment affects physiological markers is important in sustaining the current populations. It is worth noting that extreme weather patterns especially during hot season and monsoon have lately become more common due to climate change (Wassmann et al. 2009). Longitudinal studies on long-lived species, where the effects of stressful environments have been investigated are rare (Mumby et al. 2015a, b).

Noticeably, studies from Mumby et al. (2015 a & b) found that while body condition and food availability were highest during monsoon, the elephants were also the most stressed based on glucocorticoid levels. However, this study was based on low sample size. These results show clear but complex patterns of the long-term effects of seasonal ecologies in long-lived species and provide a good basis for further studies about the underlying physiology of seasonal variation in life-history and vital rates.

1.5 This study

The present study aims to determine seasonal variation in cortisol, HL ratio, oxidative stress levels and body weight in Asian elephants working in the logging camps. The unique semi-captive nature of the population enables us to measure HPA function from fecal samples, giving us an integrative measure of stress. Furthermore, the exceptional lifespan of 60 or sometimes even 80 years of Asian elephants (Robinson et al. 2012) makes them a unique model species to observe the accumulation of stress and the effects of stress on physiology. In total 1500 repeated measurements from 200 individuals were sampled for this study. Fecal cortisol samples and body weights were measured over the three seasons over five years, and leukocyte and the oxidative stress (ROS) samples were collected from blood samples over two years to address the effects of seasonality both on the three stress parameters (cortisol, HL and ROS) and on body condition (body weight). Further, I also assessed how the interaction between the stress parameters and season affected body weight. I predict that seasonal variation in climate will show increased stress in all three stress parameters during the working seasons as the seasonal effects in other mammals are common (Romero 2002b) and glucocorticoid levels usually reflect the level of oxidative damage in the individual (Bhat et al. 2008). The stress during these working seasons could also negatively affect the weight of the elephants, but this connection has not always been clear in earlier studies as for example elevated glucocorticoid levels indicate not only stress but also changes in body condition (Nunes et al. 2006) or scarce resources (Kitaysky et al. 2007).

2 METHODS

2.1 Study population

The current worldwide Asian elephant population size is around 38,500 – 52,500 individuals, of which around 16,000 are captive elephants (Sukumar 2006). Asian elephants are nowadays classified as endangered and the populations are fragmented over Southeast Asia, which makes their population growth difficult and slow. The largest Asian elephant population lives in India, followed by Myanmar, where the tropical monsoon climate has high seasonal variation in precipitation, temperature and resources. These changes affect reproduction rates from season to season, even though the elephants are considered continuous breeders (Mumby et al. 2015b).

The data used in this thesis are gathered from a government-owned elephant population in Myanmar, which is part of the largest population of captive elephants in the world. Approximately half of these captive elephants are owned by the state and are working for the timber industry under Myanma Timber Enterprise (MTE). All elephants owned by the state are monitored by local veterinarians and officers and their birth date, calving dates, body measurement information and death date have continuously been recorded in logbooks for over a century, and these records have now been computerised into an extensive database. Thus, any changes in status are covered by the database as long as the elephants are moved within Myanmar's borders. Originally around half of these working elephants are captive-born while the other half were born wild (Lahdenperä et al. 2018). The elephants live in camps and their daily tasks include riding, transport and draft power, which they do under supervision of a personal human caretaker ("mahout") (Crawley et al. 2019). Typical workday lasts from 5 to 8 hours and after which the elephants are free to roam around in the forest and interact with the other elephants unsupervised. Therefore, reproduction is not managed by humans. The mothers stop working when they are halfway through their pregnancy (after around 12 months since conception) or when there are visible tissue changes. The break continues for a year after birth so that

new-born calves can stay their first year with their biological mothers exclusively and accompany them after the mothers start working again. Nonetheless, it is common for elephant calves to also have allomothers that help biological mothers to care for their calves (Lahdenperä et al. 2016). After the mothers return to work, they start with lighter workloads and shorter workdays, and are allowed to nurse their calves when necessary. Once calves turn 4 or 5, they are separated and begin training. Workload is determined by age: elephant calves start as "training calves", but after turning 18 they are classified as "working adults" until they become "retired adults" from the age 53 and onward. The average working hours for a mature elephant (age 17-44) are approximately 6 hours a day, 3-5 days a week, and each day the elephants get a break at noon. Workloads differ from season to season, but all elephants finish working by mid-February each year to wait for hot season to pass and resume working mid-June at the beginning of monsoon season. This work schedule has altered only little over the past 40 years (Mumby et al. 2015b).

One problem with studying natural populations of animals in uncontrolled conditions outside laboratory, but still in human care, is that the study sample such as mine may be biased towards the healthiest or most reproductive individuals. In this study, this possibility is reduced by three points. First, the timber elephants are never culled, released or sold, and individuals incapable of working or reproducing are cared for the same way as working elephants. Second, veterinary care is limited to occasional vaccinations and treatment of wounds. Third, Myanmar's semi-captive elephants resemble more the individuals from the wild populations rather than individuals from fully captive populations such as zoos in both their survival and reproductive rates (Mar et al. 2012).

2.2 Dependent variables : Physiological parameters of stress

2.2.1 Cortisol

The fecal cortisol samples were collected over a five-year period from 2013 to 2017 and analysed in the laboratory in five batches, where the earliest was

analysed in August 2014 and the latest in June 2018. The glucocorticoid metabolites were extracted from fecal samples using a protocol for boiling extraction and enzyme immunoassay (EIA) for glucocorticoid metabolites (Watson et al. 2013), where for achieving consistent results the original EIA protocol was optimised by using Nunc MaxiSorp® plates, room temperature substrate reagents and dark incubation conditions. The records of the measure month were used to determine the measure season and only individuals with recorded data were selected from all variables used in the models. The cortisol samples consisted of 1712 observations from 209 individuals in total, where 771 samples are from males and 941 are from females and with a male:female ratio of 97 males to 112 females. Most of the elephants are captive-born 1948-2015, the youngest measured elephant being 3 years old and the oldest 69 years old. Cortisol varied between 0,419 and 371 ng/g/feces in Asian elephants in these samples, which showed more variation compared to previously measured levels in elephants (Foley et al. 2001). This variation is likely a result from big sample size but cortisol values <1 ng/g could also be explained by a sampling error.

2.2.2 HL ratio

Second, I assessed stress by HL ratio, which is a slow response stress indicator. The elephants were sampled during four occasions (Mar, Apr, Jul, Nov) in 2016-2017 and during two (Mar, Apr) in 2018. The elephants were measured and sampled in mornings on non-workdays. The samples were collected from blood, specifically from an ear vein in three different tubes: EDTA, heparin and serum separator tube. These blood tubes were refrigerated for a maximum of 24 hours before analysis in the laboratory. During this analysis, the samples were centrifuged, and sera was collected, frozen and sent for further analysis in a laboratory in Yangon. The EDTA blood samples were used for determining the leucocyte count (for HL ratio), where the blood cells were counted manually using a blood smear stained with Romanowsky stain. The ratios were calculated based on the amounts of heterophils and lymphocytes in the blood sample slide. This resulting heterophil and lymphocyte ratio dataset had 562 observations from 181

individuals in total. The dataset consisted of elephants born 1948-2014 and fairly balanced male:female ratio of 110 females to 71 males. HL ratios in the sampled individuals varied from 0,205 to 3,45 , which is considered as a normal range for the elephants although the normal parameters for free-ranging animals are still mostly unknown (Davis et al. 2008).

2.2.3 Oxidative stress (ROS)

Third, I studied oxidative stress damage on a cellular level caused by an increase in reactive oxygen species (ROS) levels. Oxidative stress levels were measured from blood samples by assessing the level of oxidative damage in cells caused by reactive oxygen species (ROS). The analyses were done using ROM kit for samples from 2014 to 2016, where the elephants were randomly sampled during four occasions (Apr, Jul, Nov, Dec) in 2014, during eight (Jan, Apr, May, Jun, Jul, Aug, Nov, Dec) in 2015 and three (Mar, Apr, Jul) in 2016. This d-ROMs test measures the early exposure to oxidative stress from the concentration of hydroperoxide (ROOH), which is a reactive oxygen metabolite (ROM) produced by the attack of ROS on macromolecules. The concentration of hydroperoxide was calculated by comparing the results with a standard solution, where oxidative activity on the chromogen is equivalent to the activity of H_2O_2 (0.08 mg/dL). The dataset had 286 observations from 88 individuals age 8-66, where male:female ratio is 40 males to 48 females. ROS varied between 9,24 and 35,9 mg H_2O_2/dL.

2.2.4 Body weight

Finally, I studied the effects of stress on weight. The weight data was collected monthly simultaneously with the fecal samples from the MTE elephants from 2013 to 2018 from Katha and Kawlin logging camps. For taking these measurements, an Eziweigh 3000® scale, capable of weighing up to 9000kg to the nearest 10kg, was used to measure the same individuals monthly. As calf age is linked to its size, I used standardised weights as dependent variables, to account for multiple measures of the same individuals across their lifespans - these were obtained by dividing an individual's actual size/weight measurement by the

predicted weight at that age, as obtained from von Bertalanffy growth curves from this population (presented in (Mumby *et al.* 2015a)). Separate curves were used for males and females. These standardised weights were merged with the cortisol dataset and the final dataset contained 1420 observations from 209 individuals. I also tested the effects of HL ratio and ROS on weight, however, there was not enough ROS data and weight data from the same individuals to test this effect. Moreover, the models showed no significant effect of HL ratio on weight nor any interaction between HL ratio and season or sex that significantly impacted weight (Table 1).

2.3 Independent variables

The key independent (predictor) variable used in my statistical approach (see below) to explain variation in the physiological stress parameters was the season within a year when the elephants were sampled for their stress parameters. The climate in Myanmar has three distinct seasons, which in this study have been classified according to temperature and precipitation data in the following way (Figure 1): cold (blue), hot (red), monsoon (light green). Each season has their own distinct features: cold season has lower temperatures than the others, hot season has high temperatures with moderate rainfall and monsoon has high temperatures and rainfall. These seasons are not as stationary as presented here and vary slightly in length each year.

The other independent variables used to explain variation in the physiological stress parameters were derived from a demographic database compiled, achieved and maintained by the Extraction Department of the Myanma Timber Enterprise. The state recorded logbook has information about each elephant, their life-histories, health and mother lineage: a unique registration number (ID); name; sex; camp (Kawlin and Katha in this study); date of birth; origin (captive-born or wild-caught); age; age of capture (for wild-caught); weight; height; alive or dead status; mother's ID number; last seen- date. Using this database, I constructed for

each sampled individual independent (predictor) variables concerning their ID, sex, location, origin, and season.

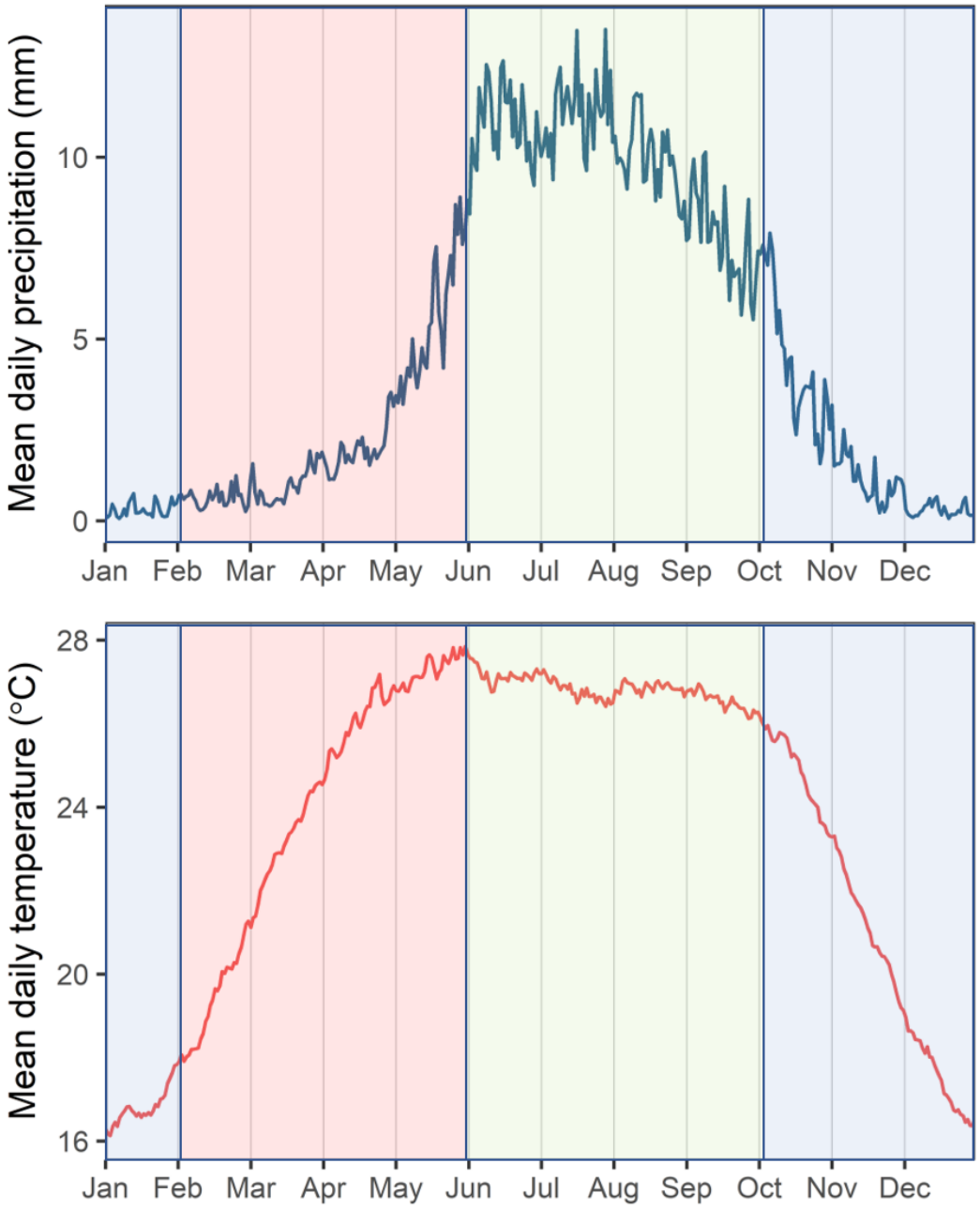

Figure 1. The mean daily temperature and precipitation across years (1951-2007 or 1961-2007) from the deciduous forest region of Sagaing and Kachin divisions. The data was obtained from the APHRODITE (Asian Precipitation - Highly Resolved Observational Data Integration Towards Evaluation) daily gridded dataset and was restricted using data from the MIMU (Myanmar Information Management Unit - A unit of the UN in Myanmar).

2.4 Statistical analyses

I investigated the influence of season on stress parameters and the influence of cortisol on body mass. First, the probability distributions of cortisol, HL ratio, oxidative stress and body mass were determined by fitting univariate distributions. All dependent variables followed gaussian distribution and for ensuring reliable comparisons of the results, the stress parameters were modelled using linear mixed modelling (LME). For all models, the season of collection was included as a fixed 3-level factor (hot, monsoon, cold) over the 2- to 5-year observation period. In order to take account of the potential effects of confounding variables, I also included the birth season, sex, age (4-level factor: taming (0-10 y.o.), training (10-20 y.o.), working (20-54 y.o), retired (54-)), camp (2-level factor: Katha, Kawlin), origin (2-level factor: captive, wild-born) as fixed factors and studied both additive and interactive effects (Table 1). Finally, the models included the individual identity (ID), the batch (only in cortisol models), and the year of collection as random factors.

2.4.1 AIC-based model selection

For each stress parameter, I compared all possible regression models with season, sex, age, origin and camp and interactions between season and age, and between season and sex as predictors (Table 1). Similarly with standardised weight models, I modelled and compared the effects of different variables and their interactions. Additionally, in the weight models after testing a linear effect of cortisol and HL ratio on body weight, I also included a quadratic effect as the baseline cortisol production may be different in each stage of individual's life.

The most competitive models were selected using the Akaike information criterion (AIC), considering each random effect as one parameter (Pinheiro and Bates 2002). Following the principle of parsimony, the model with the lowest AIC was retained as the best model. Where the difference in AIC between competing models was less than two, I retained the simplest model (Burnham and Anderson 2002). I also calculated the Akaike weight (AICw) for each model to provide the relative likelihood that the model was the best among the candidate models. The

model comparisons were carried out using maximum likelihood (ML) approach, but the results for the best models are reported using restricted maximum likelihood (REML) estimations. Only the results for the best models selected as described above are reported.

All analyses were conducted using R version 3.4.3 (R Development Core Team: A language and environment for statistical computing. R Foundation for Statistical Computing, 2017). While studying the distributions I used the function *fitdistr* (package: fitdistrplus) to fit the univariate distributions of each stress parameter. The package *nlme* was used for linear mixed model LME modelling (Pinheiro et al. 2007).

3 RESULTS

3.1 Seasonal variation in cortisol, HL ratio and ROS.

According to the AIC-based model selection, measure season, age and camp best described the variation in cortisol, HL ratio and ROS (see Table 1 and Fig. 2, 3, 4). First, the results showed strong seasonal variation in cortisol level. Each year, cortisol level during monsoon was on average 11 % lower (87 ng/g/feces) compared to cold season (93 ng/g/feces) ($F_{2,802}$= -4.34; p-value < 0.001) and 6,6 % lower compared to hot season ($F_{2,802}$= 2,78; p-value = 0,0056). The difference in cortisol level between cold and hot season did not reach statistical significance ($F_{2,802}$= -1,39; p-value = 0,16). The detected differences in cortisol levels between different seasons are unlikely driven by variation due to biased sampling of study animals in each season according age, sex, camp or status (captive-born vs. wild-caught) that were all controlled for in the model (Table 1). For example, generally, cortisol levels were mostly uniform throughout the age groups, except for the training calves group (aged 10-20), which showed approx. 6,0 % lower cortisol levels compared to taming calves and the other groups ($F_{3,332}$= -1,91; p-value = 0,057). There was also a significant difference in cortisol levels between the logging camps, with camp Kawlin population showing 13 % lower cortisol levels on average compared to camp Katha ($F_{1,363}$= -4,72; p-value <0,0001). Moreover, my study design allowed for longitudinal follow-up of the same animals across different seasons and adjusting in the analyses for any overall differences between individuals in the baseline cortisol (or the other stress parameter) levels, increasing the confidence that such confounding factors did not influence the results. The effect of season on the cortisol levels was similar for males and females and all age groups as the interactions between either season and sex ($F_{2,800}$= 0,83; p-value = 0,44) or season and age ($F_{6,796}$= 0,79; p-value = 0,58) did not reach statistical significance.

Second, I showed a significant seasonal variation in HL ratio. Indeed, elephants displayed 17% higher HL ratio, indicating higher stress levels, during cold season

compared to hot season (β= 0,86 ± 0,23, $F_{2,228}$= -4,29; p < 0,0001) along with monsoon season (β= 0,91 ± 0,24, $F_{2,228}$= -2,67; p = 0,0080). Similarly with the cortisol models, the detected differences in HL ratios between different seasons are unlikely driven by variation due to biased sampling of study animals in each season for the variables controlled for in the model (Table 1). For example, HL ratios slightly decreased with age, with the working adults (aged 20-54), who showed 15% higher HL ratios compared to taming calves ($F_{3,148}$= 2,11; p-value = 0,037), but this decrease did not show as significantly in retired elephants ($F_{3,148}$= 1,41; p-value = 0,16). Again, there was also a significant difference in HL ratios between the logging camps, with camp Kawlin population showing over 20% higher HL ratios compared to camp Katha ($F_{2,178}$= 3,71; p-value = 0,00030), while camp West Katha showed no significant changes in HL ratio compared to camp Katha ($F_{2,178}$= 0,149; p-value = 0,88). The effect of season on the cortisol levels was similar for males and females as the interactions between season and sex did not reach statistical significance ($F_{2,225}$= 0,78; p-value = 0,46). There was an interaction between age and season, with adults showing higher HL ratios during monsoon season ($F_{2,225}$= 3,31; p-value = 0,0016) and hot season ($F_{2,225}$= 3,19; p-value = 0,0011) than calves. During the cold season, calves showed higher HL ratios than adults ($F_{2,225}$= -3,19; p-value = 0,0016)**.** These differences between adults and calves displayed the differences in reactions to seasonal changes: adults showed relatively constant HL ratios throughout the year while calves showed stronger variation in their HL ratios (i.e. highest HL ratios during cold season; and lowest HL ratios during hot and monsoon seasons).

Third, as expected, the models showed seasonal variation in ROS as well, but the pattern differed markedly from those ones detected for cortisol and HL ratio. The elephants displayed 14 % higher ROS damage during monsoon than during cold season ($F_{2,124}$= 3,43; p = 0,0008). ROS damage during hot season was 12 % more severe compared to cold season ($F_{2,124}$= 2,58; p = 0,011). Once again, the detected differences in ROS damage between different seasons were clear and are unlikely driven by variation due to biased sampling of study animals in each season for the

variables controlled for in the model (Table 1). ROS damage slightly decreased with age: the working and retired adults (aged 20-) showed 15% less ROS damage compared to taming calves ($F_{2,85}$= -2,65; p-value = 0,0095). The model showed no significant overall differences between the sexes ($F_{1,85}$= 1,58; p-value = 0,12), and the effect of season on ROS damage was also similar for both sexes (interaction, $F_{2,125}$= 0,140; p-value = 0,87) and for all ages (interaction, $F_{6,118}$= 0,704; p-value = 0,65).

The range of predicted values remained in the range of observed data which confirms the good fit of the models (see Fig. 2, 3, 4). Raw data 95% confidence intervals were modelled with GLM and showed a similar pattern when compared with the model estimates. These similarities in patterns and overlapping in data points support the model and the interpretations of the results.

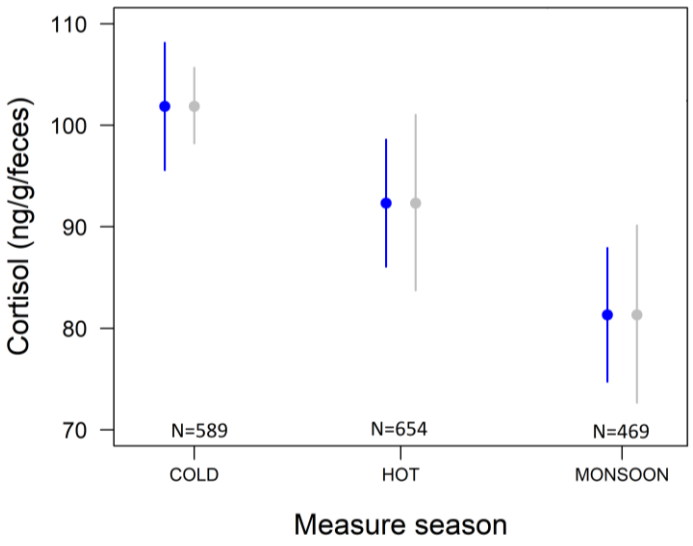

Figure 2. Seasonal variation in cortisol level. Cortisol level (ng/g/feces) of the study population is presented by season (cold, hot and monsoon). The blue dot represents the predicted cortisol level for each season while the grey dot represents the observed data. Both the predicted and observed error bars stand for 95% confidence intervals. The top left legend in the plot lists the number of repeated measurements for each season.

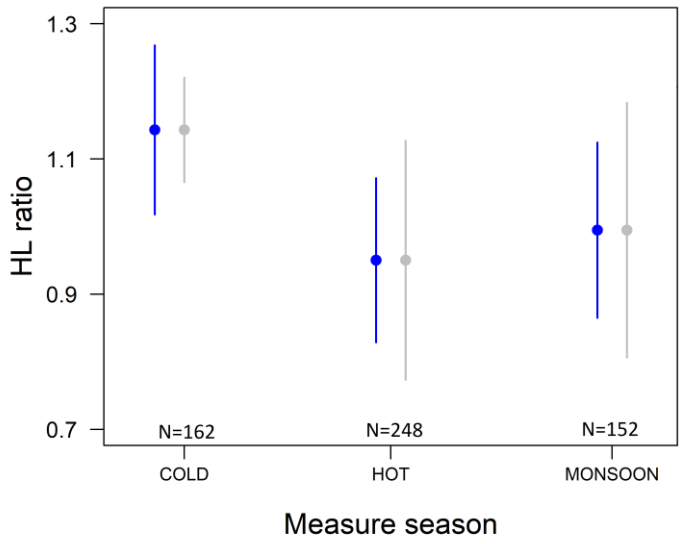

Figure 3. Seasonal variation in heterophil and lymphocyte ratio (H/L ratio). The blue dot represents predicted ratios for each season and the grey dot represents observed ratios in the population. Furthermore, the error bars for predicted and observed values are 95% confidence intervals. The top left legend in the plot lists the number of repeated measurements for each season.

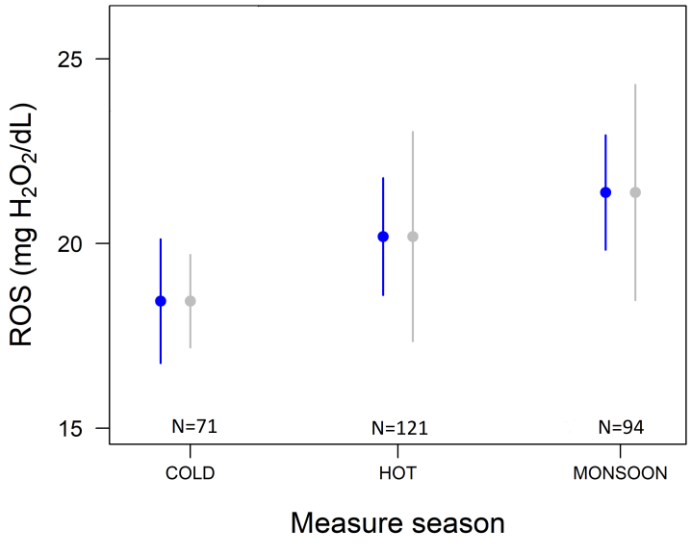

Figure 4. Seasonal variation in oxidative stress. Oxidative stress level is assessed from the level of oxidative damage (ROS) in the cells. Predicted values are marked blue in the plot and observed values are marked in grey. Both predicted and observed points have 95% confidence interval error bars. The top left legend in the plot lists the number of repeated measurements for each season.

3.2 Influence of cortisol on body mass.

After investigating the seasonal variation in the three stress variables including cortisol, HL ratio and ROS, I explored the health effects of cortisol and HL ratio variation. Body mass of the individuals included in the sample varied between 742 and 4530 kg depending on the age and body condition: in detail, variation during cold season was from 864 to 4470 kg, during hot season from 742 to 4530 kg and during monsoon season from 816 to 4280 kg. First, I investigated how seasonal variation in cortisol affected body weight. According to the AIC-based model selection, cortisol, season, sex, captive-born or wild-caught origin and camp best described the variation in body weight. There were no significant effects of either cortisol or season alone on weight, but the interaction between cortisol and season was significant. Particularly, the season of measure clearly influenced the relationship between body weight and cortisol (Table 1, Fig. 5). Body weight declined most steeply with the rising cortisol level during hot season ($F_{2,620}$= -2,29; p-value= 0,023). An individual with 300 ng/g/feces cortisol level would weigh ~4% less during hot season than an individual with 100 ng/g/feces cortisol level. In order to illustrate this decline further, for an individual weighing 1800 kg this drop would mean a loss equal to ~65 kg. This result indicates that variation in cortisol is associated with weight differently during each season. Meanwhile, there was no significant interaction between HL and season ($F_{1,41}$= 0,708; p-value= 0,40) (Table 1).

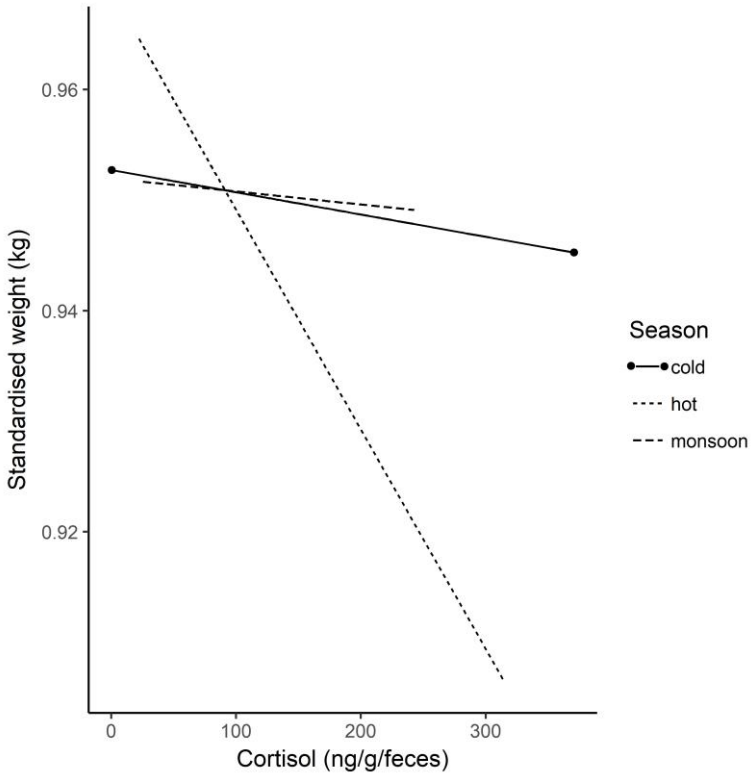

Figure 5. Cortisol's effect on body weight in function of the season of measure (cold, hot, monsoon). Body weights were standardised by controlling for the age of the elephant (see Methods for details in the residuals of weight).

4 DISCUSSION

The present study aimed to investigate the seasonal variation in markers of stress (cortisol, HL ratios and oxidative stress) and how these markers are associated with body weight in different seasons of the year in the Asian elephant. To my knowledge, this study is the first to investigate this question in such a long-lived mammal. The results provide evidence of seasonal variation in the three markers of stress. First, the cortisol model showed a decline in cortisol levels from cold season through hot season and ending in low cortisol levels during monsoon season. Second, a similar pattern was observed in the HL ratios, where cold season showed high HL ratios (indicating higher stress) while hot and monsoon seasons showed low HL ratios. Third, an opposite pattern was observed in ROS damage, where during cold season individuals experienced less ROS damage than during both hot and monsoon seasons. Therefore, stress response to seasonal variation varied between stress indicators (cortisol and HL) compared to oxidative damage (ROS). Cold season was the most stressful period when using the cortisol and HL levels as the measure of stress while it was the least stressful season when looking at ROS damage. Moreover, based on cortisol and HL results, monsoon was the least stressful season for the elephants, but during this season the elephants also displayed the highest amount of ROS in their cells. Lastly, the models showed that the association of cortisol with weight varies depending on the season. Based on these results it seems likely that seasonal variations strongly influences elephant stress physiology and these effects could be seen clearly in the steep decline of weight during hot season. These results are of broad general importance because they show how season can impact physiology and body condition in long-lived animals that typically live over several of such seasonal cycles in nature.

The results showed slight differences between cortisol and HL models, mainly for the hot season, which may not be surprising. Previous studies found that HL and cortisol stress response pathways have major differences: cortisol reacting more

quickly to stressors whilst the white blood cell response is delayed (Dale et al. 1975, Van Dijk et al. 1979). In contrast, the seasonal variation in ROS vs. cortisol and HL is robust, but the occurrence of opposite seasonal patterns between stress markers remains poorly understood. It is possible that ROS damage is delayed and takes time to accumulate before the damage is clearly visible. These types of so-called 'carry-over effects' do not immediately show the effects of stress after the perturbation but they can have significant impacts on individuals and their life-histories (Harrison et al. 2011). Likewise, the models showed increased stress levels in cortisol and HL samples, but the effects of stress manifested a few months later during the following season. For example, low stress levels during monsoon had correspondingly low ROS damage during the following season, which is the cold season. This observation supports the hypothesis that seasonal ROS damage is delayed and cumulative suggesting that the stress from one season was carried over to the other.

Poor conditions (e.g. unfavourable climate or scarcity of food) is a major cause of stress as they can affect birth rates and survival of the population (Kitaysky et al. 1999, McEwen and Wingfield 2003, Monaghan 2014). The steep decline in weight during hot season could be associated to seasonal variation in food availability. Indeed, food availability is one of the key factors impacting seasonal variation in the elephants' body condition (Mumby et al. 2015a). If this stress response significantly affects individual's health and is caused by predictable factors such as seasonal variation, this situation may lead to a trade-off between health and reproduction (Ots and Horak 1996, Doblhammer and Oeppen 2003, Shochat 2004, Jenkins et al. 2004). While stress observed in high integrative cortisol and HL levels may indicate the outcome from either a short-term or a long-term stress exposure, earlier studies have found that seasonal stress increased oxidative stress damage during summer or warm season in species such as mussels, other marine animals and ruminants (Bocchetti and Regoli 2006, Portner 2010, Bernabucci et al. 2010), which could mean that the stress during hot season is at

least partly caused by higher temperatures. Indeed, the differences in response that were observed between the seasons, where stress during hot season has a significant impact on weight but not otherwise, seems to have major consequences on health. Consequently, high temperatures during hot season could cause major stress to the elephants, which could be further amplified by climate change.

Other non-mutually exclusive hypothesis might also explain the results presented above. First, the inconsistency between the stress markers and ROS results could be also explained by the fact that these physiological markers of stress do not measure stress the same way. For instance, the level of oxidative stress during monsoon season could indicate the impacts of new, unpredictable or highly stressful environment (work) while high cortisol and HL levels during cold season may result from a hard-wired physiological reaction to seasonal variation. Besides, somewhat different animals, at different time periods, were sampled for each of the stress measures, so differences in the datasets might explain some of the variation in the results. On the other hand, the effect and sample sizes used for the analysis reinforced confidence concerning the robustness of these results. These observations could all partly explain the reverse stress pattern observed in cellular damage in ROS models. Further studies are required to better understand the mechanisms behind stress physiology and seasonal variation in long-lived animals.

These results have at least four interesting implications for further research. First, these results support earlier findings that seasonal variation has physiological impacts on animals (Romero 2002b, Nunes et al. 2006, Thitaram et al. 2008, Bhat et al. 2008) but also showcase the rarely assessed long-term effects of stress in a long-lived animal species. The issue with measuring baseline glucocorticoid levels mostly without severe interference from acute stress while taking the sample was avoided and combined with repeated measurements over five years the results

illustrate how integrative glucocorticoid levels vary over the seasons. Second, instead of assessing physiology of the stress response in one type of stress marker as many previous studies have so far (Kirschbaum et al. 1992, Touma et al. 2004, Kranendonk et al. 2005, Balestri et al. 2014), this study shows the physiological response to stress signals on multiple different physiological levels: hormonal, immunological, cellular metabolism and body condition. The patterns in the cortisol and HL ratio models were similar but the delay in ROS damage could be an indication of a 'carry-over effect' discussed earlier. Nonetheless, understanding these opposite yet intriguing patterns needs further studying. Third, the results show how all four markers of stress (cortisol, HL, ROS and body weight) may emphasise different signals: body weight may mainly correlate with food abundance while cortisol, HL, and ROS reflect broader effects of seasonality. Although elevated cortisol levels and ROS damage were observed during hot season, implying high stress levels, the changes in body condition appear to be affected by separate causes other than food availability. For example, the model showed high ROS damage during monsoon, at the same time that food abundance is at its peak (Mumby et al. 2015a). A similar pattern was observed in the study from Mumby et al. (2015a), where during monsoon the elephants showed high cortisol levels (instead of high ROS damage observed in this study) but also good body conditions at the same time. While delayed effects of the stress could explain this pattern, further investigation on the physiological pathways responding to stress is needed. Finally, these results give support for the Mumby et al. (2015a) results on seasonal body condition changes, where body condition seemed to correlate with food abundance. Unsurprisingly, the weight model showed a decline in body weight during hot season, when resources are scarce. This observation could indicate that the more the government supplies care and food for the elephants, the more their body condition will improve, especially during hot season. Perhaps securing the food supply for the elephants will help in sustaining population growth and in the conservation of Asian elephants.

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6 Appendices

Table 1. Model selection of stress parameters using mixed models (LME). *Parameter* corresponds to the dependent variable in the model. Variables cortisol, HL ratio and ROS (oxidative stress) are stress measuring variables whereas standardised weight was used as a health measuring parameter. The number of parameters is indicated by k. The best model overall is in bold.

Parameter	Distribution	k	model	AIC	ΔAIC	AIC weights
Cortisol	Gaussian	4	Season*Sex + Age + Camp	1733.923	0.000000	2.961823e-01
		3	**Season + Age + Camp**	**1735.074**	**1.150992**	**1.665805e-01**
		4	Season + Sex + CW + Camp	1735.244	1.321122	1.529963e-01
		4	Season + Age + Sex + Camp	1735.329	1.405769	1.466561e-01
		4	Season + Age + CW + Camp	1736.747	2.823806	7.217343e-02
		5	Season + Sex + Age + CW + Camp	1736.924	3.001050	6.605250e-02
		3	Season*Age + Camp	1737.519	3.596379	4.904731e-02
		4	Season*Age + Sex + Camp	1737.669	3.745897	4.551428e-02
		4	Season*Birth season + Age + Camp	1742.169	8.245884	4.797203e-03
		3	Sex + CW + Camp	1768.181	34.258227	1.077655e-08
		3	Age + Sex + Camp	1768.424	34.500977	9.544803e-09
		2	Age + Camp	1768.459	34.536540	9.376583e-09
		4	Birth season + Sex + CW + Camp	1768.984	35.061584	7.211618e-09
		4	Birth season + Age + Sex + Camp	1769.244	35.321144	6.333884e-09
		3	Season*Sex + Age	1769.422	35.499428	5.793701e-09
		3	Birth season + Age + Camp	1769.494	35.570938	5.590209e-09
		4	Age + Sex + CW + Camp	1770.112	36.188937	4.104224e-09
		3	Age + CW + Camp	1770.236	36.313155	3.857070e-09
		5	Birth season + Age + Sex + CW + Camp	1771.224	37.301713	2.352854e-09

		4	Birth season + Age + CW + Camp	1771.490	37.567369	2.060196e-09
		4	Birth season*Sex + Age + Camp	1773.208	39.285345	8.726796e-10
		3	Season*Age + Sex	1773.762	39.839092	6.616222e-10
		2	Season*Age	1775.581	41.657834	2.664865e-10
		3	Season*Birth season + Age	1777.774	43.851668	8.897957e-11
		4	Birth season*Age + Sex + Camp	1780.499	46.576576	2.278160e-11
		2	Sex + CW	1790.426	56.502885	1.592625e-13
		3	Age + CW + Sex	1792.702	58.778792	5.103950e-14
		2	Age + CW	1794.110	60.187074	2.524073e-14
		3	Birth season + CW + Age	1795.020	61.096760	1.601644e-14
		3	Birth season* Sex + Age	1795.767	61.844539	1.102016e-14
		3	Birth season*Age + Sex	1799.714	65.790978	1.531898e-15
White Blood Cells	Gaussian	3	**Season + Age + Camp**	**712.3912**	**0.0000000**	**2.599964e-01**
		4	Season + Age + Sex + Camp	712.5503	0.1591013	2.401148e-01
		3	Season + Sex + Camp	712.7095	0.3182390	2.217495e-01
		4	Season*Sex + Age + Camp	714.7879	2.3967145	7.843815e-02
		4	Season*Age + Sex + Camp	714.9368	2.5456216	7.281026e-02
		3	Season*Sex + Camp	715.0882	2.6969804	6.750336e-02
		5	Season + Birth season + Age + Sex + Camp	715.9857	3.5944725	4.309605e-02
		5	Season*Age + Birth season + Sex + Camp	718.2364	5.8452090	1.398609e-02
		2	Season*Sex	723.1228	10.7315963	1.215152e-03
		3	Season + Sex + Age	724.1450	11.7538061	7.288879e-04
		3	Sex + Age + Camp	727.1116	14.7204169	1.653748e-04
		4	Season + Birth season + Sex + Age	727.4927	15.1014820	1.366854e-04
		3	Birth season + Sex + Camp	730.0593	17.6680643	3.787877e-05
		3	Birth season*Sex + Camp	731.9237	19.5324391	1.491255e-05
		4	Birth season*Sex + Age + Camp	733.5971	21.2059198	6.458926e-06
		3	Birth season + Sex + Age	741.1730	28.7817534	1.462477e-07

ROS	Gaussian	3	**Season + Sex + Age**	**1778.140**	**0.000000**	**3.511446e-01**
		2	Season + Age	1779.142	1.001451	2.128255e-01
		4	Season + Birth season + Sex + Age	1780.512	2.371124	1.073008e-01
		2	Season + Sex	1781.331	3.190617	7.122827e-02
		4	Birth season * Sex + Age + Season	1781.490	3.349535	6.578756e-02
		3	Season * Sex + Age	1781.641	3.500805	6.099523e-02
		2	Season * Sex	1785.048	6.907435	1.110594e-02
		2	Sex + Age	1786.146	8.005991	6.412202e-03
		4	Season * Age + Sex + Birth season	1788.086	9.945396	2.431480e-03
		3	Birth season + Sex + Age	1788.485	10.344335	1.991783e-03
		2	Birth season + Age	1789.766	11.625337	1.049726e-03
		3	Birth season * Sex + Age	1792.100	13.959174	3.268058e-04
		2	Birth season * Sex	1794.482	16.341279	9.931673e-05
Standardised weight (cortisol models)	Gaussian	4	Cortisol + Season + CW + Camp	-3702.138	0.0000000	2.831052e-01
		5	**Cortisol * Season + Sex + CW + Camp**	**-3701.996**	**0.1419294**	**2.637110e-01**
		4	Cortisol + CW + Sex + Camp	-3701.216	0.9217025	1.785676e-01
		4	Cortisol * Sex + CW + Camp	-3699.794	2.3438135	8.769911e-02
		2	CW + Camp	-3699.762	2.3763205	8.628522e-02
		3	Season + CW + Camp	-3698.517	3.6208923	4.631067e-02
		3	CW + Sex + Camp	-3697.971	4.1674647	3.523663e-02
		4	Season + CW + Sex + Camp	-3696.744	5.3943819	1.907975e-02
		2	Cortisol + Season	-3677.550	24.5879901	1.296384e-06
		2	Cortisol + CW	-3676.293	25.8452047	6.914057e-07
		4	Cortisol * Season + Sex + CW	-3675.684	26.4541836	5.099116e-07
		3	Cortisol + Season + CW	-3675.557	26.5810704	4.785659e-07
		3	Cortisol + CW + Sex	-3675.090	27.0483028	3.788642e-07
		4	Cortisol + Cortisol^2 + Season + Sex	-3674.592	27.5464000	2.953406e-07
		4	Cortisol + Season + Sex + CW	-3674.388	27.7501012	2.667411e-07
		4	Cortisol * CW + Season + Sex	-3673.867	28.2706775	2.056119e-07

		4	Cortisol + Cortisol^2 + CW + Season	-3673.753	28.3852322	1.941659e-07
		4	Cortisol + Cortisol^2 + Sex + CW	-3673.224	28.9138467	1.490685e-07
		4	Cortisol * Sex + Season + CW	-3673.080	29.0577555	1.387191e-07
		2	CW + Sex	-3672.261	29.8774875	9.207334e-08
		3	Season + CW + Sex	-3671.117	31.0206888	5.198653e-08
		3	Season * CW + Sex	-3669.298	32.8405089	2.092772e-08
		3	Season * Sex + CW	-3668.128	34.0104695	1.165919e-08
Standardised weight (HL models)	Gaussian	1	Camp	-34.20857	0.000000	4.285409e-01
		2	Sex + Camp	-32.59144	1.617129	1.909137e-01
		3	HL*Sex + Camp	-31.96771	2.240858	1.397642e-01
		2	Season + Camp	-30.99645	3.212122	8.599809e-02
		3	HL + Sex + Camp	-30.72281	3.485759	7.500141e-02
		3	Season + Sex + Camp	-29.42525	4.783317	3.920200e-02
		3	HL + Season + Camp	-29.14569	5.062881	3.408801e-02
		4	HL*Season + Sex + Camp	-25.80002	8.408545	6.398821e-03
		1	HL	-14.74393	19.464637	2.542728e-05
		1	Sex	-14.14732	20.061246	1.886897e-05
		2	HL + Sex	-13.26354	20.945025	1.212936e-05
		3	HL*Sex + Season	-11.55954	22.649026	5.173910e-06
		2	HL + Season	-11.38225	22.826320	4.735000e-06
		2	HL + HL^2 + Sex	-11.33122	22.877351	4.615710e-06
		2	Sex + Season	-10.72710	23.481470	3.412368e-06
		3	HL + Season + Sex	-9.93897	24.269601	2.300993e-06
		2	HL + HL^2 + Season	-9.42504	24.783524	1.779584e-06
		2	Season*Sex	-9.23986	24.968706	1.622208e-06
		3	HL*Season + Sex	-8.93282	25.275745	1.391342e-06
		3	HL + HL^2 + Season + Sex	-7.99001	26.218559	8.683694e-07